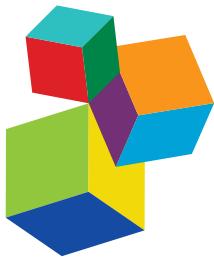


TAILORING NK CELL RECEPTOR-LIGAND INTERACTIONS: AN ART IN EVOLUTION, 2nd EDITION

EDITED BY: Ulrike Koehl, Antoine Toubert and Gianfranco Pittari

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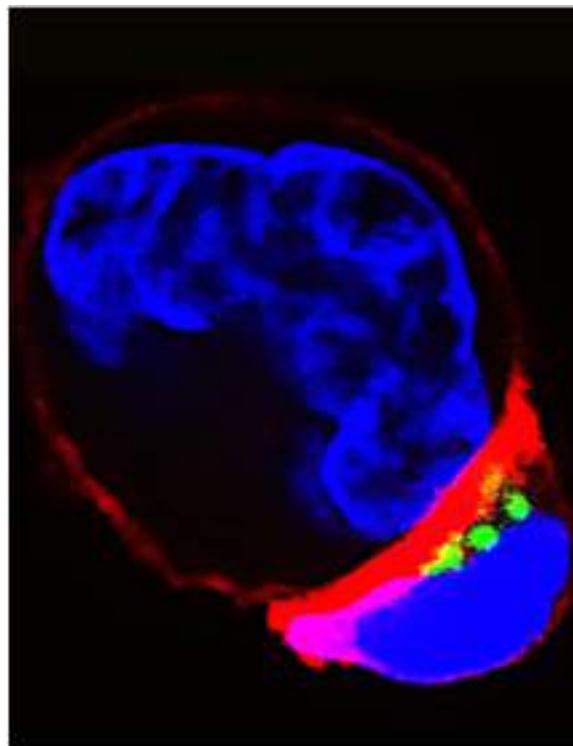
TAILORING NK CELL RECEPTOR-LIGAND INTERACTIONS: AN ART IN EVOLUTION, 2nd EDITION

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NK cell's lytic granules polarization toward the immune synapse against the K562 cell line. Green: perforin-specific antibody, red: phalloidin stain for polymerized F-actin, blue: DAPI stain for nuclei.
Image: Nicolas Dulphy.

Recognition and killing of aberrant, infected or tumor targets by Natural Killer (NK) cells is mediated by positive signals transduced by activating receptors upon engagement of ligands on target surface. These stimulatory pathways are counterbalanced by inhibitory receptors that raise NK cell activation threshold through negative antagonist signals. While regulatory effects are necessary for physiologic control of autoimmune aggression, they may restrain the ability of NK cells to activate against disease. Overcoming this barrier to immune surveillance, multiple approaches to enhance NK-mediated responses are being investigated since two decades. Propelled by considerable advances in the understanding of NK cell biology, these studies are critical for effective translation of NK-based immunotherapy principles into the clinic.

In humans, dominant inhibitory signals are transduced by Killer Immunoglobulin Like Receptors (KIR) recognizing cognate HLA class I on target cells. Conversely, KIR recognition of "missing self-HLA" - due to HLA loss or HLA/ KIR mismatch - triggers NK-mediated tumor rejection. Initially observed in murine transplant models, these antitumor effects were later found to have important implications for the clinical outcome of haplotype-mismatched stemcell transplantation. Here, donor NK subsets protect against acute myeloid leukemia (AML) relapse through missing self recognition of donor HLA-C allele groups (C1 or C2) and/or Bw4 epitope. These studies were subsequently extended by trials investigating the antileukemia effects of adoptively transferred haplotype-mismatched NK cells in non-transplant settings. Other mechanisms have been found to induce clinically relevant NK cell alloreactivity in transplantation, e.g., post-reconstitution functional reversal of anergic NK cells. More recently, activating KIR came into the spotlight for their potential ability to directly activate donor NK cells through *in vivo* recognition of HLA or other ligands.

Novel therapeutic monoclonal antibodies (mAb) may optimize NK-mediated effects. Examples include obinutuzumab (GA101), a glyco-engineered anti-CD20 mAb with increased affinity for the Fc_YRIIIA receptor, enhancing antibody-dependent cellular cytotoxicity; lirilumab (IPH2102), a first-in-class NK-specific checkpoint inhibitor, blocking the interaction between the major KIR and cognate HLA-C antigens; and elotuzumab (HuLuc63), a humanized monoclonal antibody specific for SLAMF7, whose anti-myeloma therapeutic effects are partly due to direct activation of SLAMF7-expressing NK cells. In addition to conventional antibodies, NK cell-targeted bispecific (BiKEs) and trispecific (TriKEs) killer engagers have also been developed. These proteins elicit potent effector functions by binding target ligands (e.g., CD19, CD22, CD30, CD133, HLA class II, EGFR) on one arm and NK receptors on the other.

An additional innovative approach to direct NK cell activity is genetic reprogramming with chimeric antigen receptors (CAR). To date, primary NK cells and the NK92 cell line have been engineered with CAR specific for antigens expressed on multiple tumors. Encouraging preclinical results warrant further development of this approach.

This Research Topic welcomes contributions addressing mechanisms of NK-mediated activation in response to disease as well as past and contemporary strategies to enhance NK mediated reactivity through control of the interactions between NK receptors and their ligands.

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Editorial: Tailoring NK Cell Receptor–Ligand Interactions: An Art in Evolution

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Editorial on the Research Topic

Tailoring NK Cell Receptor–Ligand Interactions: An Art in Evolution

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This research topic is inaugurated by Goh and Huntington, who revise the dynamics of surface receptor expression in murine NK cell subsets at different stages of development (Goh and Huntington). Human NK cell development is subsequently addressed. In their work, Post et al. demonstrate that the transcription factor gene ZNF683/HOBIT is critical for efficient *ex vivo* generation of CD56⁺ NK cells, but likely has limited effects on later acquisition of critical NK cell function modulators, namely NKG2A and killer immunoglobulin-like receptors (KIRs) (Post et al.).

In human disease, *in vivo* selective expansion of phenotypically defined NK cell subsets may affect disease course and response to treatment, a concept underpinned by three manuscripts in this collection. Huenecke et al. report an inverse correlation between the incidence of acute graft-versus-host disease and the frequency of reconstituted CD56 bright NK cells in pediatric patients receiving a hematopoietic stem cell transplantation (HCT) (Huenecke et al.). In their review, Pollmann et al. describe how HCV and human CMV chronic infection affect relative frequency of specific NK cell subsets. The authors specifically revise evidence supporting the concept that genetic background and NK subset composition (e.g., expression of KIR2DL3 in a HLA-C1 homozygous background) promotes HCV clearance and response to treatment (Pollmann et al.). Further elaboration on the importance of NK subpopulation analysis in predicting response to antiviral treatment is provided by Gondois-Rey et al., who report an association between NK maturation phenotype and prompt viremia decrease in response to combination antiretroviral therapy in HIV-infected individuals (Gondois-Rey et al.).

Killer immunoglobulin-like receptor and their interaction with cognate ligands are a major focus of this research topic. Heidenreich and Kröger review the effects of NK cell alloreactivity mediated by inhibitory and activating KIR in unrelated HCT (Heidenreich and Kröger). Erbe et al. analyze the differential impact of alternative HLA-Bw4 antigen groups on the clinical outcome of mAb-based immunotherapy. They previously observed that individuals with follicular lymphoma and neuroblastoma had better clinical outcome following immunotherapy if their HLA/KIR genotypes included KIR3DL1 and its cognate HLA-Bw4 ligand. The authors now show that this benefit does not extend across all HLA-Bw4 isoforms, but it is only observed for –Bw4 epitopes occurring on HLA-A alleles (HLA-A/Bw4) or HLA-B alleles with Thr amino acid substitution at position 80 (HLA-B/Bw4-T80) (Erbe et al.). Mechanisms of NK tolerance to activating KIR-specific ligands are subsequently

addressed in two manuscripts. Carluomagno et al. report that NK cells expressing KIR3DS1 may activate upon recognition of a --Bw4 I80^+ HLA-B ligand (i.e., HLA-B*51 with Ile at position 80) only if NK donor is --Bw4 I80^- , thus ensuring tolerance to the self-antigen (Carluomagno et al.). van der Ploeg et al. show that target cell infection with human CMV may potentiate KIR2DS1-mediated positive signaling *in vitro*, suggesting temporary breach of immunological tolerance to self-HLA-C2 in the presence of altered-self (van der Ploeg et al.). Finally, Maniangou et al. describe a novel next-generation sequencing technology for KIR haplotype-wide polymorphism detection, a fast and reliable tool for future studies addressing the effect of KIR allelic diversity in physiology and disease (Maniangou et al.).

Accumulating evidence indicates that positive signaling transduced by NK cell-activating receptors is subject to remarkably complex regulation involving gene expression, ligand interactions, and downstream pathways. Several contributions discuss recent insights into the mechanisms underlying NK cell activation plasticity. NKG2D activating receptor and corresponding ligands are first addressed in a series of focused review articles. Isenhardt et al. address a hot single-nucleotide polymorphism of the MICA NKG2D-binding protein (rs1051792), resulting in a Val129Met substitution. Functional implications of low-affinity 129Met and high-affinity 129Val MICA isoforms on NKG2D-mediated activation are discussed (Isenhardt et al.). Next, Mandelboim and Schmiedel illustrate mechanisms of NKG2D ligand down-regulation as a strategy of herpesvirus evasion from NK-mediated immunosurveillance (Schmiedel and Mandelboim). The role of NKG2D and MICA on the outcome of kidney transplantation is revised by Risti and Bicalho. Killer lectin-like heterodimer signaling is addressed next by Pupuleku et al., who utilized a reporter cell system to identify CD94/NKG2C-specific ligands on human CMV-infected cells (Pupuleku et al.). It is increasingly appreciated that ligand diversity and receptor alternative splice variants may potentially result in opposite (i.e., activating versus inhibitory) natural cytotoxicity receptor signaling. In their work, Pazina et al. discuss these phenomena and their potential implications in human physiology and disease (Pazina et al.).

The next section of this research topic describes strategies to enhance the cytotoxicity of cultured NK cells for adoptive immunotherapy. Granzin et al. provide a summary of methods known to promote antitumor reactivity of cultured NK cells and discuss technical and regulatory aspects relevant to NK-based cellular therapy (Granzin et al.). Three studies subsequently address the impact of specific soluble cytokines, cytokine combinations, and feeder cells on NK cell *in vitro* propagation. Sánchez-Correa et al. describe NKp30-specific upregulation and functional reversal of AML-NK cells following short term *in vitro* IL-15 exposure (Sánchez-Correa et al.). Next, Wagner et al. describe a novel NK cell culture protocol based on a two-phase sequential incubation with IL-15 (NK cell expansion) and IL-21 (NK cell functional boost). By using a rhabdomyosarcoma xenogeneic model, the authors show that this protocol may drive propagation of NK cells potentially synergizing radiotherapy antitumor effects (Wagner et al.). Delso-Vallejo et al. focus on the use of irradiated autologous PBMCs as feeders for NK cell culture. This study shows that both feeder-NK physical contact and soluble factors

are required for efficient NK cell expansion. Of interest, it also identifies differential transcriptome signatures for proliferating and non-proliferating NK cells (Delso-Vallejo et al.). Strategies to increase sensitivity of tumor cells to NK-mediated lysis are also addressed. Fischer et al. show that incubation with the SMAC mimetic BV6, a selective antagonist of inhibitor of apoptosis proteins, sensitizes rhabdomyosarcoma cell lines to NK-mediated killing (Fischer et al.). Moreover, Aquino-López et al. describe the effect of IFN γ on the expression of NK-specific ligands in a panel of tumor cell lines representing variable types of pediatric malignancies. Rationale for these studies derives from the observation that NK cells cultured in the presence of IL-15 and IL-21 secrete high levels of IFN γ upon target recognition, potentially affecting susceptibility to NK lysis (Aquino-López et al.).

Multiple clinical studies have demonstrated the safety and feasibility of allogeneic peripheral blood or cord blood NK cell adoptive immunotherapy. The potential of adoptively transferred allogeneic NK cells as a universal cell therapeutic platform in the transplant and non-transplant settings is addressed by Veluchamy et al. (Veluchamy et al.). An overview of the potential clinical applications of cord blood-derived NK cells is subsequently provided by Sarvaria et al. Tumor immune escape from NK-mediated immunosurveillance may be prevented by redirecting specificity of NK cell effectors. To this end, chimeric antigen receptor (CAR)-modified NK cells engaging tumor-associated antigens have been developed and currently represent a promising approach for clinical translation. Oberschmidt et al. address primary human CAR NK cells as an “off-the-shelf immunotherapy” and describe CAR signaling in NK cells (Oberschmidt et al.). In addition, Zhang et al. review good manufacturing practice-compliant procedures for CAR-engineered NK-92 cells redirected against ErbB2 (HER2) and other tumor epitopes (Zhang et al.). Specific antigen targeting can also be efficiently attained by cross-linking NK cells to cancer cells. In an additional manuscript, Veluchamy et al. demonstrate that lytic activity of cord blood-derived NK cells toward EGFR $^+$ colon and cervical cancer cells is strongly enhanced by the mAb cetuximab (Veluchamy et al.). Kloess et al. show that an increased NK cell cytotoxicity leading to B-cell precursor leukemia elimination can be achieved by dual-specific targeting *via* the trispecific immunoligand ULBP2-aCD19-aCD33 (Kloess et al.). Further information on NK-specific dual targeting with triple-specific antibodies to prevent escape of antigen loss variants is provided by Vyas et al. Subsequently, Messaoudene et al. address the potential of NK-based therapy as a tool to enhance potency and prolong efficacy of novel antitumor strategies (Messaoudene et al.). In a specular manner, contemporary therapeutic interventions have the potential to counter tumor-induced NK cell immunosuppression. These effects are covered by Pittari et al., who specifically address the role of NK cells in the context of multiple myeloma (Pittari et al.). To date, preclinical evaluation of NK cell-based therapies in mouse models are challenged by the inherent problem that reagents designed to trigger human immune cells do not react with murine NK cells and by the fact that human NK cell infusions in mice do not provide a human immune cell compartment. Here, Lopez-Lastra and Di Santo describe a Flt3-deficient mouse model allowing for specific enhancement of human NK hematopoiesis *via* exogenous human

Flt3 ligand-mediated dendritic cell expansion (Lopez-Lastra and Di Santo). Finally, Hofer and Koehl report some future NK cell-based strategies developed in the context of the European Union ITN NATURIMMUN network and published ahead in *Frontiers in Immunology* (Hofer and Koehl).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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UK, AT, and GP conceived, designed, and critically revised the manuscript. UK and GP wrote the manuscript. All authors approved the final version of the manuscript.

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Regulation of Murine Natural Killer Cell Development

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Natural killer (NK) cells are effector lymphocytes of the innate immune system that are known for their ability to kill transformed and virus-infected cells. NK cells originate from hematopoietic stem cells in the bone marrow, and studies on mouse models have revealed that NK cell development is a complex, yet tightly regulated process, which is dependent on both intrinsic and extrinsic factors. The development of NK cells can be broadly categorized into two phases: lineage commitment and maturation. Efforts to better define the developmental framework of NK cells have led to the identification of several murine NK progenitor populations and mature NK cell subsets, each defined by a varied set of cell surface markers. Nevertheless, the relationship between some of these NK cell subsets remains to be determined. The classical approach to studying both NK cell development and function is to identify the transcription factors involved and elucidate the mechanistic action of each transcription factor. In this regard, recent studies have provided further insight into the mechanisms by which transcription factors, such as ID2, FOXO1, Kruppel-like factor 2, and GATA-binding protein 3 regulate various aspects of NK cell biology. It is also becoming evident that the biology of NK cells is not only transcriptionally regulated but also determined by epigenetic alterations and posttranscriptional regulation of gene expression by microRNAs. This review summarizes recent progress made in NK development, focusing primarily on transcriptional regulators and their mechanistic actions.

Keywords: NK cell, transcription factors, ontogeny, maturation, homeostasis, IL-15

INTRODUCTION TO NATURAL KILLER (NK) CELLS

Natural killer cells in mice were first described in 1975 (1–3), following further investigation into splenocytes that were able to kill tumor and virus-infected cells without prior sensitization (4–6). NK cells exert their cytotoxic effect on target cells by inducing apoptosis. Upon formation of an immunological synapse with the target cell, NK cells become activated and release cytolytic granules containing perforin and granzymes (7–9). Perforin forms pores in the membrane of target cells, thereby allowing granzymes to enter the cell, activate caspases, and initiate apoptosis (8). In a similar process known as antibody-dependent cell cytotoxicity, NK cells are able to release cytolytic granules and initiate apoptosis in opsonized cells, following recognition of the opsonized cells via low-affinity Fc receptors (CD16) expressed on the surface of NK cells (10). NK cells can also initiate apoptosis in target cells through the respective engagement of Fas ligands and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on their cell surface with Fas and TRAIL receptors on the target cells (11, 12). In addition to inducing apoptosis, NK cells can indirectly mediate the clearance of

target cells by producing pro-inflammatory cytokines [e.g., interferon-gamma (IFN- γ)], which boost the innate response and recruit adaptive immune responses (13–15).

The surface markers that are commonly used to identify murine NK cells by flow cytometry vary depending on the mouse strain. C57B/6 and SJL mice express the surface markers NK1.1, NKp46, and CD49b, but not CD3, which is a surface marker of T cells. CD3 is used to exclude contaminating T cell subsets, such as natural killer T cells and NK-like T cells, that, respectively, express NK1.1 and NKp46 (16). As for other mouse strains, such as BALB/c, NK cells are identified with only CD49b and NKp46 as these strains possess allelic variants of NK1.1 that cannot be detected with the widely used PK136 antibody (16, 17).

MURINE NK CELL DEVELOPMENT

Murine NK cells can be found in all lymphoid organs and many non-lymphoid tissues, such as salivary glands, liver, and kidney. The more recent discovery of related innate lymphoid cells (ILCs) places NK cells within this family, specifically in the IL-15 dependent, IFN- γ producing group 1 ILCs. ILCs are lymphoid cells that lack rearranged antigen receptors and are dependent on the transcription factors inhibitor of DNA-binding 2 (ID2) and nuclear factor, interleukin 3 regulated (NFIL3) for their development. While NK cells are phenotypically heterogeneous and previously categorized based on their tissue of origin or location (bone marrow, thymus, fetal liver, adult liver), we appreciate that some of this heterogeneity stems from NK cells (*Eomes*⁺) and other ILC1s (*Eomes*⁻) being viewed as the same cell type. As much of our current understanding of murine NK cell development is built upon studies on bone marrow-derived NK cells [referred to here as conventional NK (cNK) cells], which represent the majority of NK cells within the body, this review will focus primarily on progress made in our understanding of cNK development.

cNK Development in the Bone Marrow—Lineage Commitment

Conventional NK cells develop from HSCs in the bone marrow, through a sequential order of intermediate progenitors. The first progenitor to arise from HSCs is the lymphoid-primed multipotent progenitor, which then gives rise to the common lymphoid progenitor (CLP) (18). The earliest NK lineage committed progenitor to arise from CLPs is known as pre-pro NK (19), which was subdivided into pre-pro A and pre-pro B (19, 20). Differing only in c-kit (CD117) expression, the relationship between pre-pro A and B remains unclear and requires further investigation. Pre-pro NK cells then differentiate into the NK progenitor (NKP) (19, 21). NKPs give rise to immature NK (iNK) cells that either undergo further development within the bone marrow (22) or enter the periphery and develop into mature NK cells (23, 24).

As the early stages of murine NK development are still poorly defined, the developmental pathway outlined above is by no means the definitive model. Heterogeneity within existing progenitor populations, along with the discovery of new distinguishing cell markers, have led to the identification of new sub-populations

and, therefore, refinements to the developmental pathway of NK cells. For instance, the common innate lymphoid progenitor (CILP) was found to possess the capacity to give rise to all lineages within the ILC family, of which NK cells are the founding member, but not B and T cells, thus making it an earlier progenitor than the pre-pro NK (25). As the CILP expresses $\alpha_4\beta_7$, it is also alternatively referred to as the α -lymphoid precursor (α LP) (25). However, it has been postulated that there could be an even earlier progenitor population, as there were only about 50 CILPs per mice, and only 2.5% of CILPs efficiently developed into all ILC lineages (25).

The early innate lymphoid progenitor (EILP) is proposed to be the earliest known progenitor for ILCs and was identified using a TCF1 (T-cell factor 1, encoded by *Tcf7*) transgenic mouse strain that expresses a green fluorescent protein reporter (26). Like CILPs, EILPs give rise to all ILC lineages both *in vivo* and *in vitro*, albeit more efficiently (26). Nonetheless, EILPs have not been shown to differentiate into CILPs and hence, the relationship between these two progenitors remains unresolved. As most of the surface markers used to identify the EILP and CILP were different, a detailed comparison of the surface marker phenotype between the two progenitors may also provide further insight.

The discovery of pre-pro NKPs was the outcome of efforts to better understand why only 8–40% of NKPs had solely NK cell potential (19). Similar studies resulted in the identification of a pre-NKP population that preceded a “streamlined” population of NKPs known as refined-NKP (rNKP) (27). Even though many parallels have been drawn between the pre-NKP and pre-pro NK, likewise with NKP and rNKP, it remains to be determined if these populations are exactly identical (20). A summary of the surface markers that are expressed on the various progenitors are provided in Table 1.

cNK Development in the Periphery—NK Cell Maturation

Natural killer cell maturation is a process by which lineage committed NK cells acquire their full effector functions. This process is also accompanied by the expression of different cell surface markers, which have helped in the identification of different NK cell maturation subsets. At present, most studies use CD11b and CD27 to divide cNK cells into three maturation subsets: immature (Imm), mature 1 (M1), and mature 2 (M2). Low (lo) and high (hi) CD11b expression divides cNK cells into immature and mature subsets, respectively (22). Heterogeneity in CD27 expression further delineates the mature NK compartment into CD27^{hi} and CD27^{lo} subsets, which has also been referred to as M1 and M2 NK subsets (24, 28). CD27 and CD11b expressing M1 NK cells have also been termed double-positive NK cells (24). The three subsets differ in proliferative and cytotoxic capacity. In general, NK cells lose proliferative potential and produce less cytokine, but become more cytotoxic against target cells as they mature (22, 24, 28).

Apart from CD27 and CD11b, markers such as KLRG1 (23, 29), CD62L (30), MCAM (31), CD49b (21), CD43 (32, 33), Ly6C (34), DNAM1 (35), and CD160 (36) have further dissected maturing NK cells into various phenotypic subsets (37, 38). NK cells that express CD160 exhibit enhanced IFN- γ

TABLE 1 | Surface markers expressed by different natural killer (NK) cell populations reported in the literature.

Surface markers	Common lymphoid progenitor	Early innate lymphoid progenitor	Common innate lymphoid progenitor (CILP)/ α LP	Pre-pro A NK	Pre-pro B NK	NK progenitor (NKP)	pre-NKP	refined-NKP (rNKP)	Imm NK	M1 NK	M2 NK
NK1.1	–	–	–	–	–	–	–	–	+	+	+
CD11b (MAC-1)	–	–	–	–	–	–	–	–	–	+	+
CD127 (IL-7R α)	hi	lo	hi	hi	hi	hi	hi	int	int	lo	lo
CD117 (c-kit)	int	lo	int	int	lo	lo	int	lo	int	lo	lo
Sca-1	int	–	int	+	+	+	+	+	–	–	–
CD49b	–	–	–	–	–	–	–	–	–	+	+
CD27	+	ND	+	+	+	+	+	+	+	+	–
CD244 (2B4)	+	ND	+	+	+	+	+	+	+	+	+
CD25 (IL-2R α)	–	–	–	ND	ND	–	–	+	–	–	–
CD122 (IL-2R β)	–	–	–	–	–	+	–	+	+	+	+
CD132 (IL-2R γ)	+	+	+	+	+	+	+	+	+	+	+
CD314 (NKG2D)	–	–	–	+	+	+	+	+	+	+	+
CD226 (DNAM1)	–	ND	int	ND	ND	+	ND	ND	hi	int	lo
CD279 (PD1)	–	ND	–/+	ND	ND	hi	ND	ND	–	–	–
CD43 (Leukosialin)	–	–	–	–	–	–	–	–	lo	int	hi
CD335 (NKp46)	–	–	–	–	–	–	–	–	+	+	+
CD253 (tumor necrosis factor-related apoptosis-inducing ligand)	–	ND	ND	ND	ND	ND	ND	ND	int	lo	lo
KLRG1	–	ND	ND	–	–	–	ND	ND	–	–	+
α β ₇ (LPAM)	+	+	+	ND	ND	–	ND	ND	–	–	–
Ly49s	–	–	–	–	–	–	–	–	–/+	–/+	–/+
CD94-NKG2	–	ND	ND	–	–	–	–	–	+	+	+
CD62L (L-selectin)	–	ND	ND	–	–	–	ND	ND	–/+	+	+
CD146 (MCAM)	–	ND	ND	–	–	–	ND	ND	lo	int	hi
CXCR3	–	ND	ND	–	–	+	ND	ND	hi	int	lo
CXCR6	–	–	+	+	+	+	ND	ND	–	–	–
Ly6C	–	ND	ND	–	–	–	ND	ND	lo	int	hi

production (36), while the opposite is true for mature NK cells that express higher levels of Ly6C (34), and KLRG1 (39). Like Ly6C, MCAM is also more highly expressed on mature NK cells, although MCAM⁺ and MCAM[–] NK cells differ in their ability to kill target cells rather than cytokine production (31). Interestingly, the expression of DNAM1 appears to be independent of NK cell maturation that is defined by CD27 and CD11b, as DNAM-1⁺ and DNAM-1[–] NK cells were observed in both the immature and mature NK compartments (35). As the correlation between these markers have not been studied in detail, the relationships between these phenotypic subsets remain unclear and warrants further investigation. A summary of the surface markers that are expressed on the various mature NK cell subsets are provided in Table 1.

TRANSCRIPTIONAL REGULATION OF MURINE cNK CELL DEVELOPMENT

Transcription factors control gene expression by either activating or repressing gene transcription. This is achieved by first binding to specific DNA sequences in the enhancer or promoter regions

that lay upstream of target genes, then promoting or blocking the recruitment of RNA polymerases that transcribe those genes (40). In terms of murine NK cell development, several TFs have been shown to play crucial roles in regulating NK cell lineage specification, NK cell maturation, or even both. Conventional NK cell development occurs mostly in the bone marrow, under the coordinated control of the TFs and cytokines. TFs like ID2, NFIL3, T-box brain protein 2 (EOMES), and T-box protein 21 (TBET) to name a few, fall into the category of intrinsic factors that regulate NK cell development. A summary of the transcription factors that are implicated in NK cell development is provided in Table 2.

Transcription Factors Regulating NK Cell Lineage Specification

The TFs that are involved in regulating NK cell lineage specification include ETS proto-oncogene 1 (ETS1), NFIL3, and TCF1. ETS1 is a key regulator of early NK cell development as ETS1-deficient mice have normal CLP numbers but lack NK cells (45). Further investigation into the impact of ETS1 deficiency on NK lineage specification revealed a reduction in pre-pro NK, pre-NKP and

TABLE 2 | Transcription factors implicated in natural killer (NK) cell development and function.

Transcription factor (gene symbol)	Phenotype of germline (KO) or conditional (cKO) deficiency				
	Bone marrow NK precursor #	NK cells #	IL-15 responsiveness	Interferon- gamma (IFN- γ) production/ degranulation	Cytotoxicity (tumor/target cells)
Ikaros family zinc finger 3, Aiolos (<i>Ikzf3</i>)	ND	KO: normal; accumulation of immature NKs (iNKs)	KO: hyperresponsive to IL-2/anti-IL-2 mAB complex <i>in vitro</i>	KO: slightly impaired IFN- γ production	KO: normal <i>in vitro</i> killing, augmented <i>in vivo</i> killing
B lymphocyte-induced maturation protein 1 (<i>Prdm1</i>)	ND	KO: reduced in spleen, liver, and lung; accumulation in bone marrow and lymph nodes; loss of mNKs	KO: hyperresponsive	KO: normal IFN- γ production	KO: normal <i>in vitro</i> killing, augmented <i>in vivo</i> killing
T-box brain protein 2, EOMES (<i>eomesodermin</i>)	ND	cKO: reduced; loss of mNKs	ND	cKO: slightly impaired IFN- γ production	ND
ETS proto-oncogene 1, ETS1 (<i>Ets1</i>)	KO: lack pre-NKPs and rNKPs	KO: reduced	KO: hyperresponsive	ND	KO: impaired <i>in vitro</i> killing and degranulation
Forkhead box protein O1, FOXO1 (<i>Foxo1</i>)	cKO: normal NKP (41)	cKO: reduced; loss of mNKs (41) cKO: normal; accumulation of mNKs (42)	ND	cKO: augmented IFN- γ production (42)	cKO: augmented <i>in vitro</i> and <i>in vivo</i> killing (42)
GATA-binding protein 3 (<i>Gata3</i>)	ND	cKO: reduced in bone marrow; accumulation in spleen and liver, systemic accumulation of iNKs	ND	cKO: impaired IFN- γ production; normal degranulation	cKO: normal <i>in vitro</i> killing
Inhibitor of DNA-binding 2, ID2 (<i>Id2</i>)	KO: normal NKP	KO: reduced; loss of mNKs; cKO: systemic reduction	cKO: hyporesponsive	KO: impaired IFN- γ production cKO: normal IFN- γ production	KO: impaired <i>in vitro</i> killing cKO: impaired <i>in vivo</i> killing
Interferon Regulatory Factor 2 (<i>Irif2</i>)	ND	KO: reduced; loss of mNKs	KO: hyporesponsive	KO: impaired IFN- γ production	KO: normal <i>in vitro</i> killing (43); impaired <i>in vitro</i> killing (44)
Kruppel-like factor 2 (<i>Klf2</i>)	ND	cKO: reduced in spleen, blood, and lung; accumulation in bone marrow and liver; loss of mNKs	ND	ND	ND
Myeloid elf-1-like factor (<i>Mef</i>)	ND	KO: reduced	ND	KO: impaired IFN- γ production	KO: impaired <i>in vitro</i> killing
Nuclear factor, interleukin 3 regulated, NFIL3 (<i>E4bp4</i>)	KO: lack CILP and NKPs, pre-NKPs and rNKPs	KO: reduced	ND	ND	ND
T-box protein 21, TBET (<i>Tbx21</i>)	ND	cKO and KO: reduced in spleen and liver; accumulation in bone marrow; loss of mNKs	ND	KO: impaired IFN- γ production	KO: impaired <i>in vitro</i> killing
T-cell-specific transcription factor 1, TCF1 (<i>Tcf7</i>)	KO: lack NKPs	KO: reduced in bone marrow; normal in periphery	ND	ND	ND
Thymocyte selection- associated high mobility group box protein (<i>Tox</i>)	KO: normal NKP	KO: reduced; loss of mNKs	ND	ND	KO: impaired <i>in vivo</i> killing
Zinc finger E-box binding homeobox 2 (<i>Zeb2</i>)	ND	KO: reduced in periphery; normal in bone marrow; loss of mNKs	cKO: hyporesponsive	cKO: normal to slightly augmented IFN- γ production	cKO: impaired killing <i>in vivo</i>

rNKP, and mature NK cell numbers, thereby supporting the role of ETS1 in NK cell lineage specification (46). ETS1 is believed to impact early NK cell development by regulating the expression of ID2 and TBET, which are also important TFs for NK cell development (46).

TCF1 and NFIL3 are also key regulators of NK cell lineage specification, as marked reductions in pre-pro NK, pre-NKP, and rNKP numbers were observed within the bone marrow of NFIL3- and TCF1-deficient mice, although only the former mouse strain exhibited an additional reduction in CLP numbers (25, 26, 47, 48). NFIL3 appears to be dispensable for mature NK cell as their numbers remained unchanged after its deletion in mature NKp46⁺ NK cells (49). Despite reduced progenitor and mature NK cell numbers in the bone marrow of TCF1-deficient mice, mature NK cells have been found in the periphery at frequencies comparable to littermate controls (26, 50). As most NK cells are derived from bone marrow precursors at steady state, an investigation into the source of NK cells in TCF1-deficient mice might perhaps shed some light on alternative pathways of NK development. The mechanism by which TCF1 mediates NK lineage specification remains poorly understood. However, TCF1 has been shown to regulate T-lineage specification by promoting the expression of genes, such as *Gata3*, *Bcl11b*, *Il2ra*, and *Cd3e*, that are critical for T cell development (51). Similarly, the mechanism by which NFIL3 specifically mediates lineage specification in NK cells remains unclear, although NFIL3 was found to promote the expression of ID2 and EOMES (47), transcription factors known to be involved in the later stages of NK cell development (52, 53). The role of NFIL3 in ID2 expression remains to be clarified given a contradicting report that ID2 expression is normal in NK cells lacking NFIL3 (48).

The role of ID2 in the lineage specification of NK cells is unclear, due to contradicting reports following deletion of the encoding gene *Id2*. While Yokota et al. (54) reported poor reconstitution of NK cells following bone marrow transplantation, implying a defect early on during NK cell development, Boos et al. (55) did not observe any reduction in NKP and iNK cell numbers. A recent demonstration of low ID2 levels in CLPs but consistently high levels in pre-pro NK, NKP, and immature and mature NK cells lends further support for the hypothesis that ID2 could indeed be important for NK lineage specification/maintenance (53). Interestingly, ID2 has been found to suppress T and B cell development through heterodimer formation with the E-box protein E2A (55–57), although how the interaction specifically promotes commitment to the NK cell lineage remains unknown.

Transcription Factors Regulating NK Cell Maturation

A greater number of TFs have been shown to play a role in NK cell maturation. These factors include ID2, TBET, EOMES, Zinc finger E-box-binding homeobox 2 (ZEB2), Thymocyte selection-associated high mobility group box (TOX), IKAROS family zinc finger 3 (AIOLOS), Interferon regulatory factor 2 (IRF2), B lymphocyte-induced maturation protein 1 (BLIMP1), Forkhead box O1 (FOXO1), Kruppel-like factor 2 (KLF2), and GATA-binding

protein 3 (GATA3). An overview of the expression of these TFs during NK cell maturation is presented in **Figure 1**.

The role of ID2 in NK cell maturation is better understood than its role in early NK cell development. A recent study provided additional insight into the underlying mechanism of ID2 by demonstrating that it modulates the expression of E2A target genes (i.e., *Socs3*, *Tcf7*, and *Cxcr5*) by titrating E-protein activity, thereby controlling the responsiveness of NK cells to IL-15 that is crucial for survival (53, 58, 59).

TBET and EOMES are members of the T-box family of transcription factors that appear to regulate distinct checkpoints in NK cell maturation. TBET- and EOMES-deficient mice exhibited a similar phenotype where NK cell numbers were reduced in the all lymphoid tissues (52, 60, 61), except in the bone marrow of the former where there was an increase in NK cell numbers (60, 61). Detailed analyses of the bone marrow from TBET-deficient mice revealed that the increase in NK cell numbers was due to an accumulation of iNK cells, which the authors attributed to a developmental block (60). However, the possibility of a defect in NK cell trafficking remains unaddressed, given that TBET plays a role in the expression of sphingosine-1-phosphate receptor 5 (S1P₅) that is required for NK cell egression from the bone marrow (62). Nonetheless, both TFs are crucial for maturation, as mice that are deficient for both have a systemic lack of NK cells despite normal progenitor numbers (52, 61). As TBET and EOMES are, respectively, required by immature and mature NK cells, the two TFs are believed to function in a sequential manner for NK cell maturation (52, 60). Quite fittingly, both TBET and EOMES were shown to be essential for IL-15 responsiveness by enforcing high CD122 expression, with *Il2rb* (gene encoding CD122) being a direct target of EOMES (63).

Similar to that of TBET-deficient mice, NK cell numbers in the bone marrow of mice deficient for ZEB2 or BLIMP1 were higher than littermate controls (64, 65). The unusual accumulation of NK cells within the bone marrow of ZEB2-deficient mice was due to reduced S1P₅ expression (64), while enhanced proliferation was found to be the underlying cause in BLIMP1-deficient mice (65). Profound losses in terminally mature NK cells were observed on closer examination of the bone marrow NK cells from both strains (64, 65). The lack of mature NK cells in ZEB2-deficient was further attributed to poor responsiveness to IL-15, which resulted in poor survival (64).

The lack of mature NK cells was reported in mice that were deficient for TOX (66), AIOLOS (67), IRF2 (43, 44), KLF2 (68), or GATA3 (69, 70). A similar lack of mature NK cells in FOXO1-deficient mice was reported most recently (41), although this remains to be clarified against an earlier study, which instead found an accumulation of mature NK cells (42). Interestingly, the iNK cells in AIOLOS-deficient mice retained their expression of KLRG1, which is typically expressed on terminally mature NK cells (67). NK cells from IRF2-deficient mice have been shown to undergo accelerated apoptosis, indicating a role for IRF2 in regulating NK cell survival as well as maturation (43). Unlike the foregoing TFs, the temporal requirement for myeloid elf-1-like factor (MEF) by maturing NK cells has not been determined, as only an overall reduction in NK cell numbers was reported (71).

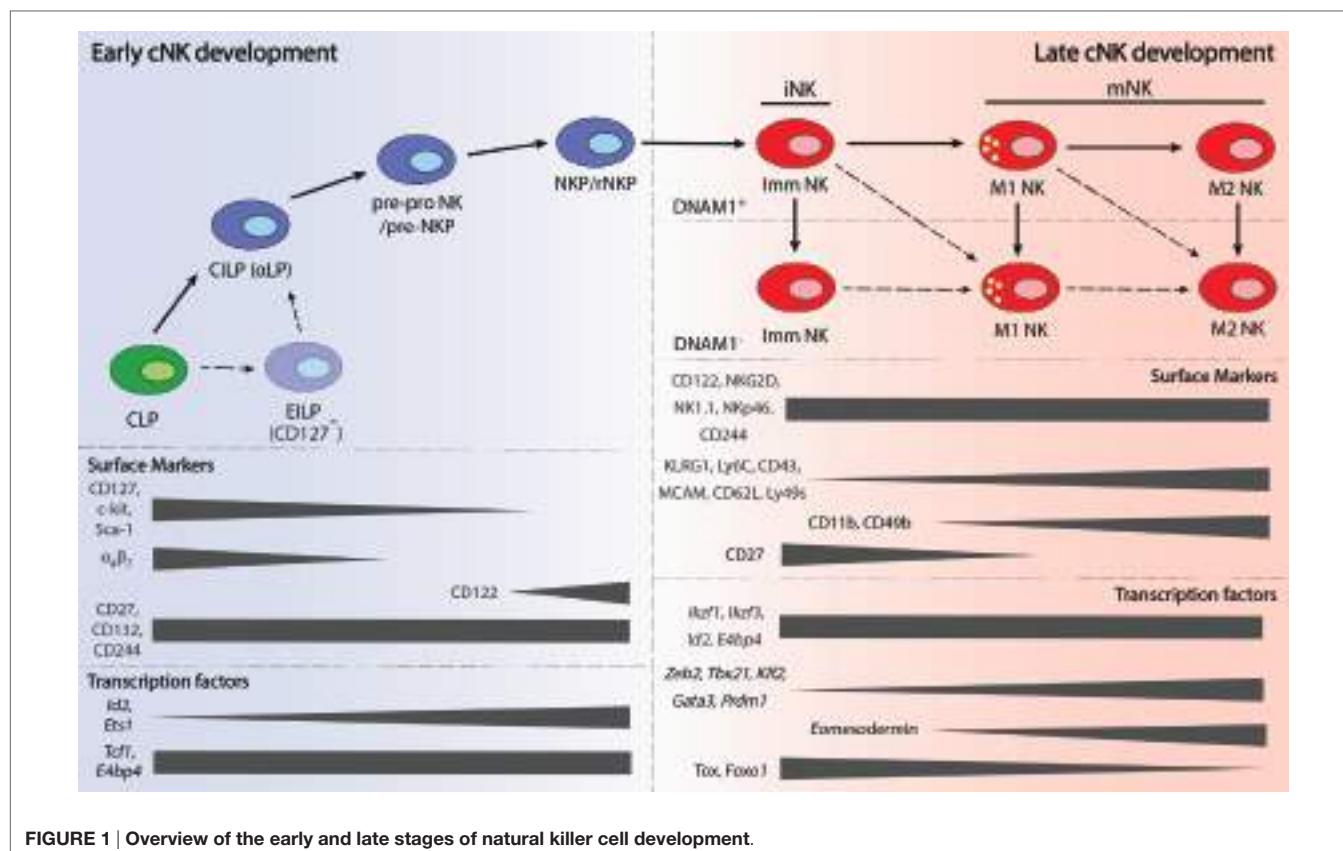


FIGURE 1 | Overview of the early and late stages of natural killer cell development.

FOXO1 was recently found to be directly involved in the initiation of autophagy in iNK cells, most likely *via* cytosolic interactions between FOXO1 and the autophagy protein ATG7 (41). KLF2 appears to regulate NK cell maturation *via* a different mechanism, influencing the expression of homing receptors such as CD62L (i.e., L-selectin) on maturing NK cells, thereby dictating their access to IL-15 signaling that is essential for survival (68). Current knowledge of how GATA3 regulates NK cell maturation is limited to disturbances in expression of the TFs ID2, TBET, and NFIL3 (70). Nevertheless, GATA3 has also been shown to regulate NK cell egression from the bone marrow in a CXCR4-dependent manner, and also NK cell proliferation in response to IL-15 *via* CD25 expression (70).

Transcription Factors Regulating NK Cell Effector Function

Natural killer cell effector function is also regulated by many of the TFs outlined above. FOXO1 has also been proposed to negatively regulate NK cell effector function, as its absence was correlated with augmented IFN-γ production in response to murine cytomegalovirus (MCMV) infections and anti-metastatic activity against the B16F10 mouse melanoma cell line (42). Augmented anti-metastatic activity against the same melanoma cell line also was reported in mice deficient for either BLIMP1 or AIOLOS, despite the lack of any significant impact on cytokine production (65, 67). On the other hand, MEF is required for normal cytokine

production and cytotoxicity, as it positively regulates IFN-γ and perforin expression, which corresponds to poorer cytotoxicity against tumor cell targets in MEF-deficient mice (71). Normal cytotoxicity but reduced IFN-γ production has been observed in IRF2-deficient mice and mice specifically lacking GATA3 in NK cells (43, 70). NK cell function is also regulated by TBET and EOMES, as TBET has been shown to bind to the regulatory regions of genes encoding granzyme B and perforin, while the expression of EOMES as NK cells mature is associated with increased transcription of mRNA (52, 60).

POSTTRANSCRIPTIONAL REGULATION OF NK CELL DEVELOPMENT BY microRNAs (miRs)

microRNAs are short non-coding RNAs (19–26 nt) that modulate gene expression at a posttranscriptional level. Recent studies have shown that miRs are also important for NK cell development and function. Using a Dicer1-deficient mouse model that abrogates miR biogenesis in NK cells, Degouve et al. (72) showed that a 10-fold global reduction in miR expression resulted in reduced NK cell numbers, aberrant NK cell maturation, along with attenuated IFN-γ production and cytotoxicity against target cells. Given that IL-15 signaling *via* the STAT5 and mTOR pathways was significantly affected, it was proposed that miRs regulate NK cell survival by modulating IL-15 sensitivity (72). Although it

remains unclear as to whether NK cell survival is dependent on specific miRs, miR-155 and miR-15/16 are unlikely candidates since mice that are deficient for either miR have normal NK cell numbers (73, 74).

Rather than NK cell survival, miR-155 and miR-15/16 appear to be essential for normal NK cell maturation, as NK cells lacking miR-15/16 are unable to fully mature into M2 NK cells (74), much like Dicer1-deficient mice, while miR-155-deficient NK cells undergo accelerated maturation (73). In contrast to the accumulation of M2 NK cells in miR-155 deficient mice, an accumulation of Imm NK cells was observed in mice that over-expressed miR-155, thereby providing additional evidence for the role of miR-155 in NK cell maturation (75). More importantly, the opposing effect that miR-15/16 and miR-155 have on NK cell maturation highlights the pleiotropic effects of miRs and suggests that there is still much to learn about the role of miRs in NK cell biology, particularly about redundancies between miRs. Nevertheless, it has been shown that miR-15/16 controls NK maturation by directly regulating levels of the transcription factor MYB, since the overexpression of miR-15/16 or MYB deficiency in miR-15/16-deficient NK cells rescues the maturation defect (74).

The mechanistic action of miR-155 can be appreciated in the context of NK cell proliferation and homeostasis, as NK cells deficient for miR-155 were unable to proliferate in response to MCMV infections and were also outcompeted by wild-type NK cells when cotransferred into homeostatic or lymphopenic environments (73). This dependency on miR-155 for proliferation under both homeostatic and infectious conditions appears to be mediated through the direct suppression of its target genes suppressor of cytokine signaling 1 (*Socs1*) and pro-apoptotic molecule phorbol-12-myristate-13-acetate-induced protein 1 (*PmaiP1*; encoding NOXA) (73). Interestingly, an accumulation of NK cells was observed in transgenic mice that over-expressed miR-155, lending further support for the regulatory role of miR-155 in NK cell proliferation (75).

EPIGENETIC REGULATION OF NK CELL DEVELOPMENT

Histone modifications have previously been shown to be essential events in B and T cell development (76, 77). Recent studies have demonstrated that defects in histone modification also impacts NK cell development with respect to lineage commitment (78) and maturation (79). Mice deficient for enhancer of zeste homolog 2 (EZH2), a H3K27 methyltransferase, were observed to have higher numbers NKP and NK cells. Microarray analysis revealed that the difference was associated with the upregulation of genes essential for NK cell development and function, thereby resulting in earlier lineage commitment and enhanced survival of NKPs (78). This increase in NK cell production was also observed when hematopoietic progenitors from human and wild-type mice were treated *in vitro* with EZH2 inhibitors (78).

A different type of histone modification, deubiquitination, also appears to be involved in the epigenetic regulation of NK

cell maturation. The histone deubiquitinase, MYSM1 (Myb-like, SWIRM, and MPN domains-containing protein 1), was found to regulate NK cell maturation as MYSM1-deficient mice possessed fewer NK cells that were mostly immature (79). Given that no defects were observed in the NKP compartment, MYSM1 was proposed to be uniquely required during NK cell maturation. Mechanistically, MYSM1 regulates NK cell maturation by binding directly to the *Id2* gene locus, as revealed by chromatin immunoprecipitation, thereby maintaining expression of the TF (79). However, the mechanism by which MYSM1 is selectively directed to the *Id2* gene locus remains unclear and thus requires further investigation.

CONCLUSION

The discovery of new members within the ILC family has rekindled efforts to better understand the development of NK cells, the founding member of the ILC family. Many of the recent breakthroughs made in the transcriptional regulation of NK cell development have been aided by key tools and techniques such as single cell RNA-seq, *in vitro* differentiation conditions, transcription factor reporter mice and conditional alleles and lineage specific Cre-expressing mouse strains. As these techniques and tools become commonplace in the field of NK cell biology, our understanding of the temporal–spatial transcriptional regulation of NK cell development and the key target genes that govern NK cell fate, homeostasis, and function becomes increasingly more complete. Recent studies have advanced our understanding of how individual TFs may be regulating NK cell commitment and NK cell lineage maintenance. However, how these various TFs form a transcriptional network and act in concert to ensure NK cell homeostasis remains unclear.

On a translational front, defining the extrinsic cues and TFs that regulate NK cell maturation, proliferation, cytokine responsiveness, and priming of effector functions represents an area of therapeutic interest. Proteins that negatively regulate NK cell maturation and fitness are tangible drug targets in cancer immunotherapy as recently evidenced by our group. Building on these potential targets will increase the likelihood of developing specific inhibitors for clinical translation.

AUTHOR CONTRIBUTIONS

WG wrote the review. NH conceived and edited the review.

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The Transcription Factor ZNF683/HOBIT Regulates Human NK-Cell Development

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We identified ZNF683/HOBIT as the most highly upregulated transcription factor gene during ex vivo differentiation of human CD34⁺ cord blood progenitor cells to CD56⁺ natural killer (NK) cells. ZNF683/HOBIT mRNA was preferentially expressed in NK cells compared to other human peripheral blood lymphocytes and monocytes. During ex vivo differentiation, ZNF683/HOBIT mRNA started to increase shortly after addition of IL-15 and further accumulated in parallel to the generation of CD56⁺ NK cells. shRNA-mediated knockdown of ZNF683/HOBIT resulted in a substantial reduction of CD56⁻CD14⁻ NK-cell progenitors and the following generation of CD56⁺ NK cells was largely abrogated. The few CD56⁺ NK cells, which escaped the developmental inhibition in the ZNF683/HOBIT knockdown cultures, displayed normal levels of NKG2A and KIR receptors. Functional analyses of these cells showed no differences in degranulation capacity from control cultures. However, the proportion of IFN-γ-producing cells appeared to be increased upon ZNF683/HOBIT knockdown. These results indicate a key role of ZNF683/HOBIT for the differentiation of the human NK-cell lineage and further suggest a potential negative control on IFN-γ production in more mature human NK cells.

Keywords: ZNF683/HOBIT, natural killer cells, CD56, ex vivo differentiation, NK-cell development

INTRODUCTION

Natural killer (NK) cells are the third largest group of lymphocytes in peripheral blood and an important component of the first line of immune defense. They act against a wide spectrum of virally infected and neoplastic cells by direct killing of these cells or production of cytokines, such as IFN-γ. As components of the innate part of the immune system, they display immediate reactivity and do not require prior sensitization (1, 2). This traditional characterization of NK cells has been expanded over the recent years as they have been described to be able to incorporate features previously thought to be restricted to the adaptive immune system, such as interaction with dendritic cells and immunological memory (3–5). In contrast to the adaptive T and B lymphocytes,

NK cells lack somatically recombined and clonally distributed antigen receptors, and their activity is controlled by a varied repertoire of germline-encoded inhibitory and activating receptors (6). Recently, additional tissue-resident subsets of innate lymphoid cells (ILCs), distinct from NK cells, became apparent and significantly expanded the complexity of innate lymphoid lineages. Whereas conventional NK cells resemble cytotoxic T lymphocytes in many aspects, ILCs 1–3 rather mirror T helper-like cells (7).

Based on currently available data, the relationship between the different innate and adaptive lymphoid lineages is reflected by their initial common differentiation from the hematopoietic stem cell (HSC) and in similarities of their transcriptional networks. According to the current hypothesis, the HSC develops through a multipotent progenitor to a common lymphoid progenitor (CLP) (8). This CLP can further differentiate into adaptive lymphocytes under the control of E-proteins, whereas the development of innate lymphocytes requires antagonism of E-proteins and likely proceeds through a common innate lymphoid progenitor (9, 10). The following pre-NK progenitor (NKP) stage restricts the differentiating cells to the NK lineage and develops into NKP and subsequently into immature NK (iNK) cells. The final step involves maturation from the iNK cells to mature NK (mNK) cells, both stages expressing the NK marker CD56 (11, 12).

Substantial data have been obtained to identify key transcription factors essential for the differentiation of adaptive and innate lymphocytes. A common theme appears to be the mutual inhibition of factors determining different lineages. For example, EBF strongly inhibits ID2 expression, thereby allowing E2A to function during B-cell development. In addition, EBF and PAX5 support B-cell differentiation by repressing additional critical regulators of T-cell and ILC differentiation, such as NOTCH1, GATA-3, and TCF-1 (8, 9). Conversely, all ILCs including NK cells are dependent for their differentiation on ID2 that heterodimerizes with E proteins and neutralizes their activity (10). Subsequently, a complex network of transcription factors guides the cells through the distinct steps of NK-cell differentiation (13). The most important transcription factors for the early stages of murine NK-cell development include STAT5, two ETS family members (PU.1 and ETS-1), and NFIL3 (also known as E4BP4) (14–17). The maturation stage from iNK to mNK cells and NK cells' function are coordinated by BLIMP-1, T-BET, EOMES, and MEF among others (18–20). Compared to the data obtained from the murine system, experimental evidence on transcription factors governing human NK-cell development is far less available. This has been partially caused by the lack of an easy and robust system to mimic human NK-cell differentiation from HSC *ex vivo*. Of the few transcription factors described so far, TOX1 and 2 are important in the early stages, T-BET and BLIMP-1 both play critical roles in the later phases and effector functions of human NK cells (21–24). From the currently available data, it appears that the precise function and sequential order of transcription factors directing NK-cell development may to some extent differ between mice and humans.

Due to their important role in immunosurveillance, NK cells and their modulation are currently being explored as a therapeutic approach in a wide variety of cancers, autoimmune diseases, allergies, and transplantation (1, 25). These attempts have led

to the development of methods for the specific modulation of endogenous NK cells as well as for *ex vivo* amplification of NK cells from patients or allogeneic donors for NK cell-infusion therapies. The obtained results show clear benefits of NK cell-based therapies, in comparison to T lymphocyte-based, including a good tolerance of allogeneic NK cells by the patients and the lack of a graft-versus-host disease (1, 25). Different strategies are exploited to obtain a sufficient number of NK cells for infusion therapies, including cytokine- and/or feeder cell-mediated expansion of peripheral blood NK cells as well as *ex vivo* differentiation from cord or peripheral blood-derived HSC (26, 27). In this regard, we have previously analyzed a feeder cell-free *ex vivo* system to generate large-scale therapeutic NK cells from cord blood stem cells that faithfully reproduces different steps of human NK-cell differentiation (28). This system is, therefore, ideally suited to investigate human NK-cell differentiation in addition to being a reliable method to generate NK cells for therapy (29, 30).

Here, we used this *ex vivo* differentiation system for a complete transcriptomic profiling of cells in different stages of NK-cell development. We identified the 20 most differentially expressed transcription factor genes and confirmed ZNF683/HOBIT mRNA as the highest upregulated transcription factor mRNA. The analysis of different human peripheral blood cell types showed preferential expression of ZNF683/HOBIT mRNA in NK cells. Then, we analyzed the role of ZNF683/HOBIT during NK-cell differentiation in more detail. In the *ex vivo* differentiation system, shRNA-mediated knockdown of ZNF683/HOBIT significantly reduced CD56[−]CD14[−] NKPs up to day 21 of culture and nearly abrogated the following generation of CD56⁺ NK cells. The few CD56⁺ cells that continued to mature displayed normal levels of NKG2A and KIR as well as degranulation capacities similar to control cells. However, the number of IFN-γ-producing cells significantly increased upon ZNF683/HOBIT knockdown. Taken together, these results support that ZNF683/HOBIT is a key regulator of early stages of human NK-cell differentiation and, in later stages, may function to repress IFN-γ production.

MATERIALS AND METHODS

Ex Vivo Differentiation of CD34⁺ Stem Cells into NK Cells

Human umbilical cord blood samples were obtained at birth after full-term delivery from the Department of Obstetrics and Gynecology of the University Hospital of Vienna, Austria. Cord blood mononuclear cells (CBMCs) were collected by Ficoll density gradient centrifugation (Lympholyte Cell Separation Media, human, Cedarlane®, Burlington, ON, Canada). Stem cells were isolated from CBMCs according to manufacturer's protocol, using a magnetic bead-based CD34⁺ isolation kit (CD34 MicroBead Kit, human; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the stem cells was evaluated by flow cytometry (CD34⁺ reached at least 95% purity) after which the cells were cultured as previously described (28, 30). In short, stem cells at an initial density of 10⁵ cells/ml were seeded into 6-well plates (Corning Incorporated, Corning, NY, USA) for 10 days in basal expansion medium

(GBGM©; Glycostem Therapeutics, Oss, The Netherlands) supplemented with stem cell factor (SCF), interleukin-7 (IL-7), thrombopoietin (TPO), and FMS-like tyrosine kinase 3 ligand (Flt3L); all factors at a concentration of 25 ng/ml (CellGro®, CellGenix GmbH, Freiburg, Germany) and granulocyte colony-stimulating factor (G-CSF, 250 pg/ml; Stemcell Technologies, Vancouver, BC, Canada), granulocyte-macrophage colony-stimulating factor (GM-CSF, 10 pg/ml; Stemcell Technologies), and IL-6 (50 pg/ml; CellGenix GmbH). At day 10, TPO was replaced with IL-15 (20 ng/ml; CellGro®, CellGenix GmbH), and at day 14, Flt3L with IL-2 (1,000 U/mL; Chiron Corporation, Emeryville, CA, USA) by refreshing half of the medium. After a total culture period of 35 days, a regular culture consisted of >95% CD56⁺CD3⁻ NK cells as evaluated by flow cytometry.

Microarray

Total RNA was extracted from cells at different time points of the *ex vivo* culture and transcribed into cDNA using the GeneChip® Whole Transcript Sense Target Labeling Kit (Affymetrix, High Wycombe, UK). The labeled cDNA was hybridized to GeneChip Human Gene 1.0ST Arrays, and the arrays were scanned and analyzed according to protocols of the manufacturer¹ as described in Ref. (31). Robust multiarray average signal extraction and normalization were performed, as detailed at the Bioconductor website² (32). The microarray data have been submitted to the GEO database under the accession number GSE95018.

RNA Sampling, cDNA Synthesis, and Real-time RT-PCR

Cultured cells (5×10^5) were lysed in Trizol (QIAzol Lysis Reagent, Qiagen Biosciences, MD, USA) and stored at -80°C . Total RNA was extracted following ThermoFisher's manual³, and 1 μg RNA was used for cDNA synthesis according to manufacturer's protocol (RevertAid H Minus First Strand cDNA Synthesis Kit, ThermoFisher Scientific, MA, USA). Two hundred nanograms of transcribed cDNA were analyzed by real-time PCR using the KAPA SYBR FAST UNIVERSAL kit (Kapa Biosystems, Inc., Wilmington, MA, USA) and primer sequences as shown in Table S3 in Supplementary Material. As internal controls, primers for either hypoxanthine-guanine phosphoribosyltransferase or β -actin were used. Samples were measured and analyzed with QIAGEN's real-time PCR cycler and corresponding software (Rotor-Gene Q, Qiagen, Hilden, Germany).

Western Blot

For protein-expression analysis, cell pellets (5×10^5 cells/sample) were resuspended in 2× Laemmli buffer, boiled for 10 min at 95°C and subsequently separated by SDS-polyacrylamide gel electrophoresis (10% Bis-Tris gels, Acrylamide:Bis 37.5:1) and transferred to a nitrocellulose membrane (Amersham Protran Supported 0.45 μm NC, GE Healthcare Europe GmbH, Eindhoven, The Netherlands) by semi-dry electrophoretic blotting in Towbin buffer with 20%

methanol (PerfectBlue, "Semi-Dry" Electroblotter Sedec, Peqlab, Southampton, UK). The membrane was blocked with 5% non-fat dry milk in TBS-T (0.2% Tween in TBS) followed by overnight incubation at 4°C with primary anti-ZNF683 antibodies (goat-anti-ZNF683 antibody C-12, 1:500, Santa Cruz Biotechnology, Heidelberg, Germany) or (mouse-anti-ZNF683 antibody, 1:500, Sigma-Aldrich, Saint Louis, MO, USA) and as control anti-GAPDH (mouse-anti-GAPDH, 1:10,000, Merck KGaA, Darmstadt, Germany). This was followed by 2 h incubation at room temperature (RT) with secondary antibodies, all 1:5,000 (donkey-anti-goat, Santa Cruz Biotechnology or goat-anti-mouse, Thermo Fisher Scientific) and corresponding washing steps with TBS-T.

Lentiviral Vectors

To generate a lentiviral construct for knockdown studies, a sense oligo, 5'-TGGAAACACATGGGCTATGACATTCAAGAGAA TGTCATAGCCCATGTGTTCTTTTC-3', corresponding with the sequence "GAAACACATGGGCTATGACAT" to position 1,402 to 1,412 from the start ATG of ZNF683/HOBIT cDNA (NCBI Reference Sequence: NM_001114759.2) and a corresponding antisense oligo, 5'-TCGAGAAAAAGAACACAT GGGCTATGACATTCTCTGAAATGTCATAGCCCATG TGTTCCA-3' were synthesized (Integrated DNA Technologies Inc., Coralville, IA, USA) as complementary overlapping oligos, with a *Xba*I overhang at the 5'end of the antisense oligo, as detailed.⁴ The complementary oligos were annealed and subsequently ligated into the LeGO-G/BSD lentiviral vector (LeGO-G/BSD was a gift from Boris Fehse, Addgene plasmid #27354) that had been digested with *Hpa*I and *Xba*I (New England Biolabs, Ipswich, MA, USA). The ligated product was transformed into the *E. coli* strain Stbl3 (New England Biolabs, Ipswich, MA, USA), recombinant colonies were selected, and plasmid-DNA isolated and verified by restriction digestion and Sanger sequencing. As a control, oligos containing a scrambled shRNA were synthesized and cloned into the LeGO-G/BSD vector using an identical strategy. The control shRNA sequence was obtained from the "Open Biosystems pGIPZ shRNAmir library" situated at University College London.⁵ To confirm the functioning of the shRNA, shRNA expression plasmids were co-transfected into HEK293 cells in the absence or presence of a commercially available ZNF683/HOBIT expression plasmid (ORF of ZNF683, transcript variant 1, in pEnter, with C-terminal Flag and His tag; Vigene Biosciences, Rockville, MD, USA) employing the CaPO₄ method using 4 μg of total DNA/6-well. Six hours after transfection medium was changed, and cells were harvested 96 h after transfection by either direct lysis in Trizol (Qiagen) for RNA isolation or 1× Laemmli sample buffer for protein-expression analysis.

Virus Production

Virus production and all experiments involving the generated recombinant viruses were performed in a separate room under biosafety level (BSL)-2 conditions following the instructions

¹Affymetrix support site; www.affymetrix.com/support/index.affx.

²www.bioconductor.org/.

³[https://www.thermofisher.com/order/catalog/product/15596026?ICID=cvc-rna-cultured-cells-c1t1](http://www.thermofisher.com/order/catalog/product/15596026?ICID=cvc-rna-cultured-cells-c1t1).

⁴<http://www.sciencegateway.org/protocols/lentivirus/cloning.htm>.

⁵[https://www.ucl.ac.uk/cancer/research/scientific-facilities-and-services/cancer-genomics-engineering-facility/rnai-library/open-biosystems-pgipz-rna-library](http://www.ucl.ac.uk/cancer/research/scientific-facilities-and-services/cancer-genomics-engineering-facility/rnai-library/open-biosystems-pgipz-rna-library).

given by the corresponding directives of the European Union (Council Directive 90/679/EEC) and the Austrian government (GTG-BGBI. Nr. 510/1994 and 114/2012). The specific work with the replication-defective human lentiviruses was registered at the Austrian Ministry of Science, Research and Economy (BMWFW-5.011/009-WF/V/3b/2015).

To produce lentiviruses, HEK293T cells (ATCC # CRL-11268) were seeded at 10^7 cells per 15 cm^2 tissue culture plate in 15 ml DMEM (DMEM/high glucose, GE Healthcare) supplemented with 10% FBS and glutamine (200 mM , Sigma-Aldrich). After 24 h, 5 ml DMEM was added, and the 60–70% dense cultures were transfected with the LeGO-G/BSD-ZNF683/HOBIT-shRNA expression plasmid plus three complementing plasmids providing the essential *Gag*, *Pol*, *Rev*, and *Tat* gene products missing in the replication-defective virus. To this end, the following 2 transfection solutions were prepared: (1) DMEM with 10% polyethyleneimine (PEI, Polysciences, Warrington, PA, USA) ($1.5\text{ ml}/15\text{ cm}^2$ plate) and (2) DMEM with $2.3\text{ }\mu\text{g}$ pCAG-KGP3R, $1.2\text{ }\mu\text{g}$ pCAG-RTR2, $2.5\text{ }\mu\text{g}$ pCAG-VSVg, and $24\text{ }\mu\text{g}$ vector of interest (in total $30\text{ }\mu\text{g}$ plasmids in $1.55\text{ ml}/15\text{ cm}^2$ plate). After 15 min incubation at RT, solution 2 was sterile filtered and added to solution 1. The combined solutions were added dropwise to HEK293T cells at a density of 70%. After 12 h, the transfection mix was replaced with fresh medium (12 ml DMEM/ 15 cm^2 plate). The supernatants were harvested after 48 and 72 h post-transfection. To remove cell debris, supernatants were centrifuged ($1,500\text{ rpm}$, 5 min, 4°C), filtered (Puradisc FP30mm cellulose acetate syringe filter, $0.45\text{ }\mu\text{m}$, sterile, GE Healthcare Life Sciences), and finally the virus particles were concentrated by ultracentrifugation for 90 min at 4°C at $25,000\text{ rpm}/76,000\times g$ in a SW-32 rotor equipped with 32 ml open-top thickwall polycarbonate tubes in a XL-70 ultracentrifuge (Beckman Coulter, Mystic, CT, USA). After ultracentrifugation, the supernatant was discarded leaving a $100\text{ }\mu\text{l}$ drop at the bottom of the tube. The virus particles were gently resuspended after keeping the tubes on ice for 1 h, aliquoted, and stored at -80°C until use.

Virus Transduction of CD34⁺ Stem Cells

After culturing freshly isolated cord blood CD34⁺ stem cells for 5 days in basal expansion medium supplemented with SCF, IL-7, TPO, and Flt3L as described under “*Ex vivo differentiation of CD34⁺ stem cells into NK cells*,” the expanded cells were transduced with lentiviral particles. To this end, 24-well plates (multi-well plate for suspension culture, Greiner Bio-one GmbH, Frickenhausen, Germany) were coated with $8\text{ }\mu\text{g}$ retinonectin/ $500\text{ }\mu\text{l}/\text{well}$ (recombinant human fibronectin fragment, Takara Bio Inc., Shiga, Japan) 24 h prior virus transduction and stored at 4°C until use. The retinonectin solution was removed, $250\text{ }\mu\text{l}$ GBGM containing virus particles (40 Transduction Units/cell) were added per well, and the plate was centrifuged at $4,000\text{ rpm}/1,900\times g$ for 2 h at 4°C . Immediately after centrifugation, the 5 days cultivated CD34⁺ stem cells were added (5×10^4 cells in $250\text{ }\mu\text{l}$ basal expansion medium/well). The following day, $500\text{ }\mu\text{l}$ of basal expansion medium was added. Two days after transduction the cells were centrifuged ($1,500\text{ rpm}$ for 5 min), suspended in fresh basal medium, and transferred to new uncoated 24-well cell culture plates (Corning® Costar®,

Sigma-Aldrich). Transduction efficiency, determined as GFP⁺ cells, was measured by flow cytometry 3 days after transduction, and cultures were continued as described in the first section (for *ex vivo* differentiation). Further cell expansion was calculated as follows. Cell numbers were obtained at the various differentiation stages and divided by the cell numbers for the corresponding GFP⁺ and GFP⁻ fractions measured 3 days after transduction, to correct for different transduction efficiencies.

Flow Cytometry

Cell surface expression of NK and monocytic markers was monitored using anti-CD56-PeCy7, anti-CD14-PerCP Cy5.5 (all BD Biosciences, San Jose, CA, USA), and anti-KIR-PE antibodies (R&D Systems, Vienna, Austria) on a FACS Canto II (BD Biosciences) and data were analyzed using both FACS DIVA software v6.0 (BD Biosciences) and Flowjo v10.0.8 (Tree Star, Yorba Linda, CA, USA). Details on antibodies are given in Table S4 in Supplementary Material.

Cytotoxicity and IFN- γ Assay

Target K562 and effector NK cells were cocultured at a 1:1 ratio (15×10^4 cells of each cell type) in $200\text{ }\mu\text{l}$ RPMI medium (RPMI 1640 medium, Life Technologies, Carlsbad, CA, USA) in an U-bottom 96-well plate (Greiner Cellstar® 96-well plates, Sigma-Aldrich) in the presence of anti-CD107a-APC (BD Biosciences). Brefeldin A and Monensin (BD GolgiPlug and GolgiStop, BD Biosciences) were added after 1 h of culture. After an additional 5 h of culture, cells were collected, stained for surface CD56 (anti-CD56-PeCy7, BD Biosciences), subsequently prepared for intracellular staining with IFN- γ (anti-IFN- γ -PE, BD Biosciences) using a fixation/permeabilization solution kit (BD Biosciences), and finally measured on a FACS Canto II.

Statistical Analysis

Statistical analysis was performed with Prism 6 software (GraphPad, San Diego, CA, USA) using Student’s *t*-test or a two-way ANOVA as indicated in Figure Legends. A *p*-value of 0.05 was considered as statistically significant.

RESULTS

ZNF683/HOBIT Is the Most Highly Upregulated Transcription Factor Gene During *Ex Vivo* Differentiation of Human NK Cells

Initially, we were interested to identify novel transcription factors potentially contributing to human NK-cell differentiation that have not been described in this function before. For this purpose, we employed a recently developed *ex vivo* differentiation system (29). In this system, cord blood CD34⁺ stem cells are initially expanded for 10 days, prior to addition of IL-15. Following further addition of IL-2 from day 14, the differentiating cells are cultured for a total period of 35–42 days, by which time a regular culture comprises over 95% NK cells. To determine the repertoire of transcription factors differentially expressed during NK-cell development, we first performed a transcriptomic profiling study

comparing samples from different time points up to day 35 with cultures at day 10 (start of the NK-cell differentiation). The 20 most highly upregulated transcription factor genes detected are shown in Table S1 in Supplementary Material. When real-time RT-PCR was performed for the corresponding transcripts, ZNF683/HOBIT mRNA was found to be by far the most highly upregulated mRNA at day 35 in relative terms (about 9,000-fold; **Figure 1A**). Among the most differentially expressed genes are many that have previously been reported to be important in NK-cell development and/or maturation such as *GATA3* (33), *TOX* (34), *ID2* (10), and *ETS1* (16) (**Figure 1A**). Based on its near absence in the stem cell cultures before addition of IL-15 and its high upregulation during differentiation, we decided to focus our further studies on ZNF683/HOBIT, especially as the role of this transcription factor in NK-cell development has not previously been reported.

Human Peripheral Blood NK Cells Preferentially Express ZNF683/HOBIT mRNA

To determine if *ZNF683/HOBIT* is also highly expressed in adult peripheral blood NK cells, we performed real-time RT-PCR on different peripheral blood leukocyte subsets of multiple donors. Indeed, compared to T and B lymphocytes as well as monocytes, *ZNF683/HOBIT* mRNA is predominantly expressed in NK cells (**Figure 1B**). CD19⁺ B lymphocytes display lower levels of *ZNF683/HOBIT* mRNA (about 7-fold less as compared to CD56^{dim} NK cells) and the levels in T lymphocytes and monocytes are even much lower or at detection limit. We were furthermore interested to compare the *ZNF683/HOBIT* mRNA levels between the more immature CD56^{bright} and the rather mature CD56^{dim} subpopulations of NK cells. The data show that CD56^{bright} cells express the highest levels of *ZNF683/HOBIT* mRNA, whereas CD56^{dim} cells display about 20% lower levels (**Figure 1B**).

ZNF683/HOBIT mRNA Is Upregulated in Parallel to the Generation of NK Cells

To define the potential role(s) of *ZNF683/HOBIT* throughout NK-cell development, we evaluated the kinetics of accumulation of *ZNF683/HOBIT* mRNA during *ex vivo* differentiation. During this process, three different cell types can be distinguished based upon the monocytic and NK-cell markers, CD14 and CD56, respectively (28). One of the subpopulations arising from the amplifying CD34⁺ stem cells consists of monocytic CD14⁺ cells that accumulate until day 14 after which they gradually disappear (**Figure 2A**). The second population consists of CD56⁻CD14⁻ cells, which peak at day 18. These CD56⁻CD14⁻ cells are presumed to comprise at least in part the NK-cell progenitors as indicated by their rapid decrease from day 18 onward, along with the concomitant rapid generation of CD56⁺ NK cells (**Figure 2A**). The CD56⁺ NK cells become the predominant population after day 25 and from day 35 onward the culture consists of over 95% CD56⁺ NK cells (**Figure 2A**).

We obtained samples at different time points during the *ex vivo* cultures and separated CD14⁻ and CD14⁺ cells by flow

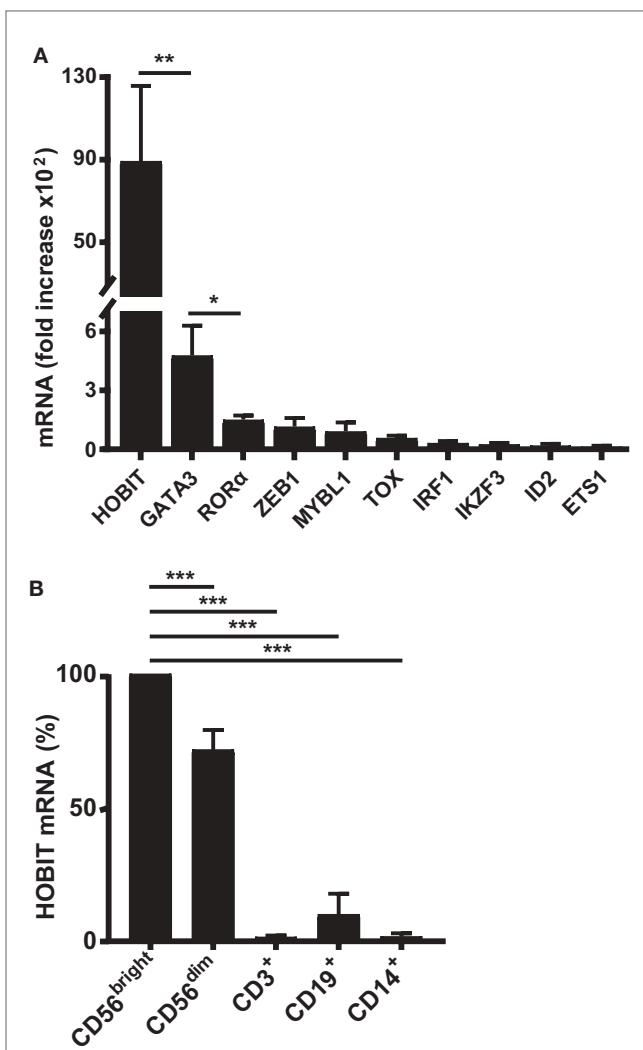


FIGURE 1 | High upregulation of *ZNF683/HOBIT* mRNA during *ex vivo* differentiation of human natural killer (NK) cells correlates with preferential expression in peripheral blood NK cells. **(A)** Ten most highly upregulated transcription factor mRNAs during *ex vivo* NK cell differentiation: CD34⁺ stem cells from cord blood were expanded and *ex vivo* differentiated into NK cells. Cells were sampled at day 10, just before the differentiation into NK cells was initiated by the addition of IL-15, and at day 35, after 25 days of differentiation. RNA was isolated and subjected to real-time RT-PCR analysis. β -actin was used as internal control. Fold upregulation of specific mRNAs at day 35 compared to day 10 is shown. Results were calculated from three series of experiments performed in triplicates with cells from different donors and are displayed as mean \pm SEM. **(B)** High expression of *ZNF683/HOBIT* mRNA in peripheral NK cells: mononuclear cells were isolated from human peripheral blood and one half of the cells used for isolation of NK cells by negative magnetic sorting. The NK cell fraction was further separated by flow cytometry into CD56^{bright} and CD56^{dim} NK cells. The second half of the mononuclear fraction was used to isolate CD3⁺ T lymphocytes, CD19⁺ B lymphocytes, and CD14⁺ monocytes by flow cytometry. RNA was isolated from the different cell samples and subjected to real-time RT-PCR analysis using β -actin as internal control. *ZNF683/HOBIT* mRNA levels within the different cell types are compared to the levels in the CD56^{bright} NK cells set to 100%. Results were obtained from three independent experiments using three different donors and are displayed as mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001).

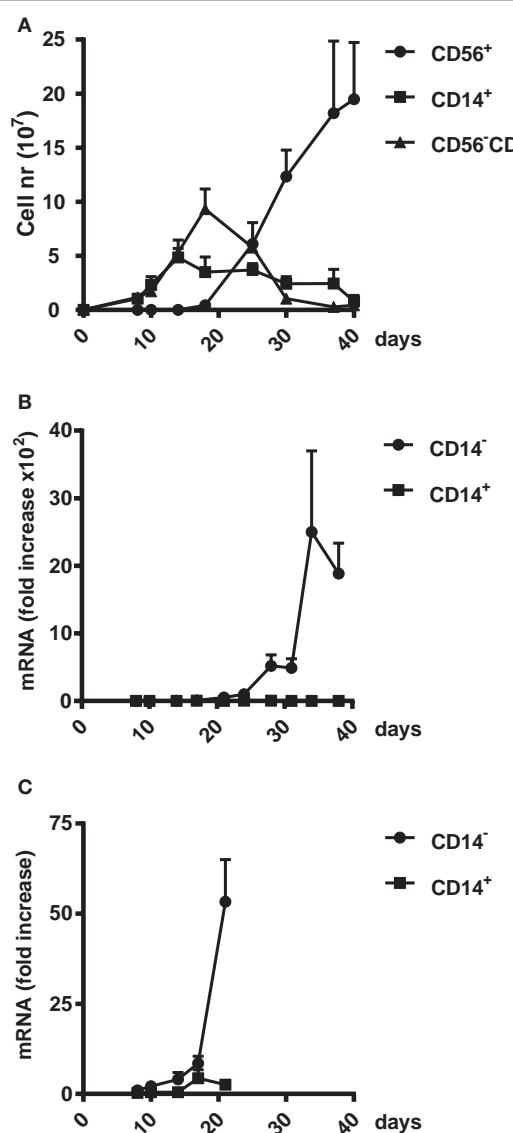


FIGURE 2 | Upregulation of ZNF683/HOBIT mRNA during natural killer (NK) cell differentiation. **(A)** Amplification of three different cell populations in the ex vivo NK-cell differentiation cultures. Cord blood CD34⁺ cells were differentiated into NK cells over a culture period of 40 days. In regular intervals, cells were analyzed by flow cytometry for expression of the monocytic marker CD14 and the NK cell marker CD56. The numbers of CD56⁻CD14⁻, CD56⁺CD14⁻, and CD56⁻CD14⁺ cells were plotted. Results are calculated from 10 independent experiments using cells of different donors and are displayed as mean \pm SEM. **(B,C)** Upregulation of ZNF683/HOBIT mRNA levels. Cell samples were taken at the indicated time points and CD14⁺ cells separated from the CD14⁻ population using magnetic sorting. RNA was isolated and subjected to real-time RT-PCR analysis with β -actin as internal control. Results are calculated from three independent series of experiments performed in triplicates using different donors. Fold upregulation in comparison to the values obtained for day 8 cells is shown as mean \pm SEM **(B)**. To display early ZNF683/HOBIT mRNA upregulation the period until day 21 is shown at a larger scale **(C)**.

cytometry. We detected that ZNF683/HOBIT mRNA is nearly absent after the initial expansion phase of 10 days. A tremendous accumulation of ZNF683/HOBIT mRNA is observed from day

24 onward in CD14⁻ cells comprising NKP and CD56⁺ NK cells (**Figure 2B**). The magnitude of ZNF683/HOBIT mRNA accumulation actually blurs the onset of expression in the display of **Figure 2B**. When plotted at a higher magnification, it is apparent that ZNF683/HOBIT mRNA increased in CD14⁻ cells already until day 17 followed by a first exponential accumulation up to day 21 (**Figure 2C**). Although we observed low expression of ZNF683/HOBIT mRNA in the CD14⁺ monocytes at day 17, the following first accumulation is absent (**Figure 2C**) as well as the exponential increase later during differentiation (**Figure 2B**).

ZNF683/HOBIT Downmodulation Substantially Reduces Expansion of NK Progenitors

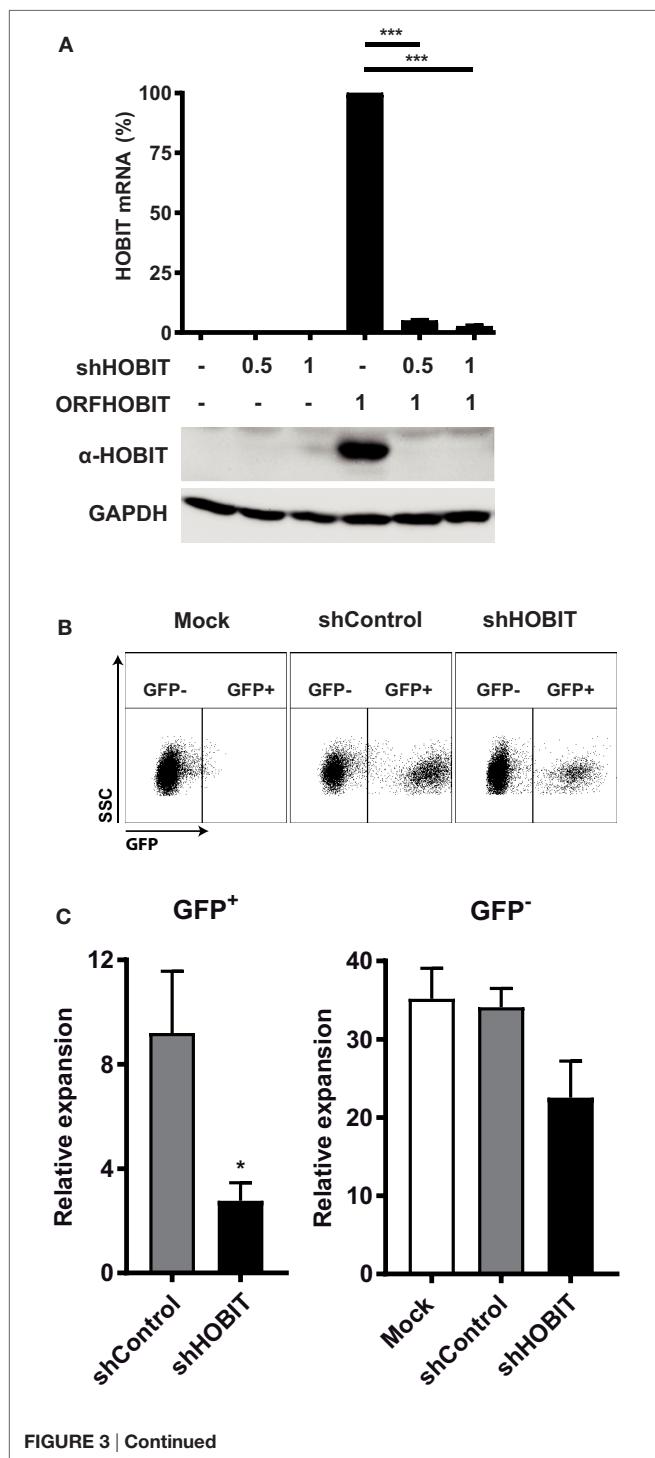
After observing that ZNF683/HOBIT mRNA is mainly present in NK cells and their progenitors, we set out to elucidate the potential role(s) of ZNF683/HOBIT during NK-cell development and maturation in more detail. For this purpose, we performed loss-of-function studies using a lentiviral-based shRNA-mediated knockdown of ZNF683/HOBIT. The appropriate functioning of the shRNA was confirmed by shRNA-mediated knockdown of ectopically expressed ZNF683/HOBIT in HEK293T cells. As shown in **Figure 3A**, expression of ZNF683/HOBIT mRNA and protein was strongly reduced (>90%) upon co-expression of the shRNA, thus confirming the proper functioning of the shRNA (**Figure 3A**).

We then continued to investigate the effects of ZNF683/HOBIT knockdown on *ex vivo* differentiation cultures. These cultures were transduced with lentiviruses expressing either ZNF683/HOBIT shRNA (shHOBIT) or a scrambled control shRNA (shControl). Cultures, mock-treated for the transduction procedure but without addition of viruses, were performed in parallel. Based on initial experiments that evaluated best transduction rates and survival in relation to days in culture, cells were transduced during the stem cell expansion phase 5 days after isolation. The presence of a GFP cassette driven by a spleen focus-forming virus (SFFV)-promoter enabled us to discriminate transduced GFP⁺ cells expressing the shRNA from non-transduced GFP⁻ cells within the same culture in parallel (35). Regularly, transduction rates between 30 and 50% were achieved. Since these varied to some extent, we normalized obtained expansion rates for the analyzed fractions to the different transduction rates measured 3 days after transduction (8 days after isolation).

Based on the fact that the expression of the NK-cell marker CD56 becomes significant only from day 24 onward, we decided to split the analysis in two parts. In the first part, we analyzed the NKP stage until day 21, in the second part the generation and maturation of CD56⁺ NK cells. At day 21, we observed a fourfold reduced expansion of the shHOBIT GFP⁺ cells compared to the shControl GFP⁺ cells (**Figure 3C**). Exemplary dot plots of the GFP staining are shown in **Figure 3B**. The GFP⁻ cells of the shHOBIT culture also seemed to be somewhat reduced in comparison to the scrambled control and regular mock cultures, but the difference did not reach statistical significance.

Since at day 21 the culture comprises mainly two different cell types, CD56⁻CD14⁻ progenitor cells and CD14⁺ monocytic

cells (**Figure 2A**), we continued to analyze these populations separately. Exemplary dot plots of this analysis are displayed in **Figure 4A**. The data showed that the effect can be mainly traced to a fourfold reduced expansion of the CD56⁻CD14⁻ progenitors in the shHOBIT GFP⁺ fraction. Although smaller inhibitory effects were also visible for CD14⁺ cells of the shHOBIT GFP⁺ pool and the GFP⁻ cells of the shHOBIT culture, both did not reach statistical significance (**Figure 4B**). However, this may

**FIGURE 3 | Continued****FIGURE 3 | Continued**

Effects of transduction with lentiviruses expressing HOBIT shRNA on total cell expansion. **(A)** HOBIT shRNA strongly reduces HOBIT expression. Lentiviral vectors expressing HOBIT shRNA were co-transfected with a HOBIT expression construct into HEK293T cells. After 96 h, cells were either used for RNA isolation or lysed in Laemmli sample buffer. The RNA was used for realtime RT-PCR analysis (upper part). Results are derived from two experiments performed in quadruplicates and shown as mean \pm SD. The proteins in the lysed samples were separated by SDS-PAGE, Western blotted, and probed with anti-HOBIT antibodies (lower part). As internal control hypoxanthine-guanine phosphoribosyltransferase was detected by respective antibodies. Two experiments with comparable results were performed. **(B,C)** HOBIT shRNA reduces expansion of cells at day 21. Cord blood CD34⁺ cells were cultured for 5 days, then cells were transduced with lentiviruses expressing either shHOBIT or a scrambled control shRNA (shControl) or were mock-treated. Transduction efficiency was measured 3 days later by flow cytometry scoring GFP-positive cells. Cells were further cultured and differentiated until day 21. Then flow cytometry was performed to evaluate expansion of transduced GFP-expressing cells. Exemplary dot plots for cells transduced with lentiviruses expressing shHOBIT or shControl or mock-transduced controls are shown in **(B)**. The numbers of the transduced GFP⁺ cells and the non-transduced GFP⁻ cells in individual cultures was calculated from the measured cell number and the respective percentages determined by flow cytometry and are shown in **(C)**. The values were normalized to the number of transduced or non-transduced cells measured at day 8 to establish the expansion rates. Results are calculated from four experiments performed in triplicates and are displayed as mean \pm SEM (*p < 0.05, ***p < 0.001).

indicate that a strong inhibitory effect on a fraction of the culture indirectly affects the whole culture, including the GFP⁻ part.

The number of CD56⁺ NK cells was too low to be analyzed in detail at this time point of the culture (**Figures 4A,B**). Together, the data clearly showed that knockdown of ZNF683/HOBIT reduces the expansion of presumptive NKP already at day 21 of the culture. This suggests a role of ZNF683/HOBIT in the early stages of human NK-cell development before the initiation of CD56 expression.

ZNF683/HOBIT Downmodulation Largely Abrogates the Generation of CD56⁺ NK Cells

The second part of our analysis focused on the formation of CD56⁺ cells and their maturation from day 21 onward. At first sight, when we analyzed the percentages of CD56⁺ NK cells formed during later stages at day 35, both GFP⁺ populations (shHOBIT and shControl) seemed comparable to regular cultures (>95% CD56⁺ NK cells, Figure S1 in Supplementary Material). However, when the number of cells was taken into account, it became apparent that the generation of CD56⁺ NK cells from the GFP⁺ progenitors in the shHOBIT pool is substantially reduced compared to the GFP⁺ control shRNA fraction. Whereas the GFP⁺CD56⁺ NK cells in the control shRNA cultures showed a strong expansion between days 21 and 35, the GFP⁺CD56⁺ NK cells in the shHOBIT cultures displayed almost no increase beyond day 21 and remained low in number (around 20% of the shControl cultures). These cells did not enter the typical expansion phase between days 24 and 35 (**Figures 5A,B**). In contrast, the GFP⁻ cells in the shHOBIT cultures displayed an expansion phase between days 21 and 30, although this expansion seemed reduced in comparison to the

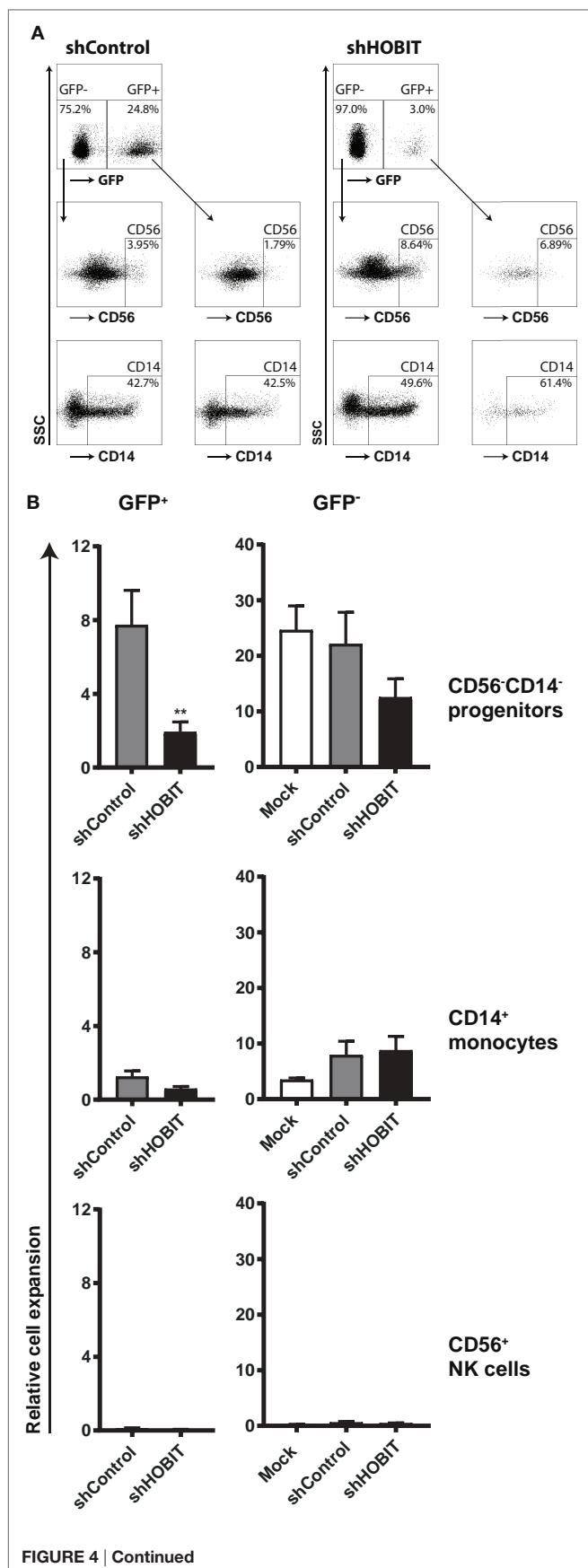


FIGURE 4 | Continued

FIGURE 4 | Continued

HOBIT shRNA strongly reduces the number of CD56⁺CD14⁻ progenitor cells at day 21.

Stem cells at day 5 of culture were transduced and further cultured until day 21. Then CD56 and CD14 expression was assessed by flow cytometry within the GFP⁺ and GFP⁻ parts of the culture.

(A) Exemplary flow cytometry dot plots displaying staining for CD56 and CD14 at day 21. **(B)** The relative expansion of CD56⁺CD14⁻, CD56⁺, and CD14⁺ cells within the GFP⁺ and GFP⁻ fractions is shown. Expansion rates were calculated from the measured cell numbers at day 21 and the percentages of the different subsets obtained by flow cytometry. Differences in transduction efficiency were corrected for by normalizing the obtained values to the number of GFP⁺ and GFP⁻ cells at day 8, respectively. Results are calculated from four independent experiments performed in triplicates using different donor cells and are displayed as mean \pm SEM (** $p < 0.01$).

shControl cultures. Again this may suggest an overall indirect effect of the strongly compromised GFP⁺ cells on the GFP⁻ part of the cultures.

Taken together, our data showed that knockdown of ZNF683/HOBIT strongly abrogates the generation of CD56⁺ NK cells. This suggests that ZNF683/HOBIT downmodulation strongly affects NK-cell progenitors shortly before the initiation of CD56 expression and prevents the progression toward CD56⁺ NK cells and/or the further proliferation of the few detected CD56⁺ NK cells.

The Few Developed CD56⁺ NK Cells Display Normal NKG2A and KIR Levels

We were further interested in the phenotype of the few detected CD56⁺ NK cells. To this end, we evaluated if these cells would further differentiate/mature *via* upregulation of NKG2A and KIR. The expression of these important NK-cell receptors was measured after 35 days of cultivation and compared between the GFP⁺ cells of the shHOBIT and the shControl cultures. We observed high levels of NKG2A in all of the GFP⁺ cells of the cultures independent of shHOBIT or control shRNA expression (Figure 6A, exemplary dot plots in Figure S2 in Supplementary Material). The GFP⁻ fractions also displayed high NKG2A levels similar to regular control cultures. Similarly, KIR expression was not significantly influenced by the presence or absence of shHOBIT or control shRNA (Figure 6A, exemplary dot plots in Figure S2 in Supplementary Material) and was comparable to the typical levels of regular cultures. In this regard, our group previously showed that *ex vivo* generated NK cells express lower levels of KIR than peripheral blood-NK cells (28). Together, these observations demonstrate that cells starting to express CD56 continue to develop normally in regard of NKG2A and KIR expression. Furthermore, it implies ZNF683/HOBIT does not influence the expression of NKG2A or KIR receptors on CD56⁺ NK cells.

Degranulation Capacities Remain Unchanged, but IFN- γ Production Is Strongly Induced Upon Virus Transduction and Further Enhanced by ZNF683/HOBIT Knockdown

Two main functions of NK cells are cytotoxicity, the direct killing of target cells, and IFN- γ production (1, 36). To evaluate

whether the CD56⁺ NK cells differentiating under knockdown conditions for ZNF683/HOBIT would functionally mature, we tested degranulation capacity in the CD107a assay and IFN- γ

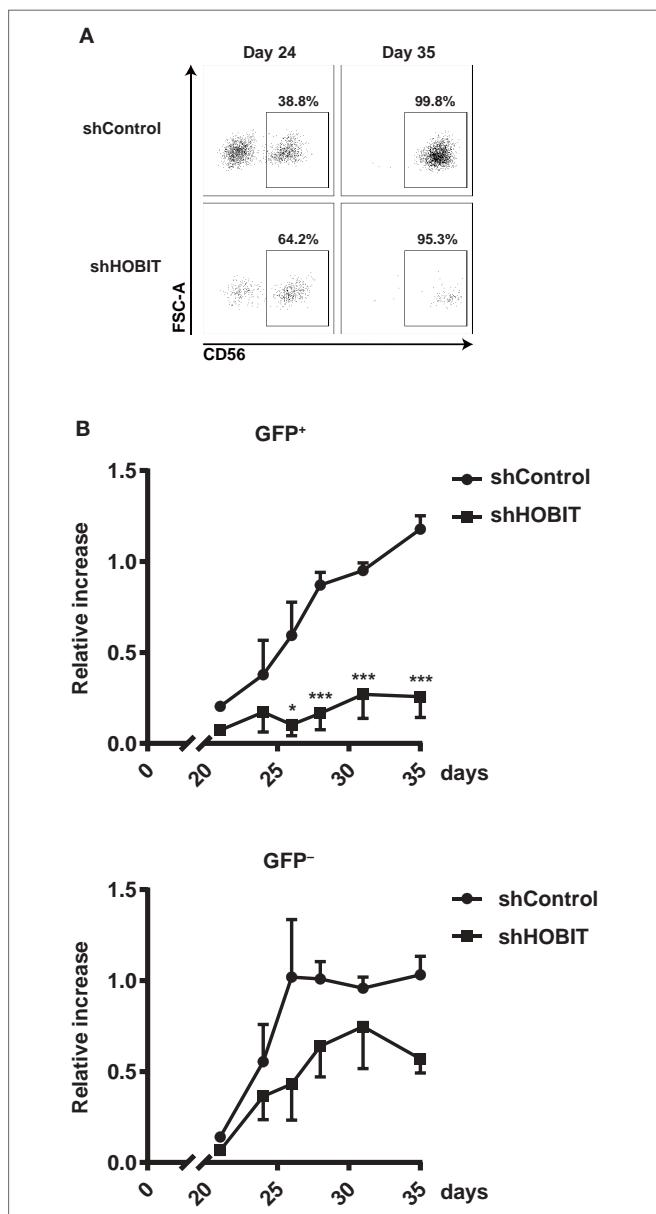


FIGURE 5 | HOBIT shRNA nearly abrogates formation of CD56⁺ natural killer (NK) cells. Stem cell cultures were transduced and cultured until day 35. The generation of CD56⁺ NK cells was monitored by flow cytometry in samples taken at days 24, 26, 28, 31 and 35. **(A)** Exemplary flow cytometry dot plots displaying staining for CD56 within the GFP⁺ fractions at days 24 and 35. **(B)** The formation of CD56⁺ cells within the transduced GFP⁺ and non-transduced GFP⁻ fractions is shown for shHOBIT and shControl cultures. Expansion rates are calculated from measured cell numbers at the respective days and the flow cytometry-derived percentages. Differences in transduction efficiency were corrected for by normalizing the obtained values to the number of GFP⁺ and GFP⁻ cells at day 8, respectively. Results are calculated from four independent experiments, two of them performed in triplicates, using different donor cells and are displayed as mean \pm SEM (* $p < 0.05$, *** $p < 0.001$).

production. Following coculture with the target cell-line K562, we detected similar levels of CD107a on the cell surface of all cells irrespective of the culture conditions (**Figure 6B**, exemplary dot plots in Figure S2 in Supplementary Material). In the same cultures, we also determined the proportion of cells with intracellular IFN- γ . Intriguingly, the transduction with the scrambled control shRNA already caused a substantial increase in IFN- γ producing cells (up to 50%). This was further enhanced upon expression of the ZNF683/HOBIT shRNA leading to 80% IFN- γ -producing cells (**Figure 6C**). In contrast, only a very small fraction of the GFP⁻ cells was competent to produce IFN- γ (**Figure 6C**). In summary, transduction with shRNA lentiviruses by itself leads to a higher proportion of NK cells with the capacity to produce IFN- γ and this is further increased by knockdown of ZNF683/HOBIT. The ZNF683/HOBIT shRNA effect is restricted to an increase in the number of IFN- γ -producing cells and not reflected in the level of IFN- γ per cell as measured by mean fluorescence intensity (MFI). However, virus transduced cells displayed higher levels of

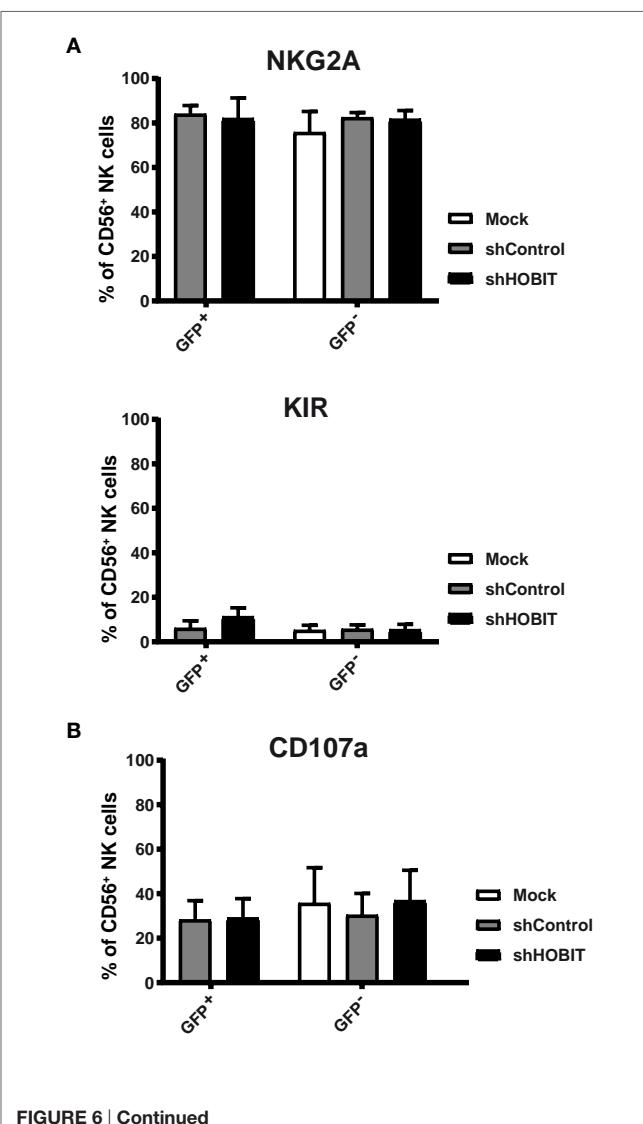


FIGURE 6 | Continued

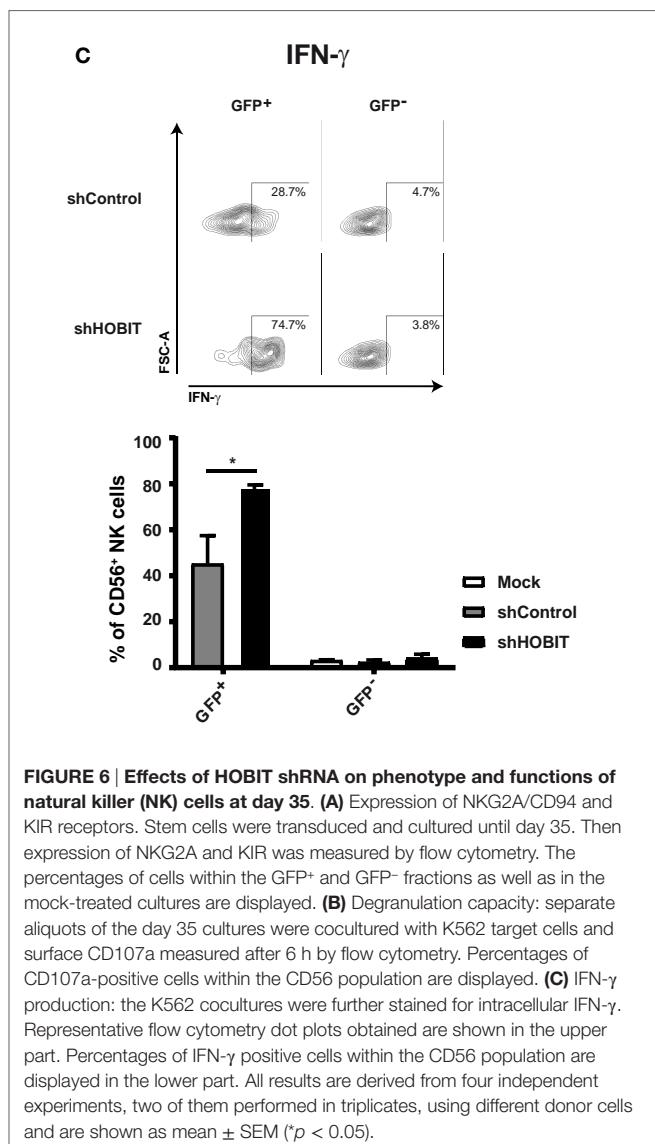


FIGURE 6 | Effects of HOBIT shRNA on phenotype and functions of natural killer (NK) cells at day 35. (A) Expression of NKG2A/CD94 and KIR receptors. Stem cells were transduced and cultured until day 35. Then expression of NKG2A and KIR was measured by flow cytometry. The percentages of cells within the GFP⁺ and GFP⁻ fractions as well as in the mock-treated cultures are displayed. **(B)** Degranulation capacity: separate aliquots of the day 35 cultures were cocultured with K562 target cells and surface CD107a measured after 6 h by flow cytometry. Percentages of CD107a-positive cells within the CD56 population are displayed. **(C)** IFN- γ production: the K562 cocultures were further stained for intracellular IFN- γ . Representative flow cytometry dot plots obtained are shown in the upper part. Percentages of IFN- γ positive cells within the CD56 population are displayed in the lower part. All results are derived from four independent experiments, two of them performed in triplicates, using different donor cells and are shown as mean \pm SEM (* $p < 0.05$).

IFN- γ per cell in comparison to non-transduced cells (Figure S3 in Supplementary Material).

DISCUSSION

The recent decade has witnessed an enormous increase in the understanding of the development of different immune-cell lineages including the various forms of innate and adaptive lymphocytes from HSCs (37). Most of this knowledge is derived from the murine system due to the possibility of genetic manipulation of the animal models. Therefore, detailed knowledge on the specificities of the equivalent human differentiation and maturation pathways and factors are still elusive. Although many factors may function in identical or similar ways in murine and human immune-cell development, certain differences are to be expected based on a much more rapid evolution of the immune system when compared to other tissues. For example, this has led to the convergent evolution of distinct classes of important

NK-cell receptors with similar function but encoded by structurally different genes such as the human KIR and murine Ly49 receptors (38, 39). It is conceivable that this is also reflected in the transcription factor circuitry defining human and murine NK cells.

Due to the recent increasing interest in NK cells as therapeutic agents, especially for the treatment of leukemia and potentially also solid cancers (40, 41), a more complete understanding of the control of human NK-cell differentiation and maturation is desirable. Here, we used a feeder cell-free *ex vivo* system for the generation of human NK cells from cord blood HSCs (29). This system can generate therapeutic NK cells that have been proven to be safe in a phase I clinical trial (42). We have, furthermore, demonstrated that the NK cells formed using this system display the typical NK cell receptors, potent ADCC and produce IFN- γ similar to peripheral blood NK cells (28). So this provided an ideal system to study transcription factors during human NK-cell differentiation and maturation.

Identity of cell types and their differentiation and maturation is controlled in large part by the action of transcription factors. Normally, important core transcription factors can be identified by the characteristics of upregulation during differentiation and their high expression in a relatively high cell type-specific fashion (43). In this regard, our transcriptomic profiling has identified a large number of transcription factors strongly upregulated during human NK-cell differentiation. Many of these have previously been described in the context of murine NK-cell development. For example, in earlier stages of NK-cell differentiation, ID2 and ID3 contribute via suppression of E proteins, such as E2A, the B-cell promoting factor. GATA3, TOX, and IKZF3 (AIOLOS) have been reported to promote later maturation stages of NK-cell development (20, 44). From these TOX has also been described specifically to contribute to human NK-cell differentiation, where it seems to control T-BET expression (21, 22).

Upon re-evaluation of the 10 most highly upregulated transcription factor genes from the profiling experiment, it was apparent that ZNF683/HOBIT mRNA was the most strongly upregulated. It displayed a 10-fold higher upregulation compared to GATA-3, the second best factor. Further analysis of different immune-cell types in human peripheral blood showed preferential expression of ZNF683/HOBIT mRNA in CD56⁺ NK cells when compared to B and T lymphocytes and monocytes. We detected low expression levels in B cells, but it was very low to undetectable in T lymphocytes and monocytes. Furthermore, ZNF683 is a homolog of the PRDM1 gene encoding BLIMP-1 (ZNF683 has, therefore, also been termed HOBIT, homolog of Blimp-1 in T cells) (45). In the mouse, Blimp-1 has been shown to be a master regulator of terminal differentiation of CD8⁺ effector T cells and plasma cells (46) and to play a role in the maturation of peripheral NK cells (18). Some upregulation of PRDM1 was detected in our profiling analysis (Table S1 in Supplementary Material), but BLIMP-1 mRNA showed a much less dramatic increase during human NK-cell differentiation than ZNF683/HOBIT mRNA (Table S2 in Supplementary Material). Considering its selective upregulation and expression and its homology with BLIMP-1, an established important differentiation factor, ZNF683/HOBIT appeared to fulfill the precondition

for a new key transcription factor controlling the development of human NK cells.

We further analysed ZNF683/HOBIT expression in the classical defined NK subsets, CD56^{bright} and CD56^{dim} cells. It is thought that the CD56^{bright} cells are more immature, preferentially produce cytokines, and will mature further into CD56^{dim} cells that display potent cytotoxic activity (1). In our analysis, evaluation of ZNF683/HOBIT mRNA revealed high expression levels in both subsets with somewhat higher levels in CD56^{bright} cells. Together with the kinetic analysis of ZNF683/HOBIT mRNA accumulation, that displayed a parallel increase with the generation of CD56⁺ NK cells, this initially suggested a preferential activity between acquisition of CD56 and the CD56^{bright} cell stage. However, a closer inspection revealed significantly increased ZNF683/HOBIT mRNA levels already in day 14-progenitors, with an exponential upregulation from day 18 onward. This suggests that ZNF683/HOBIT mRNA starts to be expressed soon after addition of IL-15 in NKP s and continuously increases with highest accumulation rates in parallel to the increase in CD56 expression. In comparison, only minor ZNF683/HOBIT mRNA levels appeared to be present in the CD14⁺ monocytic subset, which develops between days 10 and 20 and disappears thereafter.

In line with the early expression of ZNF683/HOBIT at the NKP stage, shRNA-mediated downmodulation of ZNF683/HOBIT resulted in a significantly reduced expansion of transduced cells already at day 21 of the culture. The majority of this effect could be traced to the CD56⁻CD14⁻ cells containing at least in part the NKPs. CD56⁺-expressing NK cells were not yet detectable at significant amounts at this time point in the culture. This clearly supports that a first major effect of ZNF683/HOBIT downmodulation is a reduction of proliferation and/or survival of NK-cell progenitors prior to the initiation of CD56 expression. Some reduction seemed also to occur for the CD14⁺ cells in shHOBIT transduced cultures, although this did not reach significance. As very low levels of ZNF683/HOBIT mRNA appear to be expressed in CD14⁺ cells, we are unable to rule out a potential specific negative effect also upon this subset following HOBIT knockdown.

The second major effect became apparent when we monitored the acquisition of CD56 expression. CD56 or N-CAM is generally accepted as a major marker for human NK cells, despite that its function remains elusive (47). It is present on about 95% of human NK cells (47), but lacks a clear homolog in murine NK cells (48). The kinetics of accumulation of CD56⁻CD14⁻ progenitors and CD56⁺ cells in the *ex vivo* system are compatible with the start of expression of CD56 by CD56⁻CD14⁻ progenitors from day 20 onward. This is indicated by the rapid decline of CD56⁻CD14⁻ progenitors and the concomitant appearance of the CD56⁺ cells. This generation of CD56⁺ cells was nearly abrogated upon down-modulation of ZNF683/HOBIT. This supports that ZNF683/HOBIT is essential for efficient generation of CD56 expressing cells and/or their further proliferation or survival.

Important receptors in human NK cells are the NKG2A and KIR receptors (11, 49). In the *ex vivo* cultures, the expression of NKG2A starts shortly after CD56 expression (28). NKG2A

is further expressed on the majority of the more immature CD56^{bright} cells. According to the current hypothesis, when these cells mature into CD56^{dim} cells, they will reduce NKG2A and increase KIR expression (11). Despite the substantially reduced number of CD56⁺ cells formed in the shHOBIT cultures, it was astonishing that these cells appeared quite normal in respect of NKG2A expression compared to control cultures. The observed levels of NKG2A expression on about 80% of CD56⁺ cells were comparable to the levels characteristic for regular cultures (28). Also KIR levels established with a pan-KIR antibody were similar to regular cultures, in the order of 5% of CD56⁺ cells. As previously discussed (28), in the *ex vivo* cultures NKG2A levels are similarly high as in peripheral CD56^{bright} cells, probably due to the high cytokine levels in the culture. KIR levels are intermediary between CD56^{bright} cells and CD56^{dim} cells. This suggests that *ex vivo*-generated NK cells, in terms of receptor expression, do not fully mature, whereas functionally they display full cytotoxic capabilities. In this regard, we observed no differences in degranulation capabilities against K562 targets between the shHOBIT and control cultures.

In contrast, it was remarkable that virus transduction by itself caused an increase in the proportion of IFN- γ -producing CD56⁺ NK cells. About 50% of day 35 cells transduced with control shRNA viruses produced IFN- γ upon coculture with K562 target cells, whereas only a few percent for non-transduced cells. Currently, it is unclear whether this is due to a priming process at the time of transduction at day 5 that continues to act into later stages of differentiation or whether it is high expression of shRNAs that can activate the differentiated cells. We can only speculate about the underlying mechanisms for this unexpected finding. For example, it has been described that a uridine-rich part of the HIV RNA (ssRNA40) can activate NK cells via TLR7/8 signaling (50). So, maybe the presence of high levels of shRNA or RNA transcribed from the viral sequences could somehow activate the IFN- γ machinery. Irrespective of the cause of this effect, the increase in IFN- γ -producing cells initiated by the virus itself was further enhanced by knockdown of ZNF683/HOBIT. This suggests that ZNF683/HOBIT negatively controls the development of IFN- γ -producing NK cells.

We have to introduce a caveat on the interpretation of the data for the late stages of differentiation in the *ex vivo* system. Due to the low numbers of GFP⁺ cells obtained, that further appeared to be too fragile for preparative sorting, we were unable to exclude that the few late stage GFP⁺ NK cells generated are derivatives of few cells that failed to express the shRNA and, therefore, continued to differentiate. It is, however, equally conceivable that ZNF683/HOBIT knockdown is effective on proliferation and/or survival only within a certain time window, when the cells normally still proliferate, and is less effective once the cells have passed this stage and do not proliferate but rather further differentiate and mature.

While this work was in progress, a report on ZNF683/HOBIT was published describing that the factor is highly expressed in human effector-type CD8⁺ T cells, but not in naive or most memory CD8⁺ T cells or CD4⁺ helper T cells (51, 52). High levels of ZNF683/HOBIT were expressed in CMV-specific, but not in

influenza-specific CD8⁺ T cells. This may explain why in our experiments we did not detect significant expression levels in peripheral T cells as we only tested the overall population of CD3⁺ T cells comprising all CD4⁺ and CD8⁺ T cells. This report also showed highest expression in human NK cells and low expression in dendritic cells in accordance with our data on peripheral NK cells and monocytes.

Currently available evidence suggests that ZNF683/HOBIT may be special in terms of displaying substantial differences in expression pattern and possibly function in different cell types in mice and humans. In mice, it was reported to be predominantly expressed in NKT cells, a CD4⁺ T cell subset with immediate effector functions, and to some extent in CD8⁺ T cells (45, 53). In humans, ZNF683/HOBIT is mainly expressed in NK cells and, as described by others, also in effector-type CD8⁺ T cells (51). Differences also seem to occur regarding the control of IFN- γ production. In murine NKT cells, ZNF683/HOBIT represses IFN- γ and activates granzyme B production (45). In contrast, in human long-lived effector T cells, ZNF683/HOBIT was reported to induce IFN- γ and to have no effect on granzyme B production (51). Our findings in human NK cells rather support a suppressing effect of ZNF683/HOBIT on IFN- γ production. This correlates with a report on BLIMP-1 describing a similar suppression of IFN- γ production in human NK cells (51). No effect of BLIMP-1 on cytotoxicity was observed, which is also in agreement with our findings on ZNF683/HOBIT. Generally, the homology of ZNF683/HOBIT and BLIMP-1 suggests overlapping and synergistic activities. Both factors display highly conserved zinc finger domains mediating binding to DNA target sequences in enhancer regions of a number of genes. The DNA binding sites of the factors largely overlap and both can bind to target sequences in several identical genes including *TCF7*. Both factors seem to mainly act as transcriptional repressors (44, 54), although for BLIMP-1 activating properties have also been described (55).

Taken together, our data for the first time show a role of ZNF683/HOBIT during differentiation of human NK cells. They strongly support that ZNF683/HOBIT is a key regulatory factor controlling generation, proliferation, and/or survival of NK-cell progenitors (CD56⁻CD14⁻) and is essential for efficient generation of CD56⁺ cells. Once CD56 expression has been acquired the further maturation including NK receptor expression and development of degranulation capacity seems to be unaffected, whereas IFN- γ production appears to be constrained by ZNF683/HOBIT. Although a more precise identification of the developmental stage(s) affected will need additional investigations, the data are compatible with the possibility that ZNF683/HOBIT may mainly act between early factors, such as NFIL3 (56) with proposed activities at the early committed NKP stage and factors described to promote rather maturation of NK cells at later stages such as GATA-3 (33). It further remains to be established how this is achieved in potential interaction with BLIMP-1 described so far as important mainly for late stage maturation.

ETHICS STATEMENT

Human umbilical cord blood samples were obtained at birth after full-term delivery from the Department of Obstetrics and Gynecology of the University Hospital of Vienna, Austria. This was carried out in accordance with the recommendations of the “Ethical committee of the Medical University of Vienna” with written informed consent from all subjects in accordance with the Declaration of Helsinki. The protocol was approved by the “Ethical committee of the Medical University of Vienna” (protocol number 122/2010).

AUTHOR CONTRIBUTIONS

MP performed experiments and wrote the basic manuscript. AC aided in experimental design, performed experiments, and improved the manuscript. DL provided the basis of the study with initial experiments, including the transcriptional profiling, and MO contributed with part of real-time RT-PCR analyses. UR helped with Western blots and DD with generating viral constructs. BS, WE, AN, JS, and DS contributed in the design of the study, provided cell samples, viral vectors and technology, and corrected the manuscript. EC contributed to the design, supported the progress of the project, and helped with formulating the final manuscript. EH conceived the study, supported MP and AC in all aspects of the experiments, and helped to finish the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00535/full#supplementary-material>.

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Development of Three Different NK Cell Subpopulations during Immune Reconstitution after Pediatric Allogeneic Hematopoietic Stem Cell Transplantation: Prognostic Markers in GvHD and Viral Infections

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Natural killer (NK) cells play an important role following allogeneic hematopoietic stem cell transplantation (HSCT) exerting graft-versus-leukemia/tumor effect and mediating pathogen-specific immunity. Although NK cells are the first donor-derived lymphocytes reconstituting post-HSCT, their distribution of CD56⁺⁺CD16⁻ (CD56^{bright}), CD56⁺⁺CD16⁺ (CD56^{intermediate=Int}), and CD56⁺CD16⁺⁺ (CD56^{dim}) NK cells is explicitly divergent from healthy adults, but to some extent comparable to the NK cell development in early childhood. The proportion of CD56^{bright}/CD56^{int}/CD56^{dim} changed from 15/8/78% in early childhood to 6/4/90% in adults, respectively. Within this study, we first compared the NK cell reconstitution post-HSCT to reference values of NK cell subpopulations of healthy children. Afterward, we investigated the reconstitution of NK cell subpopulations post-HSCT in correlation to acute graft versus host disease (aGvHD) and chronic graft versus host disease (cGvHD) as well as to viral infections. Interestingly, after a HSCT follow-up phase of 12 months, the distribution of NK cell subpopulations largely matched the 50th percentile of the reference range for healthy individuals. Patients suffering from aGvHD and cGvHD showed a delayed reconstitution of NK cells. Remarkably, within the first 2 months post-HSCT, patients suffering from aGvHD had significantly lower levels of CD56^{bright} NK cells compared to patients without viral infection or without graft versus host disease (GvHD). Therefore, the amount of CD56^{bright} NK cells might serve as an early prognostic factor for GvHD development. Furthermore, a prolonged and elevated peak in CD56^{int} NK cells seemed to be characteristic for the chronification of

GvHD. In context of viral infection, a slightly lower CD56 and CD16 receptor expression followed by a considerable reduction in the absolute CD56^{dim} NK cell numbers combined with reoccurrence of CD56^{int} NK cells was observed. Our results suggest that a precise analysis of the reconstitution of NK cell subpopulations post-HSCT might indicate the occurrence of undesired events post-HSCT such as severe aGvHD.

Keywords: NK cells, immune reconstitution, CD56, CD16, allogeneic transplantation, children, reference values

INTRODUCTION

The reconstitution of natural killer (NK) cells following allogeneic hematopoietic stem cell transplantation (HSCT) plays an important role in the response against residual malignant cells and the control of viral infections (1, 2). Independent of the graft source, NK cells typically regenerate within the first month following HSCT (3). However, there is an overrepresentation of CD56^{bright}CD16^{neg} NK cells in the early phase post-HSCT compared to healthy individuals (4, 5), where NK cells are composed of about 90% CD56^{dim}CD16⁺⁺ and 10% CD56^{bright}CD16⁻ cells. Whereas CD56^{dim}CD16⁺⁺ NK cells mediate cytotoxicity and antibody-dependent cellular cytotoxicity, the CD56^{bright}CD16⁻ subpopulation, which is mainly present in the early period post-HSCT, primarily secretes immunoregulatory cytokines. Presumably, the development from CD56^{bright} to CD56^{dim} NK cells corresponds to sequential steps of NK cell differentiation (6). In most patients, the ratio between CD56^{bright} and CD56^{dim} NK cells normalizes within the first 6 months post-HSCT influenced by the patient's age and events following HSCT. A correlation between the reconstitution of NK cells and overall survival was described by few studies emphasizing their essential role in the defense of infections when T cell immunity is mainly absent (7, 8). Furthermore, NK cells were successfully applied as immunotherapy for patients with high-risk malignancies suffering from impending relapse following HSCT. In clinical studies, adoptively infused NK cells induced graft-versus-leukemia/tumor effect without concomitant severe graft versus host disease (GvHD) (9–11). In addition, recent studies ascribe GvHD reduction to NK cell function post-HSCT (12). In this work, we focused on the regeneration of CD56^{bright} and CD56^{dim} NK cells with a special regard on the population shifting between these subpopulations. We divided NK cells in three NK cell subpopulations. In the first step, we established reference values of CD56⁺⁺CD16⁻ (CD56^{bright}), CD56⁺⁺CD16⁺ (CD56^{int}), and CD56⁺CD16⁺⁺ (CD56^{dim}) NK cells of healthy children and adolescents ($n = 174$). These reference values were matched and compared to the NK cell reconstitution of patients, who did not suffer from any viral infection or GvHD and are still alive after allogenic stem cell transplantation. In the next step, we investigated the associations between the reconstitution of the three different NK cell subpopulations in regard to the occurrence of events such as acute graft versus host disease (aGvHD) or chronic graft versus host disease (cGvHD) and severe viral infections in the first year post-HSCT in contrast to a cohort of patients without severe events assumed as control group.

MATERIALS AND METHODS

Reference Cohort of Children and Adolescents

In this cross-sectional monocentric study (approval ethic committee Ref. No. 139/09), 174 donors (64 females and 110 males) were included. Residual peripheral blood samples of hematologically healthy children aged 1 month to 18 years were analyzed (patients were hospitalized, e.g., for cleft lip and palate correction). Inclusion criteria involved no incidence of immunodeficiency or infection (defined as >2 severe infections/year, >8 infections/year, persistent fungal infections, post-vaccinal complications, no evidence of acute bleeding, negative for CRP and normal leukocytes, lymphocytes, and neutrophil granulocytes).

Patients and Grafts

The reconstitution of NK cells and their subpopulation CD56^{bright}, CD56^{int}, and CD56^{dim} was analyzed in $n = 74$ patients ($n = 25$ females and $n = 49$ males) transplanted from 2010 to 2016 (see Table 1). Indications for HSCT were high-risk acute lymphoblastic leukemia ($n = 51$), acute myeloid leukemia ($n = 12$), and myelodysplastic syndrome ($n = 11$). Median age at HSCT was 10.4 years (range: 1.3–24.4 years). Grafts were received from matched family donors (MSD; $n = 20$), matched unrelated donors (MUD) ($n = 39$), and haploidentical mismatched family donors with <8/10 HLA matches (MMFD; $n = 15$). No significant differences in the occurrence of GvHD were available in the different donor groups (MSD: 6/20, MUD: 10/39, and MMFD: 3/15). A second or third transplant was administered to $n = 9$ and $n = 2$ patients, respectively, because of relapse after first or second HSCT. Stem cell sources consisted of (bone marrow; $n = 48$), unmanipulated PBSC ($n = 11$), and T cell-depleted PBSC ($n = 15$). Post-transplant aGvHD occurred in $n = 31$ patients with grades I ($n = 9$), II ($n = 3$), III ($n = 16$), and IV ($n = 3$), cGvHD in 22 patients. Severe viral infections occurred in 18 patients including primary infection or reactivation with cytomegalovirus (CMV) ($n = 8$), adenovirus (ADV) ($n = 5$), and Epstein–Barr virus (EBV) ($n = 5$).

Study Design

This study was carried out in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Frankfurt University Hospital (Ref. No. #198/16). Peripheral blood was collected within the framework of a post-HSCT routine sampling for clinical follow-up from 2010 to 2016. In engrafted patients, samples were collected starting at day 15, within the first

TABLE 1 | Patient's characteristics.

Groups	All	aGvHD	cGvHD	Viral infection	Control group
Patients	74	19	22 (8)	18	23
Diagnosis					
ALL	51	13	12 (4)	13	17
AML	12	3	3 (2)	5	3
MDS	11	3	7 (2)	0	3
Age at HSCT					
Median (range)	10.4 (1.3–24.4)	8.9 (1.9–17.6)	9.4 (1.3–18.2)	10.3 (3.4–17.7)	12.1 (2.3–24.4)
Sex					
Male	49	11	15 (6)	13	16
Female	25	8	7 (2)	5	7
Donor type					
MSD	20	6	11 (4)	1	6
MUD	39	10	9 (2)	9	13
MMFD	15	3	2 (2)	8	4
Stem cell source					
BM	48	12	17	7	16
Unmanipulated PBSC	11	4	3	3	3
T cell depleted	15	3	2	8	4
GvHD					
aGVHD	31	19	20	0	0
Grade I/II/III/IV	9/3/16/3	0/0/16/3	9/3/7/1	0/0/0/0	0/0/0/0
cGvHD	22	8	22	0	0
Viral infections					
ADV/CMV/EBV	5/8/5	0/0/0	0/0/0	5/8/5	0/0/0
Survival (%)	87	74	96	78	100
Follow-up (months)					
Median (range)	35 (3–105)	33 (3–77)	43 (6–92)	28 (2–103)	48 (24–105)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; HSCT, hematopoietic stem cell transplantation; MSD, matched sibling donor; MUD, matched unrelated donor; MMFD, mismatched family donor; BM, bone marrow; PBSC, peripheral blood stem cells; GvHD, graft versus host disease; aGvHD, acute graft versus host disease; cGvHD, chronic graft versus host disease; ADV, adenovirus; CMV, cytomegalovirus; EBV, Epstein–Barr virus.

year monthly, within the second year three monthly, and afterward every 6 months until 36 months post-HSCT, respectively. In total, $n = 925$ measurements of $n = 74$ patients were included in this analysis.

Depending on the occurrence of unexpected events such as aGvHD (only grades III and IV, grades I and II excluded), cGvHD, and severe viral infections, the study group was partitioned. The patient group without aGvHD/cGvHD or any viral infections was chosen for comparison as control group.

Patients showing a distinct CD56^{int} population in a sufficient absolute amount post-HSCT were elected for more detailed analysis applying a 10-color flow cytometry.

Assessment of NK Cells and Their Subpopulations

Natural killer cell subpopulations were analyzed on a FC500 flow-cytometer (Beckman Coulter, Krefeld, Germany)

applying a five-color panel to estimate CD3⁺ T cells, CD19⁺ B cell, and CD3⁻CD56⁺ NK cells, including the differentiation into CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells, respectively. Absolute cell numbers were estimated from peripheral blood samples in a dual platform lyse-no-wash procedure as described previously (13). In brief, a tube of 100 μ l EDTA-peripheral blood was labeled with CD45/CD56/CD19/CD3 tetraCHROME (clones B3821F4A/N901/J3-119/UCHT1) multi-color mAb conjugated with FITC, phycoerythrin (PE), phycoerythrin-texas red (ECD), and phycoerythrin-cyanine 5 (PC5). CD16 phycoerythrin-cyanine 7 (PC7, clone: 6607118) was additionally added. For the measurement of T cells including T helper and cytotoxic T cells, we applied the tetraCHROME multi-color reagent CD45/CD4/CD8/CD3 (clones B3821F4A/SFCI12T4D11/SFCI21Thy2D3/UCHT1) conjugated with FITC, PE, ECD, and PC5. All reagents were acquired from Beckman Coulter Immutech (Marseille, France).

Differences in the expression profiles of CD56^{bright}, CD56^{int}, and CD56^{dim} cells were analyzed applying two panels on a Navios™ 10-color flow cytometer (Beckman Coulter, Krefeld, Germany). Panel 1: CD226 = DNAM-1 (FITC; clone: KRA236), NKG2A (PE; clone: Z199), DUMP = CD3&CD14&CD19 (ECD; clones: UCHT1, RMO52, J3-119), CD117 (PC5.5; clone: 104D2D1), CD27 (PC7; clone: 1A4CD27), CD56 [allophycocyanin (APC); clone: N901], CD127 (APC-A700; clone: R34.34), CD16 (APC-A750; clone: 3G8), CD57 [Pacific Blue (PB); clone: NC1], CD45 [Krome Orange (KrO); clone: J.33]. Panel 2: CCR5 (FITC; clone: 2D7), killer cell immunoglobulin-like receptor (KIR) mix (CD158 + CD158b + CD158e1; clones: EB6B, GL183, Z27.3.7), DUMP = CD3 + CD14 + CD19 (ECD; clones: UCHT1, RMO52, J3-119), CD117 (PC5.5; clone: 104D2D1), CX3CR1 (PC7; clone: 2A9-1), CD56 (APC; clone: N901), CD62L (APC-A700; clone: DREG56), CD16 (APC-A750; clone: 3G8), CCR7 (PB; clone: G043H7), and CD45 (KrO; clone: J.33). All antibodies were purchased from Beckman Coulter Immutech except CCR5 (BD Biosciences, Heidelberg, Germany) and CX3CR1 (Biolegend, San Diego, CA, USA). Staining was performed, using 100 μ l of peripheral blood for each tube followed by 15 min of incubation at room temperature and erythrocyte lysis applying NH₄Cl reagent (Beckman Coulter, Marseille, France).

Stained Cyto-Comp™ cells were applied to compensate the fluorescence overlap. The flow cytometer fluidic stability and the optical alignment were daily tested using Flow-Check™ Fluorospheres (Beckman Coulter, Krefeld, Germany). For verification, Immunotrol cells (Beckman Coulter) were applied three times a day. Furthermore, we participate in an external quality assessment for the detection of T-, B-, and NK cells (INSTANT e.V.—provider for German round robin test, No 213).

Data evaluation was performed using Kaluza and CXP-software (Beckman Coulter).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The NK subpopulation reference values of healthy children and adolescents were calculated with non-linear exponential regression analysis

(equation: one-phase decay, least square fit; function: $Y = (Y_0 - \text{Plateau}) \times \exp(-K \times X) + \text{Plateau}$). Significant differences between groups were assessed by a non-paired two-tailed Mann-Whitney U test. p -Values <0.05 were regarded as significant and are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

RESULTS

Normalization of CD56^{bright} to CD56^{dim} Ratio Post-HSCT within the First Year Post-HSCT

The distribution of NK cell subpopulations post-HSCT is divergent from healthy individuals showing a high proportion of CD56^{bright} cells shifting toward CD56^{dim} NK cells. To estimate the reconstitution time to a normal CD56^{bright}, CD56^{int}, and CD56^{dim} NK cell distribution and absolute cell counts post-HSCT, we first generated age-matched reference values of NK cell subpopulation frequencies and absolute NK cell counts for healthy children and adolescents (Figure S1 in Supplementary

Material). Interestingly, the proportion of CD56^{bright}, CD56^{int}, and CD56^{dim} in early childhood changed from 15, 8, and 78 to 6, 4, and 90% in adults, respectively. Subsequently, we matched the NK cell reconstitution of all patients of the control group without severe events (e.g., GvHD, infections) with the newly generated reference values (Figure 1). The frequency of CD56^{bright} NK cells is elevated directly after HSCT but reaches the upper reference range following 2 months. However, the levels of CD56^{bright} appear slightly increased remaining between the 50th and 90th percentile until 12 months post-HSCT (Figure 1A). Surprisingly, the percentage of CD56^{int} cells matches the reference range within the first 2 months post-HSCT but increases starting from 3 months exceeding the 90th percentile of the reference range following 5 months post-HSCT. After this period, the CD56^{int} fraction declines and converges to the 50th percentile of the reference values 12 months after HSCT (Figure 1B). By contrast, the CD56^{dim} fraction remains below the 10th percentile until 8 months and reaches the 50th percentile of normal reference values after 12 months post-HSCT (Figure 1C). Comparable reconstitution profiles are also apparent for absolute values of CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells. However, absolute

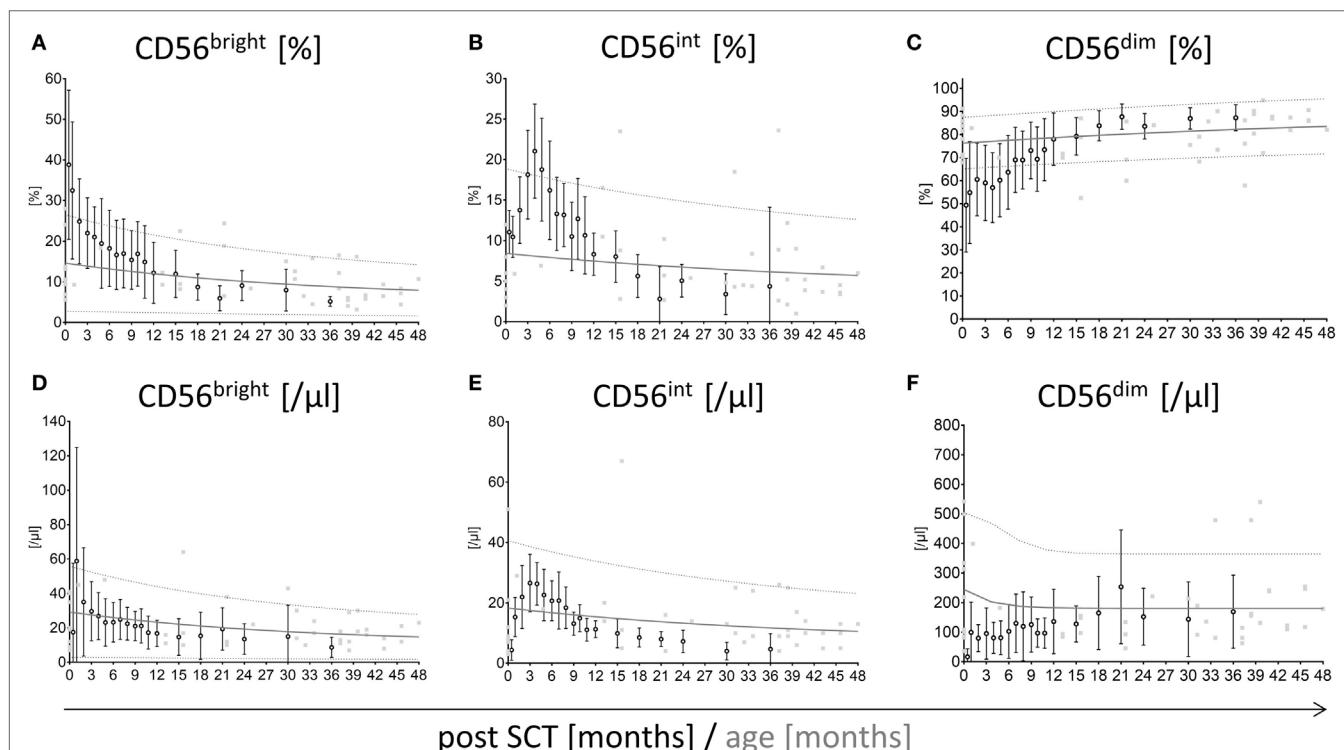


FIGURE 1 | Reconstitution of CD56^{bright}, CD56^{int}, and CD56^{dim} natural killer (NK) cells with respect to reference percentiles of healthy children.

Reconstitution of CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells plotted into a graph showing the 10th, 90th (..., dotted line), and 50th (■, solid line) percentile of NK cell reference values of healthy children ($n = 174$) as well as their underlying measurements (■, gray squares). Measurements of stem cell transplanted patients without graft-versus-host disease or viral infections ($n = 23$; mean with SD) were plotted into the reference model (○, black rimmed circles) at 19 time points until 36 months after hematopoietic stem cell transplantation (HSCT). Time points were 15 days, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, 24, 30, and 36 months after SCT. The CD56^{bright} and CD56^{dim} NK cells needed around 12 months to reach the distribution of healthy individuals NK cells. At that time point, the CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells met the 50th percentile of the reference range (A–C). CD56^{bright} NK cells were elevated post-HSCT but already reached the reference range after 2 months (A). Intermediate NK cells matched the reference range directly after HSCT but showed a fast proliferation until 5 months followed by a decline until 12 months post-HSCT (B). CD56^{dim} NK cells were underrepresented directly after HSCT, reaching the lower reference range after 8 months post-HSCT (C). The development of absolute cell counts of CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells is shown in subfigures (D–F).

values after HSCT seem to be lower than NK cells of healthy children (**Figures 1D–F**).

Reconstitution of NK Cells with Regard to GvHD Development

Patients suffering from aGvHD with grade higher than III show significant differences in the reconstitution of NK cell subpopulations compared to patients without any severe events post-HSCT. The median time of first symptoms of aGvHD was 22 days post-HSCT (range: 13–84). Within the first, second, and third month 68, 89, and 100% of the affected patients showed first signs of aGvHD, respectively. Patients without events show conspicuously higher frequency of CD56^{bright} NK cells within the first 3 months following HSCT compared to patients suffering from aGvHD with the most significant difference already 15 days following engraftment ($p < 0.0001$; **Figure 2A**). This could also be shown for the absolute CD56^{bright} NK cell amount 1 month after HSCT ($p < 0.01$; **Figure 2D**). This tendency was less pronounced when analyzing NK cells in patients with lower grades aGvHD (data not shown). Furthermore, the reconstitution of NK cell subpopulations seems to be delayed in patients suffering from aGvHD. For the CD56^{int} NK cell population, a displacement in time could be

shown, which leads to a longer increase in CD56^{int} frequency and absolute amount (**Figures 2B,E**). To reach the 50th percentile of normal reference values, patients with aGvHD need a prolonged reconstitution time taking at least two times longer compared to patients without events. Patients with lower GvHD grades were lying in between (data not shown). Even after 3 years of monitoring, a trend toward higher frequency of CD56^{bright} and CD56^{int} NK cells concomitant with lower CD56^{dim} was seen in patients suffering from severe aGvHD (not significant, **Figures 2A–C**). These differences in the NK cell development post-HSCT were also found evaluating the absolute amounts of NK cell subpopulations (**Figures 2D–F**). Noteworthy, patients affected with aGvHD following HSCT also show a reduced absolute amount of CD56^{dim} NK cells after 3 years post-HSCT (**Figure 2F**). Analyzing absolute NK cell count (including all three subgroups), we did not see a correlation between patients with and without aGvHD (Figure S2A in Supplementary Material). Analyzing cytotoxic T cells, we detected that patients reaching levels of cytotoxic T cells above 1,500/ μ l within the first year post-HSCT developed in almost all cases an aGvHD (Figure S2B in Supplementary Material). But this fact was only true for less than 25% of the total aGvHD patient cohort. Furthermore, we evaluated the NK cell regeneration of

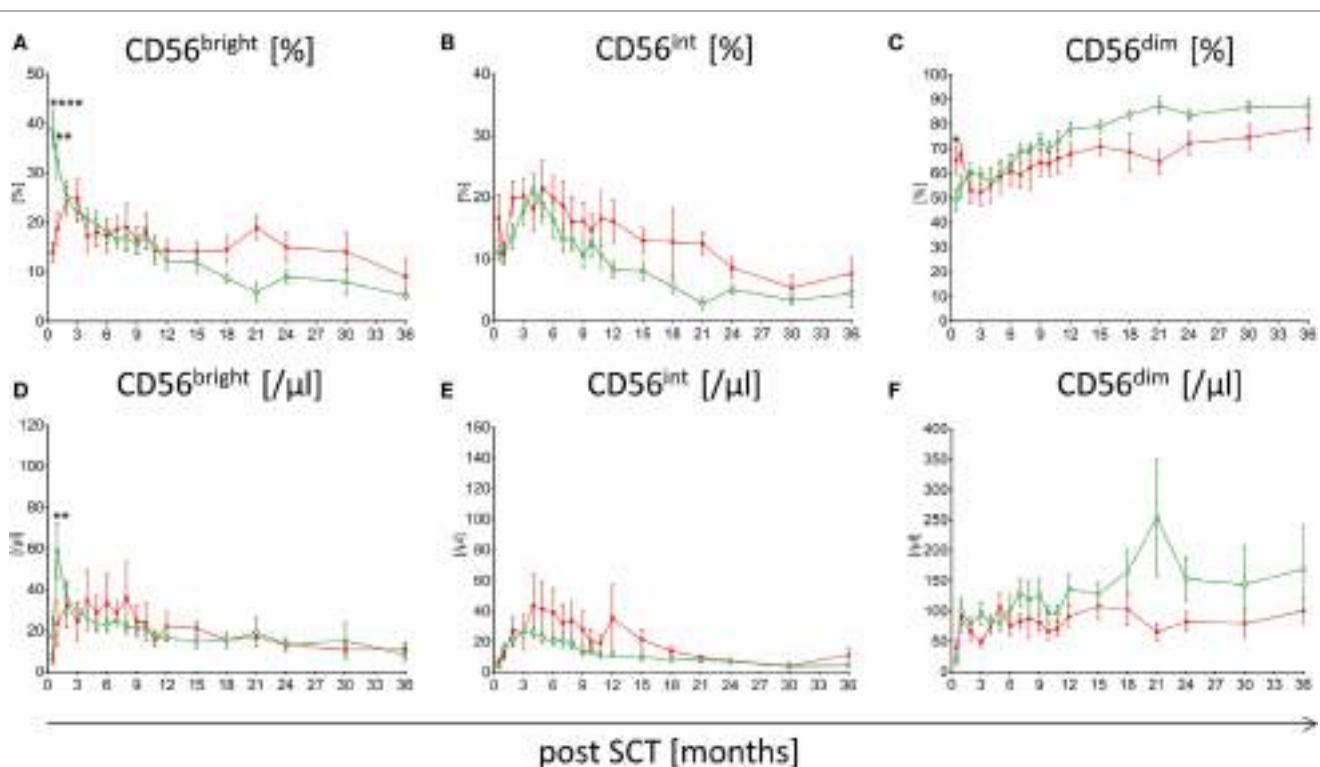


FIGURE 2 | Comparison of natural killer (NK) cell reconstitution in patients suffering from acute graft versus host disease (aGvHD) versus patients without events. Regeneration of CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells in patients affected with severe aGvHD with grade III and IV (■, red squares, $n = 19$) and patients without events post-hematopoietic stem cell transplantation (HSCT) (□, green rimmed squares, $n = 23$) at time point of 15 days, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, 14, 30, and 36 months after SCT. Especially, in the first 3 months, post-HSCT aGvHD patients showed significantly lower CD56^{bright} NK cells in both percentage (**A**) ($p < 0.0001$) and absolute amounts (**D**) ($p < 0.01$). CD56^{int} NK cells of aGvHD patients seem delayed in their development as patients without events show an increased intermediate population over a period of at least 18 months (**B,E**). Except in the beginning, CD56^{dim} NK cell levels of GvHD patients remain below those of patients without events during the whole monitoring interval (**C,F**). Measurements were available at almost all time points, except for five patients of the aGvHD group who died 2, 3, 9, 9, and 18 months post-HSCT.

patients with primary aGvHD grade III or IV that became chronic. Thereby, we detected important differences in the development of NK cell subpopulations from CD56^{bright} above CD56^{int} to CD56^{dim} NK cells. The development from CD56^{int} to CD56^{dim} NK cells is delayed for at least 2 years. Furthermore, patients with chronicification of aGvHD with grade >III have a markedly elevated CD56^{int} frequency (Figure 3A), which is also clearly visible in absolute cell count of CD56^{int} NK cells (Figure 3B).

Receptor Expression of CD56^{bright}, CD56^{int}, and CD56^{dim} NK Cells Post-HSCT

As part of the patients had low absolute cell counts post-HSCT, a detailed phenotyping of the CD56^{bright}, CD56^{int}, and CD56^{dim} NK cell populations was only possible for an elected cohort of patients. To get an understanding of the function of CD56^{int} NK cells, we analyzed surface molecules linked to NK cell cytotoxicity, adhesion, and immune regulatory functions (e.g., chemokine and cytokine receptors) and compared the expression of KIRs, CD62L, NKG2A, CD127, CD117, CX3CR1, CD226, and CD57 on all three NK subpopulations. The CD56^{dim} population showed a higher expression of KIRs, whereas the CD56^{int} and CD56^{bright} population did not. However, not all KIRs applied within the mix were expressed with equal density (Figure 4). Regarding the homing receptor CD62L, the CD56^{int}, and the CD56^{bright} fraction showed an increased expression compared to CD56^{dim} population. The expression of NKG2A was highest on CD56^{int} and CD56^{bright} cells but bipartite in CD56^{dim} NK cells. CX3CR1 is involved in adhesion and migration of NK cells and to a small extent higher presented on CD56^{dim} NK cells. As already described, we could also show that CD57 was only detectable on the CD56^{dim} subpopulation. Low expression of CD127 (IL7α chain), CD117 (c-Kit), and CD226 (DNAM-1) could be seen on all NK cell subpopulations; however, CD56^{bright} intend to have higher expression

than CD56^{dim}, whereas CD56^{int} was always lying in between. In summary, the expression profiles of CD56^{int} and CD56^{bright} NK cells were nearly congruent, but differed to CD56^{dim} cells in KIR, CD62L, NKG2A, CX3CR1, and CD57 expression (Figure 4).

Influence of Viral Infection Post-HSCT on NK Cell Reconstitution

The immune reconstitution of NK cell subpopulations post-HSCT was analyzed in patients without events and patients suffering from ADV ($n = 5$), EBV ($n = 5$), and CMV infection ($n = 8$). Infection was detected by the routine analysis of DNA copies in peripheral blood. Patients with elevated viral load at the day of transplantation were excluded from the study, resulting in a cohort of patients with occurrence of a positive viral load between 30 and 90 days post-HSCT. Interestingly, we observed a slight reduction in CD56 and CD16 expression in patients suffering from viral infection in between day 30 and day 60 post-HSCT measured by mean fluorescence intensity (Figure 5). After viral clearance in most patients, a considerable loss in absolute CD56^{dim} NK cell count occurred followed by continued regeneration of CD56^{int} NK cells, which was lower in patients without events post-HSCT on day 150 post-HSCT (Figure 5).

DISCUSSION

Especially in the early phase following allogeneic HSCT, together with neutrophils, NK cells are the first line of immune defense. Their immune reconstitution is of crucial importance for transplantation outcome with special regard to the occurrence of GvHD and viral infections. In this project, we analyzed NK cell subpopulations in detail not only focusing on CD56^{bright} and CD56^{dim} cells but also the fraction in between those subsets. With regard to NK cell subpopulation development in young healthy

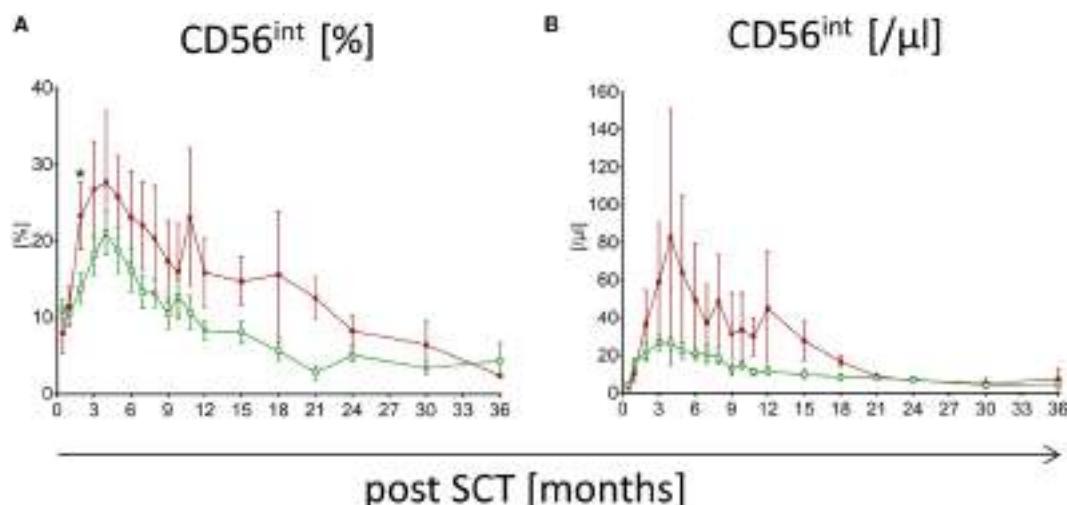


FIGURE 3 | Natural killer (NK) cell reconstitution in patients suffering from chronic graft versus host disease (cGvHD). Development of CD56^{int} NK cells of patients suffering from cGvHD after severe acute graft versus host disease (grade III or IV) is shown for frequency (A) and absolute count (B). Over a period of at least 18 months, a higher amount of CD56^{int} NK cells for patients suffering from cGvHD (■, dark red squares, $n = 8$) was detectable compared to patients without severe events post-hematopoietic stem cell transplantation (□, green rimmed squares, $n = 23$).

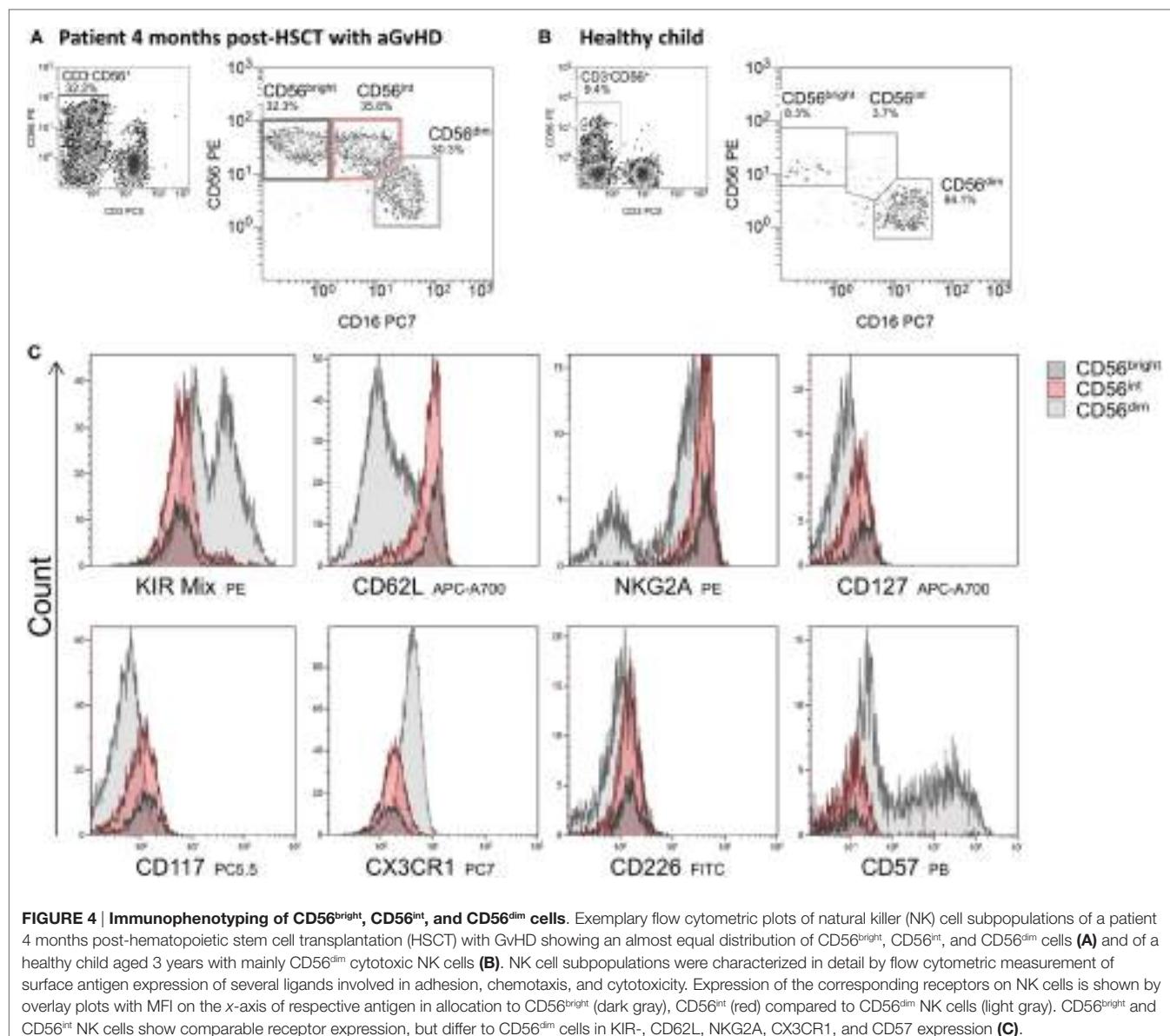
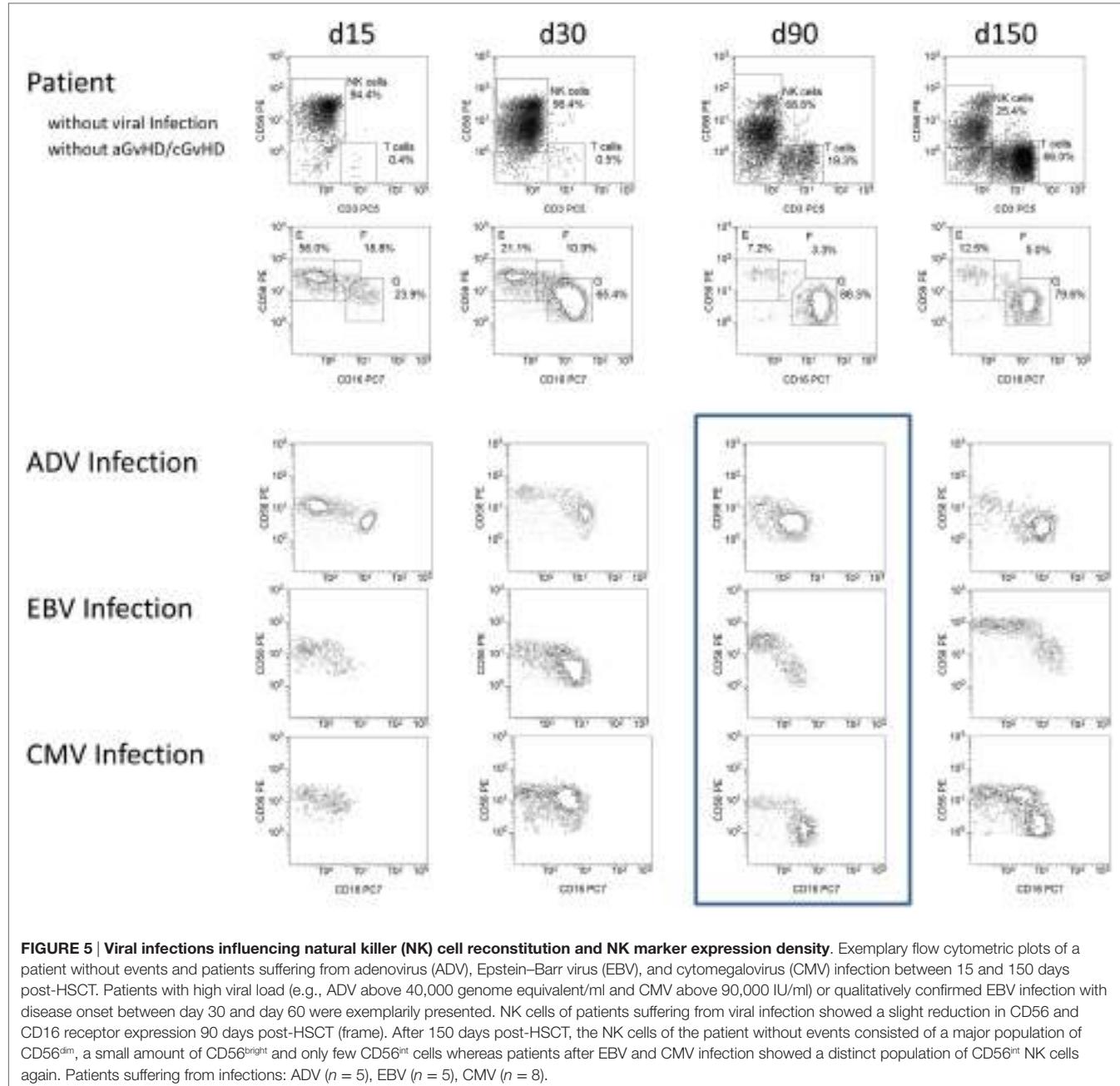


FIGURE 4 | Immunophenotyping of CD56^{bright}, CD56^{int}, and CD56^{dim} cells. Exemplary flow cytometric plots of natural killer (NK) cell subpopulations of a patient 4 months post-hematopoietic stem cell transplantation (HSCT) with GvHD showing an almost equal distribution of CD56^{bright}, CD56^{int}, and CD56^{dim} cells (**A**) and of a healthy child aged 3 years with mainly CD56^{dim} cytotoxic NK cells (**B**). NK cell subpopulations were characterized in detail by flow cytometric measurement of surface antigen expression of several ligands involved in adhesion, chemotaxis, and cytotoxicity. Expression of the corresponding receptors on NK cells is shown by overlay plots with MFI on the x-axis of respective antigen in allocation to CD56^{bright} (dark gray), CD56^{int} (red) compared to CD56^{dim} NK cells (light gray). CD56^{bright} and CD56^{int} NK cells show comparable receptor expression, but differ to CD56^{dim} cells in KIR-, CD62L, NKG2A, CX3CR1, and CD57 expression (**C**).

children, we found that it takes around 12 months until CD56^{bright}, CD56^{int}, and CD56^{dim} NK cells of patients post-HSCT reach the 50th percentile of age-matched reference range. Comparable results were published by Pical-Izard et al. describing that rapidly re-emerging NK cells remain immature for more than 6 months (8). Directly after HSCT we detected a highly increased frequency of CD56^{bright}, whereas CD56^{int} NK cells correspond to the reference range, but considerably expand within the first 3 months post-HSCT. In contrast, CD56^{dim} NK cells deserve around 8 months to enter the reference range. These results confirm the hypothesis of sequential development of NK cells with CD56^{int} NK cells representing an intermediate state from CD56^{bright} to CD56^{dim} NK cells (14, 15). These CD56^{bright} NK cells in peripheral blood are closely related to those NK cells populating secondary lymphoid tissues (16). Further evidence supporting this hypothesis was published by Freud et al. describing that the CD56^{bright} subset is the major

NK cell population that is derived early *in vitro* when CD34⁺ HPC are cultured in NK development supportive conditions, whereas CD56^{dim} NK cells develop later (17). Furthermore, CD56^{bright} NK cells display longer telomeres than the CD56^{dim} NK cells, indicating lower proliferation capacity (6).

We further characterized all three NK cell subpopulations with the finding, that CD56^{int} presented antigen expressions among CD56^{bright} and CD56^{dim} NK cells, even so CD56^{bright} and CD56^{int} NK cells showed rather equal expression profiles and seemed related more to CD56^{bright}. However, differential expression of KIRs, CD62L, NKG2A, and CD57 was observed on CD56^{dim} NK cells. This is in parallel to other findings describing an increased expression of NKG2A, the IL-7 receptor (CD127) and the lymph node homing receptor CCR7 on CD56^{bright} cells (2, 5, 8, 18, 19) whereas CD56^{dim} NK cells acquire KIR, NKG2C, and CD57 expression (20).



Promoted by the IL-15 rich cytokine milieu post-transplant, NK cells are known to be one of the first lymphocyte subpopulation recovering post-HSCT (21). Therefore, NK cell reconstitution might be the basis for generating early prognostic markers regarding the occurrence of severe events and transplantation outcome. Kim et al. published that NK cell counts after allo-HSCT, especially on day 30, were predictive markers for GvHD, non-relapse mortality, and survival (22). Furthermore, there is evidence that the speed of NK cell reconstitution correlates with transplant outcome, suggesting their important role in the early period when specific T cell immunity is absent (7, 8). Our and other findings suggest that the monitoring of NK cell subsets in

the early phase post-HSCT might provide first signs of aGvHD development (23). Interestingly, within the first 2 months post-HSCT patients without aGvHD or viral infections had significantly elevated levels of CD56^{bright} NK cells compared to patients suffering from aGvHD. This might be an early prognostic factor regarding GvHD development; however, it needs to be confirmed in a prospective study. Likewise results were also published by Kheav et al. showing an impaired reconstitution of CD56^{dim} NK cells 3 months post-HSCT (24). We also found a comparable trend for NK cell regeneration in patients suffering from cGvHD, although not significant (data not shown). This might be explained by the fact, that for aGvHD analysis, only

patients suffering from GvHD grades III and IV were considered, whereas no differentiation was available regarding cGvHD (e.g., chronicification of primary aGvHD grades I and II).

Literature is discordant whether steroids/immunosuppression have a negative impact on NK cell reconstitution. Giebel et al. proposed that the use of steroids for GvHD prophylaxis negatively affects quantitative reconstitution of NK cells after allo-HSCT (25). Although, patients suffering from GvHD grades III and IV normally receive steroids in our transplantation unit, we did not see any significant differences in the quantitative reconstitution of absolute NK cell numbers. Interestingly, Wang et al. described that CSA suppresses the *in vitro* proliferation of NK cells, especially the CD56^{dim}CD16⁺KIR⁺ NK cells, resulting in a relative increase in the number of immature CD56^{bright}CD16⁻KIR⁻ NK cells (26). This might also contribute to the delayed NK cell development in patients suffering from higher grade aGvHD that we observed within this study. However, this remains controversial as other studies analyzed the effect of CSA on NK cell function in short-term cultures and their cytokine production without finding significant differences between NK cells with and without CSA treatment (27, 28).

In patients suffering from viral infection post-HSCT, we observed a slight reduction in CD56 and CD16 expression. Notably, other publications already described the existence of CD56⁻CD16⁺ NK cells (CD56^{negative}) NK cells in viral infections (e.g., HIV, hepatitis C), where NK cells undergo numerous phenotypic and functional changes (29). This CD56^{negative} subset has been associated with high HIV viral load and has been reported to have an impaired cytolytic function and cytokine production (30). This increase occurred primarily at the expense of CD56^{dim} NK cells, whereas numbers of CD56^{bright} NK cells remained stable (31). Furthermore, we observed a considerable loss in absolute CD56^{dim} NK cells followed by continued regeneration of CD56^{int} NK cells. Alteration of NK cells upon viral infection has already been shown by other research groups, for example Pical-Izard et al. showed that in patients being affected by CMV reactivation, NK cells showed lower degranulation and TNF- α production compared to patients without CMV reactivation post-HSCT (8). In addition, it was shown that CMV reactivation is followed by an increase in the proportion of NKG2C⁺ NK cells within 2–4 weeks, which persist for at least a year (32, 33).

In conclusion, only after around 12 months, NK cells post-HSCT reconstitute to a distribution of the subpopulations CD56^{bright}, CD56^{int}, and CD56^{dim} comparable to age-matched healthy controls. The expression profiles of CD56^{int} and CD56^{bright} NK cells resemble each other but differed in KIR, CD62L, NKG2A, CX3CR1, and CD57 expression to CD56^{dim} NK cells. We observed elevated levels of CD56^{bright} directly after and CD56^{int} NK cells 3 months post-HSCT accompanied by reduced CD56^{dim} NK cells supporting the hypothesis of sequential NK cell

development. Furthermore, we analyzed alterations in NK cell development in patients with severe viral infections and GvHD. Following viral infection, there was a slight reduction in CD56 and CD16 receptor expression followed by a considerable loss in absolute CD56^{dim} NK cells and continued regeneration of CD56^{int} NK cells. Most important, within the first 2 months, post-HSCT patients without severe events had significantly elevated levels of CD56^{bright} NK cells compared to patients suffering from aGvHD. While first measurements performed as early as 15 days following HSCT revealed the most significant differences, clinical occurrence of aGvHD was observed in median on day 22 post-HSCT. Therefore, we recommend immunophenotyping of NK cell subpopulations directly following engraftment, which might be an early prognostic factor regarding GvHD development.

AUTHOR CONTRIBUTIONS

Study design: MB, SH, RE, and EK. Performed the experiments: JB-D and SBe. Provided clinical data: JS, AJ, SBa, and PB. Analyzed the data: SH and EK. Coordinated the research: SH and CC. Contributed reagents/materials/analysis tools: MB, SH, VP, JB-D, and SBe. Performed statistical analyses: SH and ES-M. Wrote the manuscript: MB and SH. Discussed data and revised the manuscript: CC, PB, VP, EU, and CK. Supervised the research: PB and TK. All the authors read and approved the final manuscript.

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The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00109/full#supplementary-material>.

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Hepatitis C Virus and Human Cytomegalovirus—Natural Killer Cell Subsets in Persistent Viral Infections

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Hepatitis C virus (HCV) and human cytomegalovirus (HCMV) are prominent examples of RNA and DNA viruses, respectively, that establish a persistent infection in their host. HCV affects over 185 million patients worldwide, who are at high risk for developing liver fibrosis, liver cirrhosis, and ultimately hepatocellular carcinoma. Recent breakthroughs in HCV therapy, using direct-acting antivirals have provided the opportunity to monitor natural killer (NK) cells after clearance of a chronic infection. There is now increasing evidence that the individual NK cell repertoire before infection is predictive for the course of disease. HCMV affects the majority of the global population. While being asymptomatic in healthy individuals, HCMV represents a severe clinical challenge in immunocompromised patients. Both viral infections, HCV and HCMV, lead to long-lasting and profound alterations within the entire NK cell compartment. This review article, will discuss the diverse range of changes in the NK cell compartment as well as potential consequences for the course of disease.

Keywords: natural killer cells, hepatitis C virus, human cytomegalovirus, chronic infection, natural killer subsets

INTRODUCTION

A wide range of viral infections challenge the immune system throughout the lifetime of its host exerting a substantial and often long-lasting impact on multiple immune parameters. Natural killer (NK) cells, vital players in the antiviral immune defense, have been shown to undergo substantial changes in phenotype, function, and subset distribution during persistent viral infections. Specific NK subsets have been associated with both efficient clearance of viruses and immune dysfunction.

Persistent viral infections can be latent or chronic. Latent infection is characterized by long periods of viral inactivity with no replication or production of new virions although stress stimuli can trigger episodes of reactivations. Prominent examples of viruses establishing latent infection are the herpes viruses [human cytomegalovirus (HCMV), herpes simplex virus (HSV), Epstein–Barr virus (EBV), varicella-zoster-virus (VZV)]. Other viruses, such as the majority of hepatitis viruses [hepatitis C Virus (HCV), hepatitis B Virus (HBV), hepatitis D Virus (HDV)] and human immunodeficiency virus (HIV), establish chronic infections in which constant replication takes place. This drives chronic inflammation, often resulting in severe tissue damage of the infected organ (1). In this review, we will focus primarily on the effects of latent HCMV and chronic HCV infection on NK cells.

Hepatitis C Virus is a hepatotropic, enveloped, (+)-strand RNA virus that is transmitted person-to-person *via* blood and establishes chronic infection in 55–85% of patients. The probability for spontaneous viral clearance depends on several factors such as age, sex, host genetic factors, coinfection with other viruses, and viral genotype (2, 3).

Currently, seven genotypes and multiple subgenotypes are described with distinct global distribution patterns. In developed countries, genotype 1 is the most common, accounting for around 50% of all HCV infections, even though it has the most favorable prognosis (4). Worldwide an estimated 2.5% of the world's population is chronically infected with HCV. Throughout the decade-long infection, the liver suffers from immunopathology, resulting in fibrosis, cirrhosis, often progressing to hepatocellular carcinoma. Each year around 500,000 people die from HCV-related liver diseases (5). To establish chronicity, the virus interferes with several innate and adaptive immune pathways, such as recognition by retinoic acid inducible gene I (RIG-I), the primary sensor for HCV-RNA in the host cell's cytoplasm (6). The emergence of viral escape variants facilitates evasion from recognition by CD8 T cells, which are the main effector cells against HCV (7).

Until 2011, standard therapy for HCV consisted of pegylated IFN- α /ribavirin. However, only around half of the patients achieved a sustained virological response (SVR) defined by no detectable HCV-RNA 24 weeks after treatment and side effects were drastic. In 2011, the first direct-acting antivirals (DAAs) were approved targeting essential viral proteins which revolutionized therapy by reaching SVR rates of >90% (4, 8, 9).

Human cytomegalovirus has a linear double-stranded DNA genome of 236 kbp. The virus spreads vertically and horizontally *via* bodily fluids by infecting epithelial and endothelial cells, macrophages, and DCs wherein it establishes life-long latency. This leads to high global prevalence rates of 60–85%, depending on socioeconomic factors, geographical location, and age. HCMV is an opportunistic pathogen, causing disease only in immunocompromised people, e.g., during transplantations or in HIV patients. Furthermore, transplacental transmission of HCMV can cause severe, primarily neurological, damage to the fetus (10).

Human cytomegalovirus has developed a plethora of strategies and dedicates a large portion of its genome to interfere with the host immune system. Many of these escape mechanisms have evolved to avoid recognition by NK cells (11–13).

Natural killer cells are important effector cells in the antiviral immune response in during HCMV and HCV infection (14–17).

The importance of NK cells in human Herpes virus infections was initially highlighted in a patient with a very rare NK cell deficiency and his enhanced susceptibility to recurrent infections (18), a clinical phenotype corroborated in later reports (19, 20). Moreover, it is also indirectly evident in the multiple immune evasion strategies that HCMV utilizes to prevent NK cell recognition (11, 12).

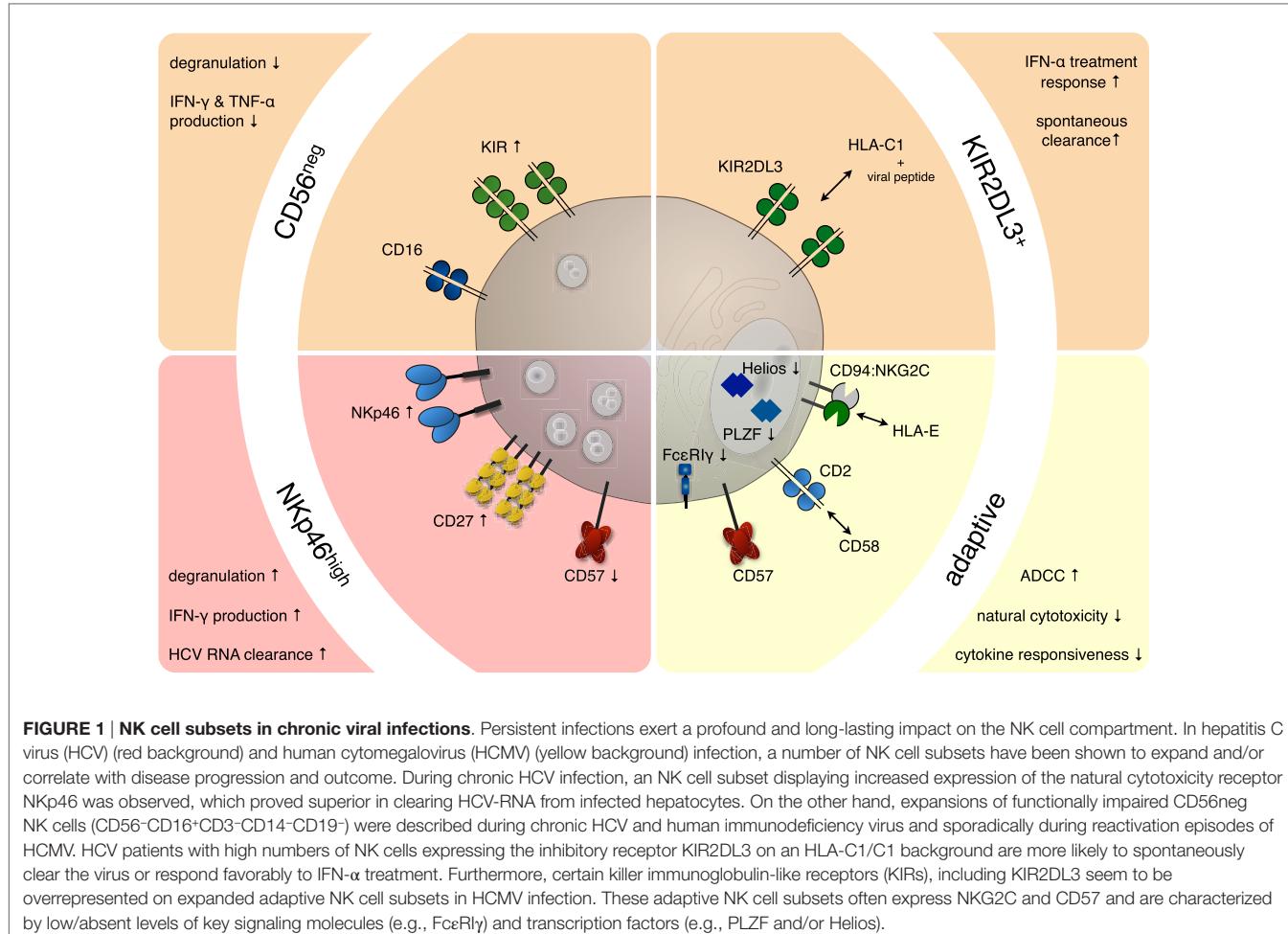
The two main strategies employed by various viruses to evade NK cells are preventing the upregulation of activating ligands or enhancing the expression of ligands for inhibitory NK cell receptors. Upon viral infection, a variety of stress-induced molecules are expressed on the surface of infected cells, which can

be recognized by activating receptors on NK cells. Important activating receptors belong to the natural cytotoxicity receptor (NCR) family, including NKp30, NKp44, and NKp46, which can recognize cellular as well as viral ligands. However, many of the NCR ligands still remain elusive. MHC class I molecules, in particular HLA-C, provide the main inhibitory signals for NK cells *via* interacting with killer immunoglobulin-like receptors (KIRs) (21). The KIRs, like their MHC ligands, are genetically highly polymorphic and expressed in a stochastic manner, leaving every NK cell with 0–4 KIR receptors (22). Furthermore, different KIR haplotypes—group A and B—have been identified. While the group A haplotype comprises almost exclusively inhibitory, the group B haplotypes also encode activating KIRs (23). Indeed, most receptors are expressed only on subsets of NK cells. Therefore, NK cells are not a uniform cell population but composed of many different subsets that differ in their mode of activation and their functional properties. In this review, we will discuss some of the NK cell subsets that have been studied in HCV and HCMV infections (Figure 1).

NK CELL SUBSETS CARRYING SPECIFIC KIRs

One receptor that has repeatedly been implicated in the anti-HCV immune response is the inhibitory KIR2DL3. In 2004, Khakoo and colleagues (24) reported a genetic association between the expression of KIR2DL3 and HCV clearance. They studied more than thousand patients infected with HCV, of whom 685 developed a chronic infection while 352 cleared the virus. Those patients, whose NK cells expressed the inhibitory KIR2DL3 homozygously on an HLA-C1/C1 background, were more likely to clear the virus spontaneously. Of note, when stratified in terms of route of infection, this association was only observed in patients with presumed low-dose viral inoculum, e.g., after a needle-stick, suggesting that the antiviral NK cell response is most efficient in situations with low viral load. Other studies in cohorts of injection drug users confirmed that exposed but uninfected individuals had a higher frequency of KIR2DL3 expression than drug users with chronic and resolved infections or healthy donors (25). This association was also reflected in the response to treatment, in which KIR2DL3–HLA-C1 expressing patients had a higher chance to achieve SVR after IFN- α -based treatment (26). However, in other studies, a correlation between KIR2DL3 and outcome of infection was not observed, albeit with smaller and different patient cohorts (27, 28). The current hypothesis for this association is that NK cells expressing KIR2DL3, which has a rather weak binding affinity to HLA-C (29), tend to receive less inhibitory signals and are, therefore, more easily activated than NK cells expressing KIRs with higher binding affinities.

Additionally, there is now increasing evidence that peptides presented in MHC class I complexes also influence binding affinities of KIRs. This has already been shown for HIV peptides (30–32) and could in 2016 for the first time be shown for an HCV peptide presented by an HLA-C1 molecule to KIR2DL3 on NK cells (33). Lunemann et al. (33) identified peptides derived from HCV NS3 and Core protein that stabilized expression of



HLA-C*03:04 on transfected 721.221 cells and facilitated binding of KIR2DL3-Fc proteins. One of the peptides derived from HCV Core protein was furthermore able to inhibit degranulation of primary KIR2DL3 $^{+}$ NK cells. When comparing core peptide sequences from different HCV genotypes, the genotype 1-derived peptide provided the strongest inhibitory signal, while peptides from other genotypes were much less effective. These results encourage speculations about possible viral escape mechanisms by modulation of KIR binding and the implication that NK cells exert evolutionary pressure on the HCV genome. Future studies should address if the identified peptides can also be presented on primary hepatocytes and if other KIRs/HLA interactions are involved as well.

Moreover, there is substantial evidence for an overrepresentation of certain KIRs in expanded adaptive NK cell subpopulations (see below) in HCMV. The currently available data yield a complex picture with some reports highlighting KIR2DL2/3 (34–36) others KIR3DL1 (37) or activating KIRs (38).

THE CD56 $^{\text{neg}}$ NK CELL SUBSET

Traditionally, NK cells are classified as CD3-CD56 $^{+}$ lymphocytes, which are further divided into a CD56 $^{\text{dim}}$ CD16 $^{+}$ and a

CD56 $^{\text{bright}}$ CD16 $^{-}$ subset. During chronic viral infections, especially in HIV and HCV, a subset of CD3-CD56 $^{-}$ CD16 $^{+}$ NK cells is detectable (39). These cells miss expression of lineage markers, such as CD14 and CD19 or markers of other cell types positive for CD16, while expressing a variety of NK cell receptors (NCR, KIR, and NKG2). They are, therefore, classified as CD56 $^{\text{neg}}$ NK cells. This NK cell subset is found at low percentages (around 5% of all NK cells) in healthy adults and even in neonates, but can expand to 10–40% of all NK cells during HIV, acute, chronic, and resolved HCV infections or HIV/HCV coinfection. Concurrently, a drop in the percentage of CD56 $^{\text{dim}}$ NK cells is observed (40–42).

Phenotypically, CD56 $^{\text{neg}}$ NK cells from healthy donors and chronic HCV patients are similar and expression of many receptors is comparable between CD56 $^{\text{neg}}$ and CD56 $^{\text{dim}}$ NK cells from chronic HCV patients. Only CD57 and to a lesser extent NKp30 were found to be expressed at lower levels in the CD56 $^{\text{neg}}$ NK subset (40). Functionally, however, CD56 $^{\text{neg}}$ NK cells appear impaired. In response to different stimuli, the CD56 $^{\text{neg}}$ NK subset of HCV patients failed to secrete significant amounts of IFN- γ and TNF- α and displayed low perforin expression and degranulation (40, 42). Yet, they showed higher TRAIL expression compared to CD56 $^{\text{neg}}$ NK cells from healthy controls (41). This functional impairment seems to be a general feature of the CD56 $^{\text{neg}}$ NK cell

subset, as it was observed in various chronic inflammatory situations. However, overnight *in vitro* culture of CD56^{neg} NK cells with IL-2, IL-12, or IL-15 resulted in cytotoxicity levels comparable to stimulated CD56⁺ NK cells, indicating that upon stimulation CD56^{neg} NK cells can effectively function (43). It was shown in HCV, as well as in HIV/HCV coinfection, that high pre-treatment levels of CD56^{neg} NK cells correlated with treatment failure (40, 44). Furthermore, after successful IFN- α /ribavirin treatment of HIV/HCV coinfected patients, absolute levels and percentage of CD56^{neg} NK cells normalized after 4 weeks (45).

So far, the role of this particular NK cell subset was not evaluated during DAA treatment of patients, but several lines of evidence suggest that the decline in CD56^{neg} NK cells after treatment is not induced by IFN- α , but results from a decrease in viral load (39). Until now, it was shown that the CD56^{dim} and CD56^{bright} NK cell subsets quickly normalize after DAA treatment in regard to numbers and functionality (46–48). This observation could indicate that the CD56^{neg} subset also normalizes. However, further studies are needed to address this and other open questions: does the CD56^{neg} subset represent a terminally differentiated or exhausted cell type or rather a specific lineage? What is the degree of plasticity in terms of other NK cell subsets becoming CD56^{neg} or CD56^{neg} NK cells acquiring CD56^{pos} phenotypes? The fact that neonates and healthy individuals already have this CD56^{neg} subset, might argue against an exhausted phenotype, although many of their properties resemble exhausted T cells.

In contrast to HCV and HIV, increased levels of CD56^{neg} cells have only been reported sporadically in HCMV infection and were observed only in a subset of patients experiencing viral reactivation (49).

THE NKp46^{high} NK CELL SUBSET

Expression of NK cell receptors was analyzed in different HCV patient cohorts (e.g., in acute or chronic infection and during treatment), but the obtained results have been highly controversial (50, 51). With regards to NKp46, however, several independent studies observed the involvement of an NKp46^{high} NK cell subset expressing multiple markers of immature NK cells (52) in protection from infection, spontaneous clearance, liver inflammation, progression of fibrosis, and outcome of treatment.

In prospective studies of injection drug users, a higher percentage of CD56^{dim} NK cells with increased levels of NKp46 was correlated with a higher percentage of individuals remaining seronegative, suggesting that high expression of NKp46 might be predictive for protection from infection (25). However, Alter et al. (42) reported lower expression of activating receptors, including NKp46, in patients with acute infection who subsequently cleared the virus than in those, who progressed to chronic infection.

In chronically infected patients, the majority of studies report elevated NKp46 expression on peripheral blood NK cells compared to healthy donors (51). Of note, this is even more pronounced in the liver (52). NKp46^{high} peripheral NK cells from healthy donors and HCV patients perform better in reducing HCV-RNA from *in vitro* infected hepatocytes, produce higher levels of IFN- γ , and degranulate more *ex vivo* in response to different stimuli (52, 53).

Likewise, intrahepatic NKp46^{high} NK cells from HCV patients were shown to degranulate more *ex vivo* than NKp46^{dim} cells (54). Furthermore, staining with an NKp46-Ig fusion protein revealed higher expression of a yet unknown NKp46-ligand on HCV-infected Huh7.5 hepatoma cells than on uninfected cells (53).

Accordingly, NKp46 levels in patients correlate positively with liver inflammation scores (54) and inversely with HCV serum levels (52), suggesting that NKp46^{high} NK cells can kill infected hepatocytes and contribute to viral control during chronic infection. The NKp46^{high} subset correlated with low fibrosis stages, possibly due to NKp46-dependent killing of hepatic stellate cells, the main drivers of fibrosis (54–56).

Even though the NKp46^{high} NK cell subset might be beneficial in reducing viral load and liver fibrosis during chronic infection, it also predicts failure to IFN- α therapy (54, 57). After successful DAA treatment, previously elevated NKp46 levels in liver and blood normalize, concomitantly with a normalization of many other NK cell receptors (47).

In contrast, in HCMV infection, there is little evidence for a direct modulation or involvement of NCRs. One report observed the dissociation of the CD3zeta chain from NKp30 after engaging the HCMV tegument protein pp65, leading to greatly reduced NKp30-mediated killing (58).

ADAPTIVE NK CELL SUBSETS

The most striking example for a long-lasting impact of a pathogenic challenge on distinct NK subsets (59) was initially identified in two key reports by Miguel Lopez-Botet's group (60, 61). In HCMV-seropositive individuals, a higher proportion of NK cells expressing the activating receptor CD94/NKG2C was detected. This expanded subset displays lower NCR levels and increased expression of CD85j/LIR-1 (60) and CD2 (38). Similar observations were made in transplant recipients who suffered from acute CMV infection/reactivation (34, 37, 49, 62, 63). *In vitro* studies recapitulated subset expansion suggesting that exposure of NKG2C⁺ NK cells to infected cells was critical for this process (61). Moreover, the interaction between HLA-E and CD94/NKG2C was defined as a critical event for subset expansion (64, 65). To date, several additional factors have been reported to contribute to the expansion and activation of NKG2C⁺ NK cells in response to HCMV infection, such as IL-15 (61), IL-12 (64), and CD14⁺ monocytes (64, 66), as well as the interaction between CD2 and upregulated CD58 on infected cells (67). As expansion of subpopulations and their subsequent longevity resemble hallmarks of adaptive immune responses, the term "adaptive NK cells" was coined for human NK cells displaying these characteristics. From here on, we will use the term in this broadly defined sense, comprising multiple subsets.

Intriguingly, while a large number of studies describe NK subset expansions in other infections, e.g., HIV (68–70) Hantavirus (65), Chikungunya virus (71), EBV (72), and HBV/HCV (35, 73), seropositivity for HCMV seems to be a necessary pre-requirement. Altered HLA-E levels and/or a certain inflammatory cytokine milieu could be common denominators permitting the (re)expansion of NK cells "primed" initially by HCMV. The initial events, however, underlying the formation of this NK cell

subpopulation in primary HCMV infection, remain enigmatic and represent a field of intense interest.

In recent years, it became clear that the initial definition as NKG2C⁺ (and CD57⁺) was not sufficient to encompass all adaptive NK cell subsets.

A study by Hwang and coworkers in 2012 (74) identified NK cell subpopulations with low or absent expression of the adaptor protein FcεRIγ in about one-third of all individuals tested. Zhang et al. (75) then established that the presence of FcεRIγ-deficient NK cells was strictly associated with prior exposure to HCMV. Expansion of an FcεRIγ⁻ subset was also observed in HCMV⁺ chronic HCV patients and correlated with low liver damage and fibrosis levels, possibly implying an involvement of this subset in protection from immunopathology (76). Further reports extended the concept of HCMV driving the expansion of adaptive NK cell populations with deficiencies in key signaling molecules to Syk, EAT-2, and DAB2 and the transcription factors PLZF and Helios (77, 78). These features are not necessarily combined at a single-cell level and instead found in different combinations creating a previously unappreciated heterogeneity among adaptive NK cells.

Intriguingly, this molecular signature partially resembles exhausted T cells and in fact a recent study described high PD-1 expression on a subset of CD57⁺ NK cells also displaying increased LIR-1 levels as well as higher NKG2C expression in some donors (79). Together with lower NCR expression in adaptive NK subsets, these features suggest a decreased functionality. While this seems to be the case for classical tumor targets (78), superior antibody-dependent cellular cytotoxicity (ADCC) responses are emerging as a prominent and distinct characteristic of adaptive NK cells (38, 75, 77, 80–82), augmented by CD2 co-stimulation (67, 83). This functional specialization is accompanied by broad epigenetic modifications, including better accessibility of the *ifn-γ* locus (77, 78, 84). Latent HCMV infection might, therefore, be a worthy trade-off for the host if the interplay of CMV-induced adaptive NK cell populations and antigen-specific humoral immunity via ADCC results in elevated resistance to heterologous infection.

Besides deciphering the generation and function of adaptive NK cell subsets, several studies focused on their localization. In mice, a subset of NK cells endowed with antigen-specific memory has been shown to reside specifically in the liver (85, 86). In human liver samples, a subset, phenotypically similar to memory NK cells in the mouse (CD49a⁺T-bet⁺Eomes⁺) that also displayed high NKG2C expression, was identified. Yet, in contrast to

peripheral NKG2C⁺ NK cells, they had an immature phenotype (CD57⁻, CD16⁻CD56^{bright}), existed in high numbers in HCMV negative donors, and were incapable of mounting ADCC responses due to lack of CD16 expression (85, 87). Therefore, besides their striking resemblance to murine liver-resident memory NK cells and some shared features with human peripheral blood adaptive NK cells, the function of this unique human liver NK subset needs to be further defined.

Very recently, another liver-resident NK cell population, characterized as Eomes^{hi}, CXCR6⁺ (88–90), and CD49e⁻ (91) was described, following up HLA-mismatched human liver-transplants. The authors demonstrated that these Eomes^{hi} NK cells survive for up to 13 years (90). This remarkable longevity makes an involvement of this subset in tissue homeostasis or antiviral responses against chronic infections a plausible scenario, which awaits further investigation.

CONCLUSION

Virus infections, especially with persistent viruses, have a remarkable impact on the NK cell compartment, shape the overall NK cell repertoire, and profoundly affect their effector functions. However, *vice versa*, the different NK subset composition and receptor distribution before infection can also be decisive how well infections can be combated. The diversity of human NK cell subsets is one of the emerging topics in the field. Especially, tissue-resident NK cells and other subsets of helper innate lymphoid cells (ILCs), their development, regulation, antiviral functions, and plasticity in tissues, such as in the liver, are currently an area of intense research. A better understanding of the development and dynamics of ILCs comprising both NK cells and helper ILCs subsets in affected tissues during chronic viral infection might help the design of improved targeted strategies for therapeutic intervention.

AUTHOR CONTRIBUTIONS

JP and AR wrote the manuscript. MH revised the manuscript. AC revised the manuscript and provided conceptual input.

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A Mature NK Profile at the Time of HIV Primary Infection Is Associated with an Early Response to cART

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Natural killer (NK) cells are major effectors of the innate immune response. Despite an overall defect in their function associated with chronic human immunodeficiency virus (HIV) infection, their role in primary HIV infection is poorly understood. We investigated the modifications of the NK cell compartment in patients from the ANRS-147-Optiprim trial, a study designed to examine the benefits of intensive combination antiretroviral therapy (cART) in patients with acute or early primary HIV infection. Multiparametric flow cytometry combined with bioinformatics analyses identified the NK phenotypes in blood samples from 30 primary HIV-infected patients collected at inclusion and after 3 months of cART. NK phenotypes were revealed by co-expression of CD56/CD16/NKG2A/NKG2C and CD57, five markers known to delineate stages of NK maturation. Three groups of patients were formed according to their distributions of the 12 NK cell phenotypes identified. Their virological and immunological characteristics were compared along with the early outcome of cART. At inclusion, HIV-infected individuals could be grouped into those with predominantly immature/early differentiated NK cells and those with predominantly mature NK cells. Several virological and immunological markers were improved in patients with mature NK profiles, including lower HIV viral loads, lower immune activation markers on NK and dendritic cell (DC), lower levels of plasma IL-6 and IP-10, and a trend to normal DC counts. Whereas all patients showed a decrease of viremia higher than 3 log₁₀ copies/ml after 3 months of treatment, patients with a mature NK profile at inclusion

Abbreviations: PBMCs, peripheral blood mononuclear cells; NK cell, natural killer cell; PTC, posttreatment controller; KIR, killer-cell immunoglobulin-like receptor; HIV, human immunodeficiency virus; PMT, photo-multiplicators.

reached this threshold more rapidly than patients with an immature NK profile (70 vs. 38%). In conclusion, a better early response to cART is observed in patients whose NK profile is skewed to maturation at inclusion. Whether the mature NK cells contributed directly or indirectly to HIV control through a better immune environment under cART is unknown. The NK maturation status of primary infected patients should be considered as a relevant marker of an immune process contributing to the early outcome of cART that could help in the management of HIV-infected patients.

Keywords: HIV, primary infection, NK cells maturation, cART, memory-like NK

INTRODUCTION

Natural killer (NK) cells are one of the major innate immune components involved in the rapid response of the host to invading virus (1). Their function is probably crucial at the time of infection and can impact the quality of adaptive immune responses and the overall outcome of infections. NK cell activity is regulated by activating and inhibitory receptors, but their effector functions are intrinsically linked to their maturation (2). Cytolysis is the typical NK cell function, but NK cell also play an antiviral role through the release of soluble factors, such as IFN- γ and TNF- α (3), which activate T cells, macrophages, and dendritic cells (DCs). CD56^{bright} NK cells are described as the progenitors of CD56^{dim}, the latter being endowed with the main NK cell effector functions (2). CD56^{dim} cells sequentially progress from an immature population, characterized by a high degranulation and proliferation potential, to a terminally differentiated population, characterized by potent cytokine production at the expense of cell division and degranulation (4). Immature NK cells express NKG2A, a C-type lectin receptor forming an inhibitory heterodimer with CD94 to interact with HLA-E on target cells (5). HLA-E is a non-classical major histocompatibility complex (MHC) class I molecule whose expression is enhanced on infected cells through the presentation of viral peptides (6). During maturation, NKG2A loss is compensated by the acquisition of self-inhibitory killer-cell immunoglobulin-like receptor (KIR) expression, while CD57, a marker of senescence, is acquired (4). NKG2C/CD94 is the activating alternative receptor of HLA-E on NK cells (7). This receptor was initially described on a subset of NK cells expanded during CMV infection (8, 9), but recently, other viruses, including human immunodeficiency virus (HIV), were also shown to drive NKG2C⁺ NK cell expansion, in the context of CMV co-infection (10, 11). The persistence of a NKG2C⁺CD57⁺ NK cell subset for more than 1 year after CMV or Hantavirus infection has led to the proposition that they are a memory-like form of NK cell (8, 12, 13). Therefore, the co-expression of CD56, CD16, NKG2A, NKG2C, and CD57 delineates sequential stages of the NK cell maturation process suggesting the acquisition of typical effector functions.

Natural killer cell functions are affected early after HIV infection (14). In addition, many modifications of the NK cell compartment, including decrease of CD56^{bright}, expansion of CD56^{dim}, and appearance of a functionally compromised

subset of CD56^{dim} expressing low levels of CD56 or CD16 were reported (15). Inversion of the ratio of NKG2A to NKG2C was described in primary HIV-infected patients (11, 16). In chronically infected patients, an overall increase of mature CD57⁺ NK cells was observed (17). In cohort of individualist risk of HIV infection, NK cell activation at the time of primary infection has been both positively and negatively correlated with the risk of HIV acquisition (18, 19). Recently, a correlation was demonstrated between NK cell repertoire diversity, linked to progression to maturity, and increased susceptibility to HIV infection (20). Therefore, while NK maturation seems to be an interesting parameter in HIV infection, so far, the impact of the overall maturation of NK cells on the outcome of primary HIV infection (PHI) remains elusive.

The Optiprim trial was designed to evaluate to what extent intensive antiviral therapy started during primary HIV infection contributes to a decrease in the size of HIV reservoirs and helps to achieve a so-called posttreatment controller (PTC) status (21). A sub-study was designed to investigate innate immune parameters. Considering the important role of NK cell maturation for their effector properties, we investigated the NK cell compartment with a combination of markers known to characterize sequential steps of the NK cell differentiation pathway. Thirty primary HIV-infected patients peripheral blood mononuclear cell (PBMC) samples were investigated at inclusion and 3 months after the onset of combination antiretroviral therapy (cART). Because cell populations expressing unexpected combinations of markers might be expanded in pathological conditions, new bioinformatics methods were applied to analyze multiparametric cytometry data in an unsupervised approach (22). This allowed the identification of a relationship between profiles of NK cells skewed to immaturity or maturity and virological and immune parameters reached naturally a few weeks after infection and after early cART.

SUBJECTS AND METHODS

Ethical Statement

All study participants provided written informed consent.

The study was approved by the Sud-Mediterranee-1 Ethics Committee and the French Health Products Safety Agency and complied with the Helsinki Declaration.

Study Population

Human immunodeficiency virus-1-infected subjects with PHI were included in a multicenter phase 3 randomized trial (ANRS-147 OPTIPRIM) (www.ClinicalTrials.gov, number NCT01033760). The endpoint was the impact of intensive vs. standard cART at month 24 on blood HIV-DNA levels. The results of this study have been published (21). Antoine Chéret was the Principal Investigator, Laurence Meyer the Methodological Investigator, and Christine Rouzioux the Virologist Investigator. We proposed a sub-study where the participants would give blood samples at day 0 (before cART initiation) and month 3. This sub-study, in which Daniel Olive and Françoise Gondois-Rey were the investigators, was designed to investigate parameters of innate immunity linked to cART efficacy. Among 90 patients in the main study, 30 patients were randomly included in this sub-study. This work shows original data on NK cells, DC, CMV, and plasma cytokines, and uses information from the main study. Patient characteristics are listed in **Table 1**.

Fifteen healthy donor samples were obtained from the French Blood Bank (EFS, Etablissement Français du Sang) as controls. PBMC samples were frozen and kept in liquid nitrogen until tested.

Flow Cytometry

Peripheral blood mononuclear cells were stained with multiparametric panels containing 9 or 12 fluorescent markers, respectively, designed to investigate NK and DC populations. The NK panel contained NKG2A-PacBlue (clone Z199, home-made), live-dead Aqua (Life Technology), CD57-FITC (Beckman Coulter; 1/30), NKG2C-PE (R&D; 1/40), CD14-PC5 (Beckman Coulter; 1/30), CD19-PC5 (Beckman Coulter; 1/30), CD56-PC7 (Beckman Coulter; 1/30), CD3-AF700 (BD Biosciences; 1/40), and CD16-APCH7 (BD Biosciences; 1/40). The DC panel contained live-dead Aqua (Life Technology), BDCA2-FITC (Miltényi; 1/30), CD123-PercpCy5.5 (BD Biosciences; 1/20), HLA-DR-ECD

(Beckmann Coulter; 1/40), CD3-PC5 (BD Biosciences; 1/40), CD56-PC5 (Beckman Coulter; 1/30), CD19-PC5 (Beckman Coulter; 1/40), CD33-PC7 (BD Biosciences; 1/40), CD14-APCH7 (BD Biosciences; 1/40), and CD16-AF700 (BD Biosciences; 1/40). Cells were incubated for 20 min at RT with reagents pre-mixed in PBS, washed, and then fixed with 4% PFA. Data were acquired on a LSRII-SORP (BD Biosciences) equipped with four lasers (405 nm/100 mW, 488 nm/100 mW, 560 nm/50 mW, and 630 nm/40 mW). Photo-multiplicators were set using unstained and fully stained samples and linked to the cytometer standardization using the acquisition setting tool. Also, $7.74 \times 10^6 \pm 3 \times 10^5$ events were recorded. Compensations were performed with beads individually stained with corresponding reagents.

Flow Cytometry Data Analysis

Data were exported and analyzed with FlowJo (version 9-2, MacOS X). NK cells were gated as CD3⁻CD14⁻CD19⁻CD56CD16⁺, CD3⁻CD14⁻CD19⁻CD56⁺CD16⁻, or CD3⁻CD14⁻CD19⁻CD56⁻CD16⁺ (Figure S1 in Supplementary Material). Contaminating non-classical monocytes represented less than 0.11% of the gated events (not shown). The remaining data ($3.5 \times 10^5 \pm 2.5 \times 10^4$ events) were exported to create new files further subjected to automated gating. DC's were defined as CD3⁻CD14⁻CD19⁻CD56⁻CD16⁻HLA-DR⁺live cells. In the DC gate, the pDC were gated as CD33^{low}BDCA2⁺CD123⁺ cells, the mDC as CD33^{high}BDCA2⁻CD123⁻ cells.

Automatic Clustering

The flowClust implementation version 3.4.11 on R version 3.1.2 under Linux Cent OS 6 was applied on the following parameters: CD56-PC7, CD16-APCH7, NKG2A-PacBlue, NKG2C-PE, CD57-FITC, to compute clusters as described (22). The number of clusters that fit the data optimally was estimated by computing flowClust on a predefined range and comparing them with the statistical criteria BIC and ICL (not shown). This number was estimated to be above 20; therefore, 27 clusters were computed in the events within the NK gate of the 60 samples (30 patients, T0, and M3) and one healthy donor. Two samples failed to be computed.

MFI values and event counts of the 1,593 clusters generated after computation were exported in an Excel table.

Multiparametric Data Management with MeV

MeV (version 4.9.0, <https://sourceforge.net/projects/mev-tm4/>) (23) was used to visualize and group multiparametric clusters using hierarchical clustering (HCL) of their centers of CD56, CD16, CD57, NKG2A, and NKG2C MFI. Euclidean distance and average linkage were chosen. Prior to MeV, centers were rescaled to adjust the 5 (respectively, 95) percentile of each dimension to -3 (respectively, +3) and normalized. The tree was cut interactively using objective MeV tools and color interpretation of the heatmap to define populations as homogeneous groups of clusters. Names were interactively given according to the comparisons of signatures between groups and the expression of known NK cell markers. The blocks were saved and imported in a spreadsheet program, leading to a matrix with

TABLE 1 | Patients' characteristics at inclusion.

Number of patients	30
Number of patients acutely infected	14
Time between estimated date of infection and enrollment (days)	34 (20–55)
Age (years)	39.4 (23–55)
Number of patients handled by intensive combination antiretroviral therapy	15
CD4 counts at T0 (count/ μ L)	550 (323–1,012)
CD8 counts at T0 (count/ μ L)	1,704 (417–8,157)
CD4 to CD8 ratio at T0	0.47 (0.08–1.32)
Human immunodeficiency virus (HIV)-RNA at T0 (\log_{10} copies/mL)	5.51 (3.2–7)
HIV-DNA at T0 [\log_{10} copies/ 10^6 peripheral blood mononuclear cell (PBMC)]	3.747 (2.78–4.68)
CD4 counts at M3 (count/ μ L)	656 (275–1,244)
CD8 counts at M3 (count/ μ L)	705 (371–1,282)
HIV-RNA at M3 (\log_{10} copies/mL)	1.88 (1.3–3.42)
HIV-DNA at M3 (\log_{10} copies/ 10^6 PBMC)	2.93 (2.25–3.62)

Mean values and (range) are indicated.

a population identifier column associated to the initial count of events. The percentages of each population of each patient sample were summarized using pivot tables.

Virus Quantification

Human immunodeficiency virus-RNA was quantified in plasma by real-time RT-PCR with the Cobas TaqMan HIV1 v2.0 assay (Roche Diagnostics). Threshold values were arbitrarily given to samples below the threshold of the assay (20 RNA copies/mL). Total HIV-DNA was quantified by ultra-sensitive real-time PCR in PBMC using the Generic HIV-DNA assay from BioCentric (Bandol, France) as described (24).

Cytokine Quantification

Plasma Cytokines

IP-10 concentrations were determined in stored plasma or serum samples (-80°C) by specific enzyme-linked immunosorbent assay, human Quantikine CXCL10 (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions. Levels of IL-6 were measured in frozen plasma samples with specific ELISA assays (Human IL-6 Platinum ELISA, eBioscience). Samples with undetectable levels of IL-6 were arbitrarily attributed half the minimal detectable value (0.46 pg/mL).

Statistics

Statistical graphics were performed with Prism 6 software. The Kruskall-Wallis test followed by multiple comparison Dunn's posttest were used to compare variables between groups. Correlations were evaluated by using simple linear regression analysis and Spearman's rank correlation test.

RESULTS

Automatic Clustering Revealed NK Cell Differentiation Subtypes within HIV-Primary Infected Patients' PBMCs

We investigated the phenotypes of differentiation of NK cells with multiparametric cytometry using CD56, CD16, NKG2A, CD57, and NKG2C, known to delineate sequential stages of NK cell maturation (2, 4). In order to discover unexpected populations, multi-stained samples were analyzed through unsupervised computation of clusters, using a method previously validated (22). Computed clusters were visualized in a MeV heatmap according to their normalized MFI for the five markers and merged to identify populations as homogeneous groups of clusters (Figure 1). The tree was interactively cut to summarize the results in 19 groups, using objective tools of MeV and a subjective overview of the heatmap (Figure 1). Six of them including only a few clusters were excluded because of their rare representation. Thirteen groups were named according to comparisons of marker MFI and homogeneity with known NK population signatures, a process similar to manual gating of cytometry data visualized in dot plots (Figure 1). Accordingly, two groups of clusters expressing the highest levels of CD56, of NKG2A, no or low levels of CD16, a typical CD56^{bright} signature, were thus named. Seven populations

expressing medium levels of CD56 and high levels of CD16 were identified as CD56^{dim}. They included several groups differentially expressing NKG2A and CD57, two markers linked to immaturity or maturity, respectively, and NKG2C, a NK cell receptor expanded during CMV infections. Four populations expressing low levels of CD56 or CD16 previously described in HIV-infected patients as dysfunctional NK populations were identified (15). Finally, phenotypes of NK cells usually found in human PBMC, including populations specifically expanded during HIV infection, and new phenotypes characterized by unexpected combinations of markers were found in patients' samples using the automatic clustering and interactive merging approach. It should be emphasized that other analyses of the same data, based on the choice of higher or lower numbers of populations could have also been pertinent. As an example, the few clusters characterized by CD56^{bright}CD16^{neg}NKG2C⁺ visible at the bottom of the tree were included in the CD56^{bright} pool while other cuts of the tree could have separated this original combination of markers.

The Distribution of the Various Phenotypes of NK Cells Defined Groups of HIV-Primary Infected Patients with Different Maturation Profiles

We next determined how these NK populations were individually distributed among the 30 primary HIV-infected patients. The blocks of clusters were imported into a spreadsheet program, leading to a matrix with a population name, associated with initial counts of events and patient number. The frequencies of the 12 populations within the total NK cells of each patient were calculated and summarized using pivot tables (Table S1 in Supplementary Material). One population (CD56^{neg}CD16⁺CD57⁺), although present in different patients, represented less than 0.5% of total NK cells and was excluded from the analysis. Frequencies of the 12 remaining populations were visualized in a MeV heatmap and used to cluster patients (Figure 2A). Three groups of patients, named X, Y, and Z appeared on the map. Group X was characterized by the highest frequencies of CD56^{dim}NKG2A⁺ and CD56^{neg}CD16⁺NKG2A⁺ NK cells. Group Y showed high frequencies of CD56^{dim}NKG2C⁺CD57⁺ and CD56^{dim}CD57⁺ NK cells. Group Z contained mainly a population of CD56^{dim}CD57⁻NKG2A⁻NKG2C⁻ (Figure 2A).

We checked whether the frequencies of the most frequent NK cell populations directing the clustering (the four populations on top of the list) were significantly different between the three groups (Figure 2B). Group X showed significant higher frequencies of CD56^{dim}NKG2A⁺ than Y and Z (34% vs., respectively, 13.8 and 14.3%). Group Y showed significant higher frequencies of CD56^{dim}CD57⁺ NK cells than X and Z (21% vs., respectively, 2.9 and 7.2%), and higher frequencies of CD56^{dim}NKG2C⁺CD57⁺ than X and Z (17.8% vs., respectively, 2.9 and 0.9%). Group Z showed 42.6% of CD56^{dim} NKG2A⁻NKG2C⁻CD57⁻ while this phenotype represented only 12 or 16% of NK cells in, respectively, X and Y. Accordingly, the three groups of patients were significantly characterized by, respectively, high proportion of CD56^{dim}NKG2A⁺ for group X, high proportion of CD56^{dim}CD57⁺ NK cells for group

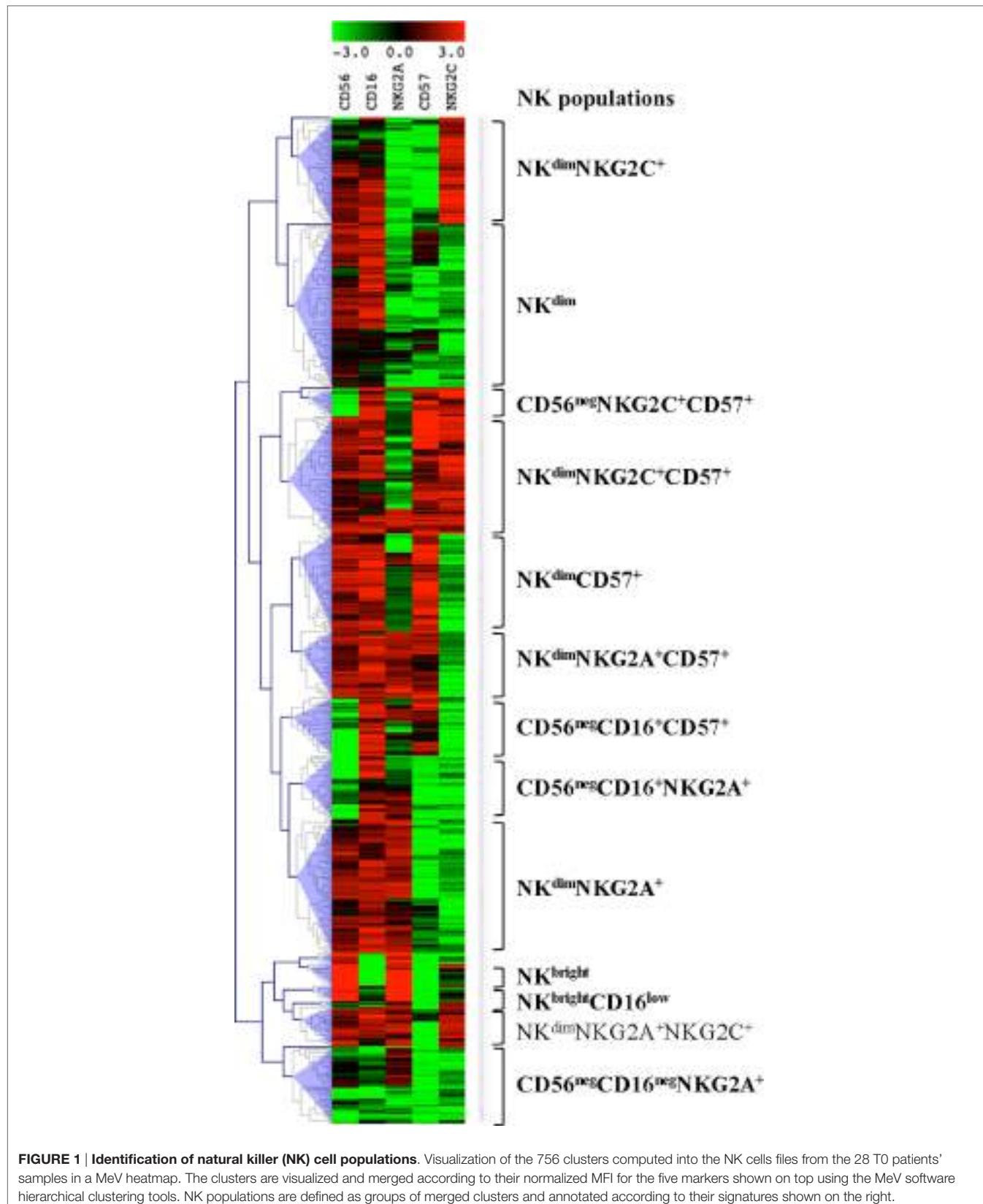


FIGURE 1 | Identification of natural killer (NK) cell populations. Visualization of the 756 clusters computed into the NK cells files from the 28 T0 patients' samples in a MeV heatmap. The clusters are visualized and merged according to their normalized MFI for the five markers shown on top using the MeV software hierarchical clustering tools. NK populations are defined as groups of merged clusters and annotated according to their signatures shown on the right.

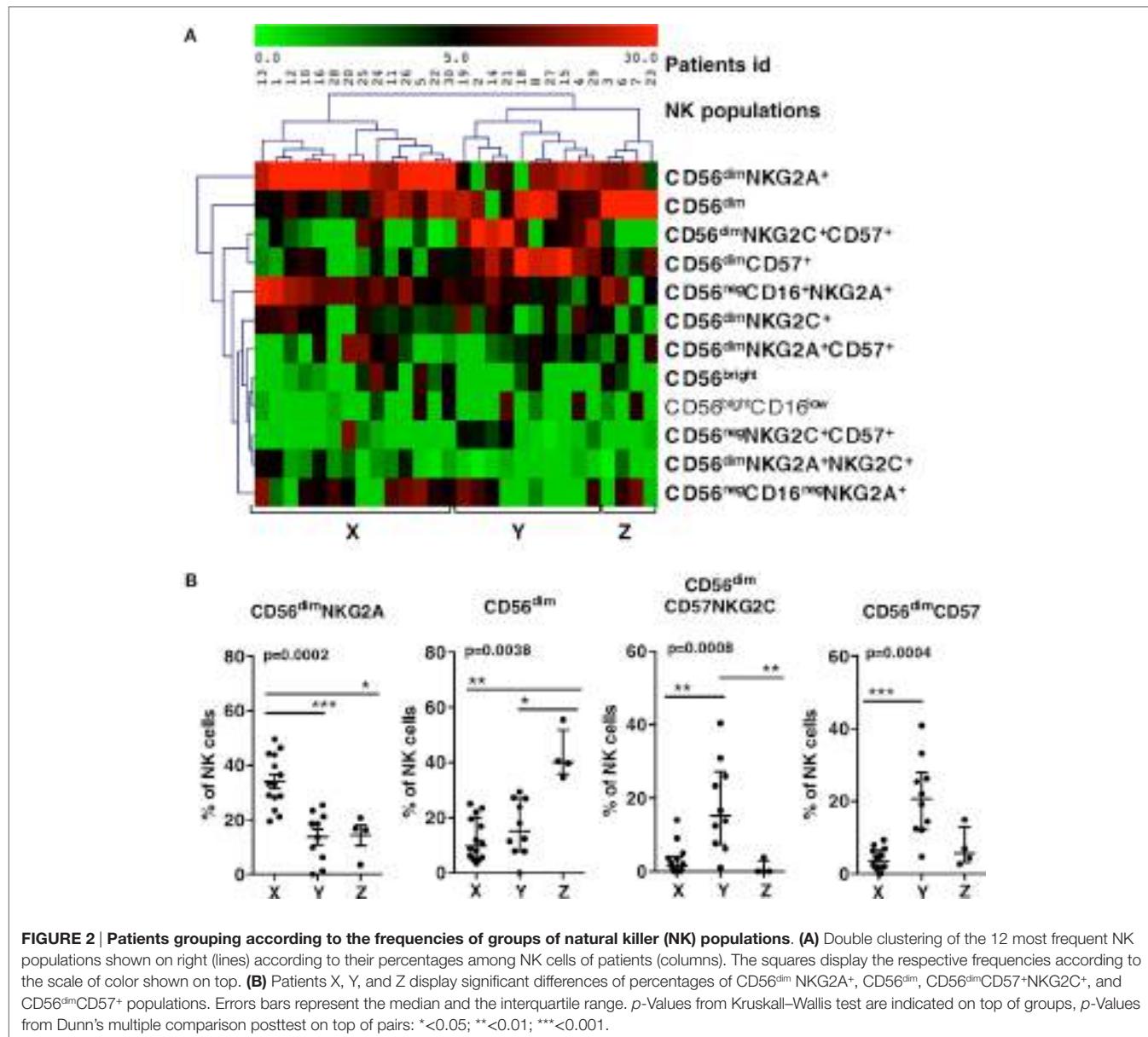


FIGURE 2 | Patients grouping according to the frequencies of groups of natural killer (NK) populations. (A) Double clustering of the 12 most frequent NK populations shown on right (lines) according to their percentages among NK cells of patients (columns). The squares display the respective frequencies according to the scale of color shown on top. **(B)** Patients X, Y, and Z display significant differences of percentages of CD56^{dim} NKG2A⁺, CD56^{dim}, CD56^{dim}CD57+NKG2C⁺, and CD56^{dim}CD57⁺ populations. Errors bars represent the median and the interquartile range. *p*-Values from Kruskall–Wallis test are indicated on top of groups, *p*-Values from Dunn's multiple comparison posttest on top of pairs: *<0.05; **<0.01; ***<0.001.

Y, and high proportion of CD56^{dim} NKG2A⁺NKG2C⁺CD57⁺ for group Z.

NK Cell Profiles of Patients Correlated with HIV Viral Load at Inclusion

To evaluate the relationship between NK profiles of patients and HIV infection, the clinical and virological characteristics of patients within groups were compared at T0 (**Table 2**). Primary infection was defined by detectable plasma HIV-RNA and incomplete HIV-1 western blot, acute infection by one band or fewer (21) and early infection by more than one band. Four out of 14 patients from group X, 4 out of 10 patients of group Y, and all 4 patients of group Z were acutely infected (**Figure 3A**). The mean age of patients from each group was not significantly different (37,

40, and 42 years for, respectively, X, Y, and Z), nor was the mean of estimated time since infection and enrollment (36.5, 35.3, and 34.7 days for, respectively, X, Y, and Z) (**Table 2**). T-CD4 counts were similar for all groups, although group Z patients showed a trend to lower levels (**Table 2**). T-CD8 counts were significantly higher in X than Y and Z (mean of 2,148 counts/ μ L vs. 1,308 and 1,415 for, respectively, Y and Z) (**Table 2**).

A striking difference was observed when the viral load was compared between the three patient groups. Patients from groups X and Z displayed significantly higher mean HIV-RNA levels than patients from group Y (respectively, $5.77 \log_{10}$ HIV-RNA copies/mL and $6.05 \log_{10}$ HIV-RNA copies/mL vs. $4.88 \log_{10}$ HIV-RNA copies/mL in group Y), while HIV-DNA were not significantly different despite a trend to lower levels for Y (**Figure 3B**). Thus, the major differences observed between the

TABLE 2 | Groups characteristics.

Characteristics	Groups of patients		
	X	Y	Z
Number of patients	14	10	4
Acute (% in the group)	28	40	100
Time between estimated date of infection and enrollment (days)	36 (23–55)	35 (22–46)	35 (32–41)
Age (years)	37 (23–64)	40 (23–62)	42 (24–55)
CD4 counts (count/ μ L)	549 (323–1,012)	584 (368–864)	430 (341–513)
CD8 counts (count/ μ L)	2,148 (502–8,157)	1,308 (417–2,716)	1,415 (1,140–1,966)
CD4 to CD8 ratio	0.41 (0.08–1.3)	0.57 (0.23–1.1)	0.31 (0.2–0.35)
Human immunodeficiency virus (HIV)-RNA (\log_{10} cp/mL)	5.77 (4.6–7)	4.88 (3.2–5.7)	6.05 (5.6–7)
HIV-DNA (\log_{10} cp/ 10^6 peripheral blood mononuclear cell)	3.8 (3.2–4.7)	3.5 (2.8–4.3)	3.9 (3.2–4.5)
Progenitors [% of natural killer (NK)]	7.7 (3–20)	4 (0–13.9)	6.3 (0.9–9.5)
Effectors (% of NK)	49.2 (29–66)	20.8 (8–28)	0
Intermediate (% of NK)	17.5 (3.5–29)	22.7 (10–39.5)	45.7 (40.5–56)
Mature (% of NK)	6.8 (0–16)	39 (21–54)	8.2 (2.7–15)
Dysfunctional (% of NK)	15 (5.7–41)	10.6 (3.2–21.6)	11.7 (2.3–16.3)

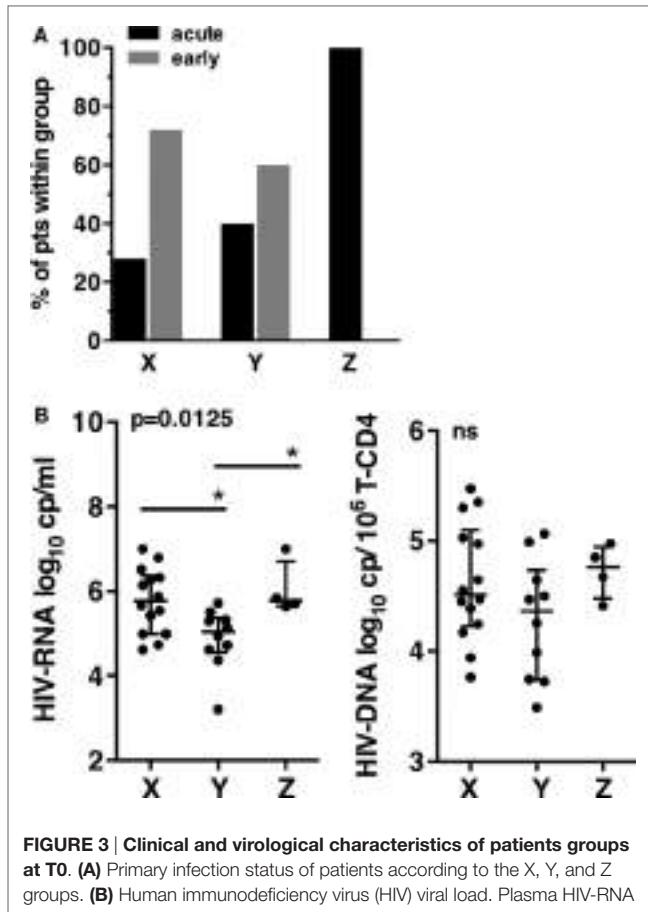
T0 mean values and (range) are indicated.

three groups of patients at inclusion highlighted the lower viral load of group Y, the group defined by an expansion of CD57⁺ NK cells.

Patients with High Frequencies of Mature NK Cells Displayed Better Immunological Parameters at Inclusion

We next addressed the question of whether patients of group X, Y, and Z were different with respect to other major disease progression markers. We analyzed plasma inflammatory markers (IL-6, IP-10) (25, 26) and also focused on markers of innate immune activation and exhaustion, including CD38 expression on NK cells, CD86 expression on monocytes, PDL-1 expression on mDC (27), and pDC and mDC frequencies (28). These markers were all described to be linked to viral load and disease progression (29). They were compared between the groups of patients and some of them were compared to a group of 15 healthy donors (Figure 4).

Patients of group X displayed higher IL-6 and IP-10 plasma levels than patients of group Y (Figure 4A). CD38 expression on NK cells was significantly increased in all patients groups as compared to healthy donors. CD86 on monocytes and PDL-1 on mDC were significantly increased in patients X as compared to healthy donors, while patients Y showed a trend to lower increase and values of PDL-1 not different from that of healthy donors (Figure 4B). All patients groups showed significant decrease of pDC as compared to healthy donors, but patients Y showed a

**FIGURE 3 | Clinical and virological characteristics of patients groups at T0.**

(A) Primary infection status of patients according to the X, Y, and Z groups. **(B)** Human immunodeficiency virus (HIV) viral load. Plasma HIV-RNA (left), HIV-DNA copies associated to millions of T-CD4 cells (right). Error bars represent the median and interquartile range. *p*-Values from Kruskall-Wallis are indicated on top of groups, *p*-Values from Dunn's multiple comparison posttest on top of pairs: **p*<0.05; ***p*<0.01; ****p*<0.001.

clear trend to higher values of pDC frequencies (Figure 4C). Only patients X and Z showed significant decrease of mDC frequencies while patients Y exhibited values similar to healthy donors (median of, respectively, 0.3 vs. 0.33%) (Figure 4C). Therefore, in addition to exhibit the lowest activation, Y patients displayed low exhaustion of DC, as shown by a trend to higher frequencies of pDC and frequencies of mDC not different from that of healthy donors (Figure 4C). As expected according to their high viremia levels, patients from groups X and Z displayed higher immune activation and exhaustion while patients Y exhibited a better immune status in PHI.

Patients with High Frequencies of Mature NK Cells Displayed a Lower Viral Load after 3 Months of cART

We then assessed the impact of the different virological and immunological status at inclusion on early cART outcome. While all patients showed a 3-log decrease of viral load after 3 months of cART (Table 2), we searched for those patients having reached a viral load below 50 copies/mL at M3. Notwithstanding the cART regimen, 6 out of 14 (57%) patients X, 1 out of 4 (25%) patients

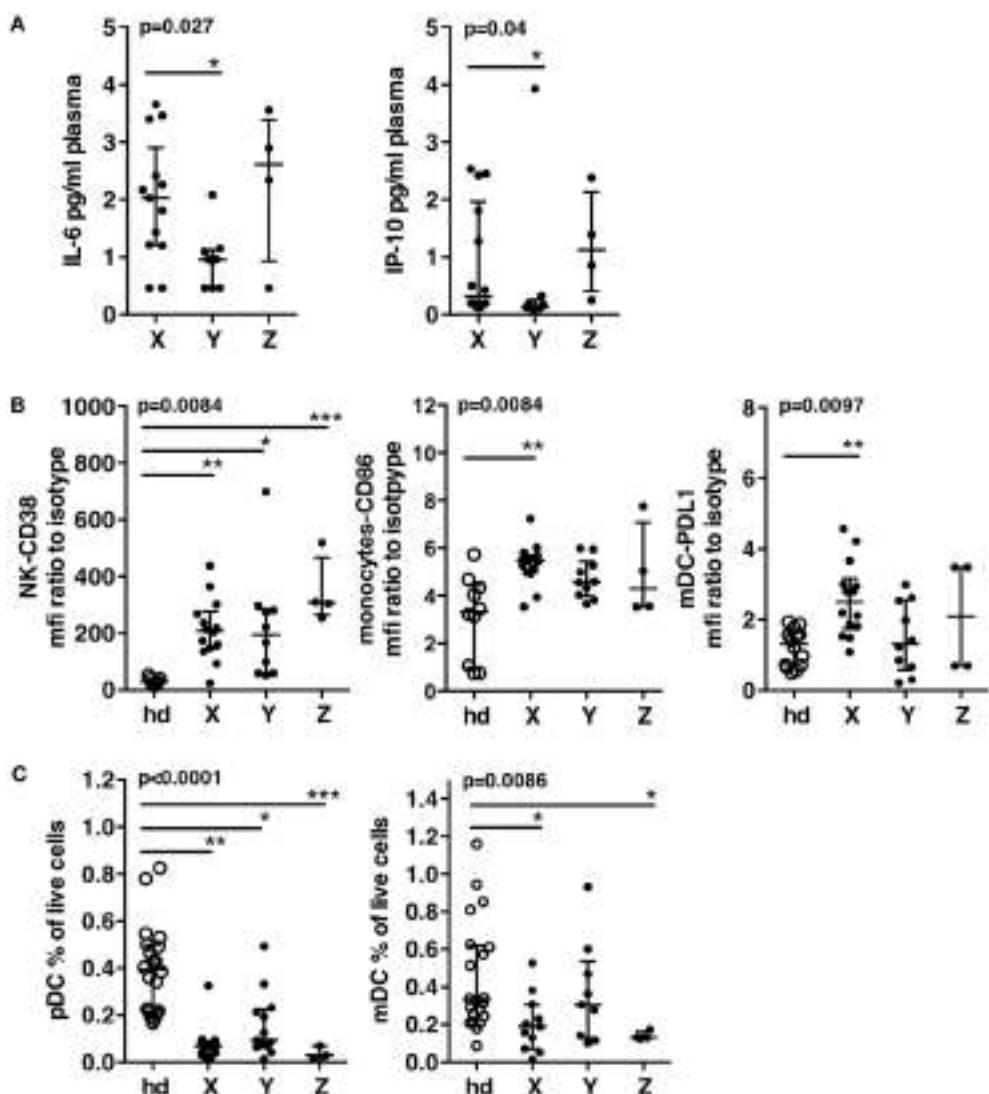


FIGURE 4 | Immune status of patient groups at T0. **(A)** Plasma levels of IL-6 and of IP-10. **(B)** Innate immune cells activation of patients groups and a group of 15 healthy donors. CD38 expression on natural killer cells, CD86 expression on monocytes, PDL-1 expression on mDC. **(C)** Dendritic cell exhaustion. Frequencies of pDC's and mDC's in the patients groups and in a group of 15 healthy donors. Errors bars represent the median and interquartile range. *p*-Values from Kruskall-Wallis test are indicated on top of groups, *p*-Values from Dunn's multiple comparison posttest on top of pairs: **p*<0.05; ***p*<0.01; ****p*<0.001.

Z, and 7 out of 10 (70%) patients Y reached a viral load below 50 copies/mL at M3. Taken together, only 38% of patients lacking significant frequencies of CD57⁺ NK cells (X + Z) reached a threshold of 50 log₁₀ HIV-RNA copies/mL at M3 whereas 70% of patients with high frequencies of CD57⁺ NK cells (Y) could reach it.

Early cART Modestly Modify the Frequencies of Mature CD57⁺ NK Cells at M3

In order to evaluate the kinetics of NK cell maturation at a short time after infection, we compared the frequencies of CD57⁺

NK cells at inclusion and after 3 months of cART in groups of patients X, Y, and Z (Figure 5). All CD57⁺ NK cells were summed. HIV-RNA showed a mean decrease of 2 logs in both groups after 1 month of treatment, one additional log being lost during the third month (left graph). In the meantime, the frequency of CD57 NK cells remained unchanged in patients from group Y (47.5% at T0 vs. 48.9% at M3), while the frequencies of CD57 populations of patients from group X and Z significantly increased, or alternatively the proportion of immature NK cells decreased (13.9% at T0 vs. 20.5% at M3 for X; 11.7 vs. 19.4% for Z). Therefore, even if the frequencies of CD57 NK cells increased during the 3 months of cART, the values reached remained far from those of patients from group Y at T0 (47.5%).

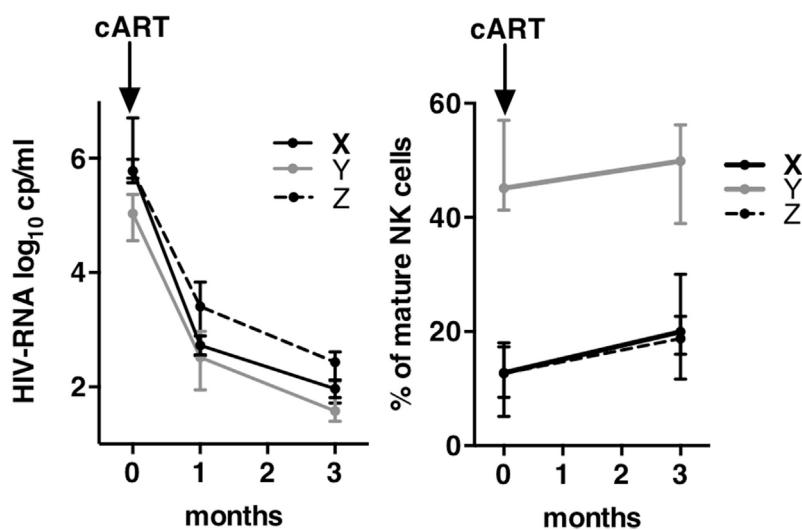


FIGURE 5 | Kinetics of viral load and of mature natural killer (NK) cells frequencies during 3 months of combination antiretroviral therapy (cART). Left graph shows the kinetics of human immunodeficiency virus-RNA between T0 and M3 after cART onset. Right graph shows the kinetics of the frequencies of mature NK cells between T0 and M3. Errors bars represent the median and interquartile range.

DISCUSSION

Natural killer cells are potent effectors of the innate immune system and key actors in the race engaged between the virus and the host. NK cell compartment is constituted by populations more or less advanced on the maturation pathway, which compose a unique landscape at an individual level. We studied the NK cell compartment of HIV-infected patients at the time of primary infection. In spite of limitations such as the number of patients and the lack of samplings before infection, our results globally support a link between NK cell compartment skewed toward maturation and decreased levels of viral load and immune activation at the time of the primary HIV infection.

While multiparametric cytometry allows deep investigation of human immune cells, discrete subsets resulting from unexpected combinations of markers are found by chance using classical manual analysis. Computation, through the consideration of all parameters at the same time allowed exploration of the full dataset and finding discrete subsets in an unsupervised approach (22). The description of the NK cells of patients into 12 subsets, whereas only six would have been searched according to previous reports (CD56^{bright}, four populations of CD56^{dim} and CD56^{neg}), provides an added value to the comprehension of the ongoing interplays between NK cell and HIV. One example is the finding of four different CD56^{neg} subsets and their clustering near four different NK cell populations, including CD56^{bright}. This result suggests that the defect affecting the NK cell can touch the lineage at different levels of maturation.

Among 12 NK populations, four co-expressed NKG2C in various combinations spanning the whole NK maturation process. The clustering of the CD56^{dim}NKG2A⁺NKG2C⁺ subset near CD56^{bright}CD16^{low}, previously described at the transition between

CD56^{bright} and CD56^{dim} (30), suggests they originate from the progenitor. NKG2C⁺ NK cell expansion was reported previously in HIV primary infection (10, 11, 16) and described during Hantavirus and Chikungunya acute infections (12, 31), always in the context of underlying CMV co-infection. The identification of discrete NKG2C⁺ NK cells subsets with a phenotype close to the NK cell progenitor suggests an ongoing generation during primary HIV infection.

The individual distribution of those 12 NK cell populations permits constitution of homogeneous and significantly different groups of patients. Group X included 14 patients whose NK cells were mainly composed of CD56^{dim}NKG2A⁺, group Y included 10 patients with high frequencies of CD56^{dim}CD57⁺ and CD56^{dim}CD57⁺NKG2C⁺, and group Z included four patients whose NK cells displayed a CD56^{dim}NKG2A⁻NKG2C⁻CD57⁻ phenotype. To understand the role of those differences on HIV primary infection, clinical, virological, and immunological characteristics of the patients groups were compared. Kinetics of HIV infection could have been involved, but this hypothesis was not sustained by the comparison of the estimated time since infection or by the status of antibodies developed to HIV, defining an acute or early primary infection. The striking difference was the viral load at inclusion: patients with high frequencies of CD56^{dim}CD57⁺ NK cells (group Y) had significantly lower levels of HIV-RNA than patients with highest frequencies of CD56^{dim}NKG2A⁺ (group X) or CD56^{dim}NKG2A⁻NKG2C⁻CD57⁻ NK cells (group Z). Although the low number of patients limits the significance of some comparisons, patients Y, as expected according to their lower levels of HIV-RNA, showed lower immune activation and lower exhaustion of immune cells than patients X and Z. cART initiated in this better environment resulted in increased efficiency at M3.

Interestingly, one of the 10 patients showing high frequencies of mature NK cells at inclusion became later a PTC (21).

CD57 is a marker of senescence highly correlated to expression of self-KIR that identifies fully mature NK cells (4). Immature KIR⁻ NK cells mostly express NKG2A to regulate their activity, only a small subset of NK cell co-express NKG2A and KIR. Accordingly, CD57⁺ and NKG2A⁺ NK cells were considered as covering, respectively, mature and immature NK populations. Accordingly, the balance of the NK cell compartment maturation is indeed the difference between the groups: NK cell of patients Y are mainly mature CD57⁺ cells whereas most NK cells of patients X are immature. Thus, the NK cell maturation profile at the time of primary infection appears to be a pertinent marker of a better immune status and response to cART started at the primary infection.

The status of the NK cells before HIV infection is not known. Because NK cell maturation is a dynamic process, imbalance of the NK cell compartment toward maturity or immaturity could be a rapid early consequence of HIV infection. CD57⁺ NK cells proliferate only slightly (4). The dynamics of their generation proposed by the follow-up of NKG2C subset during CMV reactivation in transplant patients (32) suggested that NKG2C⁺CD57⁺ resulted from the contraction of effector NKG2C⁺ several months after control of CMV viremia. During the 3 months of cART, while HIV viremia was controlled, the frequencies of CD57⁺ NK cells slightly increased in the Optiprim patients with low levels at inclusion but remained far below the values exhibited by patients with high frequencies at inclusion, suggesting that the dynamics of NK cell maturation is slower than that of viral load. An expansion of CD56^{dim} was reported during HIV primary infection (14). According to the inverse relationship between proliferation and maturation, this expansion should result in decreasing even more the frequencies of mature CD56^{dim}CD57⁺. Taking into account the limited proliferation of mature CD56^{dim}CD57⁺ and the slow dynamics of their generation, it is reasonable to speculate that the imbalance of the NK cell compartment toward maturation found at inclusion corresponded to its status at the time of HIV infection, whereas the imbalance toward immature NK cells might be a consequence of early NK cell expansion.

Accordingly, mature CD57⁺ NK cell already present at the time of HIV infection must contribute better to immune control of the virus than CD56^{dim}NKG2A⁺ NK cells does. As demonstrated in the NKG2C/CMV model, memory-like NKG2C⁺CD57⁺ NK cells expanded after CMV reactivation are potent producers of IFN- γ (8). Accumulation of NK cells expressing self-KIR was demonstrated in HIV PTCs of the Visconti study (33). Those NK cells were potent producers of IFN- γ upon stimulation with HIV-infected targets, suggesting that this function was crucial for virus control. IFN- γ can induce maturation and activation of T cells, DC, and macrophages (3), which cooperate in virus control. Indeed, DC frequencies in patients with high frequencies of mature CD57⁺ NK cells were similar to healthy donors, suggesting indeed that innate immunity overall was involved.

Paradoxically, a prospective study on a cohort of prostitutes demonstrated an increased risk of HIV infection among those displaying highly diverse NK cell repertoire before infection,

diversity being intrinsically linked to maturation (20). The high efficiency of NKG2A⁺ NK cell to kill HIV-infected CD4-T cell targets *in vitro*, recently demonstrated (34) is an underlying mechanism that could be involved in the decreased susceptibility to HIV infection of prostitutes displaying an immature NK cell compartment before infection. Taken together, these observations suggest that if immature NK cells are efficient at the time of infection, once infection is established, other mechanisms involving mature CD57⁺ NK cells contribute better to virus control.

Natural killer cell diversity increases with aging and the number of stimulations encountered, resulting in accumulation of mature NK cells heterogeneous with respects to functional activating and inhibitory receptors (20). Groups of patients showed similar mean age, their NK cell profiles should rather be attributed to individual histories resulting in a unique shaping of their NK cell repertoire. NKG2C⁺CD57⁺ NK cell could be considered as a particular subset of the mature CD57⁺ NK cell compartment whose ligand is known. Increased expression of HLA-E on HIV-infected cells suggest a possible mechanism involving NKG2C⁺ NK cells (35). Other subsets of the mature CD57⁺ pool might share unknown receptor specificities diversely able to target HIV-infected cells. Besides their functional receptors characteristics, NKG2C⁺CD57⁺ NK cells expanded after CMV infection are now considered as a memory-like or adaptive form of NK cells (8, 13). Recently, this subset has been characterized by a low expression of FcR γ (36) and enhanced potential for broad antiviral responses in the presence of virus-specific antibodies (37). While we cannot identify unambiguously adaptive NK cells, the CD57⁺ NK cells found in patients partially controlling their viral load certainly overlap them. Irrespective of the various mechanisms possibly underlying the contribution of mature NK cell to HIV control, once infection is established, evaluation of this parameter during the primary HIV infection appears relevant in the search for prognostic markers to monitor HIV-infected patients.

AUTHOR CONTRIBUTIONS

AC, LM, and CR were the chief investigators of the OPTIPRIM study. AC, CG, and GP enrolled the patients. LM coordinated the data collection. DO and FG-R conceived and designed the innate immunity sub-study. FG-R and FM performed the multiparametric cytometry experiments. GB and SG developed bioinformatics tools of analysis. VA-F and CR were responsible for the virological investigations. CL, MM-T, and MP were responsible for immune investigations. FG-R, DO, AC, CR, and AM interpreted the data. FG-R generated the figures and tables. FG-R and DO wrote the paper. All authors reviewed, revised, and approved the final manuscript. AC, CR, LM, VA-F, and DO were part of the OPTIPRIM scientific committee.

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Reduction of Relapse after Unrelated Donor Stem Cell Transplantation by KIR-Based Graft Selection

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Besides donor T cells, natural killer (NK) cells are considered to have a major role in preventing relapse after allogeneic hematopoietic stem cell transplantation (HSCT). After T-cell-depleted haploidentical HSCT, a strong NK alloreactivity has been described. These effects have been attributed to killer-cell immunoglobulin-like receptors (KIR). Abundant reports suggest a major role of KIR not only on outcome after haploidentical HSCT but also in the unrelated donor setting. In this review, we give a brief overview of the mechanism of NK cell activation, nomenclature of KIR haplotypes, human leukocyte antigen (HLA) groups, and distinct models for prediction of NK cell alloreactivity. It can be concluded that KIR-ligand mismatch seems to provoke adverse effects in unrelated donor HSCT with reduced overall survival and increased risk for high-grade acute graft-versus-host disease. The presence of activating KIR, as seen in KIR haplotype B, as well as the patient's HLA C1/x haplotype might reduce relapse in myeloid malignancies.

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INTRODUCTION

Natural killer (NK) cells are considered to contribute important immune effects against leukemia [graft-versus-leukemia (GVL) effect] after allogeneic hematopoietic stem cell transplantation (HSCT). Alloreactive NK cells are considered rather save concerning the development of graft-versus-host disease (GVHD) (1–5), although a high number of activating killer-cell immunoglobulin-like receptors (KIR) (6) or extensive NK cell stimulation (7) might promote GVHD, maybe due to remaining T-cells in the graft. Shah et al. (7) found an association between infusion of activated NK cells and occurrence of acute GVHD (aGVHD): Children with ultrahigh-risk sarcoma received T-cell-replete grafts from matched unrelated donors (URDs) or matched sibling donors with subsequent infusion of IL-15 and 4-1BBL preactivated NK cells. Five of nine patients developed aGVHD. Those effects were attributed to NK cell-mediated T-cell activation (7). The biology of NK cells is complex, but activation by human leukocyte antigen (HLA) via the group of KIR is considered to be a relevant mechanism of activation. Within this review, we will provide a summary of concepts of KIR-mediated NK cell activation and an overview of GVL effects in haploidentical (haplo), but especially in URD HSCT.

Biology and Activation of NK Cells

Natural killer cells were named after their ability to kill infected or tumor cells without the need for prior antigen contact (8–10). They are defined by surface expression of CD56 and lack of CD3 (11). Unlike T cells, NK-cell receptors do not undergo rearrangement. In a process called licensing, NK

cells with inhibitory receptors for present HLA class I (HLA-I) molecules (indicating “self”) are positively selected and stimulated for proliferation, leading to a licensed and self-tolerant subset. Missing inhibitory receptors against HLA-I do not lead to depletion but to a second subset of unlicensed but self-tolerant NK cells (12). Activation of NK cells might be initiated by antigen contact, but it is executed only after integration of abundant activating and inhibitory signals (13, 14). Today, several NK-cell receptors are known. Besides KIR, other NK-cell receptors that have been shown to have the potential to positively influence outcome after allogeneic HSCT are natural cytotoxicity receptors (15–17) as well as activating NKG2D (18) and DNAM-1 (19, 20) that bind to MICA/B and ULBPs or CD112/CD155, respectively. Both can be induced by DNA damage (21) and seem to play a role in negative regulation of T-cell responses (22) and acute myeloid leukemia (AML)/myelodysplastic syndrome immune evasion (15, 23).

KIR and HLA

Killer-cell immunoglobulin-like receptors belong to type-I transmembrane proteins of the immunoglobulin-like receptor superfamily and recognize classical HLA-I molecules (14). The 15 KIR genes and 2 pseudogenes are located on chromosome 19q13.4. According to the number of extracellular immunoglobulin-like domains (D), the receptors are named KIR2D and KIR3D (24, 25). On the cytoplasmic side, they have either long (L) inhibitory or short (S) activating domains (14). Inhibitory KIR bind to the highly polymorphic regions of HLA-I molecules: HLA-A, B, and C (26), while the ligands for activating KIR are poorly defined (14, 27).

To facilitate description of KIR-ligands, HLA-C phenotypes can be grouped into HLA-C group 1 and 2 according to their respective KIR-binding motif. HLA-C group 1 contains all ligands with serine at residue 77 and asparagine at residue 80 of the α 1 helix (HLA-C^{asn80}), binding KIR2DL2/3 and 2DS2. Members of this group are HLA-C*01/*03/*07/*08/*12/*14/*16. HLA-C group 2 (HLA-C^{lys80}) has asparagine at residue 77 and lysine at residue 80 and contains HLA-C*02/*04/*05/*06/*15/*17/*18. They are ligands for KIR2DL1 and KIR2DS1 (28–31).

KIR3DL1 binds HLA-Bw4, and KIR3DL2 and 2DS2 bind HLA-A3 and A11 (14, 18, 32–38). Despite its structure, KIR2DL4 exhibits activating capacities and might bind soluble HLA-G (39–45). The KIR phenotype of an individual is his or her distinct set of inhibitory or activating KIR with an underlying distinct genotype (27, 46, 47). All genotypes can be summarized to a set of distinct haplotypes, which again result in the superordinated KIR haplotypes A or B (27, 46). KIR haplotype B is defined as the presence of KIR2DL5, 2DS1/2/3/5, or 3DS1, which have to be absent in KIR haplotype A (48). KIR2DS4 is the only activating KIR in haplotype A (46). KIR haplotype B/x (B/B or B/A) is found in about 30% of the Caucasian population (49). A more detailed analysis includes the information, whether the individual KIR is coded in the centromeric (Cen) or telomeric (Tel) gene motif of the KIR locus, resulting in Cen-A/A, Cen-B/x, and the respective Tel haplotypes (49–52). Thus, each individual expresses a certain KIR haplotype and a distinct HLA-C haplotype (C1/C1, C1/C2, or C2/C2). For prediction

of alloreactive NK cell effects, the presence of HLA-C1, C2, and Bw4, as well as their respective KIR, are investigated (53). KIR2DL4 stimulation by HLA-G is considered to induce tolerance at the maternal–fetal barrier as well as IFN-gamma release of NK cells but not cytotoxicity (39, 43). KIR3DL2 and 2DS2 stimulation by HLA-A3 and A11 is also not in the primary focus of altering NK cell alloreactivity. KIR3DL2 has been identified as a surface marker in cutaneous T-cell lymphoma (54–56). For KIR2DS2, a reduced survival after URD-HSCT is suspected due to higher incidence of GVHD (57).

Model Situations Predicting NK Cell Alloreactivity

Different definitions of a mismatch between the donor’s NK cells and the recipient’s HLA exist, depending on the method that was chosen for KIR and HLA (HLA-C1, C2, and Bw4) evaluation (Figure 1).

Missing-Self/KIR-Ligand Mismatch (Figure 1A)

Alloreactivity was initially thought to be only dependent on lack of inhibitory HLA-I molecules in the recipient that are present in the donor (“missing-self” or “KIR-ligand mismatch” or “ligand-incompatibility model”) (53, 58–60). For evaluation of KIR-ligand mismatch, donor and recipient are screened for expression of HLA: NK cells from a HLA C1/C1 donor will be alloreactive against a C2/C2 recipient. If a recipient expresses HLA-C1, C2, and Bw4, he will be resistant toward NK cell killing, as seen in one-third of the population (61). It is assumed, but not verified that the respective KIR, necessary for alloreactivity, is present in the donor.

Receptor-Ligand Mismatch (Figure 1B)

The receptor-ligand model states that donor NK cells become activated in the graft-versus-host direction; if they have inhibitory KIR, for which the HLA ligands in the recipient are missing, the NK cells become “uninhibited” (4). Thus, in addition to the HLA status of the recipient, confirmatory KIR genotyping of the patient is required. Other than in the first model, KIR on donor cells and HLA on recipient cells are investigated, not “assumed.” This model can be considered as an improvement of the “missing ligand model.”

Missing Ligand Model (Figure 1C)

Here, only the recipients’ HLA are genotyped, and missing HLA-C1, C2, or Bw4 for inhibitory KIR predict an alloreactivity of the graft; the presence of the respective KIR that would bind to the missing HLA is only assumed (53).

Presence of Activating KIR (Figure 1D)

Some theories emphasize that to achieve NK cell alloreactivity, “un-inhibititon” of NK cells by missing inhibitory HLA ligands might not be sufficient. Activation requires additional stimulation of activating KIR in the graft (62). In this model, alloreactivity can be predicted by measurement of activating KIR on donor cells. Some studies increase the predictive validity by detection of the respective activating ligands on donor cells.

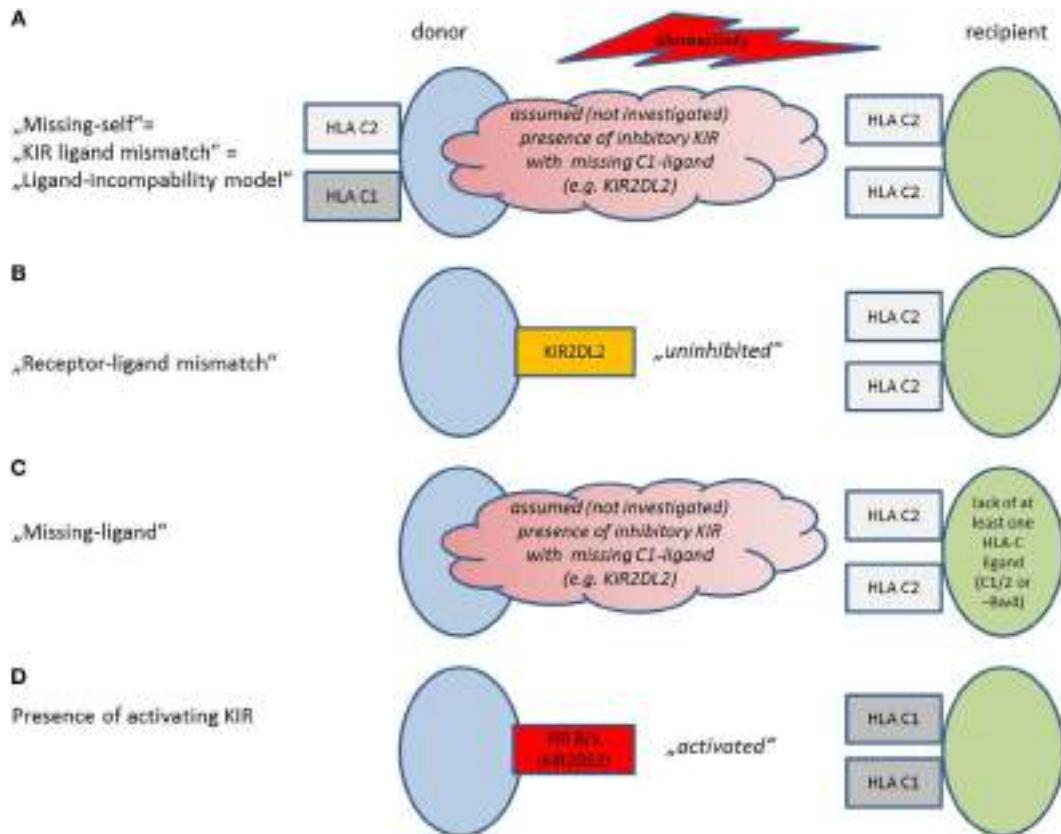


FIGURE 1 | Model situations that provoke natural killer (NK) cell alloreactivity. Models are depicted as used in the present review, adopted and modified from Symons and Fuchs (53). Details concerning the activation mechanism are provided in the text. **(A)** Missing-self model, also described as “killer-cell immunoglobulin-like receptors (KIR)-ligand mismatch” or “ligand-incompatibility model”: Potential alloreactivity in the graft-versus-host direction is predicted by investigation of human leukocyte antigen (HLA) on donor and recipient. An HLA for inhibitory KIR that is present in the donor lacks in the recipient. The presence of the respective inhibitory KIR in the donor is assumed but not verified. **(B)** Receptor–ligand mismatch: NK cells become activated in the graft-versus-host direction, if they have an inhibitory KIR, for which the HLA ligand in the recipient is missing. Thus, the NK cells are “uninhibited.” Other than in **(A)**, KIR on donor cells and HLA on recipient cells are investigated, not “assumed.” **(C)** Missing ligand: If the presence of the respective inhibitory KIR is not evaluated, but assumed in a model where at least one HLA-ligand is missing (HLA-C1/2 or Bw4). Other than in **(A)**, only HLA on recipient cells but not on donor cells are evaluated. **(D)** The presence of activating KIR predicts alloreactivity in the presence of the respective activating ligand. KIR haplotype B/x contains more activating KIR than KIR haplotype A/A.

Therefore, we determine mismatches on the donor and recipient side between ligand–ligand, receptor–ligand, and receptor–receptor or identify activating KIR in the donor (49, 53). HLA and KIR can be investigated by genotyping, phenotyping, or functional NK cell assays to predict alloreactivity. We would suggest to follow the well-described methods of Ruggeri et al. for genotyping and phenotyping (61).

All approaches were initially tested in the haploidentical setting: The Perugia group suggests donor and recipient HLA typing to identify mismatch (2), followed by confirmatory donor KIR typing to verify a mismatch between donor KIR and recipient HLA ligand (“KIR-ligand mismatch” combined with a “receptor–ligand” concept) (61). They found ligand incompatibility between donor and recipient in haploidentical HSCT to be associated with increased GVL effects and lower relapse in acute leukemia (2, 4). KIR-ligand mismatch can be prevalent either in the graft-versus-host direction when the donor’s KIR ligand is not shared

by the recipient or in the host-versus-graft direction when the recipient’s KIR ligand is not present in the donor. The St. Jude group rather focuses on receptor–ligand mismatch in the haplo setting (63), while the researchers from Minnesota implemented their strategy for the URD setting by selecting KIR B/x donors for HLA-C1-positive recipients for improved alloreactivity (50, 64, 65).

EVIDENCE OF NK CELL-MEDIATED GVL EFFECTS

Lessons Learned from the Haploidentical HSCT Setting

Much knowledge concerning NK cell-mediated alloreactivity has been collected due to the implementation of haploidentical HSCT. To reduce the risk of GVHD, T-cell depletion was

performed before graft infusion at the cost of graft rejection (66). These effects could be partially overcome by infusion of high numbers of stem cells (67). Ruggeri et al. were the first to show NK cell-mediated alloreactivity in the T-cell-depleted haploidentical graft (5). Facilitated engraftment as well as tumor lysis by NK cells occurred by donor grafts that were KIR-ligand incompatible in the graft-versus-host direction without occurrence of GVHD. Since then, many other groups have investigated the beneficial effect of alloreactive NK cells in the haploidentical HSCT (2, 68) and have refined criteria for potential donor choice (61). The results are promising for AML (4, 68, 69), while lymphoid malignancies have been shown to be resistant in some (2, 69) but not all cases (63, 70) for KIR-mediated NK cell effects. The present status of NK cell-mediated effects in haploidentical HSCT has been reviewed elsewhere (53, 71, 72).

Results in the Unrelated-Donor HSCT Setting

After the identification of beneficial NK cell-mediated alloreactivity in haploidentical HSCT, efforts were made to adopt the findings for transplantations with URD (**Table 1**). Even though many patients already have the opportunity to receive a graft from an HLA-matched donor, donor choice by KIR repertoire is useful. Since HLA and KIR are inherited separately, approximately 75% of HLA-identical sibling donors and almost 100% of matched URDs will show KIR disparities and might therefore be a potential source for alloreactive NK cells (73, 74).

KIR-Ligand Mismatch Seems to Induce Adverse Effects in URD HSCT

Davies et al. (75) were the first to perform a retrospective analysis of patients with HLA mismatched URD HSCT, comparing KIR-ligand mismatch. In the analysis, no difference in any of the primary endpoints was achieved. Concerning the subgroup of myeloid malignancies, KIR-ligand mismatch resulted in worse OS at 5 years [13 versus 38%, $P < 0.01$, no use of antithymocyte globulin (ATG)], which was even more surprising. Others confirmed worse outcome for KIR-ligand mismatch in URD HSCT after conditioning with ATG (76–79) or without ATG (80), accompanied with higher infections in the early posttransplant period (78) or increased graft rejection, TRM, and GVHD (80). A recent study confirmed higher mortality and higher TRM without difference in relapse in 3–5/8 HLA-mismatched KIR-ligand mismatched (in the host-versus-graft direction) unrelated cord blood transplants for AML and acute lymphoid leukemia (ALL) compared to KIR-matched cord blood, while no difference was found for mismatch in the graft-versus-host direction or in a higher HLA-matched subgroup or the complete patient cohort (81). The authors did suggest to not using KIR-ligand mismatch as a criterion for cord blood selection. An earlier Eurocord study (82) detected favorable outcome for KIR-ligand mismatched transplants in AML and ALL but used lower HLA-resolution techniques.

No difference in mortality after either KIR-ligand mismatched or HLA-mismatched but KIR-ligand matched donor-recipient pairs was detected by a comprehensive study of CIBMTR, EBMT, and the Dutch transplant registry (83), investigating the results of 1,571 patients with myeloid malignancies with or without T-cell depletion. KIR-ligand mismatch was associated with significantly higher high-grade aGVHD, just as HLA mismatch at HLA-C and/or B. No predictive effects of KIR-ligand mismatch on outcome after T-cell-repleted unrelated HSCT were detected in a retrospective multicenter study in France (84). Here, different models of NK cell alloreactivity were compared in a very heterogeneous cohort of patients. These investigations were partially designed as a response to the positive results in haploidentical HSCT and in a previous study by Giebel et al. (85) with different results: KIR-ligand mismatch in patients with myeloid malignancies achieved significant higher OS and RFS as well as lower TRM and relapse compared to HLA mismatch with KIR ligand match or compared to matched URD HSCT with the use of pretransplant ATG. The differing results could be only partially attributed to the use or sparing of ATG (85): Although toxic (86) or immunosuppressive (87, 88) on NK cells, ATG has been shown to accelerate NK-cell and B-cell reconstitution in some (89) but not all investigations (90, 91). It has also been shown to decelerate the recovery of CD4+ and CD8+ T cells (89, 91) while sparing effector-memory T cells and T-regulatory cells (91). The results indicated that knowledge from haploidentical cannot be transferred to unrelated HSCT without further adaptations (75). Grafts for haploidentical HSCT were mainly highly T-cell depleted and performed with high stem-cell doses as well as no or low immunosuppression, resulting in fast NK cell but slow T-cell reconstitution with low T-cell numbers and eradication of antigen-presenting cells by alloreactive NK clones (2, 67, 92). Therefore, the immunological environment during engraftment in haploidentical HSCT is much different from URD-HSCT.

Missing-Ligand Model and Presence of Activating KIR Are Predictive for Outcome

Later, Hsu et al. (60) identified not only KIR-ligand mismatch but also missing KIR ligands as protective against relapse in HLA mismatched but not in matched URD HSCT. These effects were seen in myeloid and lymphoid malignancies and supported by later investigations by other authors (93). In the study by Hsu et al. (60), the absence of HLA-C2 or HLA-Bw4 KIR ligands was associated with lower relapse. Other authors confirmed the impact of HLA-C2: Absence of HLA-C2 in recipients of KIR2DL1-positive grafts resulted in higher incidence of aGVHD after myeloablative (94) as well as reduced intensity (95) conditioning. The absence of C1 epitopes, as seen for C2/C2 recipients, has been claimed responsible for poorer outcome (57, 65, 96). In search for favorable KIR in URD HSCT, Sun et al. (97) prospectively analyzed outcome of URD AML patients without *in vivo* T-cell depletion by ATG. According to the presence or absence of activating or inhibitory KIR in donor and recipient, they calculated a new predictive algorithm

TABLE 1 | Studies on NK cell alloreactivity for unrelated donors.

Reference	N	Median age (years) ^a	Disease (n)	Tx (n)	Model	Conditioning and graft source	Immunosuppression	Main results
Davies et al. (75)	175	17	CML, AML, ALL, MDS, others	MMUD (175)	KIR-L MM ^b	Myeloablative • BM	TCD or CSA ± MTX	Adverse KIR-L MM in myeloid malignancies: Lower OS at 1 and 5 years ($P < 0.01$). No difference between KIR-L M/MM in any endpoint for total cohort
Schaffer et al. (76)	104	29	Diverse	MUD (62)/ MMUD (42)	KIR-L MM	Myeloablative • BM (80) • PB (24)	MTX + CSA, ATG	Adverse KIR-L MM: Reduced OS and RFS
Giebel et al. (85)	130	18–20.5	Diverse	MUD (61)/ MMUD (49)	KIR-L MM	Myeloablative • BM (125) • PB (5)	CSA, MTX, ATG	Beneficial KIR-L MM: Higher OS and RFS ($P = 0.0007$; 4.5 years). No influence of HLA-MM in the patients without KIR-L MM
Bornhauser et al. (77)	118	42–44	AML, CML, MDS	MUD (54)/ MMUD (64)	KIR-L MM	Myeloablative • BM (54) • PB (64)	ATG (118)	Adverse KIR-L MM: Higher relapse for KIR-L MM ($P = 0.02$), but no difference in survival after KIR-L MM, MUD, and MMUD transplantation
Schaffer et al. (78)	190	35–39	Diverse	MUD (94)/ MMUD (96)	KIR-L MM	Myeloablative (168) RIC (22) • BM (118) • PB (72)	CSA based (179) or TCD (11) plus ATG (all)	Adverse KIR-L MM: Higher infections, leading to increased TRM and reduced OS ($P = 0.01$), but no increase of relapse or GVHD
Venstrom et al. (96)	1,277	40.5–41.7	AML	MUD (664)/ MMUD (613)	Missing ligand Receptor-ligand KIR genes	Myeloablative (1,069) RIC/NMA (189) • BM (689) • PB (588)	Diverse, no ATG	Adverse absence of C1: HLAC2/C2 recipients have higher relapse than HLAC1/x recipients ($P = 0.05$) Beneficial KIR2DS1 from C1/x donor associated with lower relapse compared to absence of KIR2DS1 ($P = 0.003$) and lower mortality ($P = 0.04$) w/o higher high-grade aGVHD or TRM Beneficial KIR3DS1 associated with lower mortality ($P = 0.01$) by lower TRM and aGVHD No predictive effects in ALL patients (separate cohort)
De Santis et al. (80)	104	24	Diverse	MMUD (104)	KIR-L MM	Myeloablative • BM (65) • PB (39)	No ATG BM: CSA, MTX (59), T-cell depletion (9) PB: No CSA (39)	Adverse: KIR-L MM (HVG): Increased graft rejection Adverse KIR-L MM (GVH): Increased aGVHD grade 3–4 Adverse KIR-L MM (GVH or HVG): Increased TRM, decrease RFS Beneficial high number of donor KIR: Lower GVHD and improved survival
Giebel et al. (57)	111	18.5–21	Diverse	MUD (90)/ MMUD (21)	Missing ligand	Myeloablative • BM (96) • PB (15)	CSA, MTX, ATG	Adverse absence of C1: C2/C patients have lower OS and DFS, due to higher relapse
Sun et al. (97)	65	45–46	AML	MUD (39)/ MMUD (26)	Receptor-receptor	Diverse	CSA + MTX (65) No ATG or TCD	Prediction of incidence of aGVHD possible: Activating KIR in the donor that lack in recipient and the lack of inhibitory KIR in the donor that are present in the recipient predict increased aGVHD Indifferent results for KIR-L MM, missing ligand, number of activating KIR

(Continued)

TABLE 1 | Continued

Reference	N	Median age (years) ^a	Disease (n)	Tx (n)	Model	Conditioning and graft source	Immunosuppression	Main results
Giebel et al. (98)	25	27	ALL, AML, MDS, CML, NHL	MUD (23)/ MMUD (2)	KIR genes	Myeloablative • BM (20) • PB (7)	CSA, MTX, ATG	Adverse presence of KIR2DS1: Reduced OS and DFS due to increased GVHD and relapse <i>Indifferent</i> presence of KIR2DS1
Kröger et al. (79)	142	33	AML, MDS, CMML, CML, ALL	MUD (103)/ MMUD (39)	KIR haplotype KIR-L MM	Myeloablative • BM (67) • PB (75)	ATG, CSA, MTX	Adverse KIR B/x: Higher relapse than KIR A/A ($P = 0.03$), but only in AML/MDS/CMML/CMML, not ALL, resulting in lower OS Adverse KIR-L MM: Higher TRM, lower OS, no increase of GVHD Adverse KIR3DS1, 2DS1, 2DS5 in UVA, only 2DS5 in MVA, all resulting in higher relapse
Farag et al. (83)	1,571	59–68	AML, MDS, CML	MMUD KIR-L MM GVH (137) MMUD KIR-L MM HVG (170) MMUD KIR-L M (260) MUD (1,004)	KIR-L MM	Myeloablative • BM	± T-cell depletion	<i>Indifferent</i> KIR-L MM: For KIR-L MM (GVH/HVG) as well as KIR-L M but HLA MM at HLA B ± C versus HLA- and KIR-L M grafts: Same rates of increased aGVHD grade 3–4, TRM, treatment failure, and overall mortality compared to HLA- and KIR-L matched grafts
Hsu et al. (60)	1,770	34.5–35	AML, MDS, CML, ALL	MMUD (1,190)/ MUD (580)	Missing ligand KIR-L MM	Myeloablative • BM or PB	T-cell replete grafts	<i>Beneficial: missing ligand</i> in MMUD (defined as homozygosity of recipient HLA-B or C epitopes) resulting in lower relapse ($P = 0.004$), but not for MUD <i>Absence of HLA-C2 or Bw4 associated with reduced relapse</i> , no survival benefit <i>Indifferent</i> KIR-L MM model in subgroup of 428 patients: no difference in relapse (but also not with applied missing-ligand model in same subgroup $P = 0.07$)
Miller et al. (93)	2,062	–	AML, CML, MDS	MMUD/ MUD	Missing ligand	-	± ATG or TCD	<i>Beneficial absence of one ligand</i> in early stage AML or MDS: reduced relapse, independent from HLA match (C1/C2/Bw4) <i>Adverse absence of ≥1 ligand</i> in CML: Increased late-onset high-grade acute GVHD
Willemze et al. (82)	218	12.8–15	AML, ALL	MUD (42)/ MMUD (176)	KIR-L MM	RIC (202) Myeloablative (6) • CB (single)	CSA based (174) Other (44)± ATG (196)	<i>Beneficial</i> KIR-L MM: Improved DFS, OS, and decrease relapse
Gagne et al. (84)	264	24.5	Diverse	MUD (164)/ MMUD (100)	KIR-L MM Missing ligand Receptor-ligand Receptor-receptor	Myeloablative • BM	Unmanipulated BM	<i>Indifferent</i> KIR-L MM <i>Adverse missing-ligand</i> : Decreased survival but only in C1-deficient recipients, in myeloid malignancies <i>Adverse receptor-ligand mismatch</i> : KIR3DL1 as well as KIR3DL1/3DS1 mismatch (GVH: D+ R-, absence of recipient HLA-Bw4) from a HLA-Bw4-negative donor is correlated with low OS in HLA-identical and high relapse in MMUD HSCT

(Continued)

TABLE 1 | Continued

Reference	N	Median age (years) ^a	Disease (n)	Tx (n)	Model	Conditioning and graft source	Immunosuppression	Main results
Ludajic et al. (94)	124	42	Diverse	MUD	Missing ligand	Myeloablative (90) RIC (34) <ul style="list-style-type: none">• BM (54)• PB (70)	CSA-based (124) ± ATG (30)	Adverse absence of HLA-C2 in recipients of KIR2DL1-positive grafts or KIR A/A grafts: Increased aGVHD Beneficial absence of HLA-C2 in recipients of KIR2DS2-positive grafts: Decreased aGVHD
Cooley et al. (64)	448	33–34	AML	MUD (209)/MMUD (239)	KIR haplotype KIR-L MM	Myeloablative <ul style="list-style-type: none">• BM (397)• PB (51)	T-cell replete MMUD grafts	Beneficial KIR B/x in KIR-L M HSCT: Compared to KIR A/A higher RFS in KIR-L M (MUD and MMUD) but not in KIR-L MM (MMUD) Beneficial survival rates for KIR2DL2 and 2DS2 positive grafts
Cooley et al. (50)	1,409	19/39	ALL, AML	MUD (687)/MMUD (722)	KIR haplotype	Myeloablative <ul style="list-style-type: none">• BM (942)• PB (467)	T-cell replete MMUD grafts	Beneficial KIR B/x: Higher RFS in AML but not ALL Cen-B motifs improve outcome without increased aGVHD/cGVHD or TRM
Venstrom et al. (99)	1,087	35.3–37.5	AML, MDS, CML, ALL	MUD (670)/MMUD (417)	KIR genes KIR haplotype	Myeloablative <ul style="list-style-type: none">• BM (1,050)• PB (37)	CSA (751) No CSA (120) TCD (216)	Beneficial presence of KIR3DS1: Same rate of relapse but reduced TRM and aGVHD, resulting in lower mortality in AML and MDS. Beneficial effects increase with copy numbers of donor KIR3DS1 Beneficial effect of KIR B/x (including KIR3DS1) similar but weaker
Kröger et al. (100)	118	51	MM	Unrelated (81) Related (37)	KIR haplotype	Myeloablative (12) RIC (106) <ul style="list-style-type: none">• BM (13)• PB (105)	ATG (110)	Beneficial KIR B/x in MUD: MUD but not MMUD haplotype B/x reaches lower 1-year relapse than haplotype AA ($P = 0.005$), resulting in higher 5-year DFS ($P = 0.009$).
Venstrom et al. (96)	1,277	40.5–41.7	AML	MUD (664)/MMUD (613)	Missing ligand Receptor-ligand KIR genes	Myeloablative (1,069) <ul style="list-style-type: none">• BM (689)• PB (588)	CSA (346) Tac (428) TCD (348)	Adverse absence of C1 and beneficial KIRSDS1: Reduced risk of relapse, if the allograft was derived from an HLA-C1/x donor Beneficial presence of KIR3DS1: Not lower relapse but reduced TRM and aGVHD, resulting in lower mortality in AML
Cooley et al. (65)	1,532	Adults and children	AML	MUD (856)/MMUD (676)	KIR haplotype KIR gene content Missing-ligand	Myeloablative	T-cell replete MMUD grafts	Beneficial KIR B/x, adverse absence of C1: Relapse protection improved by high KIR-B content in recipients HLA-C1/x but not C2/C2 (significant only in MMUD, not MUD). No effect of donor HLA
Sobecks et al. (95)	909	56–57	AML, MDS	MUD (712)/MMUD (197)	Missing ligand	RIC <ul style="list-style-type: none">• BM (169)• PB (740)	Diverse ± ATG (317)	Adverse KIR2DS1 educated in a C2/C2 donor: Higher GVHD and TRM without reduced relapse (AML) Adverse ≥1 missing ligand or absence of HLA-C2: Higher aGVHD (AML) Indifferent KIR centromeric gene content or donor activating KIR
Faridi et al. (49)	281	50	AML, ALL	MSD (153)/MUD (128)	Comparison of different models	Myeloablative <ul style="list-style-type: none">• BM (10)• PB (271)	ATG, CSA, MTX	Adverse KIR-KIR mismatch: Increased cGVHD in HLA C1/x recipients Beneficial ≥1 missing ligand: Reduced relapse without improved OS Indifferent results for KIR B

(Continued)

TABLE 1 | Continued

Reference	N	Median age (years) ^a	Disease (n)	Tx (n)	Model	Conditioning and graft source	Immunosuppression	Main results
Bachanova et al. (101)	614	48–52	NHL	MUD (396)/ MMUD (218)	KIR haplotype (253) RIC (361)	Myeloablative • BM (227) • PB (387)	Diverse	Beneficial KIR B/x in MUD HSCT: Lower relapse after 5 years compared to KIR A/A donors ($P = 0.5$) with improved progression-free survival ($P = 0.007$)
Rocha et al. (81)	461	Adults and children	AML	MMUD (461)	KIR-L MM	Myeloablative • CB (single)	With (145) or w/o (35) in vivo T-cell depletion in 3–5/8 MM cohort	Adverse: KIR-L MM (HVG); At 3–5/8 HLA-MM level: Higher mortality ($P = 0.008$) and NRM ($P = 0.008$), no difference in relapse or GVHD in KIR-L MM versus KIR-L M (HVG) for AML and ALL Indifferent KIR-L MM (GVH): No differences in GVH direction or higher HLA-matched subgroup or complete patient cohort

ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globulin (rabbit); BM, bone marrow; Cen, centromeric; CML, chronic myelomonocytic leukemia; CSA, cyclosporine A; GVHD, graft-versus-host direction; GVHD, graft-versus-host disease (a, acute; c, chronic); HD, Hodgkin's disease; HLA, human leukocyte antigen; HVG, host-versus-graft direction; KIR, killer-cell immunoglobulin-like receptors; KIR-L M, KIR-L ligand mismatch; KIR-L MM, KIR-L ligand mismatch; M, matched; MDS, myelodysplastic syndrome; MM, multiple myeloma or mismatch; MSD, matched sibling donor; MMUD, mismatched unrelated donor, defined as HLA 10/10 – 8/8 or not further specified; MTX, methotrexate; MUD, matched unrelated donor; MVA, multivariate analysis; NMA, non-myoeloblastic; N.S., not significant; Tel, telomeric; PB, peripheral blood stem cells; RIC, reduced intensity conditioning; TCD, T-cell depletion; Tx, transplantation details; UVA, univariate analysis.

^aMedian age was not always declared for the total cohort but only the investigated subgroups. In this case, age-age does not describe a range but the youngest and oldest median age stated.
^bKIR-L MM in the GVH direction unless stated otherwise.

for GVHD, in which an inhibitory KIR in the donor that lacks in the patients has a negative value *vice versa* a positive value. On the other hand, they could not find other models such as KIR-ligand, missing-ligand, or high numbers of activating KIR to be predictive for aGVHD (97). In general, among the activating receptors, the presence of KIR2DS2 has been shown to be associated with lower OS and DFS as well as higher incidence of GVHD, resulting in high TRM (98). The alloreactivity of KIR2DS1 educated in a C2/C2 donor results in higher GVHD and TRM without reduced relapse (95). KIRSDS1 has been claimed responsible for reduced risk of relapse, if the allograft was derived from an HLA-C1/x donor (96), but did not show any beneficial effects in other investigations (98). The presence of KIR3DS1 was not associated with lower relapse but reduced TRM and aGVHD, resulting in lower mortality in AML patients (96, 99). KIR3DL1 and KIR3DL1/3DS1 mismatch in the GVH direction (donor positive, recipient negative, absence of recipient HLA-Bw4) from a HLA-Bw4-negative donor is correlated with low OS in HLA-identical and high relapse in HLA-mismatched URD HSCT (84). There are several other investigations apart from the environment of URD HSCT, which might be even more conflicting and difficult to transfer. Our early investigations showed the low-alloreactive KIR haplotype A to be associated with lower relapse after HSCT for leukemia (79), while in a later analysis, KIR haplotype B was associated with improved PFS and OS in patients with multiple myeloma (100). Cooley et al. (50, 64, 65) systematically investigated the influence of the KIR haplotype B. In summary, a high number of KIR haplotype B defining receptors, especially of those coded in the centromeric regions, showed beneficial effects on survival of HLA C1/x AML recipients after ATG-free HSCT without increased GVHD and without benefit of KIR-ligand mismatch. No positive influence of haplotype B was seen in recent investigations for leukemia (49) but in HLA-matched URD-HSCT of non-Hodgkin lymphoma patients, where KIR B/x grafts led to significant lower relapse after 5 years compared to KIR A/A donors ($P = 0.5$) (101). The role of KIR genotypes in matched unrelated and sibling HSCT has recently also been investigated by Faridi et al. (49). Their aim was to compare the predictive value of KIR-ligand mismatch (61) versus the "missing-ligand" hypothesis (63) or the advantage of a specific KIR haplotype (50, 64, 65). They found KIR-KIR match to be associated with lower cGVHD for HLA C1/x recipients as well as lower RFS. One or more missing ligand in the unrelated recipient for donor KIR resulted in reduced relapse (21.6 versus 63.6%, $P = 0.001$) and higher RFS without improved OS. None of the tested hypotheses had influence on OS, and no effect of donor KIR haplotype was detected.

FUTURE DIRECTIONS

During the past years, improvement in understanding NK cell alloreactivity has been made by wisely modeled analyzes (49, 60, 84). Despite clinical relevance (102–105), we still know too little about the NK cell education after HSCT (95). The interplay of NK cells and T-cells after HSCT is still subject of further investigation (105), and as we now know about KIR expression on

T-cells (106), we need to be precise in our technical methods. To overcome the problem of heterogeneity, we would suggest beginning with a simple multicenter prospective trial in adult patients with AML in first molecular complete remission, testing the hypothesis that the number of activating KIR in the unmanipulated graft improves overall survival without increasing GVHD. KIR and HLA of donor and recipient should be measured by high-resolution genotyping and phenotyping. Every patient should receive the same conditioning and first-line immune suppression.

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CONCLUSION

Due to heterogeneity of the conducted studies, a general recommendation cannot be made. In matched URD-HSCT, a donor with high numbers of activating KIR can be chosen to optimize patient's chances for survival.

AUTHOR CONTRIBUTIONS

SH and NK contributed equally to the manuscript writing.

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HLA-Bw4-I-80 Isoform Differentially Influences Clinical Outcome As Compared to HLA-Bw4-T-80 and HLA-A-Bw4 Isoforms in Rituximab or Dinutuximab-Based Cancer Immunotherapy

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Killer-cell immunoglobulin-like receptors (KIRs) are a family of glycoproteins expressed primarily on natural killer cells that can regulate their function. Inhibitory KIRs recognize MHC class I molecules (KIR-ligands) as ligands. We have reported associations of KIRs and KIR-ligands for patients in two monoclonal antibody (mAb)-based trials: (1) A Children's Oncology Group (COG) trial for children with high-risk neuroblastoma randomized to immunotherapy treatment with dinutuximab (anti-GD2 mAb) + GM-CSF + IL-2 + isotretinoin or to treatment with isotretinoin alone and (2) An Eastern Cooperative Oncology Group (ECOG) trial for adults with low-tumor burden follicular lymphoma responding to an induction course of rituximab (anti-CD20 mAb) and randomized to treatment with maintenance rituximab or no-maintenance rituximab. In each trial, certain KIR/KIR-ligand genotypes were associated with clinical benefit for patients randomized to immunotherapy treatment (immunotherapy in COG; maintenance rituximab in ECOG) as compared to patients that did not receive the immunotherapy [isotretinoin alone (COG); no-maintenance (ECOG)]. Namely, patients with both KIR3DL1 and its HLA-Bw4 ligand (KIR3DL1+/HLA-Bw4+ genotype) had improved clinical outcomes if randomized to immunotherapy regimens, as compared to patients with the KIR3DL1+/

HLA-Bw4+ genotype randomized to the non-immunotherapy regimen. Conversely, patients that did not have the KIR3DL1+/HLA-Bw4+ genotype showed no evidence of a difference in outcome if receiving the immunotherapy vs. no-immunotherapy. For each trial, HLA-Bw4 status was determined by assessing the genotypes of three separate isoforms of HLA-Bw4: (1) HLA-B-Bw4 with threonine at amino acid 80 (B-Bw4-T80); (2) HLA-B-Bw4 with isoleucine at amino acid 80 (HLA-B-Bw4-I80); and (3) HLA-A with a Bw4 epitope (HLA-A-Bw4). Here, we report on associations with clinical outcome for patients with KIR3DL1 and these separate isoforms of HLA-Bw4. Patients randomized to immunotherapy with KIR3DL1+/A-Bw4+ or with KIR3DL1+/B-Bw4-T80+ had better outcome vs. those randomized to no-immunotherapy, whereas for those with KIR3DL1+/B-Bw4-I80+ there was no evidence of a difference based on immunotherapy vs. no-immunotherapy. Additionally, we observed differences within treatment types (either within immunotherapy or no-immunotherapy) that were associated with the genotype status for the different KIR3DL1/HLA-Bw4-isoforms. These studies suggest that specific HLA-Bw4 isoforms may differentially influence response to these mAb-based immunotherapy, further confirming the involvement of KIR-bearing cells in tumor-reactive mAb-based cancer immunotherapy.

Keywords: KIR, KIR-ligand, HLA-Bw4, HLA, MHC class I, natural killer cells, cancer immunotherapy

INTRODUCTION

One modality of cancer immunotherapy utilizes tumor-reactive monoclonal antibodies (mAbs) to elicit a tumor-targeted immune response. Two recently completed clinical trials, in separate disease settings, utilized tumor-reactive mAbs to successfully target and treat the tumors: (1) the combination of dinutuximab with IL-2, GM-CSF, and isotretinoin for patients with high-risk neuroblastoma (1) and (2) rituximab for the treatment of patients with low-tumor burden follicular lymphoma (FL) (2).

Natural killer (NK) cells can contribute to the response to tumor-reactive mAb-based immunotherapeutics through antibody-dependent cellular cytotoxicity (ADCC). The ability of NK cells to elicit ADCC is regulated by activating and inhibiting signaling. Killer-cell immunoglobulin-like receptors (KIRs) are a class of receptors expressed on NK cells that influence such signaling (3,4). Most inhibitory KIRs interact with HLA class I molecules as their ligands (KIR-ligand) (5). Specifically, KIR2DL1 binds to HLA-C2, KIR2DL2 and KIR2DL3 bind to HLA-C1, and KIR3DL1 recognizes the Bw4 epitope of HLA-A and HLA-B (6, 7). The independent segregation and inheritance of KIRs and KIR-ligands help to shape NK cell function and response to immunotherapeutic agents (8–11). When inhibitory KIRs interact with class I HLA molecules on target cells, NK cell-mediated lysis and ADCC are inhibited. During development, KIR/KIR-ligand interactions lead to self tolerance and NK cells become “licensed NK cells” (12–14). Licensed NK cells have augmented cytotoxicity against class I negative tumors compared to unlicensed NK cells (15, 16).

Killer-cell immunoglobulin-like receptors and KIR-ligands segregate independently: KIR genes are located on chromosome 19; HLA genes (KIR-ligands) are located on chromosome 6. Several studies have shown that genotypic differences of KIR

and KIR-ligands can influence clinical outcome of certain cancer immunotherapies (8, 11, 17–19). We recently showed in two clinical trials that KIR3DL1 and its KIR-ligand, HLA-Bw4, appear to influence clinical outcome.

In a phase III trial (ANBL0032) of high-risk neuroblastoma patients, conducted by the Children’s Oncology Group (COG) (1), patients who inherited the KIR3DL1 gene and the gene for its HLA-Bw4 ligand (KIR3DL1+/Bw4+ genotype) and were treated with an immunotherapy regimen [dinutuximab (anti-GD2), IL-2, GM-CSF, and isotretinoin] had improved event-free survival (EFS) and overall survival as compared to those treated with isotretinoin alone (20, 21). In a separate Eastern Cooperative Oncology Group (ECOG) Phase III clinical trial of low-tumor burden FL (2), patients who were KIR3DL1+/HLA-Bw4+ and treated with a continuous regimen of maintenance rituximab had improved duration of response and % tumor shrinkage compared to KIR3DL1+/HLA-Bw4+ patients who were randomized to not receive maintenance rituximab (22, 23). Conversely, we did not observe improved outcome for patients that were *not* KIR3DL1+/HLA-Bw4+ when randomized to immunotherapy, in either study (22, 23). Furthermore, in both the COG and ECOG studies, patients who were randomized to the immunotherapy regimen that were KIR3DL1+/HLA-Bw4+ had better outcome compared to patients who were *not* KIR3DL1+/HLA-Bw4+.

Given these similar associations with outcome for the KIR3DL1/HLA-Bw4 interaction in these two clinical trials, we chose to evaluate these more deeply by evaluating the potential influence of distinct HLA-Bw4 isoforms. Polymorphisms in the $\alpha 1$ helix (positions 77–83) of HLA class I correspond to the sequence site of the Bw4 epitope that is recognized by KIR3DL1 (24). In KIR/KIR-ligand associations, we analyzed in these COG and ECOG trials, individuals were considered positive for HLA-Bw4 if they were found to have at least one of the three

isoforms of HLA-Bw4: (1) HLA-B allele with a threonine at amino acid position 80 (B-Bw4-T80), (2) HLA-B allele with an isoleucine at amino acid position 80 (B-Bw4-I80), or (3) HLA-A with a Bw4 epitope (A-Bw4). Patients were negative for HLA-Bw4 if they did not have any of these three isoforms. These polymorphisms of this Bw4 epitope can impact KIR3DL1 recognition (25–29). As such, we describe the impact of the genotype status of B-Bw4-T80, B-Bw4-I80, and A-Bw4, together with the genotype status of KIR3DL1, on the clinical outcome, based on a clinical outcome parameter that measured the duration of response to the treatment regimen (EFS in COG; duration of response in ECOG).

MATERIALS AND METHODS

Patients

COG ANBL0032 Patients

The phase III neuroblastoma clinical trial (ANBL0032; Clinicaltrials.gov # NCT00026312) evaluated the efficacy of isotretinoin alone as compared to an immunotherapeutic regimen consisting of dinutuximab (anti-GD2), aldesleukin (IL-2), sargramostim (GM-CSF), and isotretinoin (1). Of the 226 patients randomized, 174 patients (immunotherapy: $n = 88$; isotretinoin: $n = 86$) had DNA available, allowing evaluation of KIR/KIR-ligand genotype association with updated clinical outcome (>5-year follow-up if no event). All analyses in this study were conducted utilizing an intent-to-treat approach. All patients signed IRB approved consent forms enabling lab-based immune correlative analyses, and the genotyping done at UW-Madison was approved by the UW-IRB.

ECOG E4402 Patients

The Phase III ECOG clinical trial (E4402; ClinicalTrials.gov #NCT00075946) evaluated the efficacy of single agent, rituximab therapy for adults with low-tumor burden FL. Clinical results from this study have been reported elsewhere (2). A total of 408 patients with FL were entered, with 289 patients responding and randomized to no-maintenance or maintenance rituximab regimens. Disease measurements were obtained every 13 weeks (2). Of the 289 randomized patients from this trial, 213 patients had evaluable DNA and clinical data for this study, and 159 of them were randomized to no-maintenance ($n = 80$) or maintenance rituximab ($n = 79$) treatment. Of these 79 patients treated with maintenance rituximab, 75 patients had clinical data available for duration of response. All patients signed IRB approved consent forms enabling lab-based immune correlative analyses, and the genotyping done at UW-Madison was approved by the UW-IRB.

Genotyping

KIR3DL1 gene status was determined by a SYBR green real-time PCR reaction (30, 31). The genotype for HLA-Bw4, which includes three known HLA-Bw4 epitopes (B-Bw4-T80, B-Bw4-I80, and A-Bw4) were determined by PCR-SSP reactions using the KIR HLA Ligand SSP typing kit (product number 104.201-12u from

Olerup, West Chester, PA, USA) with GoTaq DNA polymerase (M8295, Promega, WI, USA). All genotyping was conducted in a blinded manner, whereby individuals who determined the genotype of the patients did not have access to the clinical outcome data.

Statistical Methods

The goal of these analyses was to evaluate the association of KIR3DL1 in combination with each HLA-Bw4 isoform (B-Bw4-T80, B-Bw4-I80, and A-Bw4) on response to therapy (EFS or duration of response). For the COG trial, EFS time was defined as the time from study enrollment until the first occurrence of relapse, progressive disease, secondary cancer, or death or until the last contact with the patient if none of these events occurred (censored). For the ECOG trial, the duration of response was defined as the time from randomization (following an initial response to the induction rituximab treatment) to documented disease progression (2).

Cox proportional hazards regression models and log-rank tests were used to compare EFS/duration of response curves by treatment and genotype combinations. The proportional hazards assumption was tested, and when the assumption was not met, adjustments were made by incorporating time-dependent covariates into the model. For both trials, only randomized patients were included in the analyses. Statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, NC, USA).

RESULTS

HLA-Bw4 Isoforms, Together with KIR3DL1, Differentially Influence the Impact of mAb-Based Immunotherapy on Clinical Outcome of Neuroblastoma Patients

In our analyses of associations of KIR/KIR-ligand genotypic influence on clinical response in the neuroblastoma study (ANBL0032), we reported on differences in clinical outcome for those KIR3DL1+/Bw4+ (immunotherapy $n = 58$; isotretinoin $n = 61$) and those *not* KIR3DL1+/Bw4+ (immunotherapy $n = 30$; isotretinoin $n = 25$), and differences in response were observed dependent upon treatment type (20, 21). Since not all of the isoforms of HLA-Bw4 may interact with KIR3DL1 to the same degree, we further assessed patients with different HLA-Bw4 isoforms in this setting.

To better understand the KIR/KIR-ligand genotypic influence on clinical outcome, we evaluated the effect of Bw4 epitope on either an HLA-A or HLA-B allele. In this study, patients who were KIR3DL1+/A-Bw4+ had a trend toward improved EFS if they were treated with immunotherapy as compared to those treated with isotretinoin alone ($p = 0.06$; **Figure 1A**) (Table S1 in Supplementary Material). In contrast, we did not find a significant difference in EFS for patients receiving the immunotherapy vs. those randomized to not receive the immunotherapy (i.e., isotretinoin alone) in the patients that were *not* KIR3DL1+/A-Bw4+ ($p = 0.35$; **Figure 1A**).

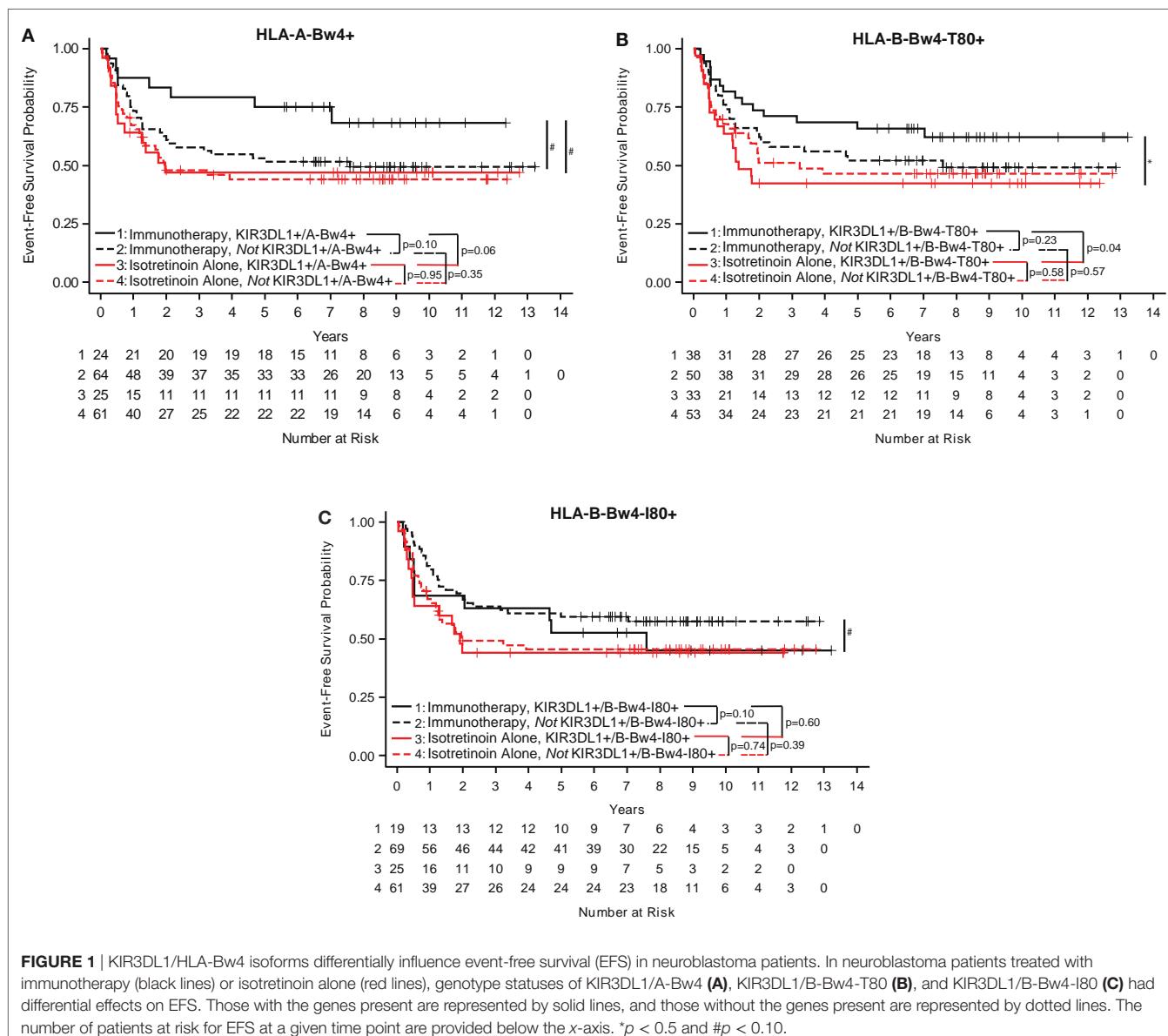


FIGURE 1 | KIR3DL1/HLA-Bw4 isoforms differentially influence event-free survival (EFS) in neuroblastoma patients. In neuroblastoma patients treated with immunotherapy (black lines) or isotretinoin alone (red lines), genotype statuses of KIR3DL1/A-Bw4 (A), KIR3DL1/B-Bw4-T80 (B), and KIR3DL1/B-Bw4-I80 (C) had differential effects on EFS. Those with the genes present are represented by solid lines, and those without the genes present are represented by dotted lines. The number of patients at risk for EFS at a given time point are provided below the x-axis. * $p < 0.5$ and # $p < 0.10$.

We found that B-Bw4-T80 and B-Bw4-I80 differentially influenced EFS in these neuroblastoma patients (Table S1 in Supplementary Material). Similar to results in **Figure 1A**, patients who were KIR3DL1+/B-Bw4-T80+ showed significantly improved EFS if they received immunotherapy compared with isotretinoin alone ($p = 0.04$; **Figure 1B**), whereas those that were *not* KIR3DL1+/B-Bw4-T80+ showed no difference in EFS for patients receiving the immunotherapy vs. those randomized to receive isotretinoin alone ($p = 0.57$; **Figure 1B**). However, for B-Bw4-I80+, the results were converse. Patients who were KIR3DL1+/B-Bw4-I80+ showed no sign of improved EFS if they received immunotherapy compared with isotretinoin alone ($p = 0.60$; **Figure 1C**). Furthermore, and in contrast to results in **Figures 1A,B**, while not significant, there appears to be improved EFS for patients receiving the immunotherapy vs. isotretinoin alone in the patients who were *not* KIR3DL1+/B-Bw4-I80+ ($p = 0.10$; **Figure 1C**).

These findings suggest that the different isoforms of HLA-Bw4 differentially influence the impact of anti-GD2-based immunotherapy on EFS for high-risk neuroblastoma patients.

HLA-Bw4 Isoforms, Together with KIR3DL1, Differentially Influence the Impact of mAb-Based Immunotherapy on Clinical Outcome of FL Patients

The ECOG E4402 Phase III clinical trial sought to optimize the rituximab treatment regimen for low-tumor burden FL patients (2). As such, different from the design of the neuroblastoma COG trial described above where one treatment arm was treated with immunotherapy and the other was not, in E4402 *all* patients were initially treated with rituximab. In E4402, all FL patients received induction rituximab, consisting of four weekly rituximab

treatments. After 13 weeks, those patients who achieved $\geq 50\%$ tumor shrinkage were randomized to two separate treatment regimens: (1) “maintenance” rituximab was given every 13 weeks or (2) “no-maintenance” where rituximab was given only upon disease progression (2). Thus, for the parameter of disease progression, the no-maintenance group received no rituximab between randomization and disease progression. Similar to the COG findings regarding the genotype status of KIR3DL1/Bw4, in this ECOG study, we also found that those KIR3DL1+/Bw4+ (maintenance $n = 49$; no-maintenance $n = 53$) had different clinical outcome than those not KIR3DL1+/Bw4+ (maintenance $n = 27$; no-maintenance $n = 26$), which was also influenced by the treatment arm.

Analyses of the three separate HLA-Bw4 isoforms suggest that the isoforms of HLA-Bw4 differently influenced the impact of maintenance rituximab. FL patients who were KIR3DL1+/A-Bw4+ that

were treated with maintenance rituximab had a longer duration of response (0 of 23 progressed, **Figure 2A**) as compared to patients who were *not* KIR3DL1+/A-Bw4+ [13 out of 53 progressed ($p = 0.008$, **Figure 2A**) (Table S1 in Supplementary Material)]. Separately, patients who were KIR3DL1+/B-Bw4-T80+ also showed significantly prolonged duration of response if they received maintenance as compared with no-maintenance rituximab ($p = 0.007$; **Figure 2B**). In addition, those patients who were *not* KIR3DL1+/B-Bw4-T80+ had a trend toward improved duration of response if treated with maintenance as compared with no-maintenance rituximab ($p = 0.07$; **Figure 2B**) (Table S1 in Supplementary Material). However, patients who were KIR3DL1+/B-Bw4-I80+ did not show prolonged duration of response if they received maintenance as compared with no-maintenance rituximab ($p = 0.40$; **Figure 2C**). Similar to the trends for improved EFS observed in neuroblastoma patients treated

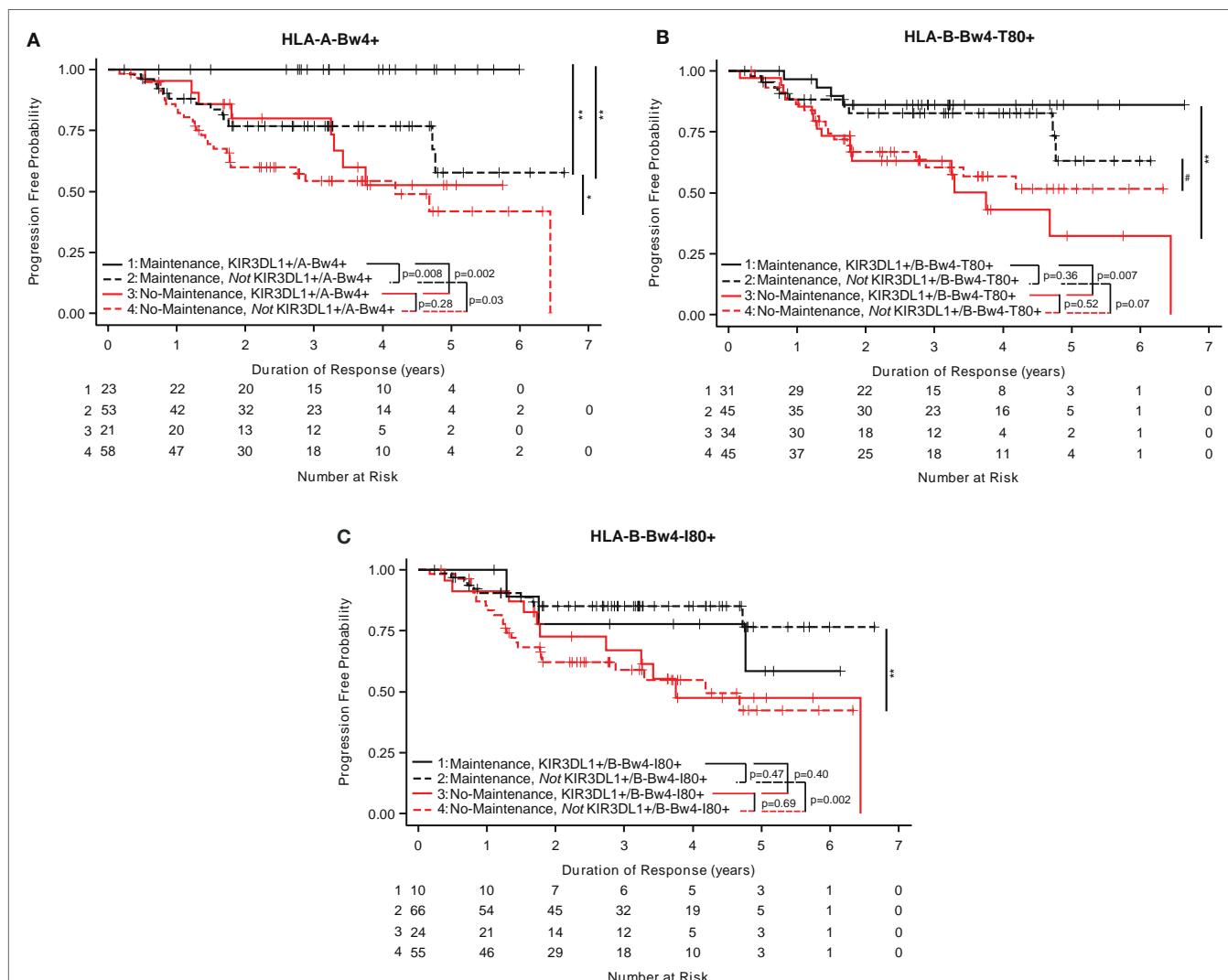


FIGURE 2 | KIR3DL1/HLA-Bw4 isoforms differentially influence duration of response in follicular lymphoma (FL) patients. In FL patients treated with maintenance rituximab (red lines) or no-maintenance rituximab (black lines), genotype statuses of KIR3DL1/A-Bw4 (**A**), KIR3DL1/B-Bw4-T80 (**B**), and KIR3DL1/B-Bw4-I80 (**C**) had differential effects on duration of response. Those with the genes present are represented by solid lines, and those without the genes present are represented by dotted lines. The number of patients at risk for duration of response at a given time point are provided below the x-axis. ** $p < 0.01$, * $p < 0.5$, and # $p < 0.10$.

with immunotherapy (**Figure 1C**), those FL patients who were *not* KIR3DL1+/B-Bw4-I80+ had improved duration of response if treated with maintenance rituximab as compared to no-maintenance ($p = 0.002$; **Figure 2C**) (Table S1 in Supplementary Material).

These findings suggest that the different isoforms of HLA-Bw4 differentially influence the impact of rituximab maintenance treatment for these low-tumor burden FL.

DISCUSSION

In both of these clinical trials, in separate disease settings, tumor-reactive mAbs were used to treat the tumors. In the analysis of KIR/KIR-ligand genotypes in each of these studies, we found similar associations with outcomes based upon the influence of KIR3DL1/HLA-Bw4. Specifically, those patients who had both KIR3DL1 and HLA-Bw4 had improved clinical outcomes if they were treated with either the COG immunotherapy regimen or the maintenance rituximab regimen in ECOG as compared to those who did not receive these same immunotherapeutic regimens (20, 21). Here, we report on the analyses of the specific HLA-Bw4 isoforms in both trials. In the ECOG trial of FL patients, patients with a KIR3DL1+/A-Bw4+ genotype or a KIR3DL1+/B-Bw4-T80+ genotype showed improved outcome when randomized to the maintenance regimen rather than to the no-maintenance regimen. In contrast, patients with a KIR3DL1+/B-Bw4-I80+ genotype showed no evidence of improved outcome when randomized to the maintenance treatment vs. no-maintenance regimen. We also observed similar trends for these same analyses in the COG trial of neuroblastoma patients.

Although other mechanisms, such as antibody-dependent cellular phagocytosis and complement-dependent cellular cytotoxicity (32, 33), could also contribute to the anti-tumor efficacy of tumor antigen-specific monoclonal antibodies, we hypothesize that the anti-tumor effect of rituximab and dinutuximab in these FL and neuroblastoma patients, respectively, is primarily through ADCC. NK cells are major contributors to ADCC, and their activity is regulated *via* the interactions between KIRs/KIR-ligands (34). As such, we hypothesize that the KIR/KIR-ligand genotypes could influence the degree that patients respond to antibody-based immunotherapies. Besides NK cells, KIRs are also expressed by a subset of T cells as well as NKT cells (35, 36). Therefore, it is possible that these other cell types may also be influenced by KIR/KIR-ligand genotypes.

Besides inherited genetic differences in KIR and KIR-ligand genotypes, other individual genetic differences, such as polymorphisms in Fc gamma receptors (FCGRs), may influence patient outcome to immunotherapy. FCGR polymorphisms can alter the affinity of FCGRs for the Fc portion of antibodies (mAbs or endogenous antibodies) (37). For example, in a separate study of patients with metastatic renal cell carcinoma treated with high-dose IL-2, we found that patients with a “higher affinity” FCGR genotype had improved clinical outcome as compared to those patients with a “lower affinity” FCGR genotype (38). In our analysis of those same metastatic renal cell carcinoma patients for KIR/KIR-ligand genotype influence on outcome,

we did not observe differences in clinical outcome associated with KIR3DL1 and HLA-Bw4 genotype status (39). The influence of FCGR polymorphisms on clinical outcome to rituximab is variable (40–42). For the FL patients analyzed here from this ECOG study, Kenkre and colleagues reported no association of FCGR genotype polymorphisms with patient outcome (43). In addition, some groups have found associations of FCGR genotype with clinical outcome for patients treated with anti-GD2 immunotherapy (8, 44, 45). For the neuroblastoma patients from this COG trial, FCGR genotype associations with clinical outcome are still under investigation. In addition, it has been reported that the influence from KIR/KIR-ligand interactions on NK cells may be affected by the affinity of the Fc portion of different therapeutic mAb used (46), the rituximab used in this ECOG trial and the dinutuximab used in this COG trial have similar human IgG1 Fc components, which may also help account for why we observed similar influences from HLA-Bw4 epitopes in these two separate studies where two different therapeutic mAbs were used.

These clinical data are consistent with the B-Bw4-I80 isoform functioning somewhat differently than the B-Bw4-T80 or A-Bw4 isoforms, and potentially making the tumor cells less responsive to the potential benefit of the anti-GD2 or anti-CD20 mAb-based immunotherapy. *In vitro* analyses have shown that a subset of HLA-Bw4 alleles (those with an B-Bw4-I80 isoform) show relative protection from lysis by NK cells (47, 48). The data presented here are consistent with these *in vitro* results; mAb-based immunotherapy may provide more benefit for patients with weaker NK cell inhibition from B-Bw4-T80 or A-Bw4, than for patients with stronger NK inhibition from B-Bw4-I80.

Given that patients assessed in either trial could be positive for more than one of the HLA-Bw4 epitopes, we did consider whether the HLA-Bw4 epitopes were in linkage disequilibrium. We found that A-Bw4 was not in linkage disequilibrium with either B-Bw4-I80 or B-Bw4-T80 (Table S2 in Supplementary Material). Thus, the influence that each of these HLA-Bw4 epitopes had on the length of patient response in either trial is presumably not due to linkage disequilibrium with each other.

We also considered whether the interaction of KIR3DL1 with these three different HLA-Bw4 isoforms showed any association of outcome among patients randomized to receive the immunotherapy regimens. Within the COG study, we observed a trend for improved outcome for those KIR3DL1+/HLA-A-Bw4+ vs. those not KIR3DL1+/HLA-A-Bw4+ (**Figure 1A**), and we also observed a trend in the opposite direction for HLA-Bw4-I80, namely, there was a trend for improved outcome for those not KIR3DL1+/HLA-B-Bw4-I80+ vs. those who were KIR3DL1+/HLA-B-Bw4-I80+ (**Figure 1C**). Although only a trend, this difference in **Figure 1A** and **Figure 1C** is consistent with differential function of HLA-A-Bw4 and HLA-B-Bw4-I80. No significant differences or trends were noted when we evaluated among the FL patients randomized to receive the maintenance rituximab regimen (**Figures 2A–C**).

The interaction of KIR3DL1 with the Bw4 epitope is dependent not only on the architecture of Bw4 but also on the sequence of the bound peptide (25, 28, 49–51). Additionally, the differences

we observed between A-Bw4, B-Bw4-T80, and B-Bw4-I80 may be due to the different inhibition strength for KIR3DL1 from these isoforms. For instance, HLA-A*32:01, HLA-B*51:01, and HLA-B*58:01 strongly inhibit target cells from lysis by KIR3DL1+ NK cells, yet HLA-B*15:13 and HLA-B*27:05 have weaker inhibitory effects, despite all being HLA-Bw4 alleles (6, 25, 26, 48, 52–54). In addition, depending on the KIR3DL1 allele, expression of KIR3DL1 can vary; different HLA-A-Bw4 alleles have differential affinity for KIR3DL1 that is attributed to high vs. low expression of KIR3DL1 (55). Furthermore, the specific Bw4 allele, as well as the KIR3DL1 allele, the strength of KIR3DL1/HLA-Bw4 interaction and the binding avidity can vary (29). For example, Saunders et al. recently showed that HLA-A*24:02 acts as a poor ligand for KIR3DL1, and the strength of its interaction with KIR3DL1 differed depending on the allele of KIR3DL1 (29). The genotyping methodology employed for analyzing the many patients in these two clinical trials reported here was not able to address these more subtle allele-specific or peptide-related issues.

Another possible cause of the differences observed in these HLA-Bw4 isoforms may be due to genetic polymorphisms of KIR3DL1 (26, 28, 29, 56–60). More than 100 alleles of KIR3DL1 have been described. Phylogenetically, these alleles span three lineages based on the polymorphism of the three extracellular domains (D0–D1–D2) (53, 61). In both of these clinical studies analyzed, we did not determine the allelic differences of the KIR genes, but rather we determined their presence or absence. Thus, we cannot assess how different KIR3DL1 alleles may affect the interactions between different isoforms of HLA-Bw4. We did, however, assess if KIR3DL1 allelic status could influence the interactions of KIR3DL1 with HLA-Bw4 and with the separate HLA-Bw4 isoforms. KIR3DL1 and KIR3DS1 are alleles, thus individuals can have 2, 1, or 0 copies of KIR3DL1 (2 copies: KIR3DL1/KIR3DL1, 1 copy: KIR3DL1/KIR3DS1, or 0 copies: KIR3DS1/KIRDS1). Although KIR3DS1 has not been shown to utilize HLA-Bw4 as a ligand *in vitro*, whether KIR3DS1 may still interact with HLA-Bw4 *in vivo* is controversial (62–65). We assessed whether the allelic status of KIR3DL1/KIR3DS1 together with HLA-Bw4 (and HLA-Bw4 isoforms) influenced patient response. We found that there was no evidence of an association with outcome in either the COG or the ECOG study that could be linked to the allelic status of KIR3DL1/KIR3DS1 (data not shown), nor was there evidence of an association of clinical outcome linked to KIR3DL1/KIR3DS1 status together with the HLA-Bw4 ligand isoforms (data not shown). Rather, the mere presence of KIR3DL1 together with its ligand, HLA-Bw4, seemed to influence patients' response to immunotherapy in both clinical trials. These observations will require validation in a separate study.

In conclusion, this work sheds further light on the role of KIR receptors on NK cells in the antitumor response to immunotherapeutic mAbs. We demonstrate that the KIR3DL1/HLA-Bw4 axis influences response to tumor-targeted mAbs in two separate clinical trials and that the presence of the B-Bw4-T80 isoform or the A-Bw4 isoform is associated with improved response to mAb-based immunotherapy, while the presence of the B-Bw4-I80 isoform is not.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of University of Wisconsin Health Sciences Institutional Review Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Wisconsin Health Sciences Institutional Review Board.

AUTHOR CONTRIBUTIONS

Each author made substantial contributions to the conception and/or design of this research, including the acquisition, analysis, and/or interpretation of data for the work; the drafting of this manuscript, including critical revisions important intellectual content, were shared duties by all authors; each author submitted final approval of this manuscript as submitted to be published; each author is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (AE, WW, PR, LC, KK, EM, YS, JH, WL, AN, FH, MH, JM, JP, MO, JM, AG, BK, AY, and PS).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00675/full#supplementary-material>.

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KIR3DS1-Mediated Recognition of HLA-*B51: Modulation of KIR3DS1 Responsiveness by Self HLA-B Allotypes and Effect on NK Cell Licensing

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Several studies described an association between killer-cell immunoglobulin-like receptor (KIR)/HLA gene combinations and clinical outcomes in various diseases. In particular, an important combined role for KIR3DS1 and HLA-B Bw4-I80 in controlling viral infections and a higher protection against leukemic relapses in donor equipped with activating KIRs in haplo-HSCT has been described. Here, we show that KIR3DS1 mediates positive signals upon recognition of HLA-B*51 (Bw4-I80) surface molecules on target cells and that this activation occurs only in Bw4-I80^{neg} individuals, including those carrying particular KIR/HLA combination settings. In addition, killing of HLA-B*51 transfected target cells mediated by KIR3DS1⁺/NKG2A⁺ natural killer (NK) cell clones from Bw4-I80^{neg} donors could be partially inhibited by antibody-mediated masking of KIR3DS1. Interestingly, KIR3DS1-mediated recognition of HLA-B*51 could be better appreciated under experimental conditions in which the function of NKG2D was reduced by mAb-mediated blocking. This experimental approach may mimic the compromised function of NKG2D occurring in certain viral infections. We also show that, in KIR3DS1⁺/NKG2A⁺ NK cell clones derived from an HLA-B Bw4-T80 donor carrying 2 KIR3DS1 gene copy numbers, the positive signal generated by the engagement of KIR3DS1 by HLA-B*51 resulted in a more efficient killing of HLA-B*51-transfected target cells. Moreover, in these clones, a direct correlation between KIR3DS1 and NKG2D surface density was detected, while the expression of NKp46 was inversely correlated with that of KIR3DS1. Finally, we analyzed KIR3DS1⁺/NKG2A⁺ NK cell clones from a HLA-B Bw4^{neg} donor carrying cytoplasmic KIR3DL1. Although these clones expressed lower levels of surface KIR3DS1, they displayed responses comparable to those of NK cell clones derived from HLA-B Bw4^{neg} donors that expressed surface KIR3DL1. Altogether these data suggest that, in particular KIR/HLA combinations, KIR3DS1 may play a role in the process of human NK cell education.

Keywords: human natural killer cells, activating killer-immunoglobulin-like receptors, KIR3DS1, natural killer cell education, HLA-B alleles, KIR/KIR-L interaction

INTRODUCTION

Natural killer (NK) cells are lymphocytes of innate immunity that are involved in the host immune defenses against viruses and tumor cells. NK cells can exert cytotoxicity against transformed cells and release soluble factors important for regulating innate and adaptive immune responses (1, 2).

The NK cell function is controlled by an array of activating and inhibitory receptors, including the family of killer-cell immunoglobulin-like receptors (KIRs) (3). Inhibitory KIRs (iKIRs) possess a long cytoplasmic tail, containing ITIM motifs, responsible for the transduction of an inhibitory signal (3, 4), while activating KIRs (aKIRs) are characterized by a short tail and by a positively charged amino acid residue in their transmembrane region, which allows recruitment of the DAP12 signaling adaptor molecule (5, 6).

KIR3DL1/S1 is the *KIR* gene characterized by the highest degree of polymorphism, and it is the only one including alleles coding for either inhibitory (*KIR3DL1*) or activating (*KIR3DS1*) receptors. In particular, *KIR3DL1* is highly polymorphic, whereas *KIR3DS1**013 is the most represented in all examined populations (7). Notably, *KIR3DS1* is the only activating receptor with three extracellular domains (8). Its inhibitory counterpart, *KIR3DL1*, recognizes HLA-A and HLA-B alleles, sharing the Bw4 public epitope (3, 4, 9, 10). Despite the high degree of homology between these two *KIR3D* receptors, knowledge about *KIR3DS1* function and ligand specificity is not completely defined so far.

In this regard, several studies have suggested that certain HLA-B Bw4 alleles characterized by isoleucine in position 80 (Bw4-I80) may be putative ligands for *KIR3DS1*. In particular, the carriage of a *KIR3DS1* allele in conjunction with *HLA-Bw4-I80* alleles in patients with chronic HIV-1 infection has been associated with a slower progression to AIDS (11, 12). In addition, in individuals affected by acute HIV-1 infections and carrying *HLA-Bw4-I80* alleles, expansion of *KIR3DS1*⁺ NK cells (13), killing of HIV-1 infected cells, and inhibition of viral replication have been reported (12).

Carr and colleagues (14) could not detect any *KIR3DS1*-Fc binding to LCL721.221 cells transfected with HLA-B Bw4-I80 alleles (HLA-B*57:01, HLA-B*58:01) or with HLA-B Bw4-T80 (B*27:05) and HLA-Bw6 (B*15:02). Nevertheless they did not exclude the possibility that binding occurred below their detection limits or required the presence of additional factors, such as the presence of specific peptides in the HLA-B peptide-binding groove. In this regard, it has been shown that *KIR3DS1* can interact productively with HLA-Bw4 in the context of HIV infection. Indeed, two HIV-derived peptides have been described to enable HLA-B*57:01/*KIR3DS1* interaction (15).

Recent studies have also reported that HLA-F open conformers (OCs) are high-affinity ligands of *KIR3DS1* and ligands of lower affinity for the inhibitory receptors *KIR3DL1* and *KIR3DL2* (16, 17). However, *KIR3DS1*/HLA-F interaction cannot fully explain the control of HIV infection in *KIR3DS1*⁺ HLA-B Bw4-I80⁺ patients only (16).

A protective role of *KIR3DS1* in controlling certain tumors promoted by chronic viral infections has also been observed. For example, a protective effect of *KIR3DS1* in combination with

HLA-B Bw4-I80 alleles has been observed against hepatocellular carcinomas developed in chronically HCV-infected patients (18).

Remarkably, despite several attempts to define the specificity of *KIR3DS1*, the role of this receptor in the process of NK cell education has not been considered yet. In the present study, by the analysis of distinct NK clones, we show that certain self HLA-B allotypes can modify the functional responsiveness of *KIR3DS1*, thus providing evidence for an effect on the education of NK cells expressing this aKIR.

MATERIALS AND METHODS

KIR Gene Profile and *KIR-Ligand* (*KIR-L*) Analyses

DNA of the tested donors was extracted using QIAamp DNA Blood Mini Kit (Qiagen, GmbH, Germany). The *KIR* gene profile and *KIR-L* analyses were performed using sequence-specific primer PCR (SSP-PCR) *KIR* genotyping kit and *KIR* ligand kit, respectively (GenoVision, Saltsjöbaden, Sweden) following the manufacturer's instruction. SSP-PCR analysis of *KIR* gene repertoire has been integrated with sequence of *KIR3DL1* codon 86 in order to distinguish *KIR3DL1* alleles coding for surface receptors from those coding for polypeptide retained into the cytoplasm (19).

KIR3DL1 and *KIR3DS1* Gene Copy Number (GCN)

KIR3DL1 and *KIR3DS1* GCN was measured using a quantitative PCR method and a comparative C_t method ($\Delta\Delta C_t$). The used amplification protocol, as well as primer and probe sequences, has been published by Jiang and coworkers (20). *RNaseP* was used as two copies reference gene (TaqMan Copy Number Reference Assay, human RNase P; Applied Biosystems).

HLA-B High-Resolution Typing

Genomic DNA was used to perform *HLA-B* high-resolution typing. Some *HLA* typings were performed by sequence-based typing (SBT) using ATRIA kits according to the manufacturer's instructions (Abbott-Celera Corporation, Alameda, CA, USA). Exons 2, 3, and 4 were bidirectionally sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the sequences were analyzed by Assign 3.5+ HARPS software (Conexio Genomics, Applecross, Australia). Some *HLA* typings were performed using sequence-specific oligonucleotide probes (PCR-SSOP; One Lambda, Canoga Park, CA, USA), and the results were analyzed by the software Fusion 3.0 (One Lambda, Canoga Park, CA, USA).

Antibodies and Flow Cytometry

The following mAbs, all produced in our lab, were used in this study: c218 (IgG1, anti-CD56), c127 (IgG1, anti-CD16), AZ20 and F252 (IgG1 and IgM, respectively, anti-NKp30), BAB281 and KL247 (IgG1 and IgM, respectively, anti-NKp46), Z231 (IgG1, anti-NKp44), ON72 and BAT221 (IgG1, anti-NKG2D), KRA236 and F5 (IgG1 and IgM, respectively, anti-DNAM-1), 11PB6 (IgG1, anti-KIR2DL1/S1), GL183 (IgG1, anti-KIR2DL2/

L3/S2), ECM41 (IgM, anti-KIR2DL3), DF200 (IgG1, anti-KIR2DL1/L2/L3/S1/S2/S5), FES172 (IgG2a, anti-KIR2DS4), z27 (IgG1, anti-KIR3DL1/S1), AZ158 (IgG2a, anti-KIR3DL1/L2/S1), Q66 (IgM, anti-KIR3DL2), z199 and Y9 (IgG2b and IgM, respectively, anti-NKG2A), 6A4 and A6/136 (IgG1 and IgM, respectively, anti-HLA-class I), D1/12 (IgG2a, anti-HLA-DR), 5A10 (IgG1, anti-PVR), L14 (IgG2a, anti-Nectin-2), and BAM195 (IgG1, anti-MICA). F278 (IgG1, anti-LIR-1/ILT2) mAb was kindly provided by Dr. Daniela Pende. Anti-NKG2C (IgG2b, 134522 clone), anti-ULBP-1 (IgG2a, 170818 clone), anti-ULBP-2 (IgG2a, 165903 clone), anti-ULBP-3 (IgG2a, 166510 clone), and anti-KIR2DL1-PE, -FITC, -APC, or non-conjugated (IgG1, 143211 clone) mAbs were purchased from R&D System Inc. (Abingdon, UK). Anti-KIR2DL5-PE or non-conjugated (UP-R1 clone), anti-KIR3DL1-FITC and -APC (DX9 clone) mAbs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD3-FITC (UCHT-1 clone), anti-CD56-PC7 (N901 clone), anti-NKG2A-APC (z199 clone), IgG1-PE (679.1Mc7 clone, isotype control), anti-KIR3DL1/S1-PE (z27 clone), and anti-KIR2DL2/L3/S2-PC7 (GL183 clone) mAbs were purchased from Beckman Coulter Immunotech (Marseille, France). Anti-KIR2DL2/L3-S2-FITC (CHL-clone) mAb was obtained from BD Bioscience Pharmingen (San Diego, CA, USA). Anti-HLA-Bw6-FITC and anti-HLA-Bw4-FITC mAbs were purchased from One Lambda (Canoga Park, CA, USA). Anti-human HLA-E (IgG1, 3D12 clone) and anti-human HLA-G (IgG1, MEM-G/9 clone) mAbs were purchased from BioLegend (San Diego, CA, USA) and Abnova (Taipei, Taiwan), respectively.

For cytofluorimetric analyses, cells were incubated with appropriate mAbs, followed by PE-, FITC-, or APC-conjugated isotype-specific goat anti-mouse secondary reagents (Southern Biotechnology Associated, Birmingham, AL, USA; Jackson ImmunoResearch Laboratories, Suffolk, UK) and/or fluorochrome-conjugated mAbs. Cytofluorimetric analyses were performed on FACSCalibur (Becton Dickinson & Co., Mountain View, CA, USA), and data were analyzed by the CellQuest Pro software.

Mean fluorescence intensity ratios (MFIRs) were calculated by dividing the mean fluorescence intensity of stained molecules by the mean fluorescence of the respective isotype control.

Generation of Resting NK Cells and NK Cell Clones

Buffy coats from healthy donors were obtained from the Immunohematology and Transfusion Center at the S. Martino Hospital (Genova, Italy). Approval was obtained by the ethical committee of IRCCS S. Martino-IST (39/2012) of Genova (Italy). Informed consent was provided according to the Declaration of Helsinki. Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Hypaque gradients. PBMCs from 120 healthy donors were screened for their KIR3DS1 expression by cytofluorimetric analysis. Donors characterized by the expression of a clearly detectable KIR3DS1⁺ NK cell subset (i.e., Z27⁺ DX9^{neg}) were further typed for their KIR repertoires and KIR-Ls ($n = 40$ donors). Based on KIR-L analysis, three groups of donors were selected: Bw4-I80 donors ($n = 12$), Bw4-T80 donors ($n = 7$), and Bw4^{neg} donors ($n = 8$). Individuals carrying both HLA-B

Bw4-I80- and Bw4-T80-coding alleles were not considered in this study.

Highly purified NK cells (97–99% purity) were isolated by depletion of non-NK cells, using Miltenyi NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) from some of the selected KIR3DS1⁺ donors (two Bw4-I80, two Bw4-T80, and three Bw4^{neg} donors). NK cells were cultured on irradiated feeder cells in the presence of 100 U/ml rhIL-2 (Proleukin; Chiron Corp., Emeryville, CA, USA) and 2 µg/ml phytohemagglutinin (PHA; Life Technologies, Paisley, UK) in round-bottomed 96-well microtiter plates to obtain activated polyclonal NK cell populations or, after limiting dilution, NK cell clones as previously described (21). After 2–4 weeks of culture, the expanded NK cells were used for the phenotypic analysis and NK cell cytotoxicity experiments. The relatively limited number of donors used to generate NK cell clones reflects the difficult collection of individuals with similar characteristics in terms of KIR and KIR-Ls. Only KIR3DS1⁺ NK cell clones expressing the inhibitory receptor NKG2A as HLA-specific receptor and characterized by a sufficient growth to perform phenotypic and functional tests were selected and analyzed in the study. The number of KIR3DS1⁺/NKG2A⁺ NK cell clones used is indicated in the legends to the figures.

Analysis of HLA-F Transcript

Total RNA was extracted from LCL721.221, C1R, C1R-B51, and JA3 cell lines using RNeasy mini kit (Qiagen) according to the manufacturer's instruction, and cDNA synthesis was performed using oligo-dT primers. *HLA-F* transcript analysis was performed using Hs04193807_g1: HLA-F human kit (Applied Biosystems, Foster City, CA, USA). GAPDH transcript was used to normalize the *HLA-F* quantity (Human GAPDH Endogenous Control Kit, Applied Biosystems, Foster City, CA, USA). The normalized *HLA-F* mRNA transcript of the tested samples was calculated as time-fold mRNA detected in the LCL721.221 cell line (chosen as reference in this study). Each cell line was analyzed in four independent experiments, and each reaction was performed at least in triplicate.

⁵¹Cr Cytolytic Assays

The NK-mediated cytotoxicity was assessed in a 4-h ⁵¹Cr-release assay as previously described (22). Cells used as targets in the various cytolytic assays were the following: P815 (murine mastocytoma cell line), C1R (human EBV-transformed lymphoblastoid cell line), and C1R transfected with HLA-B*51 (Bw4-I80) allele (Figure S1 in Supplementary Material). For redirected killing assays, P815 were used as target cells in the presence of mAbs of IgG isotype at a concentration of 0.5 µg/mL. For masking experiments, NK cells were pre-incubated with mAbs specific to the various NK receptors 10 min before addition of target cells; mAb concentration was 10 µg/mL. The E:T ratios are indicated in the figure legends. Δ and Δ-B51 indicate the variations of C1R or C1R-B51 lysis in the absence or presence of anti-KIR3DS1 mAb calculated for each NK cell clone.

Three different types of NK cell clones were used as effector cells for cytolytic assays: (1) NKG2A⁺, KIR3DS1⁺, KIR2DL2/S2/L3^{neg}, KIR2DL1/S1^{neg}, KIR3DL1^{neg}, and NKG2C^{neg}; (2) KIR3DL1⁺, NKG2A⁺, KIR3DS1^{neg}, KIR2DL2/S2/L3^{neg}, KIR2DL1/S1^{neg}, and

NKG2C^{neg}; and (3) KIR3DL1^{neg}, NKG2A⁺, KIR3DS1^{neg}, KIR2DL2/S2/L3^{neg}, KIR2DL1/S1^{neg}, and NKG2C^{neg}.

Statistical Analysis

Wilcoxon–Mann–Whitney non-parametric tests were employed. The statistical significances (*p* value: **p* < 0.1, ***p* < 0.01, ****p* < 0.001) are indicated. Graphic representations and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

RESULTS

KIR3DS1 Surface Expression on Resting NK Cells Is Not Affected by Differences in HLA-B Allotypes

Healthy donors were screened for their KIR3DS1 expression by cytofluorimetric analysis. To distinguish between KIR3DS1⁺ and KIR3DL1⁺ cells, peripheral blood NK cells were stained in double fluorescence analysis with two different mAbs: an anti-KIR3DL1-specific mAb (clone DX9) and an mAb specific for both KIR3DL1 and KIR3DS1 (clone Z27) (Figure 1A). Donors characterized by the expression of a KIR3DS1⁺ NK cell subset (i.e., Z27⁺ DX9^{neg}) were further typed for their KIR repertoires and KIR-Ls (Figure S2 in Supplementary Material).

KIR gene analysis allowed us to define the telomeric regions of the tested donors. In order to compare donors characterized by similar KIR3DL1/3DS1 locus, we restricted our analysis to TelA/TelB donors, namely, individuals characterized by one KIR3DL1 and one KIR3DS1 gene copy. KIR3DL1^{neg} donors (i.e., individuals characterized by two B telomeric regions) were excluded from this study according to recent evidences showing hyporesponsive and less frequent KIR3DS1⁺ NK cells in this type of donors (23). Subsequently, based on KIR-L analysis, three groups of donors were selected: (a) Bw4-I80 donors, typed as Bw4-I80^{pos}

and Bw4-T80^{neg} (including both Bw4-I80/Bw4-I80 and Bw4-I80/Bw6 donors); (b) Bw4-T80 donors, typed as Bw4-T80^{pos} and Bw4-I80^{neg} (including both Bw4-T80/Bw4-T80 and Bw4-T80/Bw6 donors); and (c) Bw4^{neg} donors, lacking both Bw4-I80 and Bw4-T80. Individuals carrying both HLA-B Bw4-I80- and Bw4-T80-coding alleles were not considered in this study. Also some HLA-A molecules are characterized by a Bw4-80I motif (e.g., HLA-A*23, -A*24, -A*25 or -A*32) (24), and their frequency in the European population is ~16% (<http://www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi?ID=9&cmd=PRJOV>). Nevertheless, since not all HLA-A Bw4-I80 molecules are KIR3DL1 ligands (10, 25), we restricted our analyses to HLA-B alleles with Bw4-I80.

Comparison of KIR3DS1 surface expression on resting NK cells derived from these three donor groups did not show any significant difference in terms of percentage, MFIR, and median values (Figure 1B). These data are in line with previous results showing no substantial differences in KIR3DS1 mRNA levels between HLA-B Bw4-I80 and HLA-Bw6 healthy individuals (13).

Thus, similar to what had already been demonstrated for HLA-C alleles in relation to KIR2DS1 (26), expression of different HLA-B allotypes does not influence the overall frequency of KIR3DS1⁺ NK cells and the KIR3DS1 surface expression density in peripheral blood NK cells.

Phenotypic and Functional Differences in KIR3DS1⁺/NKG2A⁺ NK Cell Clones Derived from Donors Carrying Various HLA-B Allotypes

Several NK cell clones were generated from donors belonging to each of the three above-mentioned groups (Bw4-I80: SiCa and J76 donors, Bw4-T80: J13 donor, and Bw4^{neg}: GL115 and U58 donors). These donors were all characterized by a TelA/TelB KIR genotype, nevertheless, since KIR haplotype characterized by KIR3DL1/3DS1 duplication has been described (20, 27),

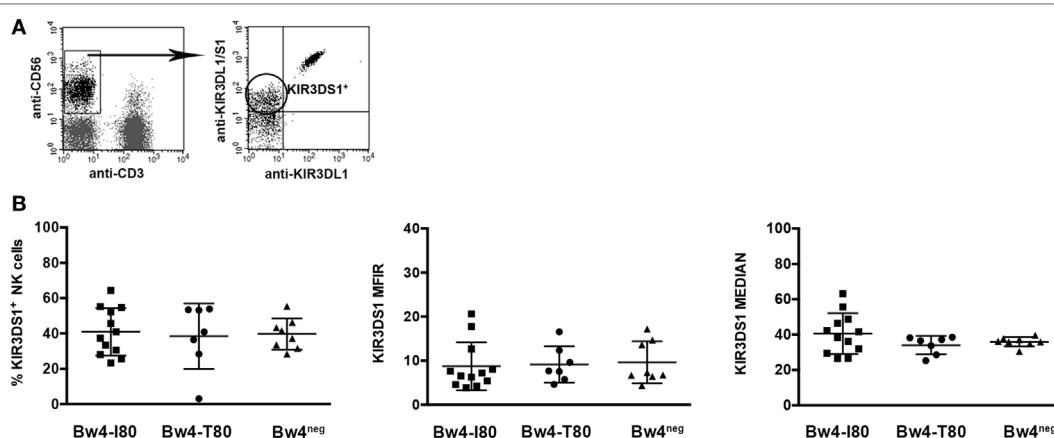


FIGURE 1 | KIR3DS1 surface expression on resting peripheral blood natural killer (NK) cells. **(A)** Gate strategy for selecting KIR3DS1⁺ NK cell subset (CD3^{neg} CD56⁺ KIR3DS1⁺ KIR3DL1^{neg}) in peripheral blood mononuclear cell. For staining, the following mAbs were used in combination: anti-CD56 (N901), anti-CD3 (UCHT-1), anti-KIR3DL1/S1 (z27), and anti-KIR3DL1 (DX9). **(B)** KIR3DS1 surface expression was evaluated on peripheral blood resting NK cells from HLA-B Bw4-I80 (*n* = 12, full squares), Bw4-T80 (*n* = 7, full circles), or Bw4^{neg} (*n* = 8, full triangles) healthy donors in terms of frequency, mean fluorescence intensity ratio, and median values. Summarized results are compared in three scatter dot plots, respectively. In each plot, average and standard deviation are represented.

GCN analysis was performed to verify, in these five donors, the presence of one *KIR3DL1* and *KIR3DS1* copy (**Figure 2**). To exclude the potential educational effects of different KIR/KIR-L interactions on *KIR3DS1* expression and function, *KIR3DS1⁺* NK cell clones expressing the inhibitory receptor NKG2A as the only known HLA-specific receptor were selected.

As shown in **Figure 2A** (left panel), cytofluorimetric analysis revealed that *KIR3DS1* expression was significantly heterogeneous among NK cell clones derived from individuals with different HLA-B allotypes. Indeed, NK cell clones derived from Bw4-I80 donors, and to a lower extent those from Bw4-T80 donors, showed a reduced expression of *KIR3DS1* as compared to NK cell clones derived from Bw4^{neg} donors (**p < 0.0001 and **p = 0.0018, respectively). On the contrary, no differences in NKG2A surface expression could be detected among the same series of NK cell clones analyzed (not shown).

KIR3DS1⁺/NKG2A⁺ NK cell clones were then analyzed in redirected killing assays using mAbs specific for several activating or inhibitory receptors. Notably, the magnitude of cytolytic responses to *KIR3DS1* mAb-mediated triggering correlated with the levels of *KIR3DS1* surface expression. In particular, as shown in **Figure 2B** (left panel), NK cell clones from Bw4-I80 donors expressing a significantly lower *KIR3DS1* MFIR displayed a reduced increment of cytotoxicity upon *KIR3DS1* mAb-mediated cross-linking as compared to those derived from Bw4^{neg} donors (*p = 0.0287). On the contrary, mAb-mediated triggering of other activating receptors, including NKp46, CD16 (**Figure 2B**, left panel), NKp30, NKp44, NKG2D, and DNAM-1 (not shown), did not display any significant variation between *KIR3DS1⁺* NK cell clones derived from Bw4-I80 and those derived from Bw4^{neg} donors. Notably, the expression of these (non-HLA-specific) activating receptors did not display any significant difference in *KIR3DS1⁺* NK cell clones derived from Bw4-I80 or Bw4^{neg} donors (**Figure 3**).

Phenotypic and Functional Analysis of *KIR3DS1⁺/NKG2A⁺* NK Cell Clones from Donors with Peculiar *KIR3DL1/S1* and HLA-B Combinations

Considering that it has been shown that the number of *KIR3DL1* and *KIR3DS1* gene copies plays an important role in modulating the HIV-1 control and that this effect seems to be detectable only after epistatic interactions between HLA molecules and KIRs (23, 28), we extended the phenotypic and functional analyses to additional NK cell clones derived from donors with peculiar *KIR3DL1/S1* and *HLA-B* combinations [**Figures 2A,B** (right panels) and **Figure 2C**]. In particular, NK cell clones were derived from: (a) an HLA-B Bw4-T80/Bw6 donor equipped with 2 *KIR3DS1* and 1 *KIR3DL1* GCN (P61, referred to as T80-3DS1-2GCN); (b) an HLA-B Bw4-T80/Bw6 donor carrying HLA-B*37:01, a particular Bw4-T80 allotype characterized by D77-T80 sequence (K9, referred to as HLA-B*37:01); and (c) an HLA-Bw4^{neg} donor equipped with a *KIR3DL1* allele coding for a polypeptide retained into the cytoplasm (K7, referred to as Bw4^{neg} *KIR3DL1^{intra}*) (29). As shown in **Figure 2A** (right panel), comparison of *KIR3DS1* MFIR among NK cell clones of this second set (T80-3DS1-2GCN, HLA-B*37:01, Bw4^{neg} *KIR3DL1^{intra}*

donors) did not reveal significant differences. On the contrary, some differences could be detected by comparing the first and the second set of donors analyzed. As shown in **Figure 2A**, *KIR3DS1⁺* NK cell clones derived from T80-3DS1-2GCN donor displayed a more heterogeneous *KIR3DS1* surface expression as compared to NK cell clones derived from T80 donor carrying one *KIR3DS1* GCN. Nevertheless, NK cell clones derived from T80-3DS1-2GCN donor (but also those from B*37:01 donor) did not display significant differences in terms of *KIR3DS1* MFIR as compared to NK clones of the first set.

On the contrary, NK cell clones from the Bw4^{neg} *KIR3DL1^{intra}* donor displayed a significantly lower *KIR3DS1* surface expression (**p < 0.0001) (**Figure 2A**) but a similar cytotoxicity upon *KIR3DS1* mAb-mediated triggering (**Figure 2B**) as compared to NK cell clones derived from Bw4^{neg} donors expressing surface *KIR3DL1*.

Moreover, the comparison of the cytotoxicity in redirected killing assays between the first and second sets of donors revealed that NK cell clones derived from the second set were characterized by higher increments of cytotoxicity upon *KIR3DS1* mAb-mediated triggering than NK cell clones from Bw4-I80 donors (*p = 0.0243 with T80-3DS1-2GCN donor, *p = 0.0014 with HLA-B*37:01 donor, and **p = 0.0059 with Bw4^{neg} *KIR3DL1^{intra}* donor) (**Figure 2B**).

Correlation Analysis between Expression of *KIR3DS1* and Non-HLA-Specific Activating Receptors in Donors Characterized by Different HLA-B Allotypes

The existence of a possible correlation between the expression of *KIR3DS1* and that of relevant non-HLA-specific activating receptors (i.e., NCRs, NKG2D and DNAM-1) was analyzed in all *KIR3DS1⁺/NKG2A⁺* NK cell clones considered in the present study. Of interest, only in T80-3DS1-2GCN donor, *KIR3DS1* surface density correlated inversely with NKp46 and directly with NKG2D MFIR (*p = 0.0154 and *p = 0.0107, respectively) (**Figure 4**). Notably, this result was detected only in NK cell clones from these donors characterized by higher and heterogeneous values of *KIR3DS1* MFIR (**Figure 2A**, right panel). Based on this observation, it cannot be ruled out that, in given HLA/KIR haplotype settings, *KIR3DS1* may influence the surface density of NKp46 and NKG2D.

KIR3DS1-Mediated Recognition of HLA-B*51 Allele on Transfected Cells

According to previous data suggesting a possible interaction between *KIR3DS1* and HLA-B Bw4-I80 alleles (30) and considering the high level of homology between the extracellular domains of *KIR3DS1* and *KIR3DL1* as well as the ability of *KIR3DL1* to recognize in most instances HLA-B Bw4-I80 alleles with higher efficiency than HLA-B Bw4-T80 alleles (9, 25), all *KIR3DS1⁺/NKG2A⁺* NK cell clones were assessed for their capability of killing HLA-B Bw4-I80 target cells. In particular, the killing activity of *KIR3DS1⁺/NKG2A⁺* NK cell clones was assessed against the C1R cell line transfected or not with HLA-B*51 allele (31).

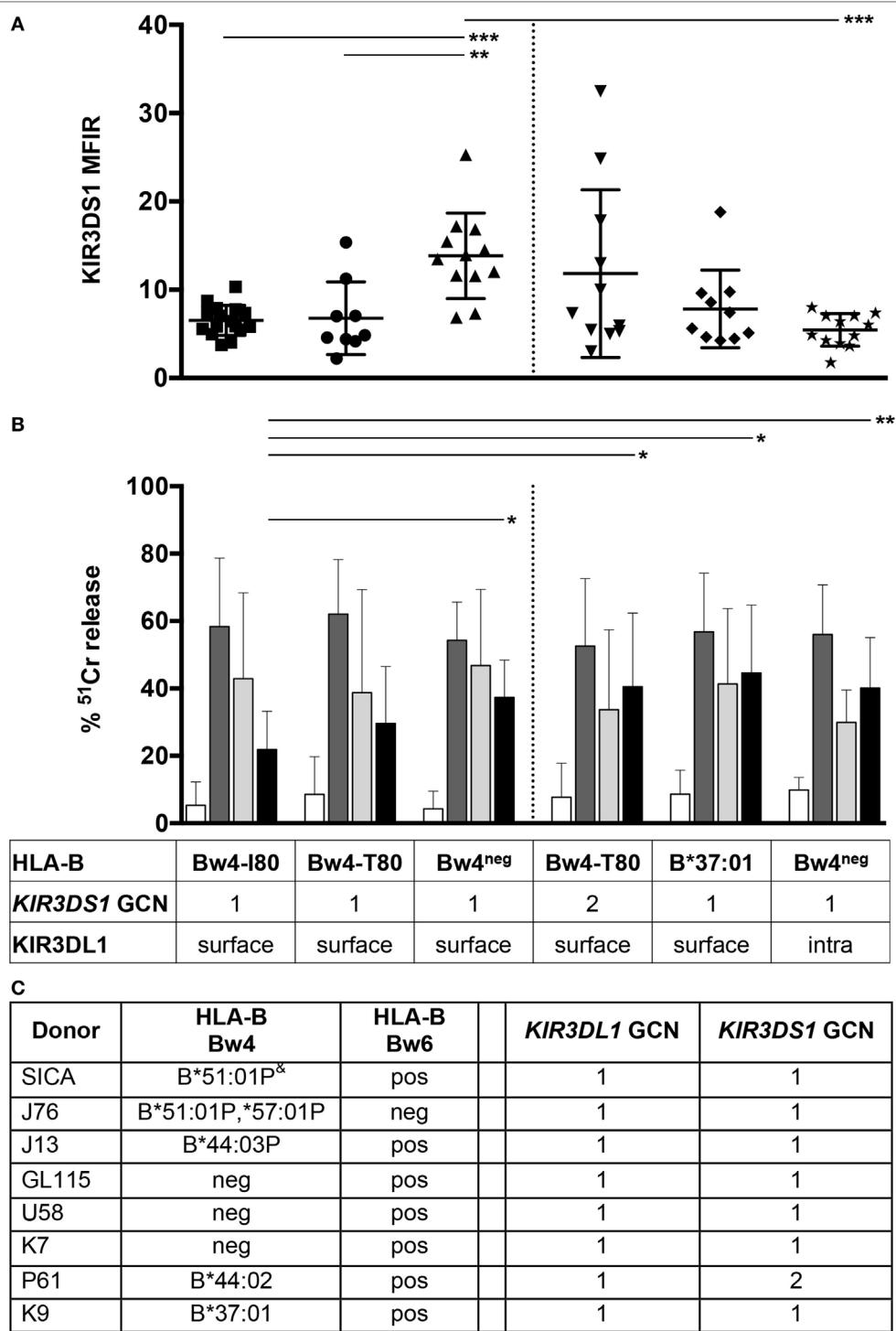


FIGURE 2 | KIR3DS1 surface expression and mAb-mediated cross-linking of KIR3DS1 in KIR3DS1+/NKG2A+ natural killer (NK) cell clones. KIR3DS1+/NKG2A+ NK cell clones derived from HLA-B-typed donors were analyzed for KIR3DS1 surface expression (**A**) and for ^{51}Cr release in redirected killing assay against P815 cell line (**B**). Data related to HLA typing and surface/intra *KIR3DL1* presence are indicated in the box below panel (**B**). (**A**) The NK cell clones of the first set (left panel) were derived from Bw4-I80 ($n = 10$, full square), Bw4-T80 ($n = 9$, full circle), and Bw4^{neg} ($n = 12$, full triangles) donors, whereas NK cell clones of the second set (right panel) were derived from T80-3DS1-2GCN ($n = 11$, overturned full triangle), HLA-B*37:01 ($n = 10$, full rhombus), and Bw4^{neg} *KIR3DL1*^{intra} ($n = 12$, full star) donors. (**B**) The cytolytic activity of the first (left panel) and second (right panel) set of KIR3DS1+/NKG2A+ NK cell clones was evaluated in redirected killing assays upon mAb-mediated cross-linking of different NK receptors. Cytolytic assays were performed in the absence of mAbs (white bar) or in the presence of anti-CD16 (c127, dark gray bar), anti-NKp46 (BAB281, light gray bar), or anti-KIR3DS1 (z27, black bar) mAbs. Average, standard deviation, and p values are indicated (* $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$). (**C**) HLA-B typing, *KIR3DL1*, and *KIR3DS1* gene copy number (GCN) analyses of the indicated donors are shown. ^xP: indicated a group of alleles with identical sequence at exons 2 and 3 and therefore sharing the same antigen-binding domains.

Considering recent studies showing that HLA-F OCs are high-affinity ligands of KIR3DS1 (16, 17), the *HLA-F* transcript was analyzed in C1R and C1R-B51 target cells to assess whether these cells would express this KIR3DS1 ligand. JA3 (a Jurkat clone) (32) and LCL 721.221 cell lines were used as negative control and positive control, respectively (16, 33). As shown in **Figure 5** and Figure S3 in Supplementary Material, C1R and C1R-B51 expressed very low levels of *HLA-F* transcript similar to JA3 cells, whereas LCL 721.221 expressed *HLA-F* mRNA five times more than C1R-B51. Thus, possible differences of lysis between C1R and C1R-B51 may not be attributed to recognition of HLA-F by KIR3DS1.

A representative cytolytic experiment against C1R/C1R-B51 target cells performed using as effector cell a KIR3DS1⁺/NKG2A⁺ NK cell clone derived from the T80-3DS1-2GCN donor is shown in **Figure 6**. As controls, KIR3DS1^{neg}/NKG2A⁺ and KIR3DL1⁺/NKG2A⁺ NK cell clones derived from the same donor were analyzed. In the absence of mAbs, C1R-B51 cells were killed slightly more efficiently than un-transfected C1R by the

KIR3DS1⁺/NKG2A⁺ NK cell clone (* $p = 0.0159$). Notably, this difference of lysis was abolished upon mAb-mediated masking of KIR3DS1, suggesting a possible positive recognition of HLA-B*51 by KIR3DS1 (**Figure 6A**). Interestingly, this result was more evident when the experiment was performed in the presence of anti-NKG2D mAb. Thus, as shown in **Figure 6B**, the difference between C1R and C1R-B51 killing was amplified by NKG2D mAb-mediated blocking (** $p = 0.0022$). Importantly, further masking of KIR3DS1 abrogated this difference (** $p = 0.0065$) (**Figure 6B**). Similar data were obtained upon additional mAb-mediated masking of NKG2A.

A similar experimental approach was applied to the NK cell clones generated from the other donors analyzed (**Figure 7**). These clones were characterized by a substantially homogeneous NKG2D expression. The cytolytic assays were performed not only in the presence of blocking NKG2D but also upon mAb-mediated masking of NKG2A in order to further reduce possible functional variations caused by different intensities of inhibitory signals consequent to NKG2A/HLA-E interaction. Then, cytotoxicity was evaluated upon additional blocking of KIR3DS1 (**Figure 7**). In this set of experiments, in order to better appreciate the KIR3DS1 contribution to target killing, each NK cell clone was also analyzed for possible variations in the lysis of C1R or C1R-B51 targets in the absence or in the presence of KIR3DS1 mAb-mediated blocking (Δ_{C1R} and $\Delta_{C1R-B51}$).

In Bw4-I80 donors, lysis of C1R and C1R-B51 was not affected by the addition of anti-KIR3DS1 mAb (as shown by the raw data as well as Δ_{C1R} and $\Delta_{C1R-B51}$ values) (**Figure 7A**). On the other hand, in Bw4^{neg} donors, the addition of anti-KIR3DS1 mAb slightly decreased C1R-B51 killing and a significant gap between Δ_{C1R} and $\Delta_{C1R-B51}$ could be detected (* $p = 0.0188$).

KIR3DS1⁺/NKG2A⁺ NK cell clones from the T80-3DS1-2GCN donor killed more efficiently C1R-B51 than C1R. Moreover, the addition of anti-KIR3DS1 mAb abolished this difference. Remarkably, in this donor, the comparison between Δ_{C1R} and $\Delta_{C1R-B51}$ showed a highly significant difference (** $p < 0.0001$), **Figure 7B**). Significant differences between Δ_{C1R} and $\Delta_{C1R-B51}$ could also be observed in KIR3DS1⁺/NKG2A⁺ NK cell clones derived from both HLA-B*37:01/Bw6 and Bw4^{neg} KIR3DL1^{intra} donors

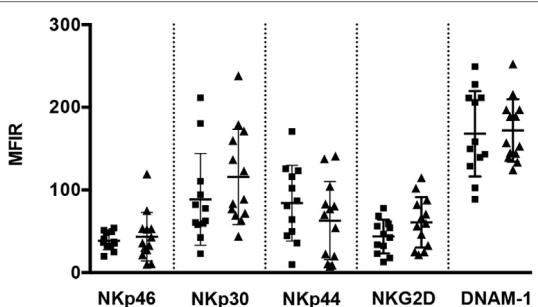


FIGURE 3 | Surface expression of several non-HLA-specific activating receptors. Staining of NKp46 (BAB281), NKp30 (AZ220), NKp44 (z231), NKG2D (ON72), and DNAM-1 (KRA236) molecules on KIR3DS1⁺/NKG2A⁺ natural killer (NK) cell clones derived from Bw4-I80 ($n = 12$, full squares) or Bw4^{neg} carrying surface KIR3DL1 ($n = 13$, full triangles) donors were compared in terms of mean fluorescence intensity ratio (MFIR) in scatter dot plot representation. Average and standard deviation are shown.

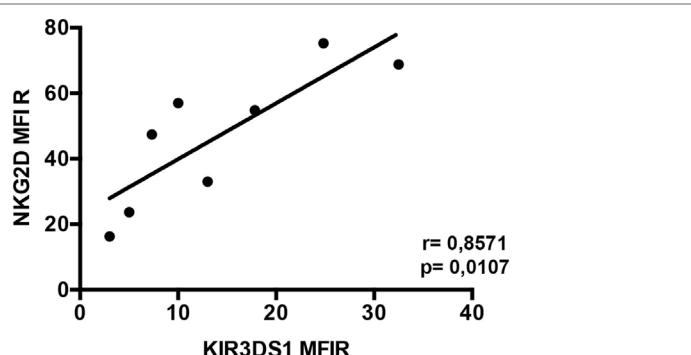


FIGURE 4 | Phenotypic analyses of KIR3DS1⁺/NKG2A⁺ natural killer (NK) cell clones derived from T80-3DS1-2GCN donor. Correlation analyses among NKp46 or NKG2D activating receptors and KIR3DS1 surface expression in several KIR3DS1⁺/NKG2A⁺ NK cell clones ($n = 8$) derived from a T80-3DS1-2GCN donor are represented. Linear regression values (r) and p values are shown.

($^{**}p = 0.0073$ and $*p = 0.0110$, respectively). All together, these data suggest a direct involvement of KIR3DS1 in the recognition of HLA-B*51 (Bw4-I80) target cells. Remarkably, this result can be detected only when NK cells were generated in particular KIR/HLA combination settings. Indeed, KIR3DS1-mediated recognition of HLA-B*51 occurs only when NK cell clones were derived from Bw4-I80^{neg} donors (Bw4-T80-2GCN, B*37:01/Bw6, or Bw4^{neg}), suggesting a role for HLA-B/KIR3DS1 interaction in the process of NK cell education.

Notably, when comparing KIR3DS1⁺/NKG2A⁺ and KIR3DS1^{neg}/NKG2A⁺ NK cell clones, a significant difference in Nkp46 surface expression was observed only in Bw4-I80 donors. Thus, KIR3DS1⁺/NKG2A⁺ NK cell clones expressed lower levels of Nkp46 than KIR3DS1^{neg}/NKG2A⁺ NK cell clones ($^{***}p = 0.0005$), further corroborating the possibility that

HLA-B/KIR3DS1 interaction may be involved in the process of NK cell education (Figure 8).

DISCUSSION

A number of studies described an association between given KIR/HLA gene combinations and clinical outcome in various immune challenges and reported a possible perturbation of KIR/HLA interactions by the presented peptide. In particular, an important combined role played by KIR3DS1 and HLA-B Bw4-I80 in controlling HIV infection (11, 12) and the recognition of specific HIV-derived peptides associated with HLA-B*57 alleles by KIR3DS1 have been described (15).

In the present study, we provided the first evidence of a direct involvement of KIR3DS1 in the NK-mediated recognition of HLA-B*51 surface molecules expressed on target cells. This capability is manifested only when KIR3DS1 is expressed by NK cells derived from individuals carrying particular KIR/HLA combinations. Although our study was performed on a limited number of donors (due to the difficulty in the collection of individuals with similar characteristics in terms of KIR and KIR-Ls as well as in the generation and expansion of appropriate NK cell clones), our results suggest a role for this activating receptor in the process of NK cell education. In particular, the KIR3DS1-mediated positive recognition of HLA-B*51 (Bw4-I80) could be detected in NK cell clones derived from Bw4-I80^{neg} donors (one Bw4-T80-2GCN, one B*37:01/Bw6, and three Bw4^{neg}) but not in those from Bw4-I80 donors (two donors). Thus, in a manner reminiscent of that previously described for KIR2DS1 (26, 34), the interaction of KIR3DS1 with its self-HLA-B class I ligand (Bw4-I80 alleles) would affect the subsequent response mediated by this aKIR. Indeed, in HLA-B Bw4-I80 donors, the process of NK cell education that is taking place via NKG2A/self-HLA-E

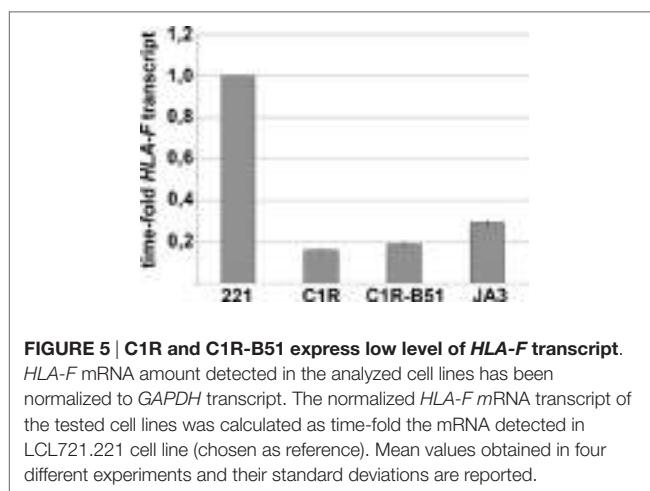


FIGURE 5 | C1R and C1R-B51 express low level of HLA-F transcript.

HLA-F mRNA amount detected in the analyzed cell lines has been normalized to GAPDH transcript. The normalized HLA-F mRNA transcript of the tested cell lines was calculated as time-fold the mRNA detected in LCL721.221 cell line (chosen as reference). Mean values obtained in four different experiments and their standard deviations are reported.

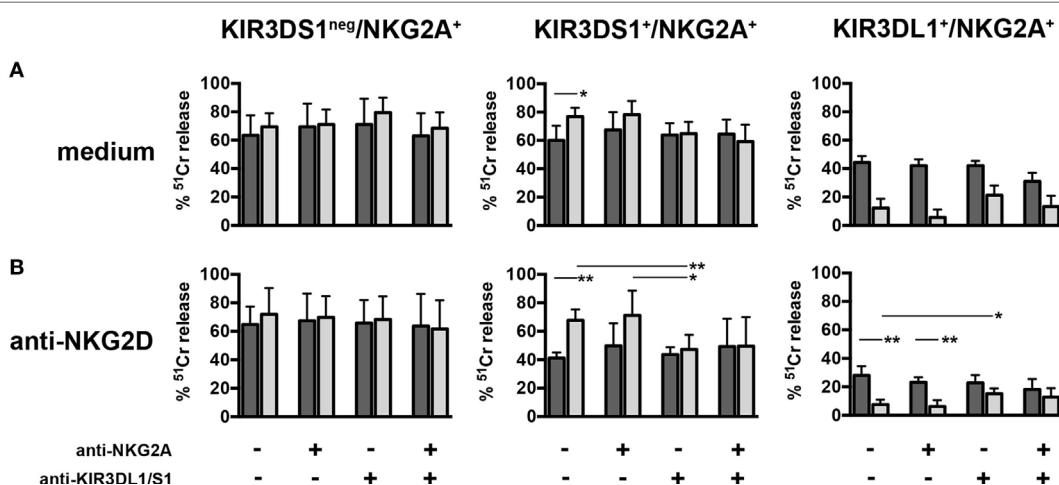


FIGURE 6 | Killing of C1R-B51 target cells by natural killer (NK) cell clones derived from a T80-3DS1-2GCN donor. Three representative NK cell clones (one KIR3DS1^{neg}/NKG2A⁺, one KIR3DS1⁺/NKG2A⁺ and one KIR3DL1⁺/NKG2A⁺) derived from the T80-3DS1-2GCN donor were tested for cytotoxic activity against C1R (dark gray histograms) and C1R-B51 (light gray histograms) target cell lines. Experiments were performed in the absence (A) or in the presence of anti-NKG2D masking mAb (B). Additional mAb-mediated maskings were carried out as indicated below panel B. Histograms summarized results of three independent experiments in duplicate. Average, standard deviation, and p values are shown ($^{*}p < 0.1$ and $^{**}p < 0.01$).

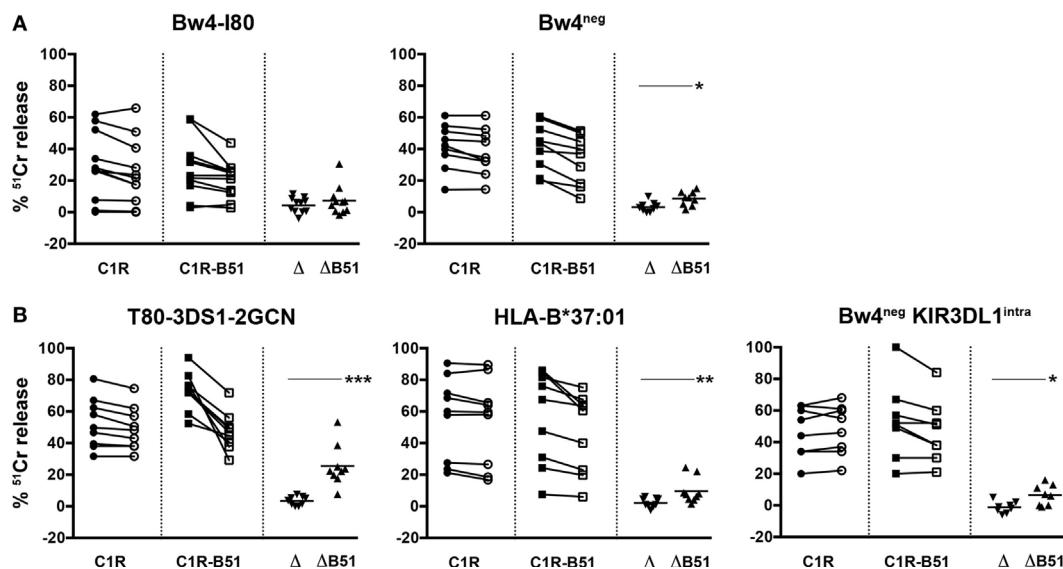


FIGURE 7 | C1R-B51 killing by KIR3DS1⁺/NKG2A⁺ natural killer (NK) cell clones. NK cell clones were tested in ⁵¹Cr-release cytotoxicity assay against C1R (circle) and C1R-B51 (square) target cell lines in the presence of anti-NKG2D + anti-NKG2A masking mAbs (full symbols) or anti-NKG2D + anti-NKG2A + anti-KIR3DS1 masking mAbs (empty symbols). Δ and Δ-B51 indicate the variations of C1R or C1R-B51 lysis in the absence or presence of anti-KIR3DS1 mAb for each NK cell clone. NK cell clones derived from Bw4-I80 ($n = 11$) and Bw4^{neg} ($n = 9$) donors are represented in panel (A), whereas those derived from T80-3DS1-2GCN ($n = 9$), HLA-B*37:01 ($n = 9$), and Bw4^{neg} KIR3DL1^{intra} ($n = 8$) donors are represented in panel (B). Plots summarized results of three independent experiments in duplicate. p values indicate a statistically significant difference between the groups ($*p < 0.1$, $**p < 0.01$, and $***p < 0.001$).

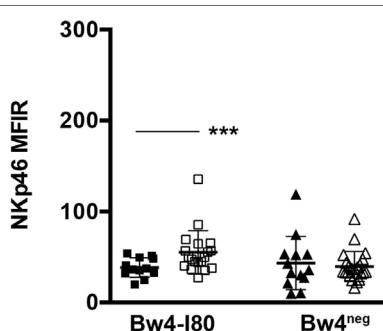


FIGURE 8 | NKp46 surface expression on KIR3DS1⁺/NKG2A⁺ and KIR3DS1^{neg}/NKG2A⁺ natural killer (NK) cell clones derived from Bw4-I80 or Bw4^{neg} donors. KIR3DS1⁺/NKG2A⁺ (full symbols) and KIR3DS1^{neg}/NKG2A⁺ (empty symbols) NK cell clones derived from Bw4-I80 (squares, $n = 12$ and $n = 19$, respectively) and Bw4^{neg} (triangles, $n = 13$ and $n = 22$ respectively) donors were stained with anti-NKp46 mAb (BAB281). Results are summarized in scatter plot analysis. Average, standard deviation, and p values are indicated (** $p < 0.001$).

interaction is characterized by a decrement in NK cell responsiveness upon KIR3DS1 engagement, thus ensuring self-tolerance. In this context, it has to be considered that KIR3DS1 responsiveness in redirected killing assays was lower in Bw4-I80 than in Bw4-I80^{neg} donors. In particular, among those analyzed, KIR3DS1 was the only receptor showing a significant differential expression and responsiveness between Bw4-I80 and Bw4^{neg} donors.

The analysis performed in the present study was limited to KIR3DS1⁺/NKG2A⁺ NK cell clones in order to evaluate whether

KIR3DS1 expression could confer alloreactivity to non-alloreactive NKG2A⁺ NK cells and to minimize the effect that other self KIR/HLA interactions would exert on NK cell education. Several studies have suggested that, in given donor/recipient pairs, the expression of aKIRs, such as KIR2DS1, can amplify the size of the alloreactive NK cell subset. This effect is particularly relevant in the successful therapy of high-risk acute leukemias in the haplo-HSCT setting (35–39). Indeed, it has been shown that the “non-alloreactive” NKG2A⁺ iKIRs^{neg} NK cells can display alloreactivity against HLA-C2⁺ recipient cells when they co-express KIR2DS1 (40). A similar effect could be true, at least in part, also for KIR3DS1. In this context, the definition of the specificity/function of KIR3DS1 would have important implications not only to identify donors capable of generating alloreactive NK cells (on the basis of the existence of a KIR/HLA-class I mismatch) but also to select the best donor, according to the size of the alloreactive NK cell subset.

On the basis of a recent study (23) showing that KIR3DL1-dependent licensing of NK cells could be involved in shaping a strong antiviral response mediated by KIR3DS1⁺ NK cells, we also analyzed KIR3DS1 expression and responsiveness in donors characterized by peculiar KIR/HLA combinations. In this context, we showed that KIR3DS1 mAb-mediated triggering of cytotoxicity in NK cell clones from a Bw4^{neg} donor carrying KIR3DL1^{intra} is similar to that detectable in NK cell clones from Bw4^{neg} donors expressing surface KIR3DL1. It is noteworthy that this increment of cytotoxicity occurred despite the low surface expression of KIR3DS1 (Figure 2). Remarkably, NK cell clones from the Bw4^{neg} donor carrying KIR3DL1^{intra} were able to kill HLA-B*51⁺ target cells with efficacy comparable to that of NK cell clones derived from Bw4^{neg} donors with surface KIR3DL1.

Moreover, different from KIR3DS1⁺ NK cell clones of classical HLA-B Bw4-T80 donors, clones derived from a donor carrying the HLA-B*37 allele (a particular HLA-Bw4-T80 allotype characterized by the D77-T80 sequence) were characterized by higher KIR3DS1 responsiveness in redirected killing assays and higher efficiency of killing HLA-B51⁺ target cells as compared to NK clones from HLA-B Bw4-I80 donors.

However, the highest increment of cytotoxicity against C1R-B51 cells was detected in NK cell clones from a T80-Bw4 donor carrying two copies of *KIR3DS1* and one copy of *KIR3DL1*. Interestingly, in this donor, an inverse correlation between KIR3DS1 and NKp46 MFIR could also be detected. This finding is relevant in the context of NK cell education. Indeed, a low surface expression of NKp46 combined with high KIR3DS1 surface density on NK cell clones may prevent possible KIR3DS1-mediated autoreactive responses in non-pathological conditions. In the same donor, a direct correlation between KIR3DS1 and NKG2D surface expression was also observed. Notably, these correlations could be observed only in NK clones from this donor in which KIR3DS1 surface expression was more heterogeneous and higher as compared to Bw4-T80 donor carrying one copy of *KIR3DS1* and one copy of *KIR3DL1*.

The direct correlation between KIR3DS1 and NKG2D surface expression is particularly interesting if we consider that NKG2D pathway is less functional in certain viral infections. Indeed, previous studies have described that several viral immune evasion strategies possibly evolved to elude NKG2D-mediated immune-surveillance (30, 41–44). For example, the HIV Nef protein prevents the expression of some NKG2D ligands at the surface of infected cells (45, 46). According to these data, in an attempt to simulate the compromised NKG2D function occurring during certain viral infections, we analyzed the effect of mAb-mediated NKG2D blocking in KIR3DS1⁺ NK clones. Thanks to this experimental approach, we could show that KIR3DS1 function may be crucial, primarily during viral infections in which other triggering signals, such as those through NKG2D, are compromised. In this context, it is also important to take into consideration that the expression of HLA-B can be modified only marginally during certain viral infections. Thus, in HIV-1-infected viremic patients, while HLA-A and -Bw6 surface molecules were significantly downmodulated in T cell blasts, HLA-B Bw4 alleles were not (47).

Considering the “discontinuity theory for immunity” (48), it is also possible that KIR3DS1⁺ NK cells in donors expressing specific HLA class I (Bw4-I80) may be less responsive under normal conditions. However, when a non-physiologic triggering signal is given to the cells (e.g., viral infections), the equilibrium that is maintaining tolerance could be disrupted and may become an important component of an efficient immune response (34). This mechanism could explain the association between the expression of *KIR3DS1* in conjunction with *HLA-B Bw4-I80* in patients with chronic HIV-1 infection and a slower progression to AIDS.

KIR3DS1 associates DAP12 ITAM-bearing molecules in its cytoplasmic tail to enable signal transduction. Surprisingly, in recent years, different studies showed that ITAMs can also

generate an inhibitory signal in addition to the activating ones. In particular, the same ITAM-coupled receptors can generate both positive and negative signals (49–51). The molecular basis of this dual function is not well understood at the present; however, it has been suggested that the avidity of receptor ligation may define the nature of response. In this regard, one may speculate that the peptide in the HLA-Bw4-I80 groove could determine change of affinity of KIR3DS1 ligation. Thus, healthy self peptides could mediate a low-affinity, tolerogenic signal, whereas viral peptides (i.e., HIV derived) would allow high-affinity activating signals. This hypothesis would be in line with the protective effect exerted by the combined presence of *KIR3DS1* and *HLA-B Bw4-I80* in patients with chronic HIV-1 infection.

All these considerations are consistent with an important role of KIR3DS1 in the control of viral infections (8, 30), primarily in post-transplantation settings (52). Since transplantation from donors displaying NK-cell alloreactivity and expressing *KIR2DS1* and/or *KIR3DS1* has been associated with a reduced risk of no relapse mortality, that is largely infection related, and with significantly better event-free survival (38, 53), our results could further improve the selection of the most suitable donor, taking into account the expression of not only *KIR3DS1* but also the self HLA-B allotype expressed.

ETHICS STATEMENT

Buffy coats from healthy donors were obtained from the Immunohematology and Transfusion Center of the S. Martino Hospital (Genova, Italy). Approval was obtained by the ethical committee of IRCCS S. Martino-IST (39/2012) of Genova (Italy). Informed consent was provided according to the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SC and SS designed and performed research, interpreted data, and wrote the paper; MF performed research, interpreted data, and wrote the paper; MB, CA, LG, and MM performed research and analyzed data; LM interpreted and critically revised the paper; and AM interpreted, critically revised data, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00581/full#supplementary-material>.

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Modulation of Human Leukocyte Antigen-C by Human Cytomegalovirus Stimulates KIR2DS1 Recognition by Natural Killer Cells

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The interaction of inhibitory killer cell Ig-like receptors (KIRs) with human leukocyte antigen (HLA) class I molecules has been characterized in detail. By contrast, activating members of the KIR family, although closely related to inhibitory KIRs, appear to interact weakly, if at all, with HLA class I. KIR2DS1 is the best studied activating KIR and it interacts with C2 group HLA-C (C2-HLA-C) in some assays, but not as strongly as KIR2DL1. We used a mouse 2B4 cell reporter system, which carries NFAT-green fluorescent protein with KIR2DS1 and a modified DAP12 adaptor protein. KIR2DS1 reporter cells were not activated upon coculture with 721.221 cells transfected with different HLA-C molecules, or with interferon-γ stimulated primary dermal fibroblasts. However, KIR2DS1 reporter cells and KIR2DS1⁺ primary natural killer (NK) cells were activated by C2-HLA-C homozygous human fetal foreskin fibroblasts (HFFFs) but only after infection with specific clones of a clinical strain of human cytomegalovirus (HCMV). Active viral gene expression was required for activation of both cell types. Primary NKG2A-KIR2DS1⁺ NK cell subsets degranulated after coculture with HCMV-infected HFFFs. The W6/32 antibody to HLA class I blocked the KIR2DS1 reporter cell interaction with its ligand on HCMV-infected HFFFs but did not block interaction with KIR2DL1. This implies a differential recognition of HLA-C by KIR2DL1 and KIR2DS1. The data suggest that modulation of HLA-C by HCMV is required for a potent KIR2DS1-mediated NK cell activation.

Keywords: natural killer cells, human cytomegalovirus, killer Ig-like receptor, KIR2DS1, HLA-C

INTRODUCTION

Since the discovery of natural killer (NK) cells more than 40 years ago (1–3), the interaction of inhibitory NK cell receptors with human leukocyte antigen (HLA) class I molecules has been characterized in detail. This led to new insights into NK cell differentiation, education, and function. However, ligands of most activating receptors, including activating killer cell Ig-like receptors (KIRs), are yet to be discovered.

KIR genes are members of the immunoglobulin (Ig) superfamily, encoded in the leukocyte receptor complex (LRC) on chromosome 19q14.3 (4). KIR molecules express either two or three extracellular Ig-like domains (2D or 3D) and consist of either a long (designated "L") or short (designated "S") cytoplasmic domain. KIRs with long cytoplasmic domains are inhibitory (iKIRs) and contain ITIMs. Activating KIRs (aKIRs) have a short cytoplasmic tail and transmit activating signals through the interaction with DAP12, which contains an ITAM (4).

Most iKIRs recognize certain allotypes of HLA class I. In general, allelic products of *KIR2DL1* bind to the C2 group of HLA-C molecules (C2-HLA-C) characterized by Asn77 and Lys80 (5), while KIR2DL2 and -2DL3, which are alleles at the same locus, recognize the C1 group (C1-HLA-C, Ser77, and Asn80) (6–8). These structural motifs were originally thought to be essential for the engagement of KIRs only on HLA-C. However, KIR2DL2 can also bind HLA-B46:01 and -B73:01 alleles, which have C1-related motifs at residues 77–83 (9). Furthermore, KIR2DL2 and -L3 receptors can bind many HLA-C alleles irrespective of -C1 or -C2 group (10, 11).

The extracellular parts of iKIRs and aKIRs are highly homologous and share conserved amino acid sequences, as "paired" receptors (11, 12). The balance between inhibitory and activating signaling through these paired receptors is tightly regulated by NK cells. Dysregulation of this balance might lead to autoimmunity or infectious diseases (13, 14). How the signaling is controlled by NK cells, however, is not completely understood, mainly due to uncertainty over the ligands and functions of aKIRs. The aKIR members seem to have evolved more rapidly than iKIRs, possibly through selection pressure imposed by pathogens (15, 16). If this hypothesis is true, it suggests that aKIR binding may be influenced by pathogen-derived proteins. Notably, KIR2DS1 and -2DS2 counterparts in chimpanzees, respectively, bind C2- and C1-HLA-C with high avidity compared to their inhibitory paired receptors (17). This indicates that the loss of binding by KIR2DS2, or highly reduced binding of KIR2DS1, to HLA-C is a product of human-specific evolution.

Most interactions of aKIRs and HLA class I molecules are very weak or undetectable (17–23). The best studied aKIR is KIR2DS1 and many studies have found that it binds C2-HLA-C (10, 11, 17, 24–35). However, this binding is much weaker compared to KIR2DL1 (10, 25, 27). Using surface plasmon resonance (SPR) analysis, Stewart and colleagues demonstrated that KIR2DS1 tetramer-binding avidity to the soluble HLA-Cw4/beta-2 microglobulin (β_2 M)/peptide complex is approximately four times lower than KIR2DL1: dissociation constants (K_d) of 7.2 and 30 μ M, respectively. In addition, amino acid substitution of the peptide at amino acid 7 or 8 drastically reduced KIR2DS1 tetramer binding to the HLA-Cw4 complex, indicating that the binding is peptide dependent (27). Both Moesta et al. and Hilton et al. demonstrated that KIR2DS1-Fc binds a range of C2-HLA-C allotypes linked to microbeads with different avidity (11, 17). Biassoni and colleagues demonstrated that the amino acid at position 70, a threonine in KIR2DL1 and a lysine in KIR2DS1, is the key residue that differentiates binding between KIR2DL1 and -2DS1. Substituting the threonine to

a lysine in KIR2DL1-Fc prevented binding to 721.221 transfected with HLA-Cw4 (221-Cw4). When lysine was substituted for a threonine in KIR2DS1-Fc, the binding to 221-Cw4 was restored (25).

The rationale for KIR2DL1 and KIR2DS1 both binding C2-HLA-C would be understandable if the activating receptor was sensitive to structural changes in the HLA molecule, or bound an alternative molecule, induced by viral infection (18, 27, 36–42). For example, in mice, the cytomegalovirus (CMV)-encoded MHC class I homolog m157 is directly recognized by the activating Ly49H receptor (43). The mouse Ly49 receptor family serves a similar role to KIRs in humans, although KIRs and Ly49 receptors are from different molecular families. KIRs play an important role in human cytomegalovirus (HCMV) infections (44–47). For instance, a recent study demonstrated that KIR2DS1⁺ decidual NK (dNK) cells degranulated after engaging with HCMV-infected decidual stromal cells (DSC), suggesting an increased ability of KIR2DS1-expressing dNK cells to respond to placental HCMV infection (47). Della Chiesa et al. have reported that HCMV can drive NK cell maturation in the absence of NKG2C in patients with hematological malignancies. These patients received umbilical cord blood transplantation from NKG2C^{-/-} donors and when HCMV reactivation occurred, an expansion of NKG2A⁻ NK cells expressing aKIRs was measured, particularly KIR2DS1 and KIR3DS1 (45). This finding is consistent with KIR2DS1 recognizing a ligand on HCMV-infected cells.

To probe the potential influence of HCMV on KIR2DS1 recognition, we designed a mouse 2B4 T cell hybridoma carrying an NFAT-green fluorescent protein (GFP) reporter. Our results suggest that modulation of HLA-C by HCMV is required for a potent KIR2DS1-mediated NK cell activation.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Reporter cells, 721.221 cells, K562 cells, and primary NK cells were cultured in RPMI-1640 (Sigma-Aldrich, Steinheim, Germany) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco, Paisley, UK), and 10% heat-inactivated Fetal Bovine Serum (Gibco). Human fetal foreskin fibroblasts (HFFs, Culture Collections—ECACC, UK) and primary dermal fibroblasts (DFs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum. Adherent cells were harvested by washing the cells once with phosphate buffered saline (PBS, Sigma-Aldrich). Then the cells were either detached from the plastic using 0.05% Trypsin/EDTA (Gibco) or AccutaseTM (Biologen, San Diego, CA, USA) for 5 min at 37°C. All cellular experiments were performed at 37°C in 5% CO₂.

721.221 cells transfected with -A23:01, -B58:01 (Bw4), -B35:01 (Bw6), -C01:02 (C1), -C02:02 (C2), -C03:02 (C1), -C04:01 (C2), -C06:02 (C2), -C07:01 (C1), and -HLA-G were generated in house. 721.221-HLA-A11:02 was provided by Parham (22) (Stanford University, Palo Alto, CA, USA).

Establishment of the Reporter Cells

The 2B4 T cell hybridoma containing an NFAT-GFP reporter gene (2B4 reporter cells) was kindly provided by Lewis Lanier (43) (University of California San Francisco, USA). KIR2DL1*003, -2DL2*001, -2DS1*002, and -2DS1^(K70T) reporter cells were generated as follows. First, pMX-neo constructs containing cDNA from a chimeric adaptor protein recombinant was used to transduce the 2B4 reporter cells. The chimeric adaptor consists of DAP12 and a cytoplasmic tail of DAP10 with spacer sequences in between. Then cDNA of the indicated KIRs was subcloned into a pMX-puro construct. For constructing 2DL1-2DS1TM (KIR2DL1 reporter) and 2DL2-2DS1TM (KIR2DL2 reporter) chimeric molecules, 5'-CCTGCACGTTCTGATTGGGACCTCAGT-3' and 5'-CCCAATCAGAACGTGCAGGTGTCGGGGTT-3' primers were used. 5'-AGTCGCATGACGCAAGACCTGGCAGGG-3' and 5'-GGTCTTGCCTCATGCGACTGATGGAG-3' primers were used for constructing the KIR2DS1^(K70T) reporter cell. Retroviruses were packaged in Phoenix-eco cells (generously provided by Lewis Lanier) using the non-modified polyethyleneimine (PEI, Sigma-Aldrich) reagent as described by Ehrhardt et al. (48). After 48 h, supernatant-containing retroviral particles was used to transduce the 2B4 reporter cells by adding Polybrene (8 ng/ml, Sigma-Aldrich) and by spin-infecting the cells at 2,500 rpm (AccuSpin 3R centrifuge, Fisher Scientific, Waltham, MA, USA) for 2 h. Cells expressing the KIRs were purified by surface staining using the PAN2D antibody (clone NKVFS1, Bio-Rad, Hercules, CA, USA), followed by single-cell sorting using the FACS sorter (BD Bioscience, Oxford, UK). The transduction success and the function of the reporter cells were analyzed by immunofluorescent staining and antibody crosslinking, as described below. The LILRB1 reporter cell was provided by Des Jones (Department of Pathology, University of Cambridge) and was constructed as described in Ref. (49).

Primary Cells

Primary NK cells and DFs were obtained from healthy individuals. Ethical approval for the use of these tissues was given by Addenbrookes National Health Service Hospital Trust institutional review board (Cambridge Research Ethics Committee) and informed written consent was obtained from all volunteers in accordance with the Declaration of Helsinki (LREC 97/092). The primary fibroblasts were fully HLA typed. HFFFs express HLA-A11:01, -A24:02, -B35:02 (Bw6), -B40:02 (Bw6), -C02:02 (C2), and -C04:01 (C2). Donor CMV307 expresses HLA-A01:01, -A26:01, -B08:01 (Bw6), -B27:05 (Bw4), -C07:01 (C1), and -C01:02 (C1). Donor CMV0005 expresses HLA-A02, -A03, -B07 (Bw6), -B13 (Bw4), -C07 (C1), and -C06 (C2). Primary NK cell donors both express KIR2DL1, -2DS1, 2DL3, NKG2A, and were C1/C1-HLA-C. In addition, donor 016 expresses NKG2C and donor 111 expresses KIR3DL1.

For functional NK cell studies, peripheral blood mononuclear cells (PBMCs) were extracted from 30 to 40 ml blood of donor 016 and 111 on a Ficoll-Hypaque density gradient (Lymphoprep, Axis-Shield, Dundee, Scotland). The PBMCs were removed from the interface of the plasma and Lymphoprep layers and washed three times with PBS before further use. NK cells were separated from the PBMCs by negative selection using the EasySep™

Human NK cell Enrichment Kit from Stemcell Technologies (Vancouver, BC, Canada).

Dermal biopsies were taken from healthy individuals by Andrew Carmichael (Department of Medicine, University of Cambridge). They were sectioned with a scalpel and were grown beneath cover slips in a six-well culture plate containing Eagle's Minimum Essential Media (EMEM, GE Healthcare, Little Chalfont, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 10% heat-inactivated fetal calf serum (Life Technologies, Carlsbad, CA, USA). The cells were grown until sufficient cell number was reached and were stored in liquid nitrogen at low passage numbers.

Coculture Experiments

Human fetal foreskin fibroblasts (10×10^3 cells/well) and DFs (10×10^3 cells/well) were seeded in 96-well flat-bottom culture plates with or without interferon (IFN)- γ (500 U/ml, PeproTech, Rocky Hill, CT, USA) for 72 h or infected with HCMV as described below. After appropriate stimulation/infection time was reached, reporter cells were added to the adherent cells at a concentration of 2×10^4 cells per well, and reporter cells were added directly. Cocultures using K562 cells and 721.221 cells were performed at an E:T ratio of 1:1 and 1:3, respectively. After an overnight coculture, the reporter cells were harvested, and GFP expression was analyzed by flow cytometry.

Antibody Cross-linking

For antibody crosslinking experiments, anti-mouse IgG-coated microplates (R&D systems, Minneapolis, MN, USA) were used. The plates were incubated for 30 min with 0.2–1 µg per well of PAN2D (clone NKVFS1) or anti-HA (clone HA-7, Sigma-Aldrich) antibodies in PBS at room temperature. After two PBS washes, the reporter cells were added.

Antibody-Blocking Assays

Unconjugated W6/32 (LEAF™ purified BioLegend), 6A4 (gift from Daniela Pende, Azienda Ospedaliera Universitaria San Martino di Genova), B1.23.2 (eBiosciences, San Diego, CA, USA), IgG2a (LEAF™ purified BioLegend), IgG2b (LEAF™ purified BioLegend), and IgG1 (LEAF™ purified BioLegend) isotype antibodies were added to pre-treated HFFFs. After an incubation of 10–15 min at room temperature and saturating concentration of 1–4 µg per well, the reporter cells were added.

Flow Cytometry

Immunofluorescence Cell-Surface Staining

The cells were harvested and rested at 37°C in 5% CO₂ for at least 30 min for the recovery of HLA class I molecules on the cell surface. Cells expressing Fc receptors or HCMV-encoded Fc receptors were first blocked with 40% human serum (Sigma-Aldrich) in PBS for at least 5 min before staining. Fractions of 721.221 cells were collected and directly incubated with the appropriate antibody. Cell-surface expression of different receptors was analyzed by immunofluorescent staining using unconjugated monoclonal primary antibodies listed in Table 1, unconjugated anti-HLA-E (3D12, IgG1, BioLegend), biotinylated anti-HLA-A11 (Abcam,

TABLE 1 | List of antibodies and their target epitopes where known.

Antibody	Isotype/supplier	Recognition	Specific HLA molecules	Reference
W6/32	IgG2a/BioLegend, Hybridoma	β_2 M bound, fully assembled human leukocyte antigen (HLA) class I molecules. The epitope is not known. It includes residues on β_2 M, α 2, and α 3 domains	HLA-A, -B, -C and HLA-E, -G	(50, 51)
B1.23.2	IgG2b/eBiosciences	β_2 M bound, fully assembled HLA-B and -C molecules. Precise epitope unknown	HLA-B and HLA-C	(52)
6A4	IgG1/Hybridoma	β_2 M bound, fully assembled HLA-B and -C molecules. Precise epitope unknown	HLA-B and HLA-C	(53)
HC10	IgG2a/Hybridoma	Free heavy chain of HLA class I. Residues 57–62, specifically residue 60 in the α 1 domain in HLA-C. The epitope is blocked by peptide binding	HLA-B and HLA-C and some HLA-A (A10, A28, A29, A30, A31, A32, A33)	(54, 55)
DT9	IgG2b/Hybridoma	Fully assembled HLA-C and HLA-E bound to β_2 M. Precise epitope unknown	HLA-C and HLA-E	(56)
L31	IgG1/MediaParma	Free heavy chain of HLA-C. Residues 66–68, with F or Y at position 67	Most HLA-C and a few HLA-B (B08, B07, B35, B51, B54, B56)	(57)

IgM, Cambridge, UK), APC-conjugated anti-HLA-Bw6 (IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany), and the appropriate isotype control antibodies. The primary antibodies were added at a concentration of 0.5–1 μ g per well in 5% FCS in PBS or 40% human serum in PBS and incubated on ice for 45 min. Following washing, the cells were incubated with secondary antibodies (0.4–1 μ g per well), AlexaFluor® 647 (AF647)-conjugated Streptavidin (Life Technologies) and polyclonal anti-mouse IgG conjugated to FITC (BD Pharmingen, San Diego, CA, USA), AF647 (Life Technologies), or R-PE (Thermo Scientific), in 5% FCS with PBS or in 40% human serum with PBS for 30 min on ice. For double staining experiments, the W6/32 antibody or IgG2a isotype control conjugated to AF647 or FITC (BioLegend) was added to the cells for 30 min on ice. The cells were fixed with 1% formaldehyde in PBS before flow cytometry analysis.

Functional Assay with Primary NK Cells Including Subsequent Multicolor Immunofluorescence Staining
 Natural killer cells were stimulated with medium alone or with IL-12 (10 ng/ml, R&D systems) and IL-15 (50 ng/ml, R&D systems) for 12 h. The following day, target cells were added at an E:T ratio of 1:1 or 10:1 (58). Cytokine-stimulated NK cells in medium alone, or cocultured with K562 cells (a gold standard NK cell target cell line, devoid of MHC class I) served as a positive control for function when HCMV-infected HFFFs were investigated for their capacity to trigger primary NK cells. After 1 h of coculture, GolgiPlug (Brefeldin A, BD Bioscience) and GolgiStop (Monensin, BD Bioscience) were added to the wells according to manufacturer's instructions, and incubated for an additional 4 h. Non-adherent cells were subsequently transferred to V-bottom well plates where multicolor immunofluorescence staining was performed (32). The following conjugated monoclonal antibodies were used: anti-KIR2DL3 FITC (REA147, Miltenyi), anti-NKG2A APC (Z199, Beckman Coulter, Brea, CA, USA), anti-CD107a APC-H7 (H4A3, BD Bioscience), anti-KIR3DL1 Brilliant Violet 421 (DX9, BioLegend), biotinylated anti-KIR2DL1 (143211, R&D Systems), anti-CD56 ECD (N901, Beckman Coulter), anti-CD3 PE-Cy5 (UCHT-1, BioLegend), and anti-KIR2DL1/S1 PE-Cy7 (EB6, Beckman Coulter). Briefly, the cells were stained with the primary antibody cocktail for 20 min. The anti-KIR2DL1/S1 antibody was added directly to the wells (dilution 1:20) for

10 min incubation. Following washing, the secondary antibody cocktail containing Live/Dead Aqua (Invitrogen, Carlsbad, CA, USA) and Qdot-605-conjugated Streptavidin (Invitrogen) was incubated for 20 min. Cells were next washed and fixed.

Data acquisition for the cellular and immunofluorescence cell-surface staining experiments was performed on FACScan (Department of Pathology), FACSCalibur or Accuri C6 flow cytometer (NIHR Cambridge BRC Cell Phenotyping Hub, BD Bioscience) depending on the experiment. The data acquisition of the multicolor primary NK cell staining experiments was performed on a BD LSR Fortessa flow cytometer, equipped with 4 lasers and 13 PMTs (Cambridge Stem Cell Institute). Flow cytometry data were analyzed using FlowJo software (TreeStar, OR, USA).

Viruses

Human cytomegalovirus strains TB40/E [isolated from a throat wash of a bone marrow transplant recipient (59)], AD169 (ATCC VR-538) and Merlin (a gift from Richard Stanton, University of Cardiff, UK) were grown, concentrated, and titrated as described previously (60). Confluent plates of HFFFs or DFs were infected with concentrated virus (TB40/E, AD169, Merlin) at a multiplicity of infection (MOI) as indicated in the experiment or with diluted (1:10) supernatant-containing virus (TB40/E-derived clones). The virus preparations were diluted in complete DMEM to obtain the required MOI. After 1 h of infection at room temperature, the cells were washed with PBS, and fresh complete DMEM was added. A coculture experiment was performed, or the cells were harvested for cell-surface staining, or western blotting, 24, 48, and/or 72 h p.i. as indicated in the experiment. Time points were chosen to reflect the temporal cascade of expression of HCMV viral proteins (immediate early, early, and late genes). Late genes, which are structural viral components, are last expressed at ~72 h post infection (p.i.) (61). UV inactivation of the virus was obtained by exposing the virus 30 min to UV light of a 30 W germicidal lamp.

Plaque Purification Assay

Human fetal foreskin fibroblasts were infected with serially diluted HCMV strain TB40/E. After 1 h of infection at room temperature, the virus-containing medium was replaced with 5 ml

per well of 2% agarose mixed 1:1 with two times concentrated DMEM medium (Millipore, Bedford, MA, USA) containing 20% FCS, 200 U/ml penicillin, 200 µg/ml streptomycin, 8 g/L sodium bicarbonate (GE Healthcare), and 1 mM of sodium pyruvate (Sigma-Aldrich). After 3 weeks, plaques (areas of dead cells) were visible by eye. Well-separated plaques, representing different viral clones of the TB40/E wild-type strain, were picked using a glass Pasteur pipette by removing the agar and plaque as a plug. The agarose plug containing viral clones were disrupted and added to freshly plated HFFs in 96-well flat-bottom culture plates to expand the viral clones. Selected clones were expanded up to a 75 cm² culture flask. After 100% infection was reached, as determined visually by microscopy, the supernatant-containing virus was harvested and stored at -80°C. Fresh complete DMEM was added to the flasks and further harvests were carried out every 72 h until approximately 90% cell lysis was visually determined. All the harvested supernatant was pooled, spun at 2,000 rpm to remove cell debris, aliquoted, and stored at -80°C.

Western Blot

Cells were washed with PBS and lysed in lysis buffer [1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, protease inhibitors (Roche, Mannheim, Germany), and 1 mM Phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) and 1.25 mg/ml N-ethylmaleimide (Sigma-Aldrich)] for 20 min at 4°C. Debris and nuclei were removed by centrifugation at 13,000 rpm (centrifuge HAWK 15/05, MSE), and a bicinchoninic acid (BCA) assay (Thermo Scientific) was used to determine protein concentrations.

Supernatants of TB40/E-derived clones were pelleted by centrifuging at 15,000 rpm for 2 h at 4°C using an Avanti J-25 Ultracentrifuge (Beckman Coulter). The virus pellets were gently washed with PBS, and 200 µl of lysis buffer was added to lyse the particles. Lysates from positive and negative virus particles were paired based on approximately the same infectivity titer. Virus titers were calculated by TCID₅₀.

Total cell or virus particle lysates were loaded onto a 10–13% SDS-PAGE gel, and proteins were transferred onto Immobilon-P PVDF membranes (Millipore). The membranes were blocked 1 h in PBS, 5% dried milk, and 0.05% Tween 20 at room temperature. The membranes were incubated overnight at 4°C or 3 h at room temperature with primary antibodies [anti-pp28 (Abcam), L31, HC10, anti-I.E.1 (Merck Millipore, Billerica, MA, USA), anti-Calnexin (Enzo Life Sciences, Farmingdale, NY, USA), and anti-Flag (M2, Sigma-Aldrich)] at concentration of 0.1–0.2 µg. The membranes were washed thoroughly, and polyclonal HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dako, 1:4,000 dilution) secondary antibody was added for 30 min at room temperature. Chemiluminescence was performed according to the manufacturer's instructions using ECL Prime (GE Healthcare) or home-made ECL (62).

Establishment CRISPR-CAS9 Knockout (KO) for β₂M in HFFs

Streptococcus pyogenes CAS9 and short-guide RNA (sgRNA) were expressed in separate lentivirus constructs: pHRSIN containing

the SFFV promoter, FLAG tag, nuclear localization signals (NLS), CAS9 and pGK Hygro (kind gift from Lehner's group, CIMR, University of Cambridge), and pKLV-containing U6 promoter, sgRNA (modified *Bbs*I), pGK Puro, 2A, and BFP tag [Addgene plasmid #50946; created by Kosuke Yusa, Wellcome Trust Sanger Institute, Cambridge, UK (63)]. The pKLV construct containing sgRNA-targeting β₂M was a gift from Dick van den Boomen (CIMR, University of Cambridge), using the sgRNA sequence 5'-GGCCGAGATGTCTCGCTCCG-3'.

The pHRSIN and pKLV lentivirus constructs (6 µg) were packaged together with pMDG and pCMV9.81 packaging vectors (4 µg) in HEK 293T cells in 75 cm² culture flasks using Opti-MEM™ and GlutaMax™ media (Gibco), and *TransIT*® Transfection reagent (Mirus, Madison, WI, USA). After 48 h, supernatant-containing lentiviral particles were used to transduce HFFs in 75 cm² culture flasks by adding Polybrene (8 ng/ml). The transduced cells were selected with 200 µg/ml hygromycin and/or 2 µg/ml puromycin and grown until a confluent 75 cm² culture flask was reached. Cells negative for total HLA class I were purified by surface staining using W6/32 antibody, respectively, followed by single-cell sorting using the FACS sorter. The CAS9 is FLAG-tagged and a HRP-conjugated anti-M2 FLAG antibody was used to detect it by western blot.

Statistical Analysis

Non-parametric one-way analysis of variance (ANOVA) using the Kruskal-Wallis test and Dunn's multiple comparisons test was used to determine the statistical significance. In these tests, a *p* value of less than 0.05 was considered significant (**p* < 0.05, ***p* < 0.01). The tests were done with GraphPad Prism version 6.00 (GraphPad software).

RESULTS

The Function and Specific Recognition of KIR2DS1 by Reporter Cells

Since KIR2DS1 binds C2-HLA-C only weakly, we aimed to investigate what might influence stronger binding. To accomplish this, we used a specifically designed mouse 2B4 T cell hybridoma carrying an NFAT-GFP reporter system, similar to that described by Arase et al. (43). KIR2DS1 was transduced into these 2B4 reporter cells, together with a modified adaptor protein. Once KIR2DS1 binds its cognate ligand, a signaling cascade is triggered through the adaptor protein, which then transcribes NFAT, resulting in GFP expression. KIR2DL1, -2DL2, and LILRB1 reporter cells were also generated. We confirmed that the reporter cells were constructed successfully by staining the surface expression of the different receptors with the relevant antibodies and by engaging the reporter cells with relevant plate-bound antibodies (Figure 1A).

There are seven amino acid differences between KIR2DL1 and -2DS1 alleles, including the threonine to lysine in KIR2DL1 and -2DS1 at position 70, respectively (Figure 1B). We wanted to investigate whether substituting the lysine to a threonine at position 70 in KIR2DS1 would result in the activation of the reporter cells as a result of the interaction with C2-HLA-C,

as demonstrated previously by Biassoni and colleagues using Fc proteins (25). We made the KIR2DS1^(K70T) reporter cell and cocultured these cells with 721.221 transfected with HLA-C03:02 (C1) or -C06:02 (C2) (221-Cw3 or 221-Cw6). Indeed, both KIR2DL1 and KIR2DS1^(K70T) reporter cells were activated after coculture with 221-Cw6 (15 and 11% GFP-positive cells, respectively) and not with 221-Cw3 (Figure 1C). This confirms the observations made by Biassoni et al. in our cellular reporter system and shows that the reporter cells are functional, specific, and sensitive.

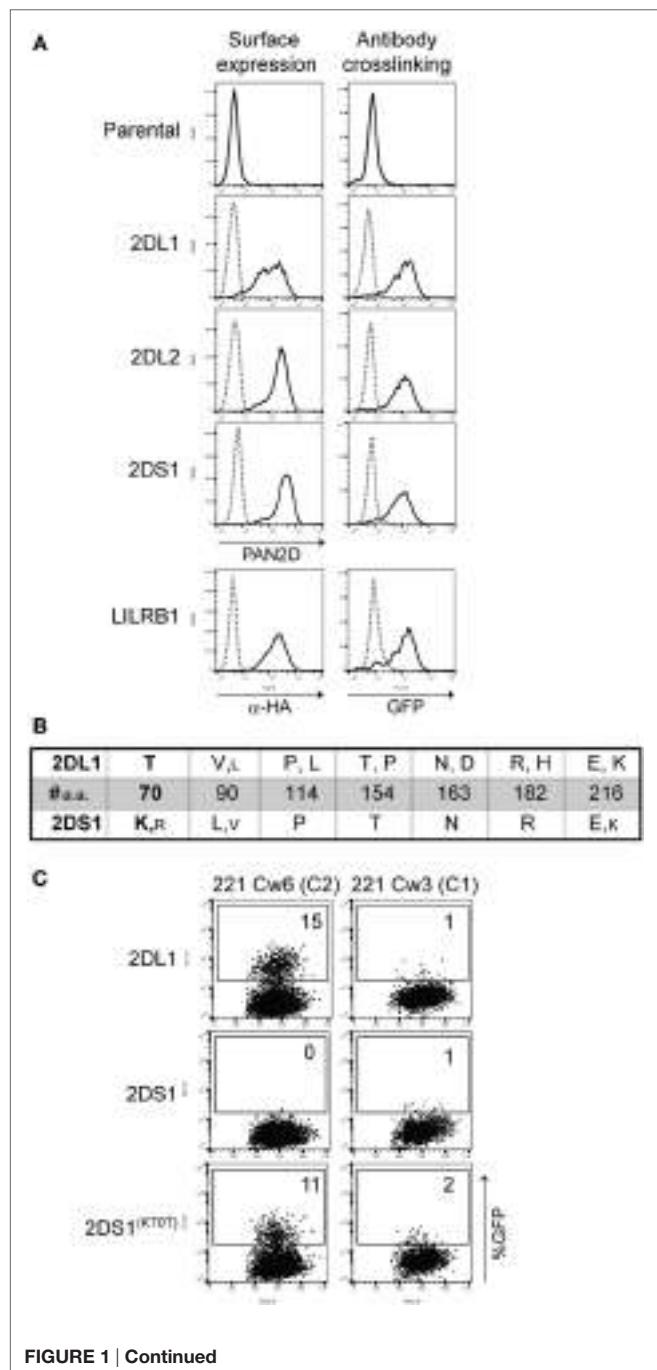


FIGURE 1 | Continued

FIGURE 1 | Continued**2B4 reporter system for activating killer cell Ig-like receptor (KIR).**

(A) Cell-surface expression on the indicated reporter cells was measured by flow cytometry after staining the cells with PAN2D (clone NKVFS1, recognizes all KIRs) or anti-HA antibody (black line, left panel). Parental 2B4 cells containing only the adaptor protein were used as a negative control. To test the function of these reporter cells, plate-bound PAN2D (clone NKVFS1) or anti-HA (LILRB1 reporter only) antibody crosslinking (black line) was used to engage KIR or LILRB1 molecules on the reporter cells during an overnight incubation (right panel). IgG1 antibody was used as isotype control (dotted line). **(B)** Table depicting amino acid positions of polymorphic sites in the extracellular domain of KIR2DL1 and -2DS1 alleles. Large letters indicate amino acids present in the majority of alleles, and small letters indicate amino acids that are present in few alleles. At position 70, a unique amino acid in KIR2DL1 and -2DS1, threonine (T) and lysine (K), respectively, was found. Allele sequences were aligned using the alignment tool in the *IPD-KIR database*. **(C)** KIR2DS1^(K70T) was transfected into the 2B4 reporter system and an overnight coculture was performed using KIR2DL1, -2DS1, or -2DS1^(K70T) reporter cells together with 721.221 cells expressing human leukocyte antigen (HLA)-Cw3 (C1) or HLA-Cw6 (C2). The next day green fluorescent protein (GFP) expression was measured by flow cytometry. The E:T ratio of the coculture was 1:3.

KIR2DS1 Reporter Cells Do Not Recognize Conventional HLA Class I

To verify whether KIR2DS1 reporter cells are activated by conventional HLA class I molecules, cocultures were performed using the KIR2DS1 reporter cells along with appropriate controls. The cocultures were done using a range of 221 cells transduced with different HLA molecules: HLA-A11:02, -A23:01, -Bw6 (B35:01), -Bw4 (B58:01), -G, -C01:02 (C1), -C02:02 (C2), -C03:02 (C1), -C04:01 (C2), -C06:02 (C2), and -C07:01 (C1). HLA expression levels on these 221 cells were confirmed by staining with W6/32 antibody (Figure 2A). KIR2DS1 reporter cells were not activated in cocultures with different 221-HLA-C or other 221-HLA class I cells. However, the positive controls KIR2DL1, -L2, and LILRB1 were activated after engaging their documented ligands (Figures 2B,C). As expected, KIR2DL1, -L2, and LILRB1 reporter cells were differentially activated, depending on the HLA-C allele they engaged. For instance, the KIR2DL2 reporter cell was weakly activated after binding HLA-C01:02 (<5% GFP-positive cells) compared to HLA-C07:01 (>20% GFP cells), as depicted in Figure 2C, lower panel. This is in line with binding studies using Fc proteins and HLA-coated beads (11, 64).

In our hands, the KIR2DS1 reporter cells did not recognize endogenously expressed HLA class I molecules on 221 cells. To investigate this further, they were cocultured with primary cells: HFFFs, CMV307, and CMV0005 DFs, which were stimulated with or without IFN- γ for 72 h. Before the coculture experiments, HLA class I surface expression levels of untreated and IFN- γ stimulated cells were measured. The primary fibroblasts all expressed high levels of folded HLA class I and HLA-C/E molecules, revealed by W6/32 and DT9 antibody staining, respectively. These HLA class I levels were further increased after 72 h of IFN- γ stimulation (Figure 3A). KIR2DS1 reporter cells remained GFP-negative when cocultured with the different untreated or IFN- γ stimulated primary fibroblasts (Figure 3B). By contrast, LILRB1 reporter cells were activated in all coculture experiments with untreated cells and were further activated in

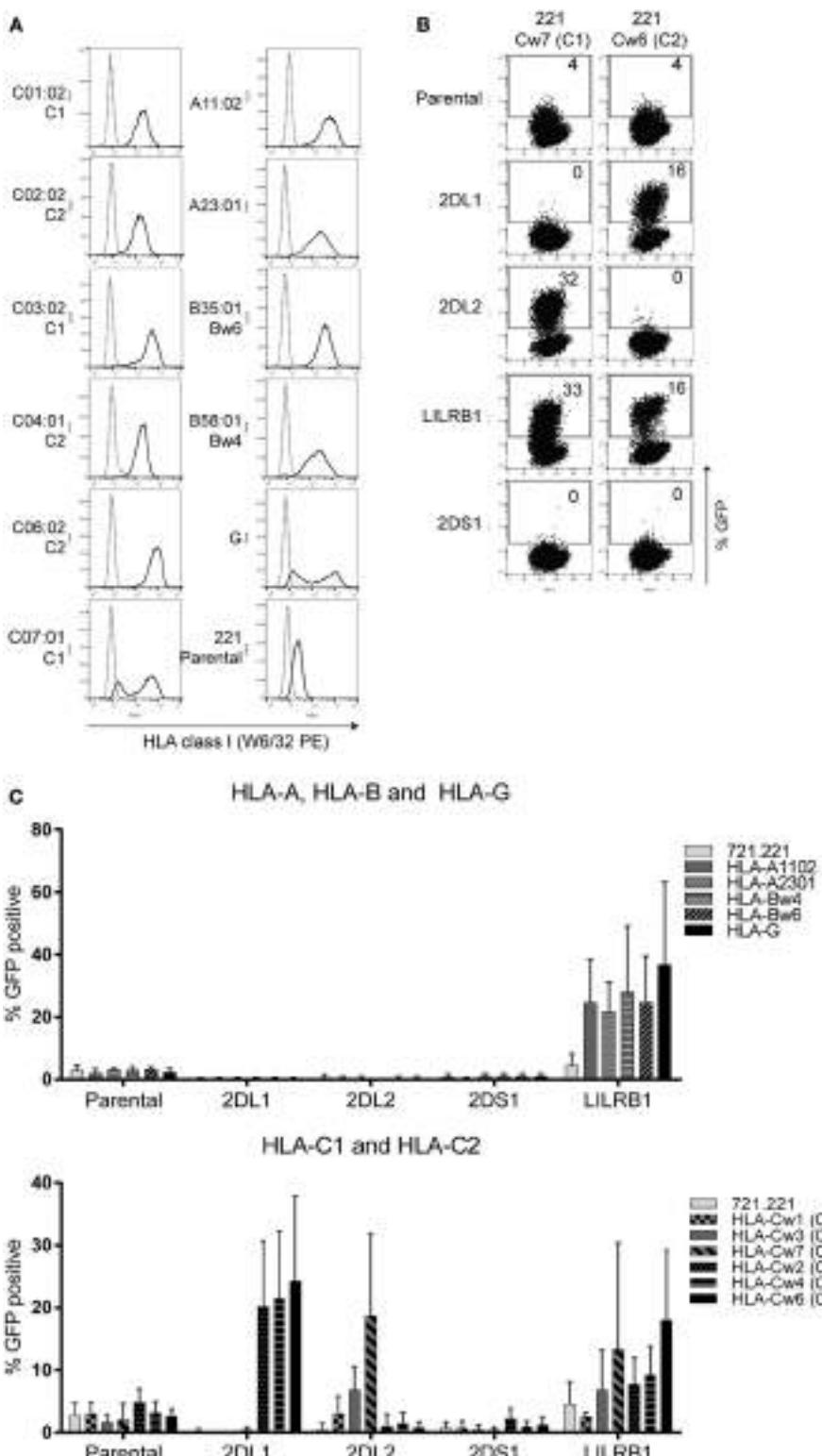
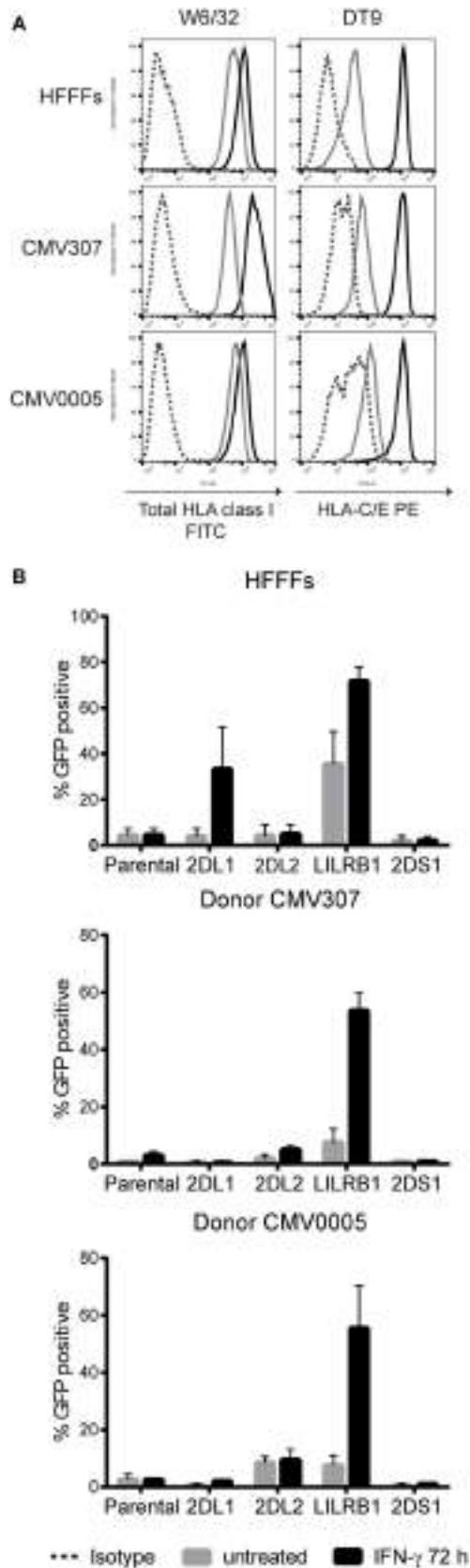


FIGURE 2 | KIR2DS1 reporter cells are not activated by conventional human leukocyte antigen (HLA) class I molecules. **(A)** The 721.221-HLA transfectants were stained with W6/32 (black line), and measured by flow cytometry. IgG2a antibody was used as isotype control (dotted line). **(B)** Dot plots of a selection of representative data from the same experiment. A coculture of the indicated reporter cells together with 721.221 cells containing HLA-C07:01 (221 Cw7, C1) or HLA-C06:02 (221-Cw6, C2) is shown. GFP expression was determined by flow cytometry. The E:T ratio was 1:3. **(C)** The data are depicted as the mean \pm SD of individual samples collected from five independent experiments.

**FIGURE 3 | Continued**

KIR2DS1 reporter cells are not activated by interferon (IFN)- γ stimulated human fetal foreskin and primary dermal fibroblasts of healthy individuals. (A) Total human leukocyte antigen (HLA) class I (W6/32) and HLA-C/-E (DT9) expression levels of untreated and IFN- γ stimulated HFFFs (C2/C2) and DFs of donor CMV307 (C1/C1) and CMV0005 (C1/C2) were measured by flow cytometry. Gray = untreated, black = IFN- γ stimulated (500 U/ml, 72 h) and dotted line = isotype control. (B) The untreated and IFN- γ -stimulated fibroblasts were cocultured overnight with the indicated reporter cells, and green fluorescent protein (GFP) expression was measured by flow cytometry. Three independent experiments are depicted in the bar graphs as the mean \pm SD of individual samples.

settings with IFN- γ stimulated cells. KIR2DL2 and KIR2DL1 reporter cells were activated, in some conditions only minimally, depending on the particular HLA-C alleles expressed by the cells (Figure 3B). KIR2DS1 reporter cells were similarly unresponsive to tumor cell lines including HeLa, Meljuso, Caski, and JEG-3 cells (data not shown). In conclusion, KIR2DS1 reporter cells were not activated after engaging with conventional C2-HLA-C molecules.

KIR2DS1 Reporter Cells Bind a Ligand on HFFFs Infected with Specific HCMV Clones

Since KIR2DS1 reporter cells were not activated by conventional HLA molecules, we considered that KIR2DS1 might recognize a pathogen-induced ligand. Several studies have suggested a role for aKIR in HCMV infection (44–47). We therefore investigated whether the KIR2DS1 ligand might be upregulated after HCMV infection. HFFFs were infected with the HCMV TB40/E strain for 24, 48, and 72 h and cocultured with the panel of reporter cells. HFFFs were used because they support full lytic HCMV infection (65) and express HLA-C02:02 and -C04:01, both C2-HLA-C.

HFFFs Infected with the TB40/E Wild-Type Strain Express a Ligand for KIR2DS1

In coculture, KIR2DS1 reporter cells increased in GFP positivity from 1% (uninfected and 24 h) to 5% (48 h) and to 21% positive cells (72 h) (Figure 4A). This indicates that KIR2DS1 recognized a ligand, which was detected by the reporter cells 48 h p.i. with HCMV. In addition, there was increased triggering of both KIR2DL1 and LILRB1 reporter cells over time (Figure 4A). This was of interest as HCMV downregulates HLA class I molecules (66–71), and a decrease in KIR2DL1 reporter activation over infection time was expected. The increase in the LILRB1 reporter cell activation might be explained by the recognition of UL18 protein, which HCMV produces as an immune evasion strategy (72, 73). Subsequently, in a total of 10 KIR2DS1 reporter cell coculture experiments with HFFFs infected with HCMV TB40/E strain were done, demonstrating the reproducibility of the KIR2DS1 reporter cell activation after infection (Figure S1 in Supplementary Material). The KIR2DS1 ligand was only expressed on HFFFs infected with the TB40/E clinical strain and not with other strains tested, such as Merlin (another clinical strain) and AD169 (laboratory strain with deletion in the ULb' region) (data not shown).

FIGURE 3 | Continued

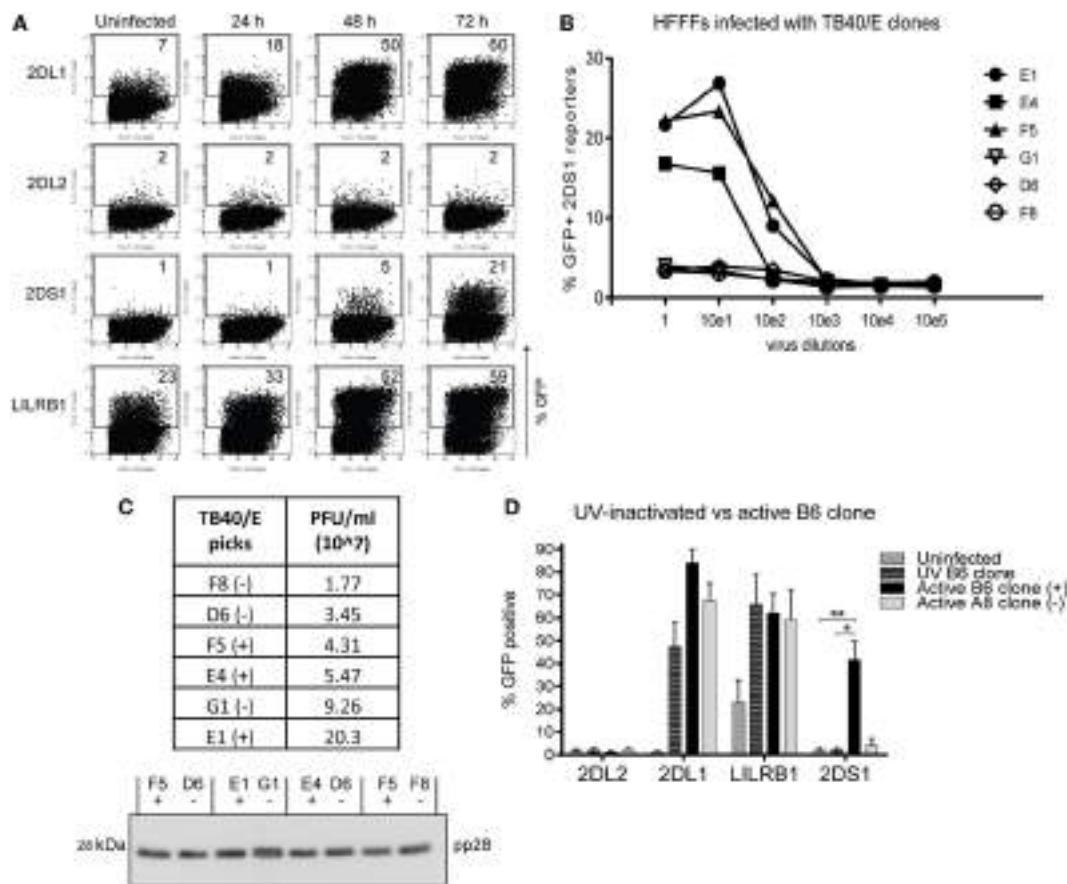


FIGURE 4 | The KIR2DS1 reporter cell recognizes a ligand on human fetal foreskin fibroblasts (HFFFs) infected with specific human cytomegalovirus (HCMV) strains. **(A)** Uninfected HFFFs and 24, 48, and 72 h infected HFFFs with HCMV TB40/E strain with an MOI of 10 were cocultured overnight with the reporter cells as indicated. A representative experiment of two independent time course cocultures is shown. **(B)** HFFFs were infected with six different TB40/E-isolated clones (E1, E4, F5, G1, D6, and F8) in 10-fold serial dilutions for 72 h and cocultured with KIR2DS1 reporter cells. A representative experiment from over three independent experiments is depicted. **(C)** The infectivities of three positive and three negative TB40/E clones were calculated by TCID₅₀ assay and are depicted in the table (virus titers in PFU/ml). Viral particles were isolated from the supernatant, lysed, and loaded onto a 12% SDS-PAGE gel for western blotting with anti-gp28 antibody. Positive and negative clones were paired based on approximately the same infectivity. Equal amounts of lysate from these pairs were loaded onto the gel. (–) indicates a negative clone, (+) positive clone. **(D)** HFFFs were stimulated with UV-inactivated TB40/E, infected with the positive B6 or negative A8 clone for 72 h. Forty-eight hours p.i. KIR2DL2 (negative control), KIR2DL1, LILRB1 (positive control), and KIR2DS1 reporter cells were added. After an overnight coculture, the GFP expression was measured using flow cytometry. The data are depicted as the mean ± SD of individual samples collected from five independent experiments. **p* < 0.05 and ***p* < 0.01 are calculated by non-parametric one-way ANOVA using the Kruskal-Wallis test and Dunn's multiple comparisons test.

Specific TB40/E Clones Activate the KIR2DS1 Reporter Cells

Tomasec and colleagues isolated two viral clones, called Lisa and Bart, from the TB40/E strain, which had different functional properties (74). We wanted to investigate whether different viral clones were also present in our TB40/E strain. Multiple viral clones were isolated using a plaque purification assay. Twelve clones were selected and used to infect HFFFs followed by coculture with KIR2DS1 reporter cells. Six clones (B6, D7, E1, E4, E5, and F5) activated KIR2DS1 reporter cells (referred to as positive clones in what follows), while the other “negative” clones (A6, A8, D6, F2, F8, and G1) did not. Figure 4B depicts a representative coculture experiment where HFFFs were infected with six randomly selected clones in a 10-fold serial dilution for 72 h and

then cocultured with KIR2DS1 reporter cells. The reporter cells were highly activated by HFFFs infected with the positive clones; E1, E4, and F5. They were not activated after infecting with negative clones; G1, D6, and F8 (Figure 4B). Infecting HFFFs with the positive clones resulted in a higher percentage of GFP-positive KIR2DS1 reporter cells (ranging from 4 to 21%, Figure S1 in Supplementary Material), indicating that the positive clones induced the KIR2DS1 ligand more efficiently. This is in line with the idea that the parental TB40/E strain contains a mixture of positive and negative viruses with respect to KIR2DS1 ligand expression.

Human cytomegalovirus infection indeed led to reduction in total HLA class I expression on most HFFFs as monitored by

binding of W6/32 antibody and this was true of both positive and negative clones (**Figure 6A**). This indirectly indicates that the positive and negative clones were equally infectious and consistently infected over 90% of the HFFFs. In addition, the differential response of the KIR2DS1 reporter cells to the positive and negative clones was not due to differences in overall viral particle numbers of the different clones. This was demonstrated by comparing the infectivity (functional virus particles) with the total number of viral particles (functional and empty/non-functional particles) by detecting a structural tegument protein pp28 by western blot after pairing positive and negative virus clones based on approximately the same infectivity titer (**Figure 4C**).

KIR2DS1 Ligand Is Only Expressed on HFFFs after Infecting with Infectious Virus

Within the investigated infection timeframe, non-infected cells could be refractory for HCMV infection, yet exposed to pro-inflammatory cytokines, such as type I interferons (IFNs), which subsequently induce HLA class I surface expression. It was therefore critically important to investigate whether KIR2DS1 reporter cells were activated by the infected cells or by the surrounding non-infected cells. To examine this, TB40/E viruses were exposed to UV light for 30 min to inactivate the virus. After exposure, viral particles will be present and able to enter the cell, but the viral genes will be inactivated and will not be transcribed. Virus inactivation was confirmed by immunohistochemistry staining of Immediate Early 1 (I.E.1) viral proteins on the treated HFFFs, as shown in Figure S2 in Supplementary Material. These UV-inactivated viruses are called “UV virus.”

After stimulating HFFFs with UV B6 clone and infecting with the positive B6 and negative A8 clones for 72 h, a coculture with parental, KIR2DL1, LILRB1, and KIR2DS1 reporter cells was performed. KIR2DS1 reporter cells were significantly activated in coculture with the active B6 clone-infected HFFFs, but not with UV B6-stimulated HFFFs nor in any other conditions, as shown in **Figure 4D**. By contrast, the KIR2DL1 reporter cells were highly activated after encountering UV B6-stimulated HFFFs, as well as with B6 clone-infected HFFFs (**Figure 4D**). Total HLA class I and HLA-C/-E cell-surface levels using W6/32 and DT9 antibodies were assessed and both HLA-A, -B, -C and HLA-C/-E surface expression levels were highly increased on UV virus-stimulated HFFFs (**Figure 6**). Since fibroblasts are known to produce IFN- α and - β (75, 76), it was expected to see such an increase in HLA class I cell-surface levels, after exposure to viral particles. This is the host response to HCMV particles without the interference of HCMV genes downregulating HLA class I molecules.

In conclusion, from the different HCMV strains tested, only the TB40/E strain activated KIR2DS1 reporter cells after infecting HFFFs. Furthermore, the TB40/E strain consists of different virus clones and these virus clones differentially activated KIR2DS1 reporter cells. Since the clones have comparable amounts of functional viral particles, the levels of activation were not governed by the number of viral particles. We may also conclude that active viral gene expression is necessary to induce the KIR2DS1 ligand.

Primary, Single-Positive KIR2DS1 NK Cells Are Only Activated in Coculture when HFFFs Are Infected with Specific HCMV Clones

After establishing that KIR2DS1 reporter cells recognize a ligand on HFFFs infected with specific clones, we asked whether primary NK cells would also interact with these infected cells. Coculture experiments were performed using freshly isolated peripheral blood NK cells from healthy individuals. NK cells expressing KIR2DS1 are hyporesponsive, if the donor is homozygous for C2-HLA-C (32). We therefore chose donors that were C1-HLA-C homozygous, bearing fully functional KIR2DS1-positive NK cells. General NK cell functionality was verified using IL-12/15 primed NK cells cocultured with the prototypic HLA class I negative NK cell target cell line K562 (positive control), or with no target cells (negative control). The KIR $^{-}$ NK cell subset represents the KIR-independent activation of NK cells, which is the background NK cell activation in this experiment.

Forty-nine percent of the cytokine-primed KIR $^{-}$ NK cells degranulated after NK cells of donor 016 encountered K562 cells, and degranulation of 4% was observed in culture without target cells, indicating that the NK cells are functional, and little background activation was observed (**Figure 5A**). In the same experiment, rested NK cells (without cytokine stimulation) were cocultured with HFFFs. NKG2A $^{-}$ cells were separated into KIR-negative (KIR $^{-}$), KIR2DL1 single-positive (2DL1sp), and KIR2DS1 single-positive (2DS1sp) NK cells. The gating strategy is described in Figure S3 in Supplementary Material. No response (1% CD107a expression) was seen from 2DS1sp NK cells cocultured with uninfected or UV B6 clone-stimulated HFFFs (**Figure 5B**). Similarly, minimal functional response from 2DS1sp NK cells was observed after coculture with HFFFs infected with the negative A8 clone (5% CD107a expression). Notably, 2DS1sp NK cells engaging HFFFs infected with the positive B6 clone showed 20% CD107a expression (**Figure 5B**). Only a slight background activation of 3% was observed in the KIR $^{-}$ population in coculture with positive B6 clone-infected HFFFs. In all the other conditions, no degranulation in the KIR $^{-}$ population was observed. Furthermore, in every condition, including the positive B6 clone condition, no degranulation of 2DL1sp NK cells was observed (**Figure 5B**). The experiment was repeated with isolated NK cells of donor 111, and these NK cells responded similarly, though slightly weaker, to the different types of treated HFFFs (**Figure 5C**). These results were reproducible in two independent coculture experiments using either isolated NK cells (Figure S4A in Supplementary Material) or PBMCs (Figure S4B in Supplementary Material).

In conclusion, similar to the reporter cells, primary NK cells expressing KIR2DS1 recognize a ligand on HFFFs infected with specific clones of the TB40/E strain.

Specific HCMV Clones Are Less Effective in Targeting HLA-C

After confirming that the KIR2DS1 ligand is expressed on HFFFs infected with positive clones of HCMV, different HLA class I surface levels of uninfected HFFFs, UV B6 clone-stimulated

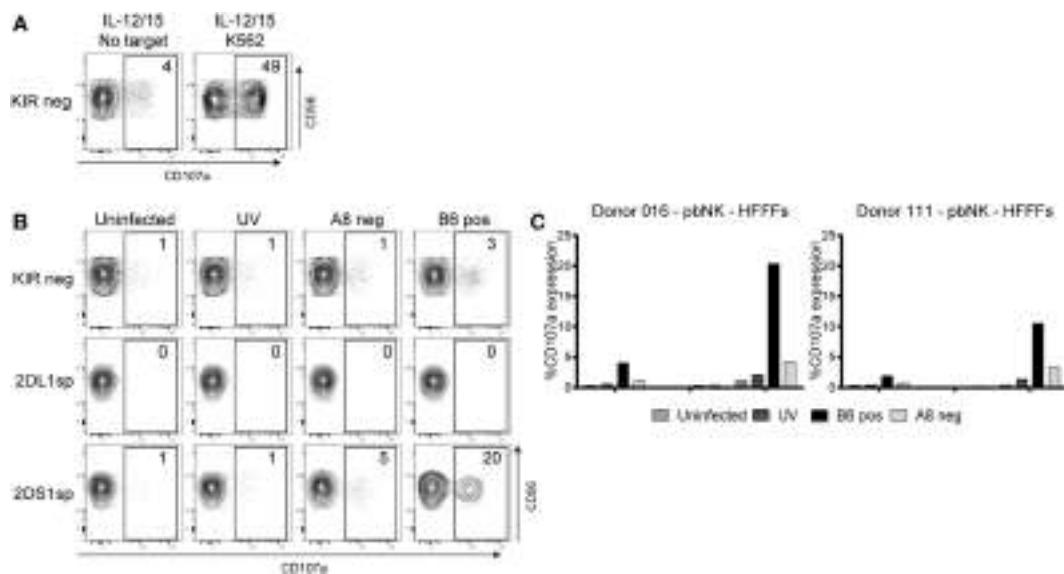


FIGURE 5 | Primary natural killer (NK) cells degranulated after coculture with human fetal foreskin fibroblasts (HFFFs) infected with the positive B6 TB40/E clone. (A) After NK cell isolation, the NK cells of donor 016 were rested or stimulated with IL-12 and IL-15 overnight. IL12/IL15-stimulated NK cells were cocultured with and without K562 cells. **(B)** Unstimulated NK cells were cocultured with uninfected HFFFs, UV B6 clone-stimulated HFFFs (UV), negative A8 clone (A8 neg), and positive B6 clone-infected HFFFs (B6 pos). After harvesting, the NK cells were stained with antibodies targeting different surface markers and the degranulation marker CD107a. NK cells from the KIR-negative (KIR), KIR2DL1 single-positive (2DL1sp), and KIR2DS1 single-positive (2DS1sp) subsets are shown. The percentage of CD107a expression is depicted. The NK subsets are grouped on NKG2A⁻ NK cells (A'). **(C)** The same coculture experiment with NK cells of donor 016 is represented in a bar graph (left) including coculture data of NK cells of donor 111 (right). The percentage of CD107a expression is shown. All cocultures were performed for 5 h at an E:T ratio of 1:1. A representative data from three independent coculture experiments are shown. The other two independent experiments are illustrated in Figure S4 in Supplementary Material.

HFFFs, and both positive and negative clone-infected HFFFs were compared. Surface expression of HLA-E (3D12), HLA-C/-E (DT9), total HLA class I (W6/32), HLA-A11, HLA-Bw6, total free heavy chain (FHC) of HLA class I (HC10), and FHC of HLA-C (L31) was analyzed. The antibodies used are listed in **Table 1** with a description of their specific recognition patterns and are discussed below.

Free Heavy Chain of HLA-C and Assembled HLA-C Remain on the Cell Surface of HFFFs Infected with Positive Clones

Positive and negative clones both reduced total HLA class I, HLA-E, HLA-Bw6, and HLA-A11 cell-surface levels on infected HFFFs compared to the UV B6 clone-stimulated HFFFs ($p < 0.05$ and $p < 0.01$, **Figures 6A,B**). However, using antibodies to FHC forms of HLA, such as HC10 and, in particular, L31 staining was higher in HFFFs infected with the positive clones compared to the negative clones. FHC HLA-C levels, as detected by L31, were close to the levels of UV B6-stimulated HFFFs, indicating that the positive clones did not effectively downregulate the FHC HLA-C surface levels (**Figures 6A,B**). This difference observed in L31 staining was reproducible in every experiment performed ($n = 11$) and in an experiment where FHC HLA-C surface levels were compared in HFFFs infected with 12 additionally isolated clones comprising six positive and six negative clones (Figure S5A in Supplementary Material). Similar differences between the clones were observed with HC10 staining (FHC of HLA class

I molecules, **Figures 6A,B**). This difference could be due to the antibody detecting the elevated FHC of HLA-C specifically. However, this remains uncertain, because specific antibodies against FHC of HLA-A and -B are not available.

The DT9 staining (detects conformational HLA-C/-E containing β_2 M) was slightly weaker and less consistent than L31, but similar differences between the clones were measurable (**Figures 6A,B**). Since DT9 antibody cross-reacts with HLA-E (56), an anti-HLA-E monoclonal antibody (3D12) was included. HLA-E surface expression in general was very low on HFFFs (**Figure 6A**) and no difference was observed between HFFFs infected with positive and negative HCMV clones (**Figure 6B**). Low HLA-E expression in the infected HFFFs is most likely due to a mutation at position 2 (Met to Val) in the canonical sequence (VMAPRTLIL) of UL40 expressed in all the TB40/E clones, as described previously (77). This result implies that the difference observed in the DT9 staining is, most likely, due to higher HLA-C expression and not HLA-E expression. In addition, assembled HLA-C and FHC of HLA-C surface levels on HFFFs infected with other viral strains, such as Merlin and AD169, were downregulated comparable to the surface levels found with the negative clones (Figure S6 in Supplementary Material). Together, these results indicate that the positive clones are less effective in down-regulating assembled HLA-C and, in particular, FHC of HLA-C in HFFFs, compared to the negative clones and other HCMV strains. However, both sets of clones downregulated other HLA molecules to similar levels.

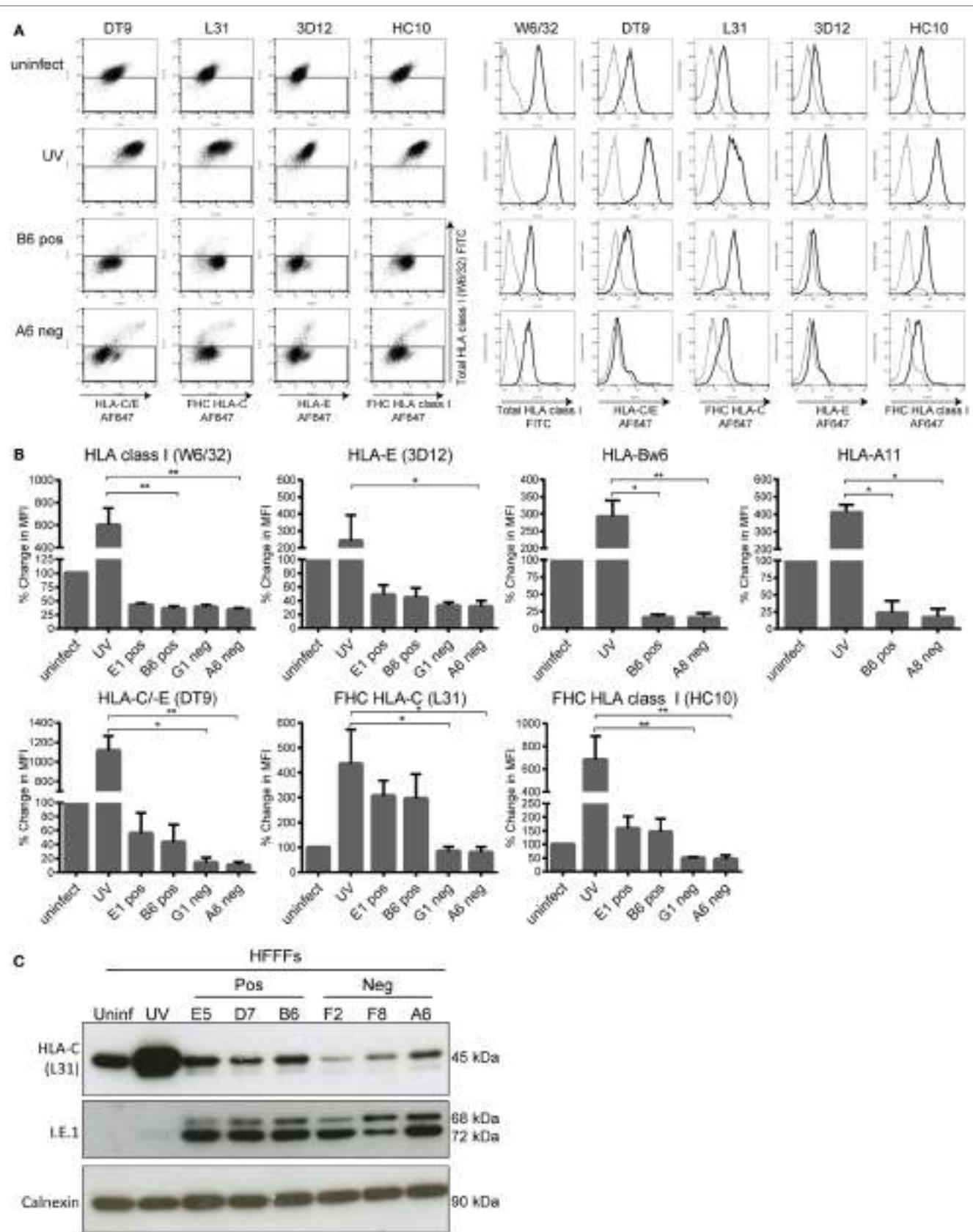


FIGURE 6 | Continued

FIGURE 6 | Continued

Human leukocyte antigen (HLA) class I surface and total HLA-C protein expression of infected human fetal foreskin fibroblasts (HFFFs). **(A)** Dot plots (left) and histograms (right) from the same representative experiment are shown. The HFFFs infected with the indicated positive or negative clones and the UV B6 clone-stimulated HFFFs (UV) were treated for 72 h. The indicated treated HFFFs were stained subsequently with W6/32 (total HLA class I), DT9 (HLA-C/E), 3D12 (HLA-E), L31 (FHC HLA-C), and HC10 (FHC HLA class I) antibodies (black line). Cells stained with the appropriate isotype control (dotted line) were included. **(B)** Collection of up to four independent cell-surface staining experiments is depicted in bar graphs as the mean \pm SD of individual samples. Anti-Bw6 and anti-A11 antibodies were also included. The percentage change in MFI is depicted with the uninfected condition at 100%. * $p < 0.05$ and ** $p < 0.01$ are calculated by non-parametric one-way ANOVA using the Kruskal-Wallis test and Dunn's multiple comparisons test. **(C)** Total lysate of uninfected, UV B6 clone-stimulated (UV), positive clones- (E5, D7, B6), and negative clones (F2, F8, A6)-infected HFFFs were loaded onto a reduced 10% SDS-PAGE gel. The membrane was blotted with the L31, anti-I-E.1 viral protein (measure of infection) and anti-calnexin (loading control) antibodies.

More Total HLA-C Protein Is Expressed on HFFFs Infected with Selected Clones of HCMV

After having found that HLA-C and, in particular, FHC of HLA-C cell-surface levels were higher on positive clone-infected HFFFs, we investigated whether differential expression of total HLA-C protein levels could be tracked by western blot. HFFFs infected with three positive clones had higher amounts of total HLA-C compared to the negative clones, although the levels in general were lower than for the uninfected and UV B6 clone samples. As expected, HFFFs stimulated with UV B6 clone contained high amounts of HLA-C protein (Figure 6C). Additionally, we tested HFFFs infected with a further 12 clones (6 positive and 6 negative, Figure S5B in Supplementary Material). From the 18 clones tested in total, 7 positive clones had high amounts of total HLA-C, while 7 negative clones had lower amounts.

In conclusion, positive TB40/E clones are defective in down-regulating HLA-C and, in particular, FHC of HLA-C in HFFF cells. Higher amounts of total HLA-C protein were found in HFFFs infected with the positive TB40/E clone compared to infection with the negative TB40/E clone. This may reflect a deficiency in degradation/turnover of HLA-C by positive TB40/E clones.

Pan HLA Class I Antibodies Block the KIR2DS1-Ligand Interaction

Antibody-blocking experiments were performed using various anti-HLA class I antibodies to obtain a better understanding of the KIR2DS1 interaction. Previously, Stewart and colleagues tested a large panel of pan HLA class I antibodies in a blocking experiment to analyze the interaction of KIR2DL1 and KIR2DS1 tetramers with 221-HLA-C transfectants (27). They concluded that W6/32 and HC10 antibodies were not able to block both KIR2DL1 and KIR2DS1 tetramer interactions with C2-HLA-C. However, other pan HLA class I antibodies, such as 6A4 and B1.23.2, did block (27). To confirm the previous findings, the interaction of KIR2DL1 reporter cells with UV B6-stimulated HFFFs, which highly expressed all HLA class I molecules, was tested. As expected, W6/32 antibody did not block the KIR2DL1 interaction with the HFFFs, but the 6A4 and B1.23.2 antibodies did (Figures 7A,B, left panels). Next, the HFFFs were infected with the positive B6 clone and the antibody blocking experiment was repeated. W6/32, 6A4, and B1.23.2 antibodies blocked the KIR2DS1 reporter cell interaction with B6 clone-infected HFFFs. Notably, the W6/32 antibody did not block and the 6A4 and

B1.23.2 antibodies only partially blocked the KIR2DL1 reporter cell interaction (Figures 7A,B, right panels). Furthermore, DT9, L31, and HC10 antibodies were also not able to block the KIR2DS1-ligand interaction and the anti- β_2 M only partially (data not shown). Together, these data are consistent with a differential mode of recognition of C2-HLA-C by KIR2DL1 and KIR2DS1.

HFFFs with a β_2 M KO Infected with Positive HCMV Clones Do Not Induce the KIR2DS1 Ligand

To confirm whether the KIR2DS1 ligand is a HLA class I molecule and specifically HLA-C, the β_2 M gene was knocked out of HFFFs. The HLA class I complex cannot be formed without β_2 M and therefore, HLA class I molecules, but also FHC of HLA class I, will not be transported efficiently to the cell surface (78). The β_2 M gene was knocked out by using the CRISPR/CAS9 genome editing tool (79). After selection and single-cell sorting, β_2 M KO HFFFs were checked for β_2 M, total HLA class I (W6/32), and FHC of HLA-C (L31) surface expression by flow cytometry and total protein expression by western blot. β_2 M KO HFFFs did not express surface β_2 M, total HLA class I, and FHC of HLA-C. B6 and A8 clone infection did not alter these expression levels (Figure 8A). Total β_2 M, total HLA class I (detected with HC10), and most HLA-C protein were also absent in the β_2 M KO HFFFs, compared to the untreated HFFFs (WT) and HFFFs containing CAS9 without the sgRNA (CAS9), indicating that the β_2 M KO was successful (Figure 8B).

Subsequently, β_2 M KO HFFFs and WT HFFFs, either stimulated with UV B6 clone or infected with a positive and negative clone, were cocultured with the KIR2DS1, -L1, -L2, and LILRB1 reporter cells. The KIR2DS1 reporter cell was not activated after encountering β_2 M KO HFFFs infected with the positive B6 clone, while, in the same experiment, 44% of KIR2DS1 reporter cells were GFP positive after coculture with B6 clone-infected WT HFFFs (Figure 8C). KIR2DL1 and LILRB1 reporter cells were not significantly triggered by β_2 M KO HFFFs in all the conditions (Figure 8C).

This result is consistent with KIR2DS1 recognizing an HLA class I molecule, including HLA-C. Together with the cell-surface staining and the antibody-blocking experiment, the data suggest that modulation of C2-HLA-C by HCMV induces a potent KIR2DS1-mediated NK cell activation.

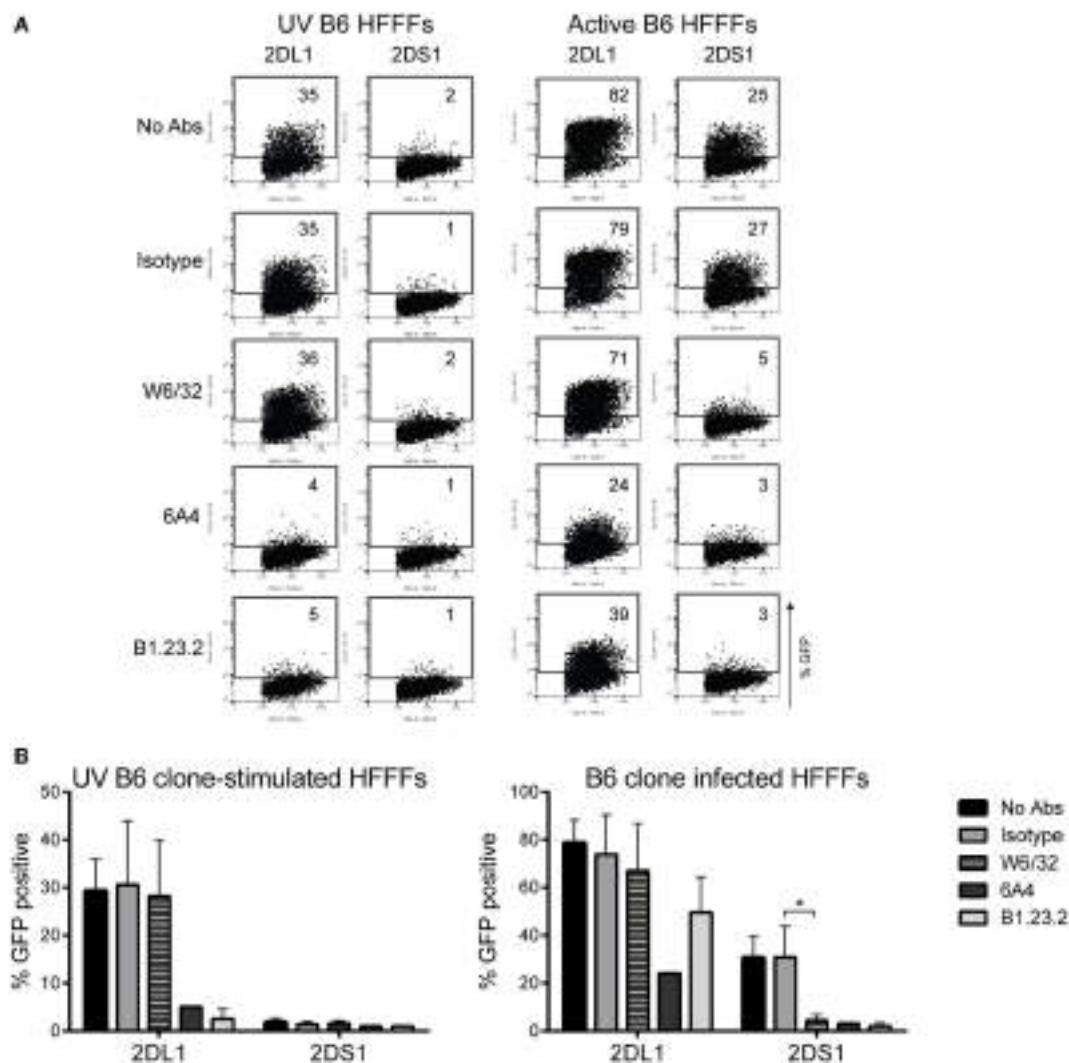


FIGURE 7 | Antibody-blocking experiment of reporter cells and HFFFs stimulated with UV B6 clone or infected with the B6 clone. W6/32, 6A4, B1.23.2, and isotype antibodies were added to 72 h UV-stimulated or infected HFFFs, before an overnight coculture with KIR2DS1 and KIR2DL1 reporter cells was performed. GFP expression was measured by flow cytometry. **(A)** The dot plots are data from a representative experiment. **(B)** Collection of up to five independent blocking experiments is depicted in bar graphs as the mean \pm SD of individual samples with $*p < 0.05$ calculated by non-parametric one-way ANOVA using the Kruskal-Wallis test and Dunn's multiple comparisons test.

DISCUSSION

We found that KIR2DS1 recognizes a ligand on HFFFs infected with the TB40/E strain of HCMV. This wild type strain consists of at least two sets of virus clones: one set that, after HFFF infection, activates KIR2DS1-expressing cells (positive clones) and one that does not (negative clones). This specific KIR2DS1 recognition was only present when the HFFFs were infected with fully functioning viruses, indicating that the virus plays a direct role in expressing the KIR2DS1 ligand. In addition, KIR2DS1 single-positive (2DS1sp) primary NK cells degranulated after engaging with positive clone-infected HFFFs. Together, this indicates that KIR2DS1 reporter cell activation correlates with physiological KIR2DS1 binding to its ligand.

The Reporter System Is a Valid Way of Examining KIR Specificity

Using the reporter system, we confirmed that a single amino acid substitution K70T in KIR2DS1 altered the recognition from no binding to binding the same cognate ligands as KIR2DL1 (**Figure 1C**). This confirms the important role of residue 70 in the binding avidity to HLA-C by KIR2DL1/2DS1. These findings together with the differential recognition of HLA-C alleles by LILRB1, KIR2DL1, and -2DL2 reporter cells (**Figure 2C**) indicate that the reporter system is sensitive to subtle differences. One possible explanation of KIR2DL1 and -L2 reporter cells responding minimally to DFs (**Figure 3B**) is that there is binding of KIR to its ligand, but this binding is not strong enough to trigger a signaling cascade to activate the reporter cell. The signaling cascade

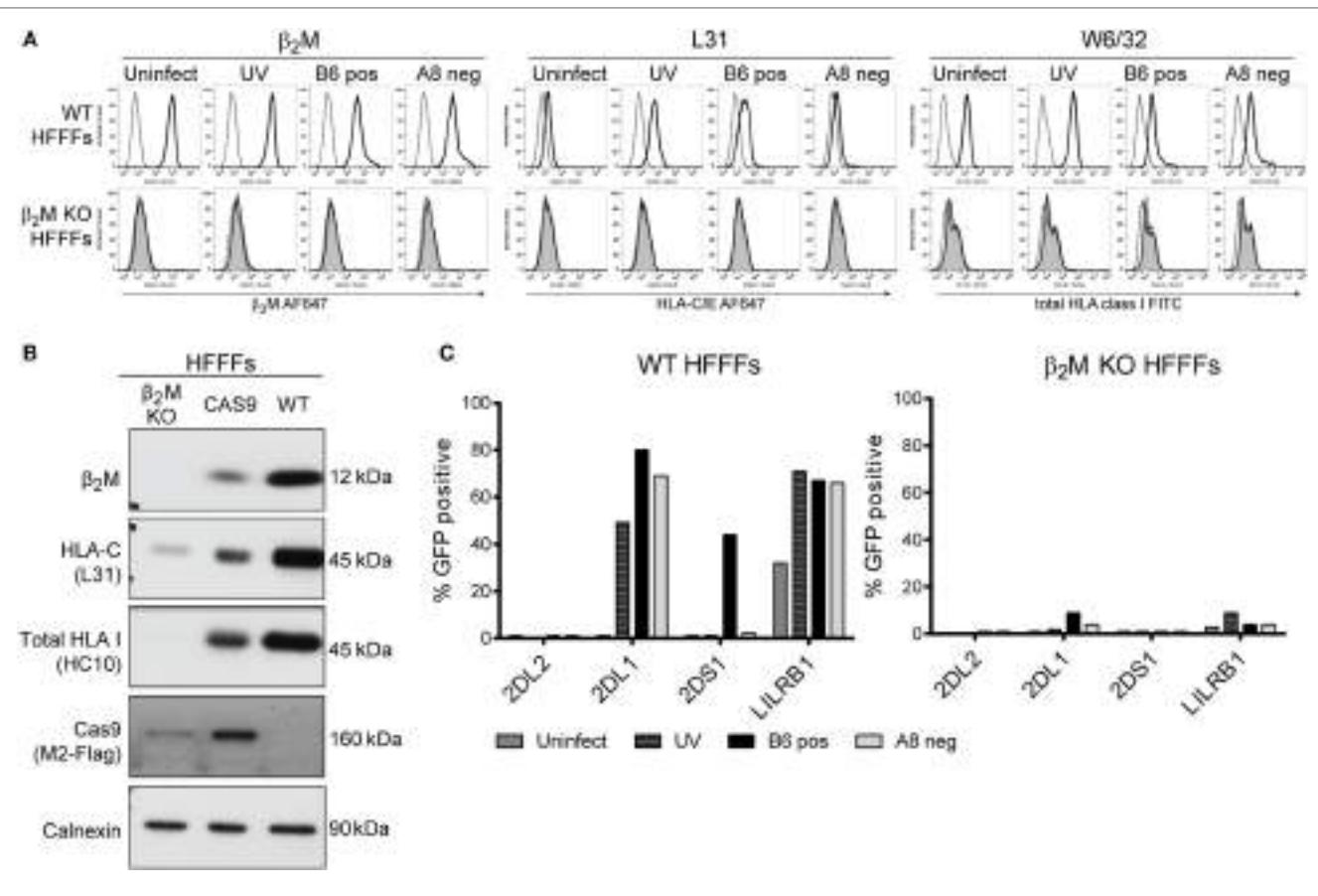


FIGURE 8 | β_2 M KO HFFFs do not activate KIR2DS1 reporter cells. **(A)** To control the success of the β_2 M knockout, untreated (WT) HFFFs (black line) and β_2 M KO HFFFs (shaded gray line) were stained with anti- β_2 M, W6/32, and L31 antibodies. Before staining, the cells were stimulated with UV clone (UV) or infected for 72 h with the positive B6 clone (B6 pos) or the negative A8 clone (A8 neg). Unstained cells were included (dotted line). **(B)** Total lysate of β_2 M KO HFFFs, HFFFs containing CAS9 without sgRNA (CAS9) and WT HFFFs were loaded onto a reduced 10% SDS-PAGE gel. The membrane was blotted with the anti- β_2 M, L31, HC10, anti-M2 Flag, and anti-calnexin (loading control) antibodies. **(C)** β_2 M KO HFFFs and WT HFFFs were stimulated with UV B6 clone (UV) and infected with the positive B6 clone (B6 pos) or negative A8 clone (A8 neg) for 72 h. An overnight coculture was performed with KIR2DL2 (negative control), KIR2DL1, KIR2DS1, and LILRB1 reporter cells. GFP expression was measured by flow cytometry. This experiment was performed twice.

will only be triggered if there is a true interaction. The degree of receptor/ligand clustering might also influence the downstream signaling, as shown by Oszmiana et al. (80). Therefore, the reporter cells may be a more physiological system compared to Fc proteins or tetramers.

KIR2DS1 Interacts with HLA-C but Not in the Same Way as KIR2DL1

There are several examples of “paired” immunoreceptors consisting of almost identical external moieties with positive and negative signaling tails, respectively (13). It is believed, but by no means proven, that this situation is driven by host-pathogen interaction. Our data are broadly consistent with this proposal. Our data fit with KIR2DL1, the inhibitory receptor, interacting with C2-HLA-C for recognition of a self-ligand in order to promote education/licensing of NK cells and subsequent loss of inhibition when the ligand is missing. By contrast, the role of aKIR has been unclear. Some groups reported weak binding, particularly for KIR2DS1, but these effects are inconsistent. In general, aKIR

appear to interact with HLA molecules weakly except in certain circumstances (27, 41, 42). We found that KIR2DS1 reporter cells were not activated after engaging conventional HLA class I molecules (Figures 2 and 3), as shown previously by others (10, 11, 17, 24–35). There is a possibility that the target cells used in previous KIR2DS1 studies did not only express conventional HLA-C molecules. These cells were EBV positive (721.221 cells, BLCLs, C1R cells), and/or tumor-derived cells, such as leukemia blasts and lymphomas. In addition, other studies included primary cells such as DCs (33), T cells (30, 33), B cells (27, 29), and MRC-5 fibroblasts (supplementary data of Stewart et al.) KIR2DS1 did not interact with or bind these primary cells, unless they were stimulated: in the case of DCs, stimulated with LPS and T cells, stimulated with PHA to form T cell blasts. Furthermore, Crespo et al. found that HCMV-infected JEG-3 cells and fetal extravillous trophoblasts (EVT) did not induce degranulation and cytokine production of dNK cells. They only found a cytotoxic response when dNK cells were exposed to HCMV-infected DSC specifically, indicating differential recognition of dNK cells.

to HCMV-infected cells (47). One explanation could be that KIR2DS1-mediated NK cell activation could only occur through an unknown synergistic engagement of other activating receptors, as proposed by Bryceson et al. (81). Additionally, perhaps a high level of C2-HLA-C is needed for a potent KIR2DS1-mediated NK cell activation, which is the case for these target cells. Primary cells might express too low levels of C2-HLA-C and in combination with the weak binding of KIR2DS1 to C2-HLA-C might result in the absence of NK cell activation. Crespo et al. also found reduced levels of HLA-C on HCMV-infected DSC leading the authors to speculate that an unknown activating ligand for KIR2DS1 is upregulated by HCMV infection which is recognized by dNK cells (47). Alternatively, our findings indicate that KIR2DS1 might recognize a modified form of C2-HLA-C, which is induced by selected TB40/E clones in HFFFs. Compared to HCMV from other sources, positive TB40/E clones were less effective in controlling FHC of HLA-C and, to a certain extent, assembled HLA-C (Figure 6). W6/32, 6A4, and B1.23.2 antibodies were able to block the KIR2DS1-ligand interaction on positive clone-infected HFFFs, while these antibodies could not block, or only partially block, the KIR2DL1 interaction (Figure 7). β_2 M KO experiments confirmed that KIR2DS1 binds a HLA class I molecule, most likely HLA-C, on these infected cells (Figure 8). It is unlikely that KIR2DS1 was binding directly to an HCMV-encoded protein, since KIR2DS1 reporter cells were not activated in coculture with β_2 M KO HFFFs infected with the positive clone. The most parsimonious interpretation is that the virus influences the balance of recognition directly of HLA-C by the KIR2DL1/S1 pair. At this stage, however, other possibilities cannot be ruled out, such as a combination of HLA class I with another protein.

What Is the Difference Between HLA-C Recognized by KIR2DS1 and by KIR2DL1?

Since all the data indicate that HLA-C forms at least part of the KIR2DS1 ligand the question that arises is how does HLA-C differ upon viral infection such that KIR2DS1 is brought into play? Figure 9 suggests various models which may be tested, namely:

- Bound peptide:** there have been reports of KIRs recognizing certain peptide motifs presented by HLA class I molecules. Stewart and colleagues have demonstrated that amino acids at position 7 and 8 of the peptide play a role in KIR2DS1 binding. KIR2DS1 has similar peptide selectivity to KIR2DL1 (27). Work from Khakoo's group has shown that peptides with certain motifs have either strong inhibitory, low inhibitory, or antagonistic effects on KIR2DL2 and -2DL3⁺ NK cells (82). These data suggest that NK cells are able to sense alterations of target cells, through selective peptide recognition.
- Modified glycosylation:** KIR3DL1 binding to HLA-B57:01 is dependent on the N-glycan on HLA-B57:01. Removing the N-glycan resulted in reduced inhibition, thus increasing degranulation of KIR3DL1⁺ NK cell clones (38). The N-glycosylation site on HLA class I is highly conserved and the glycan structures on HLA-C are relatively uniform between HLA-C allotypes (83). Perhaps viral infections could alter these glycosylation patterns and break the uniformity,

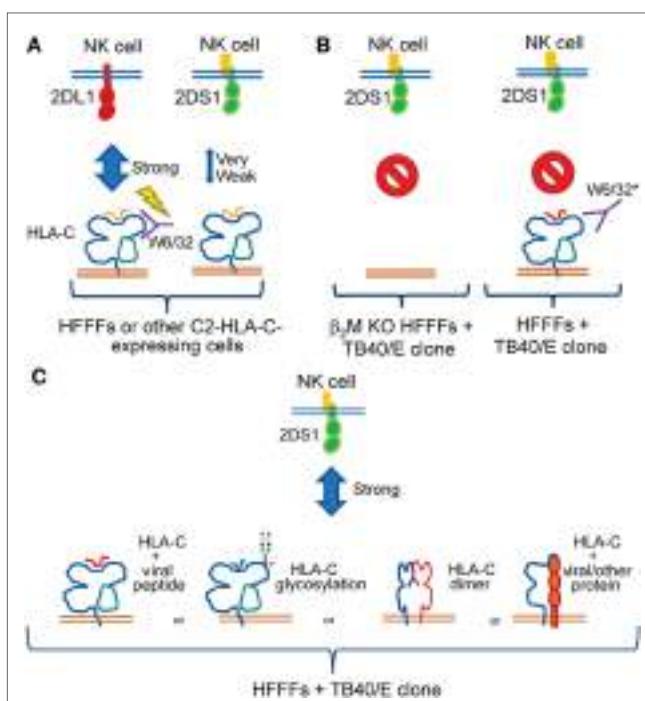


FIGURE 9 | Summary of the findings and working models. **(A)** Both KIR2DL1 (red) and -2DS1 (green) bind conventional group C2 human leukocyte antigen (HLA)-C; however, KIR2DS1 binds weakly. Our data were consistent with previous studies demonstrating that the W6/32 antibody does not block the KIR2DL1–HLA-C interaction. The lightning bolt indicates KIR2DL1 triggering even after adding W6/32 to the coculture. **(B)** Strong KIR2DS1 activation was observed after coculture with HFFFs-infected selected TB40/E clones. After infecting β_2 M KO HFFFs with selected TB40/E clones, KIR2DS1 interaction was diminished (left). In addition, W6/32 blocked the KIR2DS1–ligand interaction after coculture with HFFFs-infected selected TB40/E clones (right). *Other pan class I antibodies, such as 6A4 and B1.23.2, blocked both KIR2DL1 and KIR2DS1 interaction. The red symbol indicates no binding of KIR2DS1 after coculture in the indicated setting. **(C)** Remaining FHC of HLA-C and, to certain extent, assembled HLA-C surface expression was found on infected HFFFs. Together with the other findings, we hypothesize that a modification of C2-HLA-C is induced by HCMV, which influences KIR2DS1 recognition. This modification could relate to the following: presentation of HCMV-derived peptides; alteration of glycosylation patterns on the HLA-C molecule; formation of HLA-C homodimers; or heterodimers association with another protein.

resulting in the recognition of KIR2DS1. HIV and HCV infection has been shown to alter glycosylation in host cells, due to ER stress (84, 85). This might change the glycosylation pattern of HLA class I.

- Formation of HLA-C homodimers or heterodimers with other HLA class I molecules:** KIR3DL2 binds FHC forms of HLA-B27, including HLA-B27 dimers (86). LILRB1 interacts with different HLA class I molecules, which are able to dimerize via a cysteine residue in the cytoplasmic tail (49). Recently, it is described that the ligand for KIR3DS1 is FHC of HLA-F (41, 42). These data are evidence of the capability of Ig-like receptors to bind FHC HLA molecules or HLA homodimers.
- Modification by formation of HLA-C heterodimers with a virus-derived protein:** in mice, activating receptor Ly49P binding to an MHC-CMV protein heterodimer has been reported.

Ly49P binds CMV-infected cells expressing a complex of the m04 CMV protein together with the MHC class I molecule H-2D^k (87). Very recently, Serena et al. proposed that HIV-1 Env protein associates with FHC of HLA-C and that HIV-1 specifically upregulates FHC of HLA-C at the cell surface of infected cells (88). Their work has some parallels with our findings. Our preliminary experiments, however, did not show any evidence of HLA-C forming homo- or heterodimers (data not shown).

Based on these observations together with our findings, we argue that KIR2DS1 most likely binds C2-HLA-C either through recognizing HCMV-derived peptide or changes in glycosylation patterns. This will be the focus of future experiments.

Have Activating KIRs Evolved to Recognize Infected Cells?

Studies investigating the evolution of KIRs and other paired receptors have proposed that the activating members may be evolving more rapidly than the inhibitory members through selection imposed by pathogens (15, 16). The positively charged lysine at position 70 in KIR2DS1 is critical for the diminished binding to C2-HLA-C compared to KIR2DL1. This amino acid is conserved in all KIR2DS1 allotypes (with the exception of KIR2DS1*001, which has a charged arginine) (11). Conversely, the lysine at position 70 in KIR2DS1 could be crucial for binding modified HLA-C induced by pathogens. KIR2DL1 might have evolved to recognize HLA-C on healthy cells (induced-self), while KIR2DS1 might recognize slight structural changes on HLA-C induced by pathogens (altered-self). KIR2DS1 may still bind conventional HLA-C weakly to secure tolerance, yet recognition by KIR2DS1 of a modified form of HLA-C could overcome this tolerance. Our findings favor this hypothesis.

Why Were KIR2DL1 Reporter Cells More Activated after Encountering TB40/E-Infected HFFFs?

The GFP expression of KIR2DL1 reporter cells, after encountering TB40/E-infected HFFFs, was even higher than in coculture with UV TB40/E-stimulated HFFFs, which contain high expression levels of HLA-C (Figure 4A). HCMV downregulates HLA class I molecules, thus these findings were unexpected. A possible explanation is that KIR2DL1 binds the remaining HLA-C on the infected cell surface (Figure 6B). Ameres et al. reported that HCMV downregulates certain HLA-A and -B alleles more efficiently than HLA-C alleles (89, 90). Another explanation could be that KIR2DL1 recognizes an alternative ligand, comparable to how both inhibitory Ly49I and activating Ly49H-binding m157 (43, 91).

Differences in HCMV Isolates

Natural killer cell responses appear to differ when encountering cells infected with various HCMV strains and even clones within strains. Chen et al. also demonstrated that the ability of NK cells to control virus spread through LILRB1 was variable between HCMV viral strains, depending on the amino acid sequence

within UL18 (92). Thus, the variable effect between HCMV strains on NK cell activity and vice versa should be taken into account when setting up experiments and interpreting published data. This will also count for cytotoxic T cells and other immune cell responses.

TB40/E-derived positive clones were the only viruses that, upon infecting HFFFs, expressed the KIR2DS1 ligand. Together, these findings imply that after infection the positive clones are less successful in downregulating the KIR2DS1 ligand than TB40/E-derived negative clones or other HCMV strains. As a result, KIR2DS1 reporter cells and 2DS1sp NK cells are specifically detecting the ligand on positive clone-infected HFFFs. This suggests that the other HCMV strains are capable of downregulating the KIR2DS1 ligand as an immune evasion strategy to NK cells. Our findings could explain why this interaction has not been detected in previous studies. Identification of the differences between the positive and negative clones by whole virus genome sequencing should help to resolve this issue.

CONCLUSION

Our findings indicate that activating KIRs do not bind the same conventional HLA molecules as their inhibitory counterparts. They suggest that pathogenic infections are required for strong activating KIR binding, discriminating between healthy and unhealthy cells. To our knowledge, this is the first time that the role of HCMV on activating KIR recognition has been conclusively shown. Future work will provide new insights into the role of NK cells in HCMV infection and transplantation. This could lead to more targeted and effective therapeutic avenues in the treatments for HCMV infection in new-born babies, immuno-suppressed individuals, and patients undergoing solid organ or HSCT transplantation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Addenbrookes National Health Service Hospital Trust institutional review board, Cambridgeshire 2 Research Ethics Committee (REC 97/092) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Cambridgeshire 2 Research Ethics Committee.

AUTHOR CONTRIBUTIONS

KP, MW, and JT conceived and designed the experiments. KP and MI performed the experiments and analyzed the data. CC designed and provided the reporter cells. KP, MW, JT, CC, MI, and AM discussed the data and commented on the manuscript. KP and JT wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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Killer Immunoglobulin-Like Receptor Allele Determination Using Next-Generation Sequencing Technology

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The impact of natural killer (NK) cell alloreactivity on hematopoietic stem cell transplantation (HSCT) outcome is still debated due to the complexity of graft parameters, HLA class I environment, the nature of killer cell immunoglobulin-like receptor (KIR)/KIR ligand genetic combinations studied, and KIR⁺ NK cell repertoire size. KIR genes are known to be polymorphic in terms of gene content, copy number variation, and number of alleles. These allelic polymorphisms may impact both the phenotype and function of KIR⁺ NK cells. We, therefore, speculate that polymorphisms may alter donor KIR⁺ NK cell phenotype/function thus modulating post-HSCT KIR⁺ NK cell alloreactivity. To investigate KIR allele polymorphisms of all KIR genes, we developed a next-generation sequencing (NGS) technology on a MiSeq platform. To ensure the reliability and specificity of our method, genomic DNA from well-characterized cell lines were used; high-resolution KIR typing results obtained were then compared to those previously reported. Two different bioinformatic pipelines were used allowing the attribution of sequencing reads to specific KIR genes and the assignment of KIR alleles for each KIR gene. Our results demonstrated successful long-range KIR gene amplifications of all reference samples using intergenic KIR primers. The alignment of reads to the human genome reference (hg19) using BiRD pipeline or visualization of data using Profiler software demonstrated that all KIR genes were completely sequenced with a sufficient read depth (mean 317x for all loci) and a high percentage of mapping (mean 93% for all loci). Comparison of high-resolution KIR typing obtained to those published data using exome capture resulted in a reported concordance rate of 95% for centromeric and telomeric KIR genes. Overall, our results suggest that NGS can be used to investigate the broad KIR allelic polymorphism. Hence, these data improve our knowledge, not only on KIR⁺ NK cell alloreactivity in HSCT but also on the role of KIR⁺ NK cell populations in control of viral infections and diseases.

Keywords: high-resolution killer cell immunoglobulin-like receptor typing, allele polymorphism, next-generation sequencing, International Histocompatibility Workshop DNA samples, Natural killer cells

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) provides a curative therapy for many patients with hematological malignancies (1). Donors for HSCT are currently selected based on the level of matching for HLA-A, -B, -C, -DRB1, and -DQB1 loci. Siblings, 10/10 HLA matched, remain the gold standard. However, substantial risks of morbidity and mortality caused by disease relapse (2), graft-vs-host-disease (GvHD) (3), and infection (4) are still prevalent after related, or unrelated HSCT. Natural killer (NK) cells are the first post-HSCT cells, reconstituting antiviral and antitumoral activity (5). NK cells are able to recognize the missing-self *via* killer cell immunoglobulin-like receptors (KIRs) (6). Ruggeri et al. (7) were first to report the beneficial effect of KIR ligand mismatched donor NK cell alloreactivity after T cell-depleted HLA haplo-identical HSCT resulting in less relapse, less GvHD, and better overall survival in patients with acute myeloid leukemia. The impact of KIR⁺ NK cell alloreactivity on HSCT outcome is still controversial due to the heterogeneity of graft parameters, HLA class I environment, nature of KIR/KIR ligand genetic combinations studied, and KIR⁺ NK cell repertoire size (8–12).

As HLA class I genes, KIR genes are highly polymorphic (13). In humans, 16 KIR genes have been described including eight inhibitory genes (2DL1/L2/L3/L4/L5, 3DL1/L2/L3), 6 activating genes (2DS1/S2/S3/S4/S5, 3DS1), 2 two pseudogenes (2DP1, 3DP1). These genes are located within the leukocyte receptor cluster found on chromosome 19q13.4, spanning a region of 150 kb. Within a population, the genotypic diversity of KIR genes occurs at different levels. First, the number and nature of KIR genes vary between individuals defining different KIR haplotypes. KIR haplotypes are classified into group A and group B (14). The group A haplotype is defined by a fixed set of nine KIR genes: four framework KIR genes (3DL3, 3DP1, 3DL2, and 2DL4) that form the centromeric and telomeric part of KIR locus, three inhibitory KIR (2DL1, 2DL3, and 3DL1), a pseudogene (2DP1), and a single activating KIR gene (2DS4). The group B haplotype is defined as having a variable number of KIR genes (7–14) including the four framework KIR genes and specific KIR genes (2DS2, 2DL2, 2DL5, 2DS3, and 2DS1). Second, a variable number of copies [copy number variation (CNV)] of the gene generated by recombination and replication have also been described for some KIR genes particularly those of the B haplotype (15–17). The CNV seems to influence the licensing of KIR⁺ NK cells (18). Overall, various KIR genotypes can be observed in a population. All KIR genes, and especially for inhibitory KIR, a high degree of allelic polymorphism has been described. The latest KIR Immuno Polymorphism Database (IPD-KIR) describes 753 KIR alleles. KIR allele polymorphisms need to be investigated throughout the exon and the intron regions, and regulatory regions as shown for KIR3DL1 (19). In contrast to HLA class I genes, structure and length of KIR genes vary. KIR allele polymorphisms impact both KIR⁺ NK cell phenotype and function, as we and other groups having described for KIR3DL1 (20–25) and for KIR2DL2/L3 (26). Differences in the intensity of expression (strong, weak, or null) have been described for the KIR3DL1 receptor, defining different allotypes according to the

KIR3DL1/3DS1 allele combinations present in healthy individuals (21, 27). Furthermore, the nature of KIR3DL1 alleles does not only impact the KIR3DL1 cell density but also the strength of the KIR3DL1–HLA interactions which in turn can affect NK cell functions (28, 29). The recognition of KIR allotypes using anti-KIR monoclonal antibodies also varies depending on the KIR allele polymorphism (30).

Taking these points into account, it is therefore necessary to thoroughly investigate the phenotypic and functional impact of KIR allele polymorphisms. Until now, potential KIR⁺ NK cell alloreactivity in HSCT was mainly evaluated depending on the KIR/KIR ligand genetic combinations present and analyzed only at a generic level (i.e., presence or absence of KIR genes and KIR ligand). We speculate that KIR allele polymorphisms may alter donor KIR⁺ NK cell phenotype/function, and thus modulate their alloreactivity affecting HSCT outcome. However, the impact of KIR allele polymorphisms on HSCT outcome remains difficult to assess due to the lack of suitable allele typing methods for all KIR genes. Until recently, several standard methods are used to type KIR genes at allelic level. Those methods include sequence-specific oligoprobe hybridization (31–37), sequence-specific primer (SSP) typing (22), SNP assay (38), Sanger sequence-based typing (SBT) (20, 39–42), high-resolution melting (43), and also combined SSP/SBT (21, 44). KIR allelic polymorphisms have been investigated for a few functional KIR genes (KIR2DL1/2DL2/2DL3/2DS1 /3DL1/3DS1). Standard methods to type KIR genes at allelic level are usually single KIR locus specific and/or target a limited polymorphism. In addition, the constant increase in the number of KIR alleles described generates more and more ambiguous KIR typing in heterozygous samples since KIR polymorphism can extend over the entire gene. Recent advances in high-throughput sequencing technology [next-generation sequencing (NGS)], especially in immunology and hematology (45), enable determination of KIR alleles and KIR gene CNV. The extent of KIR allele polymorphisms, as demonstrated by exome capture, reported 37 new KIR alleles from 15 healthy South African individuals (46). Recently, whole KIR genome sequencing by NGS was used as a control method to validate CNV genotyping in the KIR locus (17). An exome capture that focused on KIR and HLA class I loci was also recently described (47). In this study, we developed a reliable NGS method for high quality DNA samples and easily implemented for the study of KIR allele polymorphisms.

MATERIALS AND METHODS

Samples

Thirty B-EBV cell lines from the 10th International Histocompatibility Workshop (IHW) were selected from a well-characterized panel known for their KIR gene content. KIR genotype information, including KIR allele typing of some KIR genes for all these B-EBV cell lines, was obtained either from the IPD/KIR database or from literature for specific KIR loci. Known KIR genotypes and allele typing of these 30 B-EBV cell lines are provided in the Table S1 in Supplementary Material.

KIR Long-Range (LR) PCR and Primers

DNA genomic extractions were performed from B-EBV cell lines using a Nucleospin blood kit (Macherey-Nagel, Duren, Germany). The concentration and the purity of all DNA samples were checked on a NanoDrop 2000C spectrophotometer (ThermoFisher, Wilmington, DE, USA) by measuring the ratio of absorbance at 260 and 280 nm. In parallel, 1.5 µg of each DNA sample was loaded on an agarose gel to check the DNA integrity. For KIR LR PCR, five intergenic KIR primers already described (17) and one additional *in-house* designed primer including four forward primers (#1, 5'-gccaataacatccgtgcgcgtcagct-3'; #2, 5'-ctcacaacatc-tgtgtcgactga-3'; #4, 5'-acggctgcgtctgcacagacagacc-3', #6, 5'-cacatcgctgcaccggtcagtcgagccg-3') and two reverse primers (#3, 5'-ttggagagggtggcagggtcaagt-3'; #5, 5'-ctccatctgagggtccc-tgtatgt-3') were used to amplify the whole KIR genome.

The KIR LR-PCR protocol was optimized using the method described by Vendelbosch et al. (17). Briefly, KIR LR-PCR was performed with 2.5 U of PrimeSTAR GXL DNA Polymerase (Ozyme, Saint-Quentin en Yvelines, France), 1× PrimeSTAR GXL buffer, 200 µM of dNTP mixture (Ozyme) and 0.2 µM final concentration of each KIR primer. The LR-PCR reaction was performed in a C1000 Touch™ Thermal Cycler (Biorad, Marnes la Coquette, France) consisted of an initial denaturation of 2 min at 94°C followed by 30 cycles of 20 s at 94°C, 12 min at 68°C and 1 cycle of final elongation of 10 min at 72°C in the final 50 µL volume. This protocol enables amplification of each KIR gene from 5' to 3' untranslated regions (UTR). The final KIR LR-PCR product was run on 0.7% Seakem agarose gel in TBE1X (Lonza, Verviers, Belgium) and visualized by staining with the SYBR® safe (Invitrogen, Villebon sur Yvette, France) using the SimplyLoad™ Tandem DNA ladder size marker (Ozyme) to confirm the amplification and correct fragment size as well as to check for non-specific amplification.

Library Preparation and Sequencing

Qubit dsDNA High Sensitivity Assay Kit (Life Technologies, Villebon sur Yvette, France) was used to quantify the starting DNA library in the Qubit® fluorometer (Life Technologies). The library preparation was performed using the NGsgo GENDX kit (Bedia Genomics, Chavenay, France). To achieve the optimal insert size and library concentration, 250 ng of each genomic DNA was randomly fragmented according to the manufacturer's instructions. Briefly, 8.25 µL of NGsgo master mix (prepared from 2 µL of NGsgo-LibrX Fragmentase buffer plus 3.25 µL of NGsgo-LibrX End Prep buffer plus 1.5 µL of NGsgo-LibrX Fragmentase Enzyme plus 1.5 µL NGsgo-LibrX End Prep Enzyme) (Bedia Genomics, Chavenay, France) was added to each genomic DNA in a final volume of 32.5 µL. The fragmentation, end-repair, and dA-tailing reactions were performed in a T100™ Thermal Cycler (Biorad, France) consisted of 20 min of fragmentation and end-repair at 25°C followed by 10 min of dA-tailing at 70°C. The dA-tailed DNA fragments of each sample were then subjected to adapter ligation in 9.25 µL of an NGsgo master mix containing 7.5 µL of NGsgo-LibrX Ligase mix, 0.5 µL of NGsgo-LibrX Ligation Enhancer, 0.25 µL of NGsgo-Indx adapter for Illumina, and 1 µL of nuclease free water. The adapter ligation reaction took place in a T100™ Thermal Cycler (Biorad, France) for 15 min at

20°C followed by a cooling step at 15°C. The first cleaning and size selecting of the samples after adapter ligation were performed in a 0.45× beads:DNA ratio by using the Agencourt® Ampure XP (Beckman Coulter, Villepinte, France) according to the manufacturer's instructions and eluted in 12.5 µL of 0.1× elution buffer (Lonza Rockland, USA). The size-selected, adapter-ligated DNA fragments of each DNA sample were then dual indexed with 15 µL of NGsgo reaction mix made from 12.5 µL of NGsgo-LibrX HiFi PCR mix plus 1.25 µL of NGsgo-Indx IN-5 and 1.25 µL of NGsgo-Indx IN-7 in a final volume of 25 µL followed by a PCR reaction in a T100™ Thermal Cycler (Biorad). PCR cycling was performed as follows: an initial denaturation of 30 s at 98°C followed by 10 cycles of 10 s at 98°C, 30 s at 65°C, 30 s at 72°C and 1 cycle of final elongation step of 5 min at 72°C in the final volume of 25 µL. A second DNA cleaning and size selecting was performed in a 0.6× beads: DNA ratio by using the Agencourt® Ampure XP beads according to the manufacturer instructions and eluted in 16.5 µL of 0.1× elution buffer (Lonza Rockland, USA).

Quality control procedure for the library preparation included verification of fragment size before and after purification by using the QiAxcel Advanced System (QiAgen, Courtaboeuf, France). The pooled and barcoded libraries were denatured with 0.2 M of NaOH and diluted in the pre-chilled HT1-buffer to obtain a final library concentration of 12 pM. The final denatured library was subsequently sequenced by using the MiSeq sequencer (Illumina, Biogenouest Genomics Platform Core Facility, Nantes, France; HLA Laboratory, EFS Nantes, France) with 500 cycles v2 kits, which generated 250-bp end sequence reads.

Sequencing Data Analysis and KIR Allele Assignment

The quality of the Illumina raw data sequences obtained was monitored by using the Sequencing Analysis Viewer Illumina software. The quality of the base calling from images and sequences was determined by the quality score (Q30) which must be $\geq 75\%$ for 2×250 bp reads. KIR reads were mapped to the human genome reference sequence hg19 (GRCh37) by using the Burrows-Wheeler Aligner Memory Efficient Mapping (BWA-MEM) tool. The binary alignment map (BAM) files containing mapped reads were then visualized on Integrative Genomics Viewer (IGV) algorithm (48).

In parallel, raw KIR sequences were aligned and visualized using the Profiler software version 1.70, initially developed by Dr. M. Alizadeh (Research Laboratory, Blood Bank, Rennes, France) for NGS-based HLA typing (49). A flowchart for data analysis using the Profiler software is provided in Figure S1 in Supplementary Material. The first step of analysis consists by merging R1 and R2 sequences to each other when at least 10 complementary bases were found between R1 and R2 of the same cluster. During this phase, for each inconsistency of base calling, the quality value was used to select the best assignment. All sequences issued from a cluster for which we could not determine complementary between R1 and R2 remained unchanged. All sequences were transformed to FASTA format at the end of this step. The second step of analysis consists of phasing each of the sequences obtained in step one by using Blast algorithm. The third step of analysis consists by merging all sequences together

using Blast information. In this step, the depth for each position and the number of sequences for each allele were calculated. The first three steps are managed in a Linux environment. The last step is presentation and assignment of each construction based on database information in a friendly interface for user, all mismatches and differences to the database are extracted and presented to the user.

For KIR allele assignment, a manual bioinformatic pipeline was first used in the absence of available softwares. This consisted of exporting from IGV, all exon sequences of each KIR gene and comparing polymorphic bases with those referenced from the IPD-KIR database. Then, two different bioinformatics algorithms were used: the first one, hereafter called “BiRD,” was developed by the BiRD platform (E. Charpentier, U. Guyet, Genomics and Bioinformatics Core Facility GenoBiRD, Nantes, France) and consists of an analysis pipeline built with Snakemake on the same logic as the manual method. A flowchart for data analysis using the BiRD software is provided in Figure S2 in Supplementary Material.

Harvesting KIR-Specific Reads

First, raw sequences from fastq files are processed through cutadapt (v1.8.1) in order to remove Illumina adapter sequences. The cleaned reads are then mapped to hg19 (GRCh37) reference genome using BWA-MEM (v0.7.12) with the default parameters.

Determining Presence/Absence or KIR Genes

Absence or presence of KIR genes is evaluated using GATK DepthOfCoverage on the BAM and using a browser extensible data (BED) file describing the chromosome position of each gene (except KIR2DP1 and KIR3DP1). Coverage mean is calculated on each gene position, and a threshold of 10 is applied in order to ascertain its absence or presence. Presence/absence of KIR genes defined by NGS is concordant to the KIR genotype of the 30 IHW samples, stratified by AA vs Bx genotypes, previously validated in our laboratory by PCR-SSP multiplex method (data not shown).

Determining KIR Alleles

Allele-specific nucleotide positions are extracted manually using IPD-KIR alignment tool.¹ For every gene, the Nucleotide—CDS of all alleles are aligned against the default reference allele. A python script is then used to reformat the multipage alignments in order to have one allele alignment per line. A second python script is utilized to extract all variations from the default reference allele and map the exon position number of these variations to the chromosome position. A file is created for each gene listing all the variations found for every allele. Bases at these positions are then called using SAMTools (v1.2-2) mpileup for all samples. Finally, KIR alleles are determined by calculating the percentage of nucleotide matches between the base calls and the allele variations for each KIR allele, the highest percentage giving the most confident allele.

The second algorithm used for KIR allele assignment was the Profiler software, previously described in Figure S1 in Supplementary Material, version 1.70 (49), which permits to

directly assign KIR alleles at the highest level resolution (seven digits) since full intron and exon sequences were considered and also provides quality data such as mean coverage for each KIR locus. The fragment size percentage of sequences for each allele/locus was also considered as well as percentage of mapping for each KIR gene.

Overall, KIR allele assignment for each locus and for all samples corresponds to the combined KIR results obtained using manual pipelines, BiRD, and Profiler softwares. KIR alleles were assigned on the basis of the known DNA sequences identity within the IPD/KIR database.² KIR alleles are named in an analogous fashion as the nomenclature used for HLA class I alleles. After the gene name, an asterisk is used as a separator before a numerical allele designation. The first three digits of the numerical designation are used to indicate alleles that differ in the sequences of their encoded proteins. The next two digits are used to distinguish alleles that only differ by synonymous (non-coding) differences within the coding sequence. The final two digits are used to distinguish alleles that only differ by substitutions in an intron, promoter, or other non-coding region of the sequence.

RESULTS

LR KIR Gene Amplifications

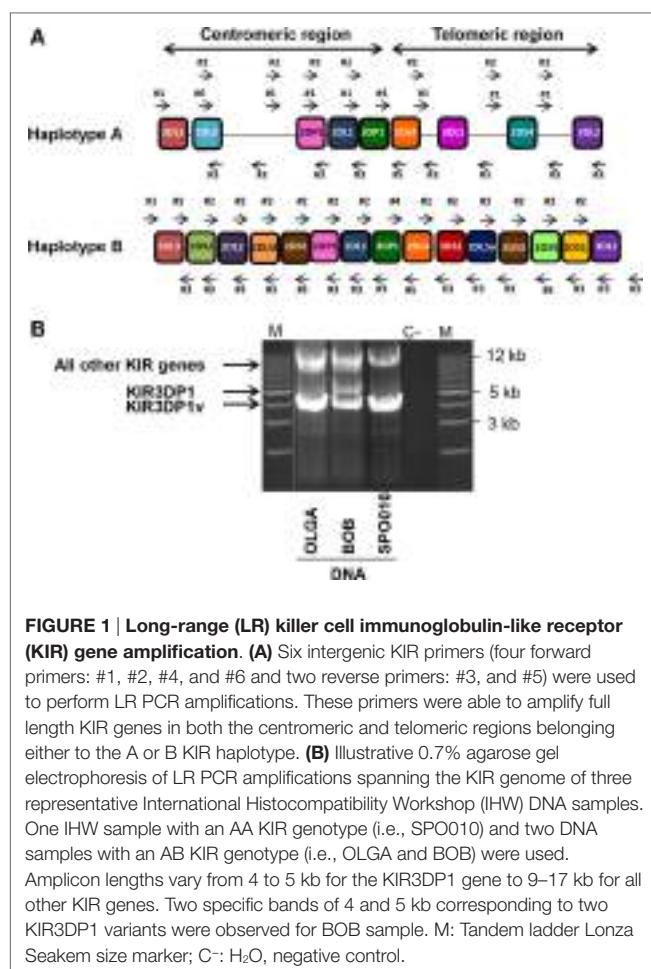
Thirty reference IHW samples with known KIR genotyping (Table S1 in Supplementary Material) were used to validate our NGS method for typing of each KIR gene at allelic resolution. DNA integrity, checked by loading each sample on an agarose gel, confirmed high quality for all samples (data not shown). In order to amplify all KIR genes from the 5' UTR to the 3' UTR, six intergenic KIR primers were chosen to allow the amplification of framework KIR genes. These intergenic primers also amplify KIR genes located either in the centromeric or telomeric region, which belong to the A and/or B specific KIR haplotype genes (Figure 1A). A robust LR amplification of KIR genes was obtained for all samples as illustrated for three representative IHW samples (Figure 1B). One specific band between 4 and 5 kb for the KIR3DP1 pseudogene and another specific band between 9 and 17 kb corresponding to a cluster of all other KIR genes were observed, irrespective of KIR AA or AB genotype (Figure 1B) as KIR genomic length varies depending on KIR genes (Table S2 in Supplementary Material). For some IHW samples such as BOB, two specific bands at 4 and 5 kb were observed for the KIR3DP1 gene corresponding to KIR3DP1*003 and KIR3DP1*001 variants, respectively, whereas only one band at 4 kb specific of KIR3DP1*003 variant was observed for OLGA and SPO010 samples (Figure 1B).

Complete Sequencing of All KIR Genes

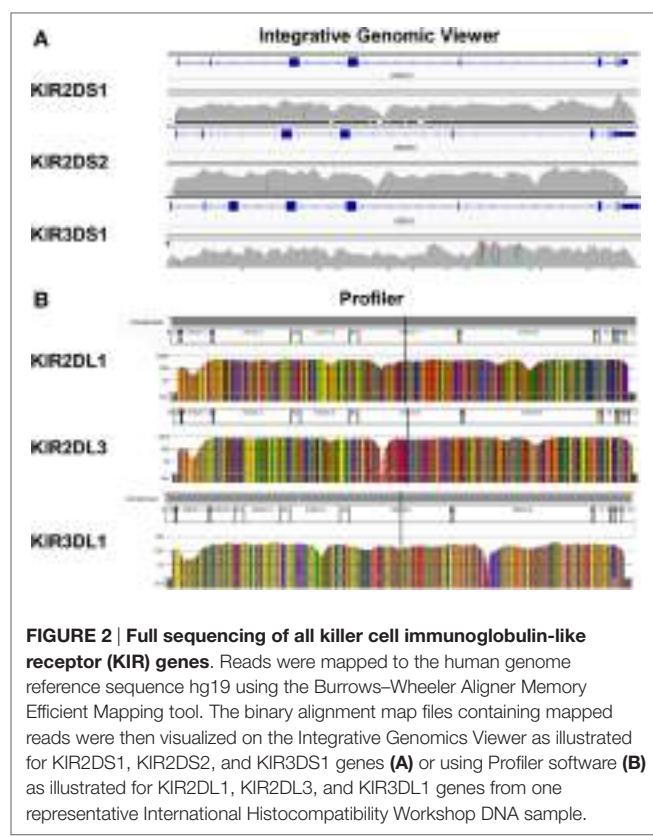
In order to check the specificity of KIR LR-PCR obtained, amplicons were further fragmented and sequenced on paired end 2×250 bp from Illumina MiSeq platform. The sequencing of all amplicons yielded a total of 6.3 Gb, which was generated from a 755 ± 31 K/mm² cluster density (data not shown). Approximately 88.2% of the clusters passed QC filters and on

¹<https://www.ebi.ac.uk/ipd/kir/align.html>.

²<http://www.ebi.ac.uk/ipd/kir/alleles.html>



average, 82.4% of both reads passed with a Q30 > 82% (data not shown). Thus, analysis of FastQ data obtained from all IHW samples reported an excellent quality control. The entire length of KIR genes was sequenced with good coverage as illustrated for KIR2DS1, KIR2DS2, and KIR3DS1 (Figure 2A) activating genes, and for KIR2DL1, KIR2DL3, and KIR3DL1 (Figure 2B) inhibitory genes using either IGV or Profiler software, respectively. For all genes, the depth of coverage varies most at the beginning and at the end of the amplicons, but all key regions were sufficiently covered. In particular, we observed that mean coverage ranged from 62.5× (KIR2DS4) to 2,373.3× (KIR3DP1) leading to a mean coverage of 316.55× for all KIR genes except for KIR2DL5A genes since not analyzed using Profiler (Table S3 in Supplementary Material). A significant correlation was observed between mean coverage and genomic KIR length ($r = 0.85$, $p < 0.0001$) as illustrated Figure 3A. Indeed, the lower the genomic length, the higher the mean coverage is as illustrated for the KIR3DP1 gene. The mean percentage of mapping, established by the coverage of amplicon, ranged from 86.2% (KIR3DL2) to 98.2% (KIR2DP1 and KIR3DP1) (Figure 3B; Table S3 in Supplementary Material) suggesting that sufficient read depth was obtained for determination of all KIR genes. However, KIR2DL5A reads could have been mapped



only using BWA-MEM software and BiRD algorithm. Overall, these results demonstrate the efficiency of our NGS-KIR allele typing approach to capture the full KIR genomic locus and the uniformity of coverage for each KIR locus confers assurance for KIR allele assignment.

Specificity of NGS-Based KIR Allele Typing

Due to the high degree of KIR polymorphisms and the fact that NGS technology generates a lot of sequencing reads, three different algorithms were evaluated to increase the reliability of KIR allele assignment as reported for NGS-based HLA typing (50). KIR allele assignment was first done manually and then confirmed using both BiRD pipeline and Profiler software. Overall, resulting KIR allele assignments of the 30 reference IHW samples were feasible for all loci and for the majority of samples without remaining ambiguities (Table 1).

We further evaluated the strength of our NGS-based method for KIR allele assignment. For all IHW reference samples tested ($N = 30$), the number of KIR alleles previously known in the IPD/KIR database and those obtained by our NGS-KIR based typing approach was compared for each KIR locus. As an example, from the 30 IHW samples tested, only 5 KIR3DL3 alleles out of 60 expected alleles for this framework gene were previously known in the IPD/KIR database (Table S1 in Supplementary Material), 54 KIR3DL3 alleles from 24 heterozygous and 6 homozygous samples (Table 1) were assigned

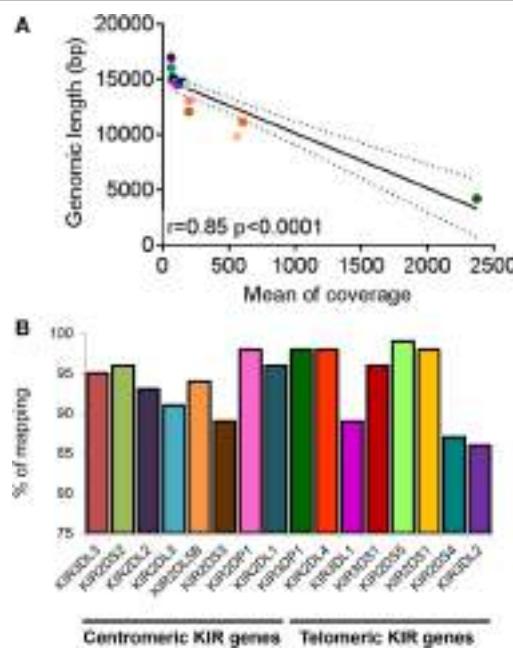


FIGURE 3 | Coverage and percentage of mapping obtained for killer cell immunoglobulin-like receptor (KIR) genes. (A) Correlation graph representing mean coverage for each KIR gene and KIR genomic length. Mean coverage was estimated for each KIR gene present for all International Histocompatibility Workshop (IHW) samples using Profiler software. Statistical significance was determined using the Pearson's rank coefficient using GraphPad Prism version 6 software (GraphPad Software, La Jolla, CA, USA). A significant *p*-value between mean coverage and genomic KIR length was observed ($p < 0.0001$). **(B)** Bars representing the percentage of mapping of each centromeric and telomeric KIR gene present for all IHW samples determined using Profiler software. KIR2DL5A locus was not included since not analyzed using Profiler.

by our NGS-based KIR allele typing approach (Figure 4). Our NGS-based KIR allele typing approach permits identification of additional framework KIR alleles, e.g., KIR3DP1 ($n = 43$), KIR2DL4 ($n = 48$), and KIR3DL2 ($n = 44$) (Table 1; Figure 4). NGS-based KIR typing method also allows the identification of polymorphisms of well-functionally characterized KIR by increasing the number of assigned KIR alleles of the 30 IHW samples available in the IPD/KIR database (Table S1 in Supplementary Material), e.g., KIR2DL1 ($n = 30$), KIR2DL2 ($n = 10$), KIR2DS1 ($n = 11$), KIR2DS2 ($n = 12$), KIR3DL1 ($n = 35$), and KIR3DS1 ($n = 11$) (Table 1; Figure 4). The number of activating KIR2DS1, KIR2DS2, and KIR3DS1 assigned alleles by NGS remained low because only IHW samples with the corresponding activating KIR gene were included in this analysis. Overall, a higher number of KIR alleles were identified from these 30 IHW samples by our shotgun NGS methodology compared to those previously characterized by other less sensitive methods, as referred to in the IPD/KIR database ($N = 422$ vs $N = 233$, respectively).

The knowledge of KIR allele typing of IHW samples, recently updated thanks to an exome capture (47), permits to evaluate the concordance of our NGS-based KIR allele results (Table 1) with

those of Norman et al. since 22 IHW samples were commonly used in both methods (Table S1 in Supplementary Material). In this case, a large number of allelic KIR typing for all loci was compared ensuring the reliability of our NGS-based KIR allele typing method. For each KIR locus and for the 22 IHW concerned samples, KIR allele typing results were divided into: concordant (one KIR allele matched for homozygous samples or two KIR alleles matched for heterozygous samples), semi-concordant (one KIR allele matched and one KIR allele mismatched), and discordant (one KIR allele mismatched for homozygous sample or two KIR alleles mismatched for heterozygous sample). For each KIR allele, only the first three digits were taken into account for the assessment of concordance. Complete concordance (100%) of KIR allele typing was demonstrated in 11 KIR genes. The concordant genes were KIR2DS2 (8 samples out of 8), KIR2DL5B (2 out of 2), KIR2DS3 (1 out of 1), KIR2DL1 (18 out of 18), KIR2DL4 (22 out of 22), KIR3DL1 (17 out of 17), KIR3DS1 (7 out of 7), KIR2DL5A (7 out of 7), KIR2DS5 (6 out of 6), KIR2DS1 (6 out of 6), and KIR2DS4 (16 out of 16) (Figure 5). Concordant results were observed, but at a lesser frequency for KIR3DL3 (20 out of 22, i.e., 91%), KIR2DL2 (4 out of 5, i.e., 80%), KIR2DL3 (7 out of 8, i.e., 88%), KIR2DP1 (16 out of 20, i.e., 80%), KIR3DP1 (16 out of 18, i.e., 89%), and KIR3DL2 (16 out of 18, i.e., 89%) (Figure 5).

Ten semi-discordant KIR allele results and two discordant KIR allele results between our NGS-based method and exome data were identified (Table 2). Except for the pseudogene KIR2DP1, with four IHW samples, these discrepancies were limited to 1 or 2 out of 22 IHW samples per locus (Table 2). KIR allele determinations using manual, BiRD algorithm, and different versions (the latest one Rev 2.0.188) of Profiler software were carefully reviewed. Only IHW samples sequenced on different runs and with the same KIR allelic results were reported (data not shown). These potential discrepancies (5%), possibly linked to the design and implementation of each algorithm, need to be further validated by another typing method such as SSP or sequencing.

Overall, our NGS-based method and exome data showed a rate of concordance of 95% for all loci, established for all KIR genes on 22 IHW samples, suggesting a reliable method.

DISCUSSION

In this study, we developed an NGS-based KIR allele typing approach to characterize the sequence of all polymorphic KIR genes. Our method of typing all KIR genes at high resolution provides an alternative, easily implemented method practice, to study the KIR allele polymorphisms. It may be a cheaper method than exome capture (47). This tool is currently adapted to the KIR gene large-scale analysis. Using our approach, the majority of KIR alleles previously uncharacterized by standard methods were clearly identified from genomic DNA of 30 B-EBV cell lines from the 10th IHW. High quality DNA samples, high fidelity of enzyme polymerase, and a reliable library preparation were needed since evaluation of different Taq polymerase enzymes

TABLE 1 | Next-generation sequencing-based killer cell immunoglobulin-like receptor (KIR) allele typings of 30 reference B-EBV cell lines from the 10th International Histocompatibility Workshop.

ID	Centromeric KIR genes									Telomeric KIR genes								
	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5A	2DS3	2DS5	2DS1	2DS4	3DL2
AMAI	*013 *041			+			*00301 *004	+	*003 *006	*0080101 *0080102	*001					*00301	*00101	
AMALA	*00402 *00802	*00101	*00301	*001			*00201	*00302	*007 *00901	*00102 *00501	*01502	*001 *01301			*00201	*00201	*001	*0020105 *0070102
BOB	*00101 *01303	*00101	*00301	*00201			*00301	*00302	*002 *00302	*001 *005	*002	*01301	*00101		*00201	*00201	*001	*0020101 *0070102
BRIP	*00801 *004	*00104	*00301	+		*00103 *00201	*0010201 *0020101	*00302	+	*0010305 *00501	*008	*01301	*00103 *00501	*00103 *00201		*002	*003	*0070102 *0070103
CALOG	*00207			+			*00201	*00302	*00302 *010	*008	*001					*00301	*00101	
ERO	*01001										*004					*00601	*00301	
COX	*00102 *00103			*00201 *007			*00301	*00201	*005 *006	*00501* *011	*005010	*055	*00101		*00201	*00201	*010	*00103 *007
DEU	*00101 *01402	*00101	*001	*00201			*00301	*00201	*001 *006	*00801 *011	*00101					*003	*01001 *010	
DKB	*00101 *006			+			*00301	*00201	*00302 *006	*0010201 *00103	*002					*00101	*0020101 *00902	
HO301	*014	*00101 *002	*00101		*010	*00103 *00201	*00102	*004 *010	*003010 *004	*00102	*002		*00103 *00201			*001	*00201	
HID	*01402 *018			*00101			*00201	*00302	*00302 *010	*00102	*01502					*00101	*00201	
HOM-2	*00101 *0090101			+			*00201	*00302	*00302 *005	*00801 *006	*001					*00301	*0010102 *00601	
HOR	*001 *048			+			*00301	*00201	+	*00501		*01301	*00101		*002	*00201		*007 *021
JHAF	*00901 *026			*00101			*002	*00302	*00302	*011	*00501					*010	*001 *01001	
JVM	*007 *00801	*00101	*00301	+			*005	*00302	*001 *00302	*00103 *00801	*00101 *008					*003010	*00101 *009	
KAS011	*00901 *01302			+			*002 *00301	*00201 *00302	*00302 *006	*00103 *005	*008	*01301	*00101		*00201	*00201	*00301	*01001 *019
KAS116	*013 *01501			+			*002	*00302	+	*011	*00501					*010	*0103 *010	
LBUF	*00301 *0090101	+	+	*001			*002	+	*00302 *0090102	*00102 *011	+					+	+	
LUY	*001 *02701			*00101 *00501			*00201 *00301	*00302	*00302 *011	*00801 *00501	*00401					*00601	*001 *010	
MOU	*00207 *00801			*001			*00201 *005	*00302	*00302	*00801	*00101 *00401					*00301	*010 *00601	

(Continued)

TABLE 1 | Continued

ID	Centromeric KIR genes									Telomeric KIR genes								
	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3	2DP1	2DL1	3DP1	2DL4	3DL1	3DS1	2DL5A	2DS3	2DS5	2DS1	2DS4	3DL2
OLGA	*00201 *00902			*00101			*00201 *006	*00302	*00302	*005 *011	*001 *00501	*01301	*00103		*002	*002	*010	*00701
PE117	*00101 *01002			*00101 *00201			*00201 *00301	*00201 *00302	*00901	*00501 *00802	*00401	*01301	*001		*00201 *00201	*00601	*00701 *018	
PF04015	*01402	*00101	*00101 *003						*001	*011	*00501					*010	*00103	
RSH	*0040202 *00901	*00101	+	+	*004		*00201 *009	*00302 *01201	*00304 *008	*0010307 *011	*00501 *017				*006	+	+	
SAVC	*00801 *00202			*00101			*008 *00201	*00302	*00302	*00102 *00802	*00401 *01502				*006010	2*00202 *00301		
SPO010	*00206		+				*00201	*00302	+	*011	*0050101				*010	*001		
T7526	*0090101			*00101			*00201	*00302	*00302	*00501 *00102	*01502	*013	*00101		*00201 *002	*001	*0020105 *0070102	
VAVY	*002 *017			*00101 *00201			*002 *003	*002 *00302	*00302 *006	*011	*00501				*010	*0010302		
WT51	*00103 *036	*00101	+	+	*00201	+	*001 *004	+	+	*00501		*01301	*00101 *00501	+	*002	*002	+	
WDV	*00301 *0090101	*00101	*003	+		*002	*002	*00302 *00901	*00302	*00501		*01301	*00501	*002		*00201	*0070103	
YAR	*00102			+			*002 *003	+	*00302 *006	*0010201 *011	+				+	+		

Results are presented according to the centromeric or telomeric localization of KIR genes on human genome. KIR alleles were named according to the last nomenclature available on the IPD/KIR database (<http://www.ebi.ac.uk/ipd/kir/>). + indicates the presence of a specific KIR gene. ID, sample identification.

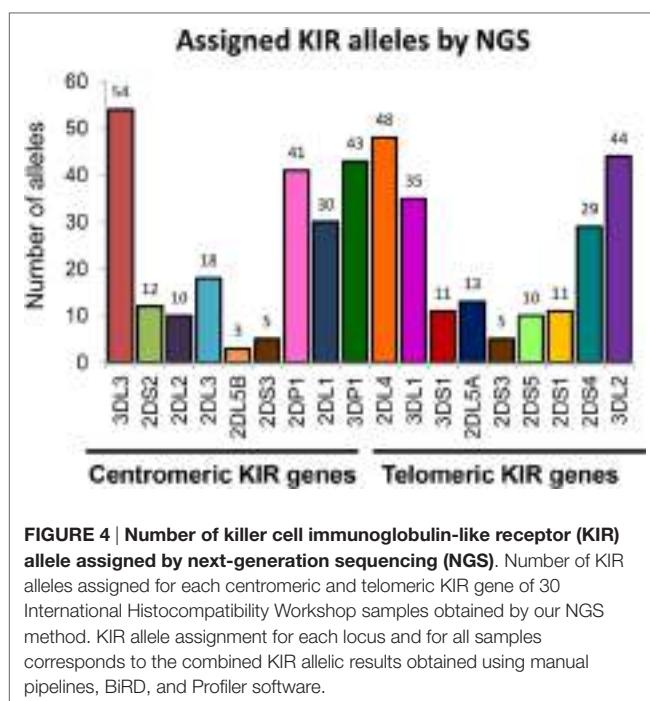


FIGURE 4 | Number of killer cell immunoglobulin-like receptor (KIR) allele assigned by next-generation sequencing (NGS). Number of KIR alleles assigned for each centromeric and telomeric KIR gene of 30 International Histocompatibility Workshop samples obtained by our NGS method. KIR allele assignment for each locus and for all samples corresponds to the combined KIR allelic results obtained using manual pipelines, BiRD, and Profiler software.

and library preparation kits gave conflicting results (data not shown).

Our study evaluated the performance of different algorithms for KIR allele assignment. Reliability of the manual, BiRD pipeline, and Profiler software was tested since neither algorithm alone was able to provide 100% accuracy for all KIR loci. Our results showed that the Profiler software was reliable to assign KIR alleles through the full length of each KIR gene, excluding KIRDL5A variants. In this case, KIR2DL5A and KIR2DL5B sequences were too closed, and Profiler software failed to accurately analyze both sequences. Since all coding, non-coding, and regulatory regions were explored, one could expect that a lot of new KIR alleles will soon be described. Analysis with Profiler consists of two distinct parts. The first part is performed in three steps in a Linux environment: the first step corresponds to the merging of each R1 and R2 issued from the same cluster to each other, each time that a complementarity of at least 10 bases is found, with correction or base calling inconsistencies using a quality value for each nucleotide. There are two interests in this step: longer sequences and lower sequences number were analyzed. The second step corresponds to the phasing of each sequence based on KIR databases using Blast algorithm. Third, the data file from Blast was used to merge all sequences together to construct each allele. In this step, calculation for depth of each position and the number of sequences used for each allele are determined. The second part is done on a Windows environment. A friendly interface presents graphics of all sequences for all studied loci. Assignment of all sequences is done using a database, highlighting all mismatches compared to reference and also differences between KIR alleles selected. Each allele is scored for quality control as per the European Federation for Immunogenetics guideline.

Killer cell immunoglobulin-like receptor alleles of all genes including KIR2DL5A, but excluding the pseudogenes KIR2DP1 and KIR3DP1, were assigned using BiRD algorithm. However, many allelic ambiguities remained when this pipeline was used alone (data not shown). It is likely that this is due to the fact that only coding regions (CDS) were taken into account for allele variation comparison. Analysis of all exon/intron polymorphisms, CNV detection, summary statistics of call accuracy for KIR gene content (presence/absence) and for KIR allele identification needs to be completed. Furthermore, the two pseudogenes KIR2DP1 and KIR3DP1 could be manually added to the BED file describing the gene positions on the genome in order to include them in the analysis pipeline.

Due to the time-consuming nature of manual KIR allele assignment, two different algorithms are needed to ensure the reliability of NGS-based typing methods for the identification of KIR allele polymorphisms.

Until now, KIR genetic population studies have often been restricted to the identification of KIR gene content, or of A and/or B KIR haplotypes (51, 52). Determination of KIR alleles in healthy individuals of a given population may provide a better definition of KIR haplotypes (52) and KIR gene linkage disequilibrium (53) and will considerably increase the IPD/KIR database.

The implementation of our suitable NGS.KIR method will enable analysis of all allelic polymorphism within KIR genes extending to all coding, non-coding, and regulatory regions. A link between KIR allelic polymorphism and the expression level and/or function of the corresponding KIR⁺ NK cells is necessary for all KIR genes as previously established for the expression level of HLA-A and HLA-Cw molecules (54–56). We speculate that KIR allelic polymorphisms may affect not only the distribution and function of these gene products but also the licensing of NK subpopulations as described for HLA class I molecules (57, 58). Deep analysis of KIR⁺ NK cell phenotype and function depending on KIR and HLA class I alleles present is needed to assess the diversity of KIR⁺ NK cell repertoire (21, 59), as well as the specificity of anti-KIR antibodies (30, 60). Overall, the analysis of KIR allelic polymorphisms combined with the autologous HLA class I environment will enable better evaluation of KIR⁺ NK cell functional subpopulations (61). This functional KIR⁺ NK cell repertoire will be better defined by taking into account the nature of KIR alleles present in addition to the autologous HLA class I environment.

Investigation of KIR allelic polymorphism may be of an immunological interest in the context of viral infections such as those related to CMV (62), HIV (63), HCV (64), and of human reproduction (65). In the context of HSCT, inclusion of KIR allele typing in addition to HLA typing may provide a better evaluation of HSC donor's KIR⁺ NK cell repertoire (21, 59, 60, 66, 67). An identification of those with the best antileukemic potential will provide a potential tool to determine an early posttransplant hematopoietic chimerism when donor and recipient have identical KIR genotypes (68) as well as the impact of KIR⁺ NK cell alloreactivity on HSCT outcome (69–73). The functional relevance of typing both KIR and HLA genes at

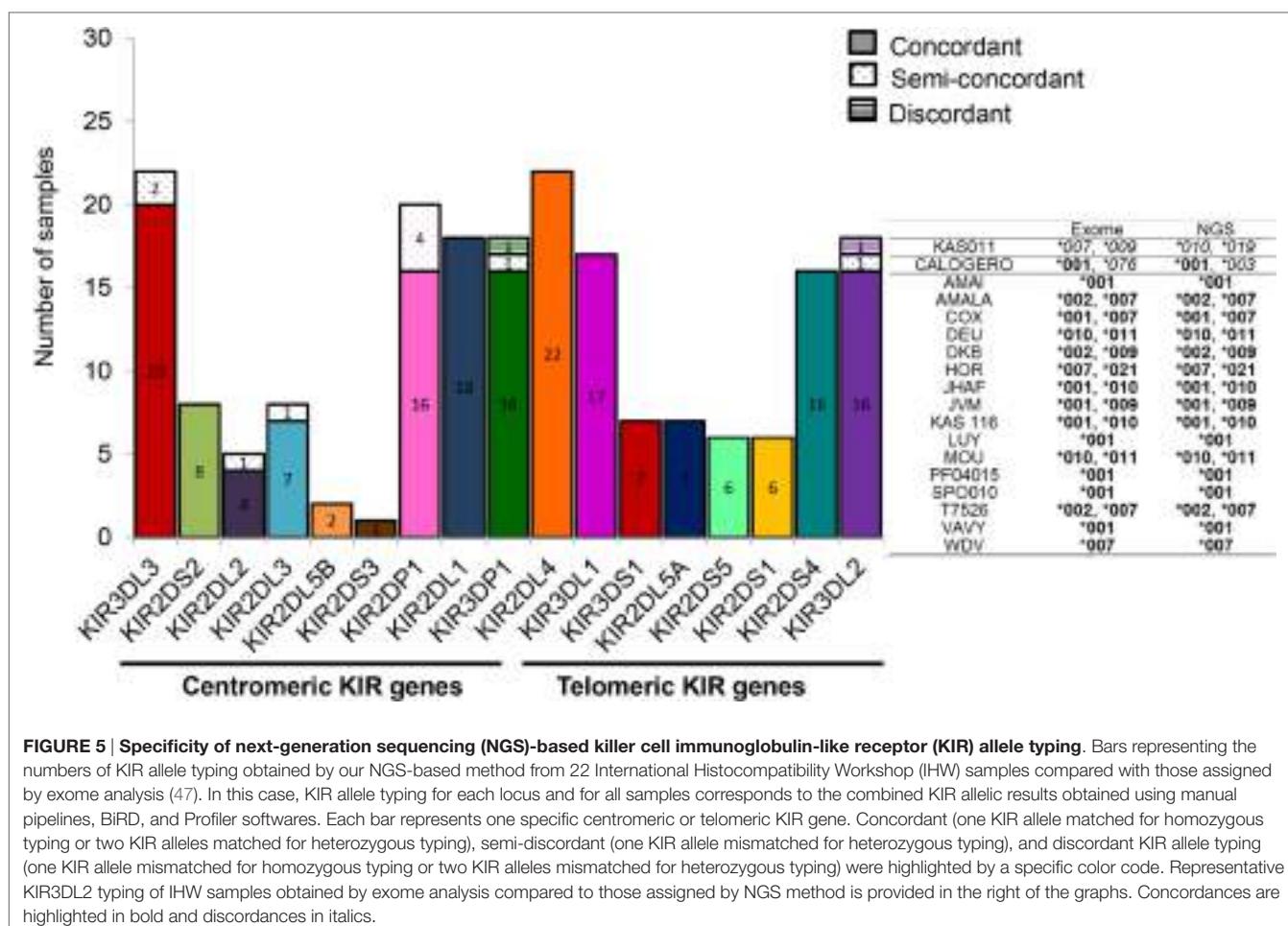


FIGURE 5 | Specificity of next-generation sequencing (NGS)-based killer cell immunoglobulin-like receptor (KIR) allele typing. Bars representing the numbers of KIR allele typing obtained by our NGS-based method from 22 International Histocompatibility Workshop (IHW) samples compared with those assigned by exome analysis (47). In this case, KIR allele typing for each locus and for all samples corresponds to the combined KIR allelic results obtained using manual pipelines, BiRD, and Profiler softwares. Each bar represents one specific centromeric or telomeric KIR gene. Concordant (one KIR allele matched for homozygous typing or two KIR alleles matched for heterozygous typing), semi-discordant (one KIR allele mismatched for heterozygous typing), and discordant KIR allele typing (one KIR allele mismatched for homozygous typing or two KIR alleles mismatched for heterozygous typing) were highlighted by a specific color code. Representative KIR3DL2 typing of IHW samples obtained by exome analysis compared to those assigned by NGS method is provided in the right of the graphs. Concordances are highlighted in bold and discordances in italics.

TABLE 2 | Discordant killer cell immunoglobulin-like receptor (KIR) typing of International Histocompatibility Workshop samples observed between typing obtained by exome capture^a (47) and those obtained by next generation sequencing (NGS) in this study.^b

ID	Centromeric KIR genes						Telomeric KIR genes						
	3DL3		2DL2		2DL3		2DP1		3DP1		3DL2		
	Exome KIR typing ^a	NGS typing ^b	Exome KIR typing ^a	NGS typing ^b	Exome KIR typing ^a	NGS typing ^b	Exome KIR typing ^a	NGS typing ^b	Exome KIR typing ^a	NGS typing ^b	Exome KIR typing ^a	NGS typing ^b	
AMAI							*003, *013		*00301, *004				
CALOGERO	*00207, *017	*00207, *01001								*002, *00302, *00302	*00302, *010	*00101, *076	*00101, *00301
COX					*002	*00201, *007							
DKB										*015	*00302, *006		
KAS011							*002	*002, *00301				*00701, *00902	*01001, *019
LUY							*002, *016		*00201, *00301				
PF04015			*003	*00101, *003									
WT51							*004, *018		*004, *001				
YAR	*00102, *044	*00102											

Discrepancies are shown in italics for each KIR locus concerned. Allelic typing in bold represent concordant alleles. KIR alleles were named according to the last KIR nomenclature. ID, sample identification.

high resolution may help determine their combined effects on outcome of HSCT.

AUTHOR CONTRIBUTIONS

BM performed KIR allele typing by next-generation sequencing, KIR allele assignment, interpretation of data, and wrote the manuscript. NL performed DNA extractions from EBV-B cell lines, KIR genotyping and KIR allele typing by next-generation sequencing and commented on the manuscript. MA upgraded Profiler software integrating a KIR module, analyzed data, and commented on the manuscript. UG developed a pipeline for KIR allele assignment, analyzed data, and commented on the manuscript. CW and GD performed DNA extractions from EBV-B cell lines and commented on the manuscript. EC supervised the development of a pipeline for KIR allele assignment, provided a bioinformatic help on KIR read mapping, and commented on the manuscript. AW provided advices on the library construction setting and commented on the manuscript. CR designed the study, analyzed and interpreted data, commented on the manuscript, and contributed to writing the manuscript. KG designed the study, analyzed data, and wrote the paper. All the authors have approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00547/full#supplementary-material>.

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Impact of the MICA-129Met/Val Dimorphism on NKG2D-Mediated Biological Functions and Disease Risks

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The major histocompatibility complex (MHC) class I chain-related A (*MICA*) is the most polymorphic non-classical MHC class I gene in humans. It encodes a ligand for NKG2D (NK group 2, member D), an activating natural killer (NK) receptor that is expressed mainly on NK cells and CD8⁺ T cells. The single-nucleotide polymorphism (SNP) rs1051792 causing a valine (Val) to methionine (Met) exchange at position 129 of the MICA protein is of specific interest. It separates MICA into isoforms that bind NKG2D with high (Met) and low affinities (Val). Therefore, this SNP has been investigated for associations with infections, autoimmune diseases, and cancer. Here, we systematically review these studies and analyze them in view of new data on the functional consequences of this polymorphism. It has been shown recently that the MICA-129Met variant elicits a stronger NKG2D signaling, resulting in more degranulation and IFN- γ production in NK cells and in a faster costimulation of CD8⁺ T cells than the MICA-129Val variant. However, the MICA-129Met isoform also downregulates NKG2D more efficiently than the MICA-129Val isoform. This downregulation impairs NKG2D-mediated functions at high expression intensities of the MICA-Met variant. These features of the MICA-129Met/Val dimorphism need to be considered when interpreting disease association studies. Particularly, in the field of hematopoietic stem cell transplantation, they help to explain the associations of the SNP with outcome including graft-versus-host disease and relapse of malignancy. Implications for future disease association studies of the MICA-129Met/Val dimorphism are discussed.

Keywords: NK cells, T cells, activating NK receptor, costimulation, single-nucleotide polymorphism, autoimmune diseases, cancer, hematopoietic stem cell transplantation

INTRODUCTION

The major histocompatibility complex (MHC) class I chain-related A (*MICA*) is the most polymorphic non-classical MHC class I gene in humans, and 105 alleles are known encoding for 82 protein variants (<http://www.ebi.ac.uk/imgt/hla/>, release 3.25.0). *MICA* is encoded within the human leukocyte antigen (HLA) complex close to HLA-B (1, 2). The protein structure is similar to classical class I molecules, but *MICA* is not associated with β 2-microglobulin and does not present peptides. *MICA* is constitutively expressed only on a few cell types, including gastrointestinal epithelium, but is induced due to cellular and genotoxic stress (3, 4), malignant transformation, or virus infection

(5, 6). MICA is a ligand for NKG2D (NK group 2, member D), an activating natural killer (NK) receptor encoded by the *KLRK1* gene (7). NKG2D is expressed on most human NK cells, CD8⁺ $\alpha\beta$ T cells, $\gamma\delta$ T cells, iNKT cells, and subsets of effector or memory CD4⁺ T cells (8, 9). On NK cells, NKG2D signaling elicits killing of target cells (10) and secretion of IFN- γ (11). On CD8⁺ $\alpha\beta$ T cells, NKG2D provides a costimulatory signal to activate naïve cytotoxic T lymphocytes (12). NKG2D contributes to the elimination of tumor cells (13) and plays a role in the defense against pathogens (14, 15). In addition to MICA, MICB and the UL16-binding proteins (ULBP) encoded by the retinoic acid early transcript 1 (*RAET1*) family function as ligands for NKG2D. *MICB* is also very polymorphic with 42 alleles encoding 28 protein variants (<http://www.ebi.ac.uk/imgt/hla/>, release 3.25.0). The *RAET1* gene family is localized on chromosome 6 outside the HLA complex and six loci encode functional proteins (16). *RAET1* genes are less polymorphic than *MICA* and *MICB*.

Polymorphisms of *MICA* have been investigated for their role in infections, autoimmune diseases, and cancer (17–21). The single-nucleotide polymorphism (SNP) rs1051792 (G/A) causing a valine (Val) to methionine (Met) exchange at position 129 in the $\alpha 2$ domain of the MICA protein has gained specific interest. It separates *MICA* alleles into two groups (22). MICA isoforms containing a methionine at position 129 bind NKG2D with high affinity, whereas those with a valine bind NKG2D with low affinity. High-affinity alleles include *MICA**001, *002, *007, and *017; among the low-affinity alleles are *MICA**004, *006, *008, *009, and *010 (23). Due to its functional consequences, the MICA-129Met/Val dimorphism has been investigated in several disease association studies. Here, we review these studies in view of new data on the functional consequences of this amino acid variation elicited after binding to NKG2D.

MICA-129Met/Val DISEASE ASSOCIATION STUDIES

In September 2016, we searched Pubmed for MICA-129Met/Val disease association studies using the key words rs1051792, MICA-129, MICA AND polymorphism AND Met, and MICA AND polymorphism AND Val. Moreover, we exchanged polymorphism by SNP, Met by methionine, and Val by valine. We identified 17 publications, in which an association of the MICA-129Met/Val dimorphism with a disease or disease complication has been investigated. One study in Chinese language (24) appeared to be not independent of a larger study published in English (25). Thus, we analyzed 16 independent studies published between 2005 and 2015 (Table S1 in Supplementary Material). Three studies are small with less than 100 cases. All others are of a medium size with more than 100 but less than 1,000 patients included, and most studies used a case-control design.

Eight studies investigated associations with autoimmune diseases, i.e., ankylosing spondylitis (AS) (26), rheumatoid arthritis (RA) (27–29), inflammatory bowel disease (IBD) (25, 30) [including ulcerative colitis (UC) and Crohn's disease], systemic lupus erythematosus (SLE) (28), type I diabetes (31), latent autoimmune diabetes in adults (LADA) (31), and psoriasis (32). In one study, the MICA-129 SNP has not been determined directly. Instead, the

SNP rs1051794 was typed and reported to be in complete linkage disequilibrium with the rs1051792 (27). Five studies reported on malignancies, i.e., nasopharyngeal cancer (33), hepatitis B virus (HBV)-induced hepatocellular carcinoma (HCC) (34), cutaneous malignant melanoma (35), and relapse of malignancy after hematopoietic stem cell transplantation (HSCT) (36, 37). Three studies investigated infections or their complications, i.e., HBV infection and HBV-induced HCC (34), left ventricular systolic dysfunction (LVSD) in chronic Chagas heart disease (38), and ocular toxoplasmosis (39). One study investigated an association of the MICA-129Met/Val dimorphism with recurrent miscarriage (40). The two studies on HSCT (36, 37) investigated besides relapse also other outcomes including graft-versus-host disease (GVHD).

Three studies, on recurrent miscarriage (40), ocular toxoplasmosis (39), and malignant melanoma (35), failed to demonstrate an association with the SNP. Thus, 81% of the studies showed an association at least for a subgroup, e.g., juvenile AS, whereas in all patients with AS, the association was dependent on HLA-B27 (26), or a sub-phenotype, e.g., severe LVSD (38). However, we must assume that other negative association studies have not been published. In seven studies, a MICA-129 allele and the corresponding homozygous genotype were both associated with a disease risk (25, 28, 29, 31, 32, 34, 38). The odds ratio (OR) was then always higher for the genotype than the allele. In six studies, the *Met* allele and/or the *Met/Met* genotype were found to be associated with a risk, including autoimmune diseases [juvenile AS (26), UC (30), SLE (28), and psoriasis (32)], a malignancy (HBV-induced HCC) (34), and a complication of an infection (severe LVSD in chronic Chagas disease) (38). In three studies, the *Val* allele and/or the *Val/Val* genotype has been identified to confer a risk for autoimmune diseases [including RA (27), UC (25), and diabetes (31)] and for nasopharyngeal carcinoma (NPC) (33). Moreover, rheumatoid factor (RF) positivity in RA patients has been associated with the *Val* allele and the *Val/Val* genotype (29). In the studies on HSCT, different outcomes showed different associations. In one study (36), the *Met/Met* genotype was associated with an increased risk of relapse and the *Val/Val* genotype with an increased risk of chronic GVHD. In our recent study (37), the *Met/Met* genotype conferred a risk of acute GVHD, whereas having *Met* alleles reduced the risk to die from acute GVHD. Overall, the *Val* allele was associated with a higher mortality after HSCT (37).

The results of these disease association studies do not allow for a simple unifying interpretation, such as the high-affinity MICA-129Met variant being associated with an activation of the immune system resulting in a lower risk of infections and cancer but higher risk of autoimmunity (Figures 1A,B). Autoimmune diseases are associated with both variants even within the same disease entity. UC, e.g., has been associated with the *Met/Met* genotype in a small study from Spain (30) but with the *Val* allele and *Val/Val* genotype in a larger study from China (25). RA has been associated with the *Val* allele in a study from France and Germany (27), but no association was found in cohorts from Japan (28) and Tunisia (29). Notably, a role of the NKG2D pathway has been reported for the pathogenesis of RA (41) and SLE (42), although this has not been linked to

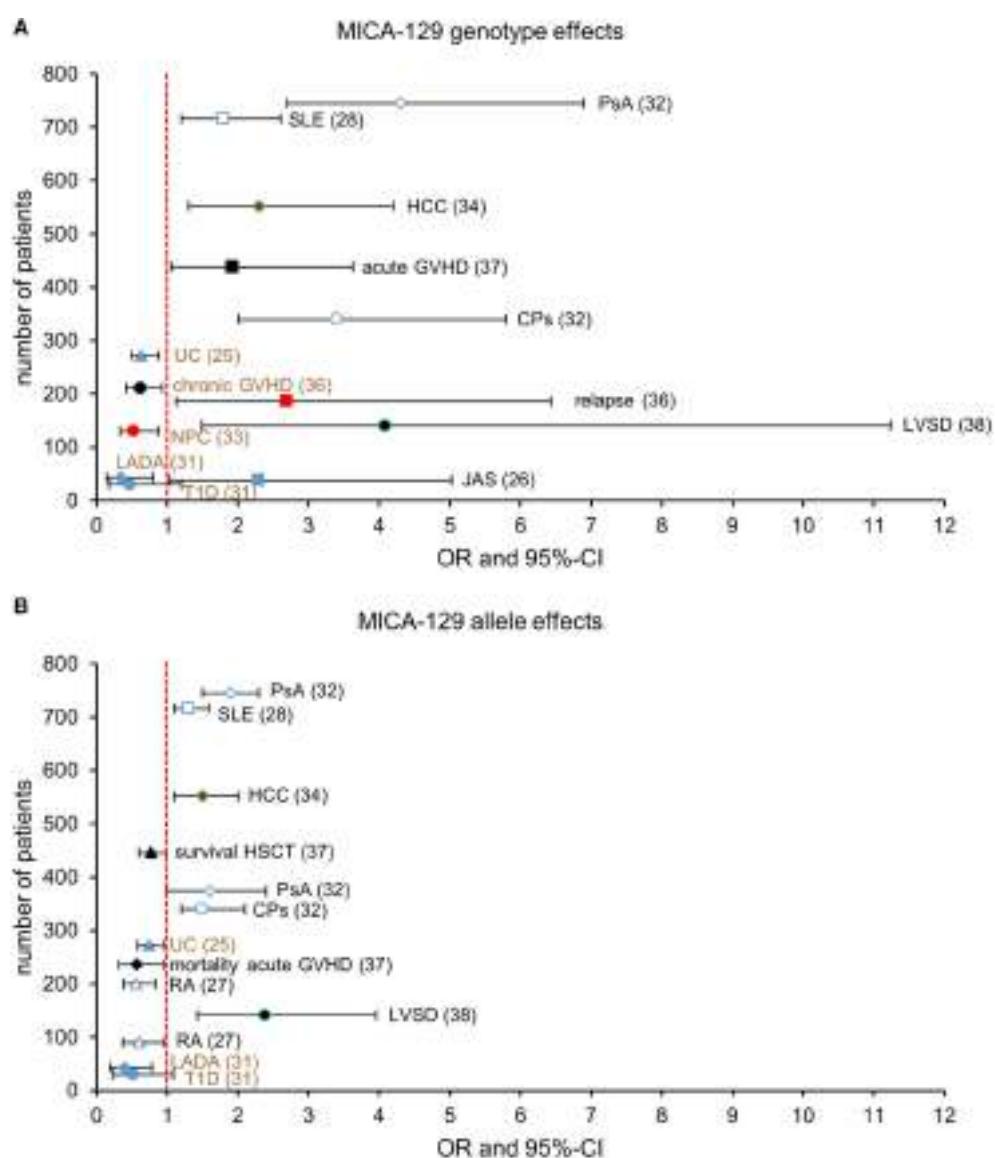


FIGURE 1 | Reported associations of the homozygous MICA-129 genotypes (A) and the MICA-129 alleles (B) with health risks [odds ratio (OR) > 1] or advantages (OR < 1). Shown are ORs with 95% confidence intervals (CI) or hazard ratios reported by Boukouaci et al. (36) and Isernhan et al. (37) (overall survival) in event-time data. The number of patients analyzed in the studies is indicated at the y-axis. Studies reporting on autoimmune diseases are shown by open and closed blue symbols and malignancies by red symbols; studies reporting complications of infections (LVSD, Chargas disease; HCC, hepatitis B virus infection) are shown by green frames, and others are displayed by black symbols. The investigated diseases or complications and the references for the studies are indicated. **(A)** MICA-129Met/Met genotype effects are directly displayed. For studies that reported MICA-129Val/Val genotype effects [chronic GVHD (36), NPC (33), UC (25), RA (27), T1D, and LADA (31), indicated by brown font], the graph displays the corresponding effect of the pooled MICA-129 Met/Met and MICA-129Met/Met genotypes to allow for a direct comparison. **(B)** MICA-129Met allele effects are directly displayed. For studies that reported MICA-129Val allele effects [UC (25), T1D, and LADA (31), indicated by brown font], the graph displays the corresponding effect of the MICA-129Met allele; ORMet = 1/ORVal and 95%-CIMet = (1/CIVal, upper, 1/CIVal, lower). Abbreviations: CPs, cutaneous psoriasis; GVHD, graft-versus-host disease; HCC, hepatocellular carcinoma; JAS, juvenile ankylosing spondylitis; LADA, latent autoimmune diabetes in adults; LVSD, left ventricular systolic dysfunction; NPC, nasopharyngeal carcinoma; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes; UC, ulcerative colitis.

polymorphisms. Juvenile AS has been associated in a small study with the *Met/Met* genotype (26), and a larger sequencing study identified the *MICA*007:01* allele that encodes a methionine at position 129 as a risk allele for AS in both Caucasian and Han Chinese populations (43). However, *MICA*019*, encoding

a valine-129, has been identified as the major risk allele in Han Chinese (43). Malignancies were found to be associated with *Val/Val* genotype in the case of NPC (33) but with the *Met/Met* genotype in the case of relapse after HSCT (36). These different associations could suggest that the observed associations are

random or dependent on the population studied. However, since the MICA-129Met/Val dimorphism is functional, it could also indicate that we need to better understand this function to predict its consequences in the pathophysiology of different diseases in various populations, which might be exposed to different interfering environmental factors. This assumption is supported by genome-wide association studies (GWAS), which have assigned disease risks for NPC (44), HCC (45, 46), cervical cancer (47, 48), and asthma (49) or advantages, such as HIV long-term non-progression (50) to the *MICA* gene region in an unbiased manner.

FUNCTIONAL CONSEQUENCES OF THE MICA-129Met/Val DIMORPHISM

It has been shown by Steinle and colleagues that MICA-129Met isoforms bind NKG2D with high affinity in contrast to MICA-129Val isoforms that bind with low affinity (22). Yoshida and colleagues combined the MICA-129Met variant with the A9 variant of a microsatellite polymorphism in the transmembrane (TM) region and the MICA-129Val variant with the A5-TM variant in GST-fusion proteins (28). NK92MI cells showed a reduced NKG2D expression and killed K562 cells less efficiently when exposed to the MICA-129Met-A9-TM variant, but IFN- γ production was increased (28). We recently studied the consequences of binding of the two MICA-129 variants to NKG2D on primary NK cells and CD8 $^{+}$ T cells using cell lines transfected with expression constructs and recombinant Fc-fusion proteins differing only in amino acid 129 (37, 51). The recombinant MICA-129Met variant stimulated a stronger phosphorylation of SRC family kinases in NK cells than the MICA-129Val variant. Subsequently, the MICA-129Met ligand triggered more degranulation and IFN- γ production than the MICA-129Val ligand (**Figure 2A**). We then exposed NK cells to target cells expressing different amounts of the MICA-129 variants. The extent of degranulation and IFN- γ secretion correlated positively with the MICA expression intensity on the target cells but only for the MICA-129Val isoform. The expression intensity of the MICA-129Met isoform, in contrast, had either none or even a negative effect on the extent of degranulation, target cell killing, and IFN- γ release (37). On CD8 $^{+}$ T cells, the MICA-129Met isoform induced an earlier costimulatory activation than the MICA-129Val isoform (**Figure 2B**). Importantly, the MICA-129Met ligand induced also a stronger downregulation of NKG2D on both NK and CD8 $^{+}$ T cells than the MICA-129Val ligand. This downregulation of NKG2D impaired the capability of NK and CD8 $^{+}$ T cells to receive signals via NKG2D (37). Thus, MICA-129Met ligands, which elicit strong NKG2D responses, stimulate in parallel a robust negative feedback signal by downregulation of NKG2D that limits the initially stronger effects of MICA-129Met ligands. These data show that the biological effect of the MICA-129Met/Val dimorphism changes with the MICA expression intensity. Variant MICA-129Met triggers more NKG2D signals at low expression intensities, whereas variant MICA-129Val elicits more NKG2D effects at high expression, at which the MICA-129Met variant already downregulates NKG2D leading to impaired function. Thus, the biological effect

of the SNP can hardly be predicted without information on the expression intensity of MICA.

It is known that expression intensities vary for certain *MICA* alleles (52, 53). The G allele of the SNP at -1878 (rs2596542) in the promoter region of the *MICA* gene region, e.g., was found to have a higher transcriptional activity (54). Biological effects of the MICA-129Met/Val dimorphism can be expected to be modified by polymorphisms affecting *MICA* gene expression. We have investigated whether the Met/Val dimorphism itself affects MICA expression. In transfected cells, more of the MICA-129Met variant was retained in intracellular compartments (51). A similar alteration of the intracellular transport has been described for *MICA*-A5.1 variants (55). Thus, the combination of polymorphisms affecting transcription and intracellular transport of MICA could modify the effect of the Met/Val dimorphism.

Another important aspect of MICA is the generation of soluble MICA (sMICA) by proteolytic shedding. sMICA can induce NKG2D downregulation (56, 57) resulting in tumor immune escape (58). Some *MICA* polymorphisms have been reported to affect the amounts of sMICA in sera of patients including the SNP at -1878 (rs2596542) in the promoter region (34, 45, 59) that affects transcription (54), a microsatellite in exon 5 encoding the TM region (60, 61), and the MICA-129Met/Val dimorphism. In patients with UC, the *MICA*-129Val/Val genotype was associated with higher sMICA serum levels (25), and the *MICA*-129Val allele was also associated with higher sMICA serum levels in HBV patients and controls (34). In transfected cells, we found that the MICA-129Met isoform was more susceptible to shedding than the MICA-129Val isoform (51). However, due to the intracellular retention of the MICA-129Met variant (51), less sMICA might appear in sera (25, 34). Notably, intracellular retention and preferred shedding both appear to limit the expression of the high-affinity MICA-129Met isoform at the plasma membrane.

MICA-129Met/Val DISEASE ASSOCIATIONS IN VIEW OF BIOLOGICAL FUNCTIONS

Recent data on the MICA-129Met/Val variation demonstrate the complexity of the functional consequences of this exchange of a single amino acid (37, 51). There are several layers of this complexity, which are as follows: (1) the function of the variant is not constant but dynamic (37); it depends on the MICA expression intensity, and the direction of the biological effect can invert for the MICA-129Met variant at higher expression. (2) Epistatic effects must be expected for this SNP as polymorphisms affecting the expression of MICA will modify the functional effects of the MICA-129Met/Val isoforms. Moreover, the expression intensity of NKG2D can be modified by SNPs in the *KLRK1* gene (62) and those might interact with the *MICA*-129 variants. Other genes within the NKG2D pathway including other ligands might also show epistatic effects (63). (3) MICA can target NKG2D on several cell types, and biological effects on different cell types might be synergistic or antagonistic. An activation of NK cells and a costimulation of CD8 $^{+}$ T cells both can promote antitumor

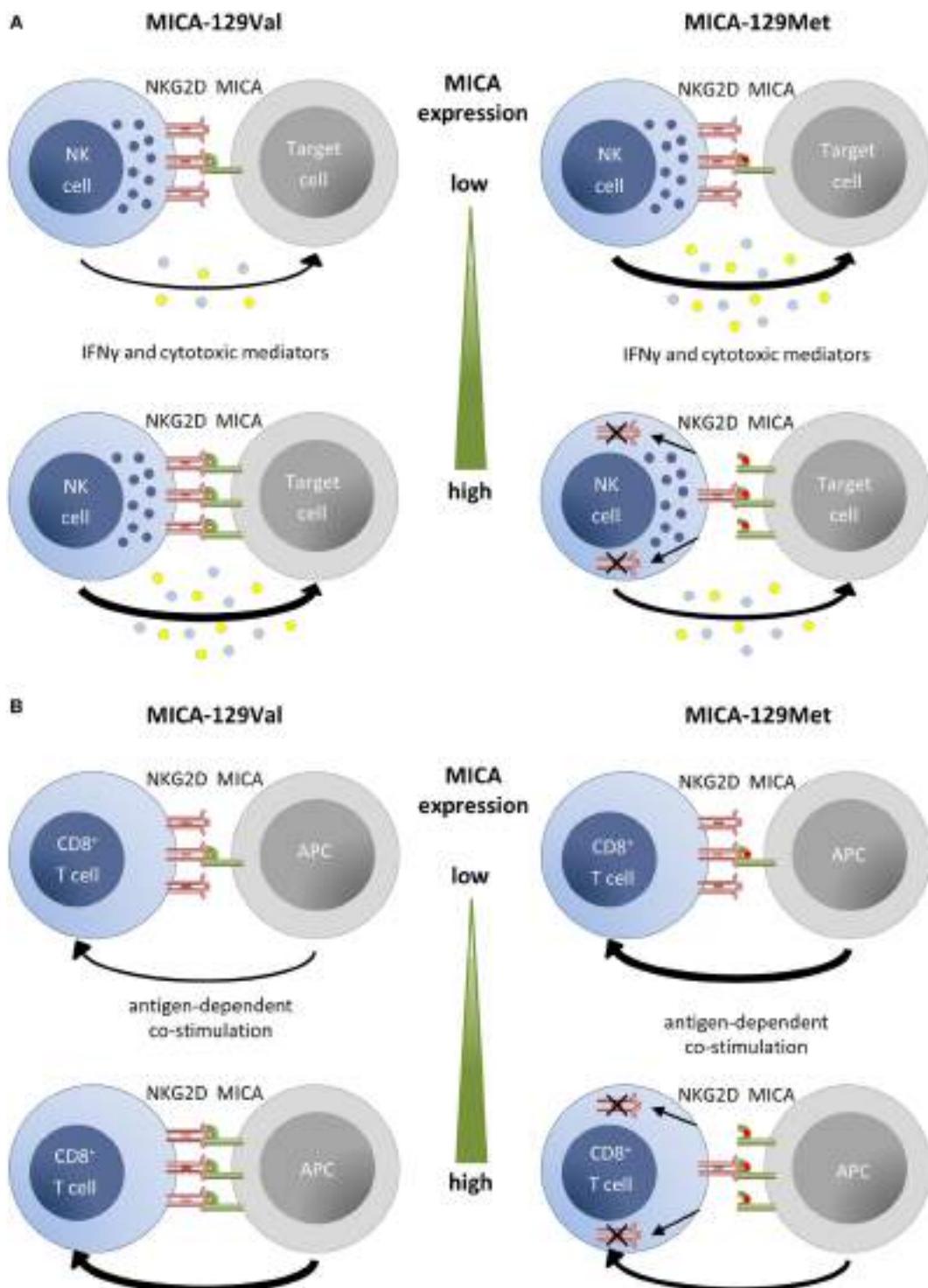


FIGURE 2 | Summary of functional effects of MICA-129 variants depending on expression intensity. (A) For target cells expressing the MICA-129Val variant, the degree of natural killer (NK) cell cytotoxicity and IFN- γ production increases steadily with the MICA expression intensity. Augmented expression of the high-affinity MICA-129Met isoform, in contrast, has none or even a negative effect on these NK cell functions due to a rapid downregulation of NKG2D on NK cells. **(B)** Antigen-dependent costimulation of CD8 $^{+}$ T cells with the MICA-129Met variant allows for an earlier antigen-dependent activation than costimulation with the MICA-129Val variant. However, the downregulation of NKG2D in response to MICA-129Met ligands impairs any subsequent NKG2D-dependent costimulation and T cell activation. The downregulation of NKG2D on CD8 $^{+}$ T cells is augmented with MICA-129Met expression intensity. The figure is reproduced from Isernhagen et al. (37).

immunity. By contrast, a strong activation of NK cells might polarize an immune response to a Th₁ reaction and reduce the risk to develop a Th₂-mediated autoimmune disease. (4) Additional factors, such as sMICA or anti-MICA antibodies (36) that might neutralize sMICA, have been shown to be functionally important and have been determined in some of the disease association studies (25, 34, 36).

Currently, we mostly have not sufficient clinical and biological information to interpret the MICA-129Met/Val disease association studies in view of the complex function of this polymorphism. However, the two HSCT studies do provide more information and illustrate the clinical effects of the MICA-129Met/Val dimorphism as explained previously in detail (37). In our study (37), the homozygous carriers of *Met* alleles had an increased risk to experience acute GVHD, possibly due to immediate strong effects of MICA-129Met variants on NKG2D signaling. Having at least one *Met* allele reduced the risk to die from acute GVHD likely due to a rapid downregulation of NKG2D on alloreactive CD8⁺ T cells mediated by engagement of a high-affinity MICA-129Met variant. Carrying a *MICA-129Met* allele increased in consequence the chance of survival in all patients and in patients receiving a *MICA-129*-matched graft (37). Boukouaci and colleagues reported an increased risk of chronic GVHD for recipients with the *Val/Val* genotype, whereas the *Met/Met* genotype was associated with the risk of relapse (36). Sustained NKG2D-mediated activation of alloreactive CD8⁺ T cells would be expected if only MICA-129Val variants are present that fail to efficiently downregulate NKG2D, and this could increase the risk of chronic GVHD but reduce the risk of relapse. Thus, the different risk associations reported in the two studies are not arguing against the relevance of the MICA-129 dimorphism for the outcome of HSCT. The principal relevance of the NKG2D pathway for HSCT is further emphasized by studies showing an effect of the genotype of the NKG2D ligand *RAET1L* (64) and NKG2D itself (65) on the survival of patients. Moreover, matching for *MICA* alleles (66–69) and specifically for the *MICA-129* polymorphism (70) is beneficial in HSCT. The huge effect of *MICA-129* matching appears hardly explainable solely by the avoidance of a potential minor histocompatibility antigen. A “tuning” of the threshold of NKG2D signaling toward the affinity of NKG2D ligands present in an individual (52) and disturbance of this balance by mismatching could be considered as an alternative explanation.

Despite the functional relevance of the *MICA-129* SNP, it cannot be excluded that some of the associations reported are random or caused by linkage disequilibrium with classical HLA genes. The association of MICA-129 with psoriasis (32) has been disproven in large GWAS cohorts (71). However, associations with NPC (33) and HCC (34) are supported by GWAS data pointing to the *MICA* gene region (44–46).

CONCLUSION

Information on functional consequences of a polymorphism is indispensable for understanding disease associations. The variation in the disease associated allele or genotype of *MICA-129* in the published studies must not indicate random associations. For *MICA-129*, the biological function can change with expression

intensity, epistatic interactions can be expected, the effect on different lymphocytes can vary, and modifying factors, such as sMICA, have to be considered. Notably, as expected for a functional SNP with a minor allele (*MICA-129Met*) frequency ranging from 48% in Africans to 30% in Asians (72), and being even the major allele reported in one of the analyzed studies (26), both alleles appear to confer advantages and disadvantages in specific situations suggesting balancing evolution of the *MICA* alleles. Since the MICA-129 dimorphism is considered as decisive for distinguishing low- and high-affinity variants (22), the frequency of alleles encoding high-affinity MICA variants is expected to match the frequency of the MICA-129Met variant. However, other *MICA* polymorphisms and their interaction need to be studied further (73).

In future studies, the MICA-129Met/Val dimorphism should be analyzed in larger cohorts. Detailed clinical information would help to understand why associations might differ in cohorts. Additional biological information should be obtained in parallel to genetic data. Most important would be data on MICA expression intensities in relevant tissues at relevant time points. Due to the complexity of MICA-129Met/Val effects, this polymorphism is unlikely to become a simple genetic biomarker for prediction of disease risks. However, it still may provide highly important information. We found that *Val/Val* genotype carriers undergoing HSCT specifically profited from a treatment with antithymocyte globulin to deplete T cells (37). This might be explained by a lack of a high-affinity MICA variant that efficiently downregulates NKG2D on alloreactive donor CD8⁺ T cells. Moreover, the *MICA-129* dimorphism might be relevant when considering therapies aiming at upregulation of MICA on tumor cells to sensitize them for NK cells (74, 75). Increasing the expression of MICA-129Met variants could result in opposite effects than intended.

AUTHOR CONTRIBUTIONS

RD searched the literature; RD and AI interpreted the functional data; RD and DM interpreted the genetic association data; RD drafted the manuscript; AI, DM, and HB commented the draft; and all the authors approved the final version.

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SUPPLEMENTARY MATERIAL

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Disarming Cellular Alarm Systems—Manipulation of Stress-Induced NKG2D Ligands by Human Herpesviruses

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The coevolution of viruses and their hosts led to the repeated emergence of cellular alert signals and viral strategies to counteract them. The herpesvirus family of viruses displays the most sophisticated repertoire of immune escape mechanisms enabling infected cells to evade immune recognition and thereby maintain infection. The herpesvirus family consists of nine viruses that are capable of infecting humans: herpes simplex virus 1 and 2 (HSV-1, HSV-2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), roseoloviruses (HHV-6A, HHV-6B, and HHV-7), and Kaposi's-sarcoma-associated herpesvirus (KSHV). Most of these viruses are highly prevalent and infect a vast majority of the human population worldwide. Notably, research over the past 15 years has revealed that cellular ligands for the activating receptor natural-killer group 2, member D (NKG2D)—which is primarily expressed on natural killer (NK) cells—are common targets suppressed during viral infection, i.e., their surface expression is reduced in virtually all lytic herpesvirus infections by diverse mechanisms. Here, we review the viral mechanisms by which all herpesviruses known to date downmodulate the expression of the NKG2D ligands. Also, in light of recent findings, we speculate about the importance of the emergence of eight different NKG2D ligands in humans and further allelic diversification during host and virus coevolution.

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HERPESVIRUSES—CONSTANT COMPANIONS DURING HUMAN LIFE AND EVOLUTION

Herpesviruses have accompanied humankind since the dawn of evolution. Herpesvirus infections date back at least 6 million years, even before evolutionary split between hominids and chimpanzees (1). From that time on, viral strategies to ensure survival and dissemination coevolved together with the immune system that continuously developed new measures to clear viral infections.

To date, nine different herpesviruses capable of infecting humans have been identified: HSV-1, HSV-2, varicella zoster virus (VZV), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), HHV-6A, HHV-6B, HHV-7, and Kaposi's-sarcoma-associated herpesvirus (KSHV) (2).

Despite their different life cycle and growth properties, cellular tropisms and although they cause different diseases, all herpesviruses share common features. They are enveloped and contain a linear, double-stranded DNA genome, ranging from 125 kb (for VZV) to 235 kb (for HCMV) (2).

Furthermore, all have the ability not only to infect lytically but also to establish life-long infection in their host, a status called latency, which is a dormant infection lacking pathology and viral replication (2, 3).

Most herpesviruses are widely spread in human populations. Serological tests reveal that HSV-1, VZV, EBV, HHV-6, and HHV-7 have the highest prevalence of the herpesvirus family and infect about 90% of the adult population (4–10). Notably, the prevalence of herpesviruses varies geographically and is influenced by socioeconomic status (2, 11, 12). HCMV prevalence can therefore vary between 50 and 100% dependent on the population studied (13). Some herpesviruses reactivate symptomatically and frequently in healthy individuals for as yet unknown reasons, while others only cause symptomatic reactivation in immunodeficient patients (3). However, research over the past few years revealed that all herpesviruses use common strategies during primary infection, reactivation, and sometimes even during latency, in order to evade the immune surveillance during the different phases of herpesvirus life cycle. The interactions between adaptive immunity and herpesviruses are described elsewhere (14–17). In this review, we will focus on the interaction of herpesviruses and natural-killer group 2, member D (NKG2D)-expressing immune cells. The human-activating receptor NKG2D is expressed on all natural killer (NK) cells as well as on most T cells including $\gamma\delta$ T cells and NK T cells (18). Its importance was shown for tumor surveillance (19) and inflammatory diseases (20). The significance of NK cells in herpesvirus immune surveillance becomes clear by looking at NK cell-deficient individuals who suffer from recurrent, severe, potentially life-threatening herpesvirus infections (21, 22).

GENETICS OF NKG2D LIGANDS

In the course of human evolution, eight different, functional ligands for the NKG2D receptor emerged: MHC class I polypeptide-related sequence A and B (MICA and MICB, respectively) and the unique long 16 binding protein 1–6 (ULBP1–6) (23). Also known as “stress-induced ligands,” they are barely found on healthy cells in order to avoid auto-reactivity toward normal tissues. These ligands, however, are upregulated and expressed on the cell surface following various stresses including genotoxic stress, oncogene activation or hypoxia that are commonly seen in tumorigenesis, or following viral infection (24, 25).

All NKG2D ligands belong to the MHC class I-like protein family. ULBP family members have an $\alpha 1/\alpha 2$ domain structure, whereas the MIC proteins possess an $\alpha 1/\alpha 2/\alpha 3$ domain structure (26). Interestingly, classical MHC class I proteins serve mainly as inhibitory ligands for NK cells, whereas the NKG2D ligands activate NK cells (27, 28).

Up until now, 16 different allelic variants were identified for the 6 members of the ULBP family (29). More than 100 different MICA alleles and more than 40 MICB alleles were identified to date; a finding that demonstrates the striking superior evolutionary plasticity of the MIC family [[http://hla.alleles.org/alleles/classo.html](http://hla.alleles.org/alleles/classo.html;); (30)] (Figure 1). The reason behind this enormous diversity of the MIC family is still unknown. MIC genes lack hypervariable regions; point mutations and genetic shuffles occur over all

three domains (31). Comparing amino acid sequence homology, MICA and MICB are very similar (about 85% identity), whereas the similarity to ULBP family proteins is comparatively low (only about 20–25% identity between MIC and ULBP proteins). ULBP family members share about 60% amino acid sequence identity with each other (32–34). Interestingly, MICA, MICB, ULBP4, and ULBP5 contain a transmembrane domain and a cytoplasmic tail, whereas ULBP1, ULBP2, ULBP3, ULBP6, and one particular allelic variant of MICA (allele *008) are glycosylphosphatidylinositol (GPI) anchored (30).

Only recently, post-transcriptional cellular mechanisms that control stress-induced ligand expression by RNA-binding proteins (35–37) and microRNAs (miRNAs) (38, 39) began to be unraveled; however, the regulatory circuits and expression patterns in normal cells remain incompletely understood. By contrast, much information was gathered about the suppression of NKG2D ligands during herpesvirus infection, emphasizing the importance of the receptor NKG2D for anti-viral immunity.

ALL HERPESVIRUSES SUPPRESS EXPRESSION OF STRESS-INDUCED LIGANDS DURING INFECTION

HSV-1 and -2—HHV-1 and -2

Herpes simplex virus 1 and 2 can cause orofacial and genital infections in otherwise healthy individuals with a competent immune system (40). Reactivation is believed to be triggered by stress, sunlight, fever, or skin traumas, e.g., caused by surgery (40, 41).

The effects of HSV-1 infection on the expression of NKG2D ligands were first studied by Schepis et al. [(42); Figure 1; Table 1]. Both ULBP2 and MICA surface expression levels were found to be decreased following infection with HSV-1 strain F. Concurrent with a loss of surface expression, MICA messenger RNA (mRNA) levels were decreased. Since MICA downregulation was abrogated by inhibiting the viral DNA polymerase, the authors concluded that a late viral gene is responsible for the reduction of MICA expression. However, in this study, none of the cell lines tested expressed other ligands besides MICA and ULBP2. Another study, performed by Campbell et al., confirmed the decrease in MICA and ULBP2, but could additionally show a downregulation of ULBP1 and ULBP3 (43). Interestingly, MICA, ULBP2, and ULBP3 were shown to be reduced at the overall protein level, whereas ULBP1 was retained intracellularly, proving that different mechanisms act on these ligands (43).

In a subsequent study, Enk et al. added some mechanistic detail about the regulation of ULBP2 and ULBP3 (44). They reported that the viral miRNA miR-H8 interferes with the generation of GPI-anchored proteins by targeting PIGT, a key protein in the GPI-anchoring process (45, 46). Consequently, both ULBP2 and ULBP3 levels were reduced in miR-H8 overexpressing cells. Interestingly, ULBP1 is also GPI anchored but not affected by this pathway, explaining the necessity for another mechanism of downregulation—intracellular retention. However, since both MICA (except the allele MICA*008) and MICB are transmembrane proteins and were not affected by the miR-H8 overexpression,

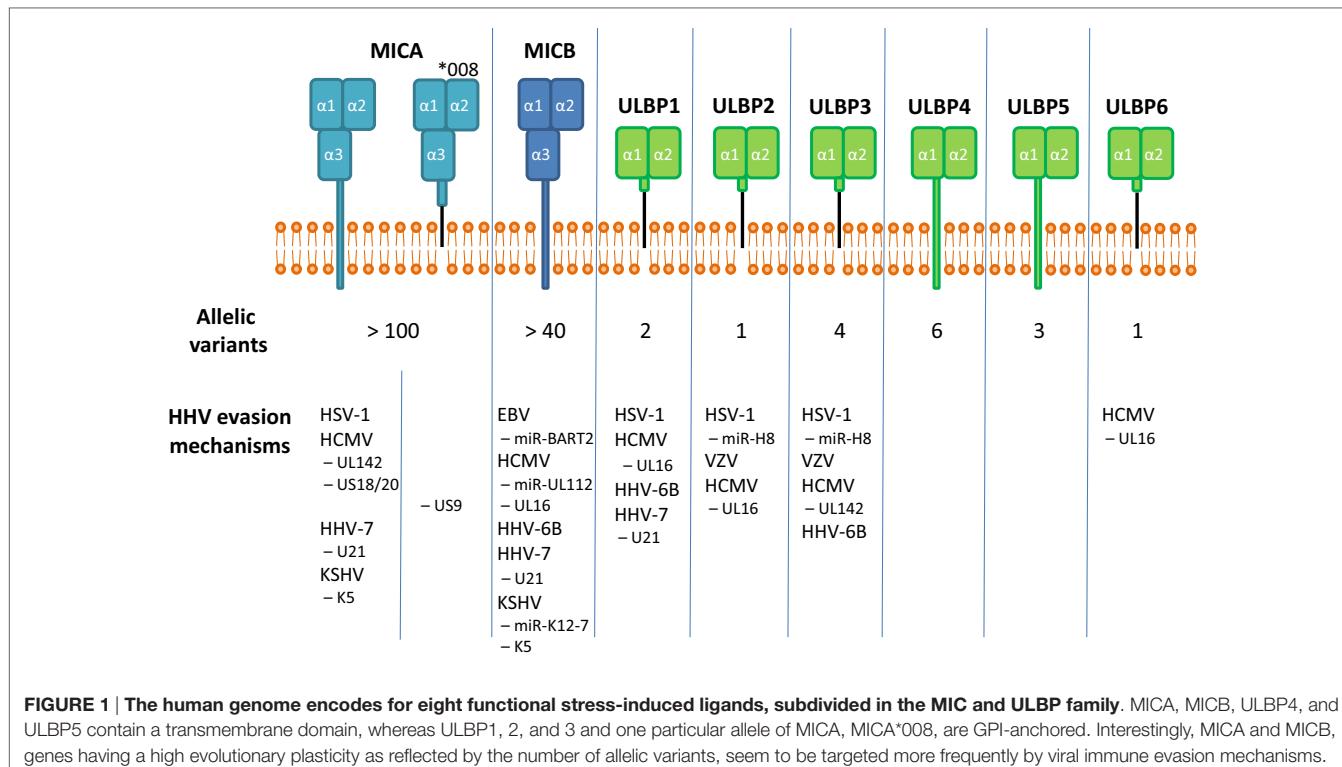


FIGURE 1 | The human genome encodes for eight functional stress-induced ligands, subdivided in the MIC and ULBP family. MICA, MICB, ULBP4, and ULBP5 contain a transmembrane domain, whereas ULBP1, 2, and 3 and one particular allele of MICA, MICA*008, are GPI-anchored. Interestingly, MICA and MICB, genes having a high evolutionary plasticity as reflected by the number of allelic variants, seem to be targeted more frequently by viral immune evasion mechanisms.

the viral mechanism responsible for these downregulations are still unknown.

VZV—HHV-3

Varicella zoster virus is the causative agent of varicella (chickenpox) in primary infection (47). In the elderly or immunosuppressed patients, VZV can reactivate and cause herpes zoster (shingles), which is characterized by painful skin lesions as well as neurological and ocular disorders (47, 48).

By infecting retinal epithelial cells with a clinical VZV strain, Campbell et al. revealed a down-modulation of the surface expression of the NKG2D ligands ULBP2 and ULBP3 [(43); Figure 1; Table 1]. By contrast, MICA surface expression increased during the course of VZV infection; ULBP1 and MICB were not expressed in the studied cells. Due to the overall reduction of surface expression of NKG2D ligands, reduced activation of NK cells in the presence of VZV-infected cells as compared to mock infected cells was observed. Interestingly, the total protein levels of ULBP2 and ULBP3 were not reduced in infected cells (43), indicating intracellular retention of these ligands by a yet unknown viral factor.

EBV—HHV-4

Epstein–Barr virus is usually acquired asymptotically in childhood (2, 49). Infection during adolescence can lead to infectious mononucleosis (in about 50% of primary infections), a weakening and sometimes painful but self-limiting disease associated with the occurrence of atypical lymphocytes in the blood stream (2, 49). Reactivation can occur in immunocompromised individuals and is, among others, linked not only to lympho-proliferative

diseases such as Burkitt's and Hodgkin's lymphoma but also to nasopharyngeal carcinoma (50, 51).

A sensitization of EBV-infected cells switching from latent to lytic infection to NK cell killing was reported by Pappworth et al. (52). They showed the induction of ULBP1 following this switch in a Burkitt's lymphoma-derived cell line, whereas all other NKG2D ligands were absent from the cell surface. Later on, an overexpression study performed by Nachmani et al. revealed that the latency-associated viral miRNA miR-BART2-5p is capable of binding MICB mRNA and suppressing its translation [(53); Figure 1; Table 1]. Interestingly, they showed that the binding site in the MICA mRNA sequence was mutated in such a way that prevented the miRNA from suppressing MICA as well.

Remarkably, to the best of our knowledge, there are no immune evasion mechanisms regarding NKG2D ligands during lytic EBV infection described to date. This phenomenon might be explained by a study published by Song et al. (54). They showed that EBV-transformed B cells produce and release the tryptophan-derived metabolite L-kyurenine that downmodulates NKG2D receptor expression on bystander NK cells. Therefore, the suppression of NKG2D ligands on infected cells might be of little importance if the effector cells themselves are effectively disarmed.

HCMV—HHV-5

While being a harmless pathogen for immunocompetent individuals, HCMV constitutes a major risk for the elderly, patients after organ transplantation and AIDS patients (55). Additionally, primary infection in pregnant women can cause miscarriage, stillbirth, or developmental retardation of the child (55). HCMV possesses the largest genome of all HHVs of about 235 kb (2).

TABLE 1 | Overview of known suppression mechanisms for NKG2D ligands by HHVs.

	Viral effector	Ligand	Mechanism	Reference
HSV-1	?	MICA	?	(42, 43)
	?	ULBP1	Intracellular retention	(43)
	miR-H8	ULBP2	Interferes with protein maturation	(42–44)
	miR-H8	ULBP3	Interferes with protein maturation	(43, 44)
HSV-2	?	?	?	?
	?	ULBP2	Intracellular retention	(43)
Varicella zoster virus	?	ULBP3	Intracellular retention	
Epstein–Barr virus	miR-BART2-5p	MICB	Translational repression	(53)
	miR-UL112	MICB	Translation repression	(56)
Human cytomegalovirus	UL16	MICB	Intracellular retention	(60)
		ULBP1	Intracellular retention	(32, 57)
		ULBP2	Intracellular retention	(32, 57)
		ULBP6	Intracellular retention	(61)
	UL142	MICA	Intracellular retention	(62)
		ULBP3	Intracellular retention	(63)
	US18/US20	MICA	Lysosomal degradation	(64)
	US9	MICA*008	Proteasomal degradation	(68)
		?	?	?
		MICB	Proteasomal degradation	(71)
HHV-6A	?	ULBP1	Proteasomal degradation	
	?	ULBP3	Proteasomal degradation	
HHV-6B	?	ULBP1	Proteasomal degradation	
	?	ULBP3	Proteasomal degradation	
HHV-7	U21	MICA	?	(72)
		MICB	?	
		ULBP1	Lysosomal degradation	
		MICA	Ubiquitylation/intracellular retention	(75)
Kaposi's-sarcoma-associated herpesvirus	K5	MICB	Ubiquitylation/intracellular retention	(75)
		MICB	Translational repression	(53)

?, no published data available.

Therefore, it might not be surprising that HCMV has the greatest number of viral mechanisms dedicated to the immune evasion by manipulating NKG2D ligands described to date.

The first viral miRNA identified to target immune molecules in general and NKG2D ligands in particular was miR-UL112, discovered by Stern-Ginossar et al. [(56); **Figure 1; Table 1**]. By binding to the 3'-UTR of the MICB mRNA, it represses translation, and surface levels are rapidly reduced, leading to decreased NK cell activation. UL16 was the first HCMV viral protein found to bind and retain ULBP1, ULBP2, ULBP6, and MICB intracellularly (“ULBPs” were named for being UL16-binding proteins) (32, 57–61). Later, UL142 was shown to sequester both MICA and ULBP3 intracellularly, they colocalized with markers of the *cis*-Golgi apparatus inside infected cells (62, 63).

Fielding et al. showed that the viral proteins US18 and US20 are capable of both independently and synergistically downregulating MICA expression by targeting it for lysosomal degradation (64).

Notably, the GPI-anchored allele MICA*008 was not found to be targeted by the abovementioned viral mechanisms and was therefore considered as HCMV-resistant escape variant. Since the MICA*008 allele is a highly prevalent in human populations worldwide, the hypothesis was formed that its prevalence is the result of viral selective pressure (65–67). However, Seidel et al. showed that this supposed escape variant is specifically targeted by the HCMV protein US9 during its maturation process, prior to its egress from the ER, instead forcing MICA*008 to proteasomal degradation (68).

Roseoloviruses—HHV-6A, HHV-6B, and HHV-7

HHV-6A, -6B, and -7 have long been neglected in research. Only in the past years have these viruses gained attention since it became obvious that they not only cause a common children’s disease (roseola infantum) but might also be involved in severe illnesses, especially in immuno-incompetent individuals like neuroinflammatory diseases (HHV-6A), transplant rejection, myocarditis (HHV-6B), or encephalitis (HHV-6A, -6B, and HHV-7) (69, 70). For this reason, immunomodulatory features of these viruses were studied only relatively recently.

We showed that HHV-6B strain Z29 is capable of suppressing the surface expression of the NKG2D ligands ULBP1, ULBP3, and MICB, but not MICA or ULBP2 [(71); **Figure 1; Table 1**]. This was true both in primary T cells and in T cell lines. As a cellular response to the viral infection, mRNA levels of all stress-induced ligands rise following infection; however, the virus suppresses the three abovementioned ligands on protein level and degrades them rapidly in a proteasome-dependent pathway shortly after the start of infection. Also, we showed that the degradation of the three ligands is mediated by at least two different viral proteins.

As for HHV-7, Schneider et al. showed that U21, which was previously shown to target HLA class I for lysosomal degradation, also causes lysosomal degradation of ULBP1 resulting in a mild downregulation. Additionally, they observed a major downregulation of MICA and MICB (72). These findings were

established using the overexpression of the viral protein U21. However, the exact mechanism for MIC proteins degradation remained unclear. Probably, U21 interferes with proper protein glycosylation rendering the MIC proteins unstable and causing them to be targeted for cellular degradation. Due to the mild loss of ULBP1, this degradation was proposed to be the result of a “side-effect” of U21-mediated HLA class I degradation, since these related proteins were targeted to lysosomal degradation with higher affinity and to a greater extent.

However, since the study was limited to overexpression of a single gene and no studies were conducted using an actual infection model, it is possible that additional stress-induced ligands are affected by HHV-7 or that additional mechanisms targeting the same ligands exist.

KSHV—HHV-8

Kaposi's-sarcoma-associated herpesvirus is the human herpesvirus with the lowest seroprevalence in the Western world with only about 1–3% of individuals infected (73). Still, this virus is a significant cause of cancer, primarily in AIDS patients, whereas immunocompetent individuals do not experience KSHV reactivation (73, 74). In developing countries, seroprevalence is substantially higher (73).

During lytic infection, KSHV evades NK cell recognition by expressing the viral E3 ligase K5. Thomas et al. showed that K5 modifies lysine residues within the cytoplasmic tails of both MICA and MICB with ubiquitin. Consequently, these molecules are internalized from the cell membrane and intracellularly sequestered, but not degraded [(75); **Figure 1; Table 1**]. Notably, the fact that the MICA allele *008 as well as ULBP1, ULBP2, and ULBP3 are GPI anchored and therefore lack a cytoplasmic tail, render them resistant to K5-mediated ubiquitinylation. Additionally, Nachmani et al. reported that the viral miRNA miR-K12-7 specifically represses the translation of MICB by binding to the 3'-UTR of its mRNA (53). Interestingly, MICA mRNA was shown not to be targeted by miR-K12-7 since the 3'-UTR is significantly shorter than the MICB equivalent and does not contain the binding site (53).

EIGHT LIGANDS, FURTHER ALLELIC DIVERSIFICATION: HOST-PATHOGEN EVOLUTION AT FULL SPEED

As emphasized above, herpesvirus family members developed numerous mechanisms to interfere with the expression of the stress-induced ligands. However, most of these studies still leave unanswered questions. More mechanisms and viral effectors are still waiting to be discovered. The viral protein repertoire is probably much larger than currently known; by using ribosome profiling of HCMV and KSHV, numerous new open reading frames (ORFs) have been identified (76, 77). The functions of many viral proteins and ORFs are yet unknown and we are just on the verge of understanding the importance of viral non-coding RNAs, including long non-coding RNAs (78, 79).

While the NKG2D receptor itself is conserved among species, its ligands are not. Interestingly, having eight functional ligands of two different families (MIC and ULBP) and various alleles, the human NKG2D ligand repertoire is more complex than that of other species. Mice possess even nine functional ligands (MULT1, Raet1 α - ϵ , H60a-c) (80). However, their domain structure reveals them to be ULBP family homologs with low allelic diversity. Non-human primates were shown to have homologs of the MIC proteins (81, 82). Still, compared to humans with more than 100 allelic variants, even great apes seem to possess lower allelic variation (83).

Herpesviruses might be a major driving force for diversification of stress-induced ligands and further mutagenesis within alleles leading to allelic variations. None of the described viral mechanisms is capable of eliminating the expression of all stress-induced ligands, the evolutionary pressure rendered these ligands so diverse that no single viral protein or RNA is sufficient to regulate all of them.

As described earlier, viral miRNAs of HCMV, EBV, and KSHV target MICB mRNA at different sites of its 3'-UTR and suppress protein translation (53, 56). Despite the high degree of sequence homology in their 3'-UTRs, MICA is not targeted by any of these miRNAs. The binding sites for the viral miRNAs of HCMV (miR-UL112) and EBV (miR-BART2-5p) are modified by a single-nucleotide insertion, thus abolishing miRNA-induced translation repression. The sequence that is targeted by the KSHV encoded miR-K12-7 is completely absent due to a major deletion in the MICA 3'-UTR.

A similar mutagenesis apparently occurred in the MICA protein to escape UL16 binding. UL16 binds to an α -helical structure in the α 2 domain of MICB. By substituting single amino acid residues in MICB with their MICA equivalents, Spreu et al. could show that a single substitution (at two different positions) is sufficient to abolish UL16 binding (84); hence, MICA is spared from UL16-mediated intracellular retention by virtue of very few mutations. Additionally, Klumkrathok et al. suggested that even different allelic variants of MICB are bound with different affinities by UL16 due to amino acid substitutions in the α 2 domain (85).

A third piece of evidence for a herpesvirus-driven coevolution is the emergence of MICA*008, a highly prevalent, GPI-anchored MICA variant. MICA*008 is not targeted by UL142, by US18, or by US20. Only recently, it was discovered that the evolutionary relatively novel US9 is capable of targeting solely this distinct allele, but none of the full-length alleles containing a transmembrane domain (68).

These few examples illustrate well the human capability to adjust to viral immune evasion strategies. Accordingly, this strong selective, coevolutionary pressure necessitates modification of viral effector molecules targeting the immune surveillance system as well. By comparing different isolates from HCMV-infected individuals, Renzette et al. and Sijmons et al. indeed showed on a global level that genes involved in immune evasion within the HCMV genome are strongly diversified and contain high numbers of single-nucleotide polymorphisms (86, 87). Among others, one particular mutable gene was found to be UL142, which interacts with NKG2D ligands as pointed out before (87).

DIVERSIFICATION: AN EVOLUTIONARY NECESSITY?

It seems obvious to conclude that herpesviruses and ligands for NKG2D continuously shape each other during coevolution, whereas the NKG2D receptor itself remains conserved.

Particularly MICA took the lead in this race on the human side, its 3'-UTR became shortened and modified and numerous allelic variations emerged to withstand herpesvirus infection. The diversity of MICA alleles might thereby even create a “population level resistance” by making it difficult for newly emerging viral mechanisms to successfully target all MICA variants at once.

However, in contradiction to this theory and the supposed importance of stress-ligand evolution, several reports showed a wide distribution of a MICA-MICB null haplotype (also described as MICA-del-MICB-null), a phenotype that occurs mainly, but not exclusively, in East Asia (88–91), apparently with no major evolutionary disadvantage or clinical manifestations.

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In fact, there are several known MICA-null alleles also independent of this haplotype. If and how MICA and MICB functions are compensated in these individuals, e.g., by the redundancy of the other NKG2D ligands that are still present, has yet to be elucidated; however, this phenomenon teaches us that we are still far from a complete understanding of the complex families of NKG2D ligands.

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DS outlined, wrote, referenced the manuscript, and prepared the figure and table. OM supervised and carefully edited the work.

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MICA and NKG2D: Is There an Impact on Kidney Transplant Outcome?

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This paper aims to present an overview of MICA and natural killer group 2 member D (NKG2D) genetic and functional interactions and their impact on kidney transplant outcome. Organ transplantation has gone from what can accurately be called a “clinical experiment” to a routine and reliable practice, which has proven to be clinically relevant, life-saving and cost-effective when compared with non-transplantation management strategies of both chronic and acute end-stage organ failures. The kidney is the most frequently transplanted organ in the world (transplant-observatory¹). The two treatment options for end-stage renal disease (ESRD) are dialysis and/or transplantation. Compared with dialysis, transplantation is associated with significant improvements in quality of life and overall longevity. A strong relationship exists between allograft loss and human leukocyte antigens (HLA) antibodies (Abs). HLA Abs are not the only factor involved in graft loss, as multiple studies have shown that non-HLA antigens are also involved, even when a patient has a good HLA match and receives standard immunosuppressive therapy. A deeper understanding of other biomarkers is therefore important, as it is likely to lead to better monitoring (and consequent success) of organ transplants. The objective is to fill the void left by extensive reviews that do not often dive this deep into the importance of MICA and NKG2D in allograft acceptance and their partnership in the immune response. There are few papers that explore the relationship between these two protagonists when it comes to kidney transplantation. This is especially true for the role of NKG2D in kidney transplantation. These reasons give a special importance to this review, which aims to be a helpful tool in the hands of researchers in this field.

Keywords: transplantation, kidney, allograft, MICA, MICA-129, NKG2D, LNK1, HNK1

INTRODUCTION

Genetic diversity is the hallmark of MHC genes (1). The main antigenic barrier to transplantation is molecules, which are polypeptide products of a cluster of genes known, in humans, as human leukocyte antigens (HLA). In addition, a family of highly glycosylated MHC-encoded molecules, the *MHC class I chain-related (MIC)* genes, has been identified (2) as a second lineage of mammalian MHC I genes, which could constitute an antigenic barrier to transplantation as well (3). The MIC molecules possess a low degree of homology to other *MHC class I* encoded genes and interact with

¹<http://www.transplant-observatory.org>.

both T-cell and natural killer (NK)-cell receptors (2). MIC proteins act as ligands for NK cells, $\gamma\delta$ T cells, and $\alpha\beta$ CD8 $^{+}$ T cells, which express natural killer group 2 member D (NKG2D) ligand (4). The importance of the MICA protein in kidney transplantation has been acknowledged in recent years, and the role they play in graft rejection has been intensely pursued.

MICA GENE: STRUCTURE, POLYMORPHISMS, AND FUNCTION

The *MIC* gene family consists of seven members (*MICA–MICG*) (Figure 1), five of which are pseudogenes, and two, *MICA* and *MICB*, of which are functional (5, 6). *MICA* and *MICB* are the most divergent members of the human MHC-encoded class I genes identified to date, having an average of 19, 25, and 35% similarity in the extracellular $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains, respectively, to those of other MHC α -polypeptides (7).

The *MICA* gene is located 46.4 kb centromeric to *HLA-B* on the short (p) arm of chromosome 6 at position 21.33 (3).

MICA and *MICB* have been shown to differ in the transcriptional control regions from common HLA class I genes. *MICA/B* genes lack the prototypic MHC class I gene promoter regulatory elements, the SXYY module [heterotrimeric X-box-binding factor—regulatory factor X; X2-box-binding factor—cyclic-AMP-responsive-element-binding protein; Y-box-binding factor—nuclear transcription factor Y (NF-Y); and an as-yet-unidentified S-box-binding factor]. In contrast, the regulatory promoter module of *MICA/B* contains heat shock elements resembling those of HSP70 genes, a CCAAT box that binds to nuclear transcription factor Y (NF-Y), and a GC box that binds to Sp1, Sp3, and Sp4 transcription factors (8).

There are 12 known possible haplotypes of *MICA* 5' promoter regions, including a null haplotype due to a deletion of the entire *MICA* gene (*MICA-P12*), which are more densely distributed in both ends compared to the central portion of 5' promoter (8, 9).

MICA has six exons separated by five introns (Figure 2): exon 1 encodes the leader peptide, exons 2–4 encode three extracellular globular domains, exon 5 encodes the transmembrane domain, and exon 6 encodes the cytoplasmatic tail (6, 10). An intron of 6,840 bp follows exon 1 and is unusually large for a class I gene. The remainder of the *MICA* gene has a quite similar organization to classical class I genes, except for the presence of a relatively long intron 5 and the fusion of the cytoplasmic tail and 3' UTR sequence in a single last exon (11).

It is considered that *MICA* gene has a codominant expression, and the presence of heat shock elements within the promoter

suggests that *MICA* transcription is induced under stress conditions, and that therefore the *MICA* protein functions as an indicator of cell stress (11–13). The first intron of the *MICA* gene contains an NFkB-binding site that binds p65 (RelA)/p50 heterodimers and p50/p50 homodimers of the NFkB transcription factor family. The role of the proximal –130 bp NFkB site was reported as necessary and sufficient for transcriptional transactivation of *MICA* in response to TNF α in primary endothelial cells (ECs) (14).

Gene transcription isoforms are mRNAs transcribed from the same locus that differs in their transcription start sites and/or untranslated regions or protein coding DNA sequences (CDSs) also producing different protein isoforms. The alternative splicing of *MICA* leads to the formation of four isoforms. Two of them were described by Zou and Stastny (15) (*MICA* isoforms 1 and 2), and they did not appear to be tissue specific.

MICA isoform 1 (1*001) is the longest isoform, derived from the *MICA*001* allele. *MICA* isoform 2 (1*008:01) is a variant isoform derived from the *MICA*008:01* allele that contains a four-nucleotide insertion (rs9279200), which causes a frameshift mutation and subsequent truncation of the CDS, compared to isoform 1 (allele *MICA*001*) (15). The other two isoforms of *MICA*, isoforms 3 and 4, are described only in the *ncbi.nih.gov/gene*² website. *MICA* isoform 3 is, like isoform 2, encoded by the *MICA*008:01* allele; however, it is shorter than isoform 2 at the N-terminus, containing an alternate 5' exon, differences in the 5' UTR, and lacking a portion of the 5' coding region, with translation being initiated from a downstream in frame start codon. *MICA* isoform 4 contains an alternate 5' exon and uses an alternate splice site in an internal exon. It differs in the 5' UTR, lacks a portion of the 5' coding region, and initiates translation from an alternate start codon, compared to variant 1 (*MICA*008:01* allele). Isoform 4 has a distinct and shorter N-terminus, compared to isoform 2.

The *MIC* genes are transcribed in keratinocytes, ECs, fibroblasts, monocytes, epithelial cell lines and epithelial tissues of cell lines, and freshly isolated cells (2, 16) and are not usually transcribed in CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, and CD19 $^{+}$ cells (17). MIC protein is only expressed on the cell surface of freshly isolated ECs, fibroblasts (17), and gastric epithelium (12). MIC protein acts as a ligand for NK cells, $\gamma\delta$ T cells, and $\alpha\beta$ CD8 $^{+}$ T cells, which express NKG2D ligand (NKG2DL) (4).

²<https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=100507436#>.

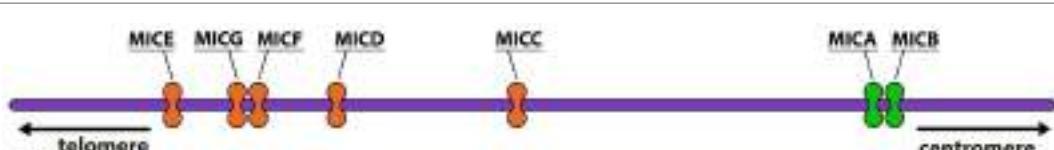
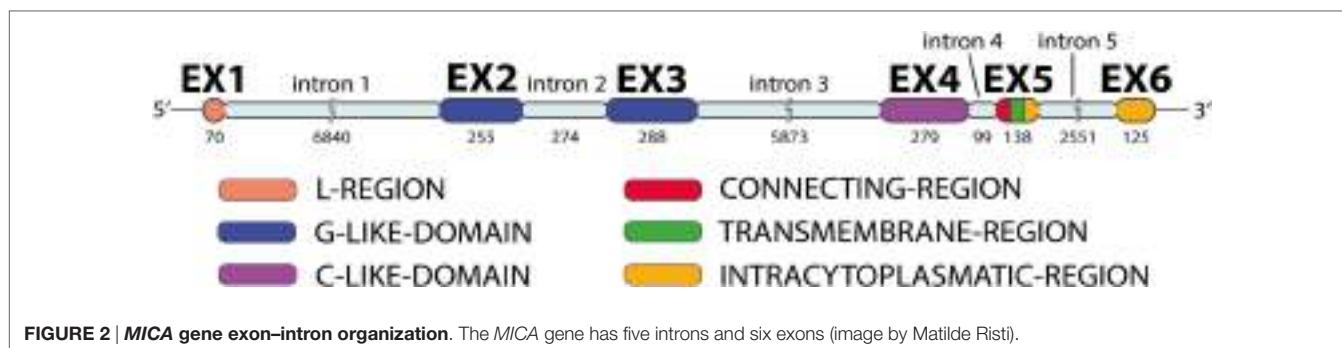


FIGURE 1 | Representation of *MHC class I chain-related (MIC)* genes. The functional genes are represented in green and the pseudogenes are in orange (image by Matilde Risti).



MICA Polymorphism and -129Met/Val Dimorphism

Bahram et al. (3) first described *MICA**01 to *MICA**05 alleles with a total of 18 nucleotide substitutions resulting in 14 amino acid changes in the final polypeptide. Fodil et al. (7) described the alleles, *MICA**06 to *MICA**16, with nine nucleotide substitutions and eight amino acids changes. One year later, Mizuki et al. (18) showed a variable number of trinucleotide GCT repeats that encode 4, 5, 6, 7, 9, or 10 alanine (A, Ala). The short tandem repeats or microsatellite alleles were labeled as A4, A5, A6, A7, A8, A9, and A10. There is also an A5.1 allele that contains five triplet repeats of GCT plus an additional guanine nucleotide insertion (GGCT). This insertion causes a frameshift mutation leading to a premature intradomain stop codon within the transmembrane region, which deletes the MICA cytoplasmic tail. The A4, A5, A6, A7, A8, A9, A10, and A5.1 sizes are, respectively, 179, 182, 185, 194, and 183 bp (18–20). At the time of writing (October 2016) hla.alleles.org³ reports 105 *MICA* alleles, 2 of which considered null, result in 82 different *MICA* proteins. All *MICA* alleles from *001 to *087 producing different proteins and their nucleotides variations on exons 2–6 are shown in Table 1.

Several studies have documented *MICA* allele frequencies within different populations (Figure 3), and the frequency distribution varies between them. For example, the same group of three alleles (*MICA**008, *MICA**002, and *MICA**004) accounts for more than 50% of the allele frequencies commonly found in several Caucasoid populations (21–24) but at the same time *MICA**027's frequency is extremely different in a comparison between the South American Indian and Caucasoid populations (25). Single high-frequency *MICA* alleles are each associated with more than one different *HLA-B* allele, but this pattern is not reciprocal. Most specific *HLA-B* alleles, including *B**07:02 and *B**08:01 variations, are usually linked to a single *MICA* allele. This pattern suggests that the *MICA* alleles had an earlier origin than major branches of *HLA-B* alleles (26).

The evolutionary history of *HLA-B* alleles is recognizable in the linkage relationship between *HLA-B* and *MICA* genes. The high degree of sequence similarity between three *HLA* alleles (*B**35, *B**53, and *B**58) indicates that they were all generated from the same progenitor allele, and the observation that globally they are all linked to the *MICA**002 allele further supports

TABLE 1 | Nucleotide variations on exons 2–6 for *MICA* alleles from *001 to *087.

EXON 2 $\alpha 1$		
CODON 6	CTG (LEU)	CGC (ARG) CTC (PRO)
CODON 14	TGG (TRP)	GGG (GLY)
CODON 23	CTC (LEU)	GTT (LEU)
CODON 24	ACT (THR)	GCT (VAL)
CODON 26	GTA (VAL)	GGA (GLY)
CODON 36	TGT (CYS)	TAT (TYR)
CODON 38	AGG (ARG)	AGC (SER)
CODON 39	CAG (GLN)	TAG (Stop)
CODON 55	GGA (GLY)	GGC (GLY)
CODON 56	AAT (ASN)	AAC (ASN)
CODON 64	AGA (ARG)	AAG (ARG)
CODON 69	AAC (ASN)	AAT (ASN)
EXON 3 $\alpha 2$		
CODON 90	CTC (LEU)	TTC (PHE)
CODON 91	CAG (GLN)	CGG (ARG)
CODON 93	ATT (ILE)	ATG (MET)
CODON 102	AAC (ASN)	AGC (SER)
CODON 105	AAG (ARG)	AAG (LYS)
CODON 112	TAC (TYR)	TAT (TYR)
CODON 114	GGG (GLY)	AGG (ARG)
CODON 122	CTG (LEU)	GTG (VAL)
CODON 124	ACT (THR)	TCT (SER)
CODON 125	AAG (LYS)	GAG (GLU)
CODON 129	ATG (MET)	GTG (VAL)
CODON 130	CCC (PRO)	TCC (SER)
CODON 139	GCC (ALA)	GCA (ALA)
CODON 142	GTC (VAL)	ATC (ILE)
CODON 151	ATG (MET)	GTG (VAL)
CODON 156	CAC (HIS)	CTC (LEU)
CODON 169	CGG (ARG)	TGG (TRP)
CODON 173	AAA (LYS)	GAA (GLU)
CODON 174	TCC (SER)	TCT (SER)
CODON 175	GGC (GLY)	AGC (SER)
CODON 176	GTA (VAL)	GGT (GLY)
CODON 181	ACA (THR)	ATA (ILE)
EXON 4 $\alpha 3$		
CODON 190	CGC (ARG)	TGC (CYS)
CODON 191	AGC (SER)	AGT (SER)
CODON 193	GCC (ALA)	GCA (ALA)
CODON 198	ATT (ILE)	ATC (ILE)
CODON 205	TCT (SER)	TCC (SER)
CODON 206	GGC (GLY)	AGC (SER)

(Continued)

³<http://hla.alleles.org/nomenclature/stats.html>.

TABLE 1 | Continued

EXON 4 α_3		
CODON 208	TAT (TYR)	TGT (CYS)
CODON 210	TGG (TRP)	CGG (ARG)
CODON 213	ACA (THR)	ATA (ILE)
CODON 215	AGC (SER)	ACC (THR)
CODON 221	GTA (VAL)	CTA (LEU)
CODON 230	TGG (TRP)	TCG (SER)
CODON 244	TGG (TRP)	TGA (Stop)
CODON 247	AAC (THR)	ACT (THR)
CODON 250	TGC (CYS)	CGC (ARG)
CODON 251	CAA (GLN)	CGA (ARG)
CODON 253	GAG (GLU)	AAG (LYS)
CODON 254	GAG (GLU)	AAG (LYS)
CODON 256	AAG (ARG)	AGT (SER)
		AAG (LYS)
CODON 265	GGG (GLY)	AGG (ARG)
CODON 268	AGC (SER)	GGC (GLY)
CODON 269	ACT (THR)	ATT (ILE)
CODON 271	CCT (PRO)	GCT (ALA)
EXON 5 TM		
CODON 295	CGT (ALA)	CGCT
CODON 304	TAT (TYR)	TAC (TYR)
CODON 306	CGT (ARG)	TGT (CYS)
EXON 6		
CODON 350	GAT (ASP)	GCT (ALA)
CODON 354	ACT (THR)	GCT (ALA)
CODON 359	GGC (GLY)	GGT (GLY)
CODON 360	GCC (ALA)	ACC(THR)

Codons are shown in the first column. The second column shows the triplets and their corresponding amino acids in the consensus sequence (MICA*001). The third column lists that triplet's possible variations in other alleles compared with the consensus sequence. Amino acid substitutions in MICA on the three external protein domains (exons 2–4), on the transmembrane domain TM (exon 5) and carboxy-terminal cytoplasmic tail (exon 6). The G nucleotide insertion is represented in red in the exon 5 TM.

this conclusion. Specific MICA alleles also tend to associate with serological HLA-B groups. A rare exception can be found in *B*44*, whose two subgroups *B*44:02* and *B*44:03* have exclusive associations with *MICA*008* and *MICA*004* (26).

The *MICA-129Val/Met* dimorphism, caused by an SNP (rs1051792) at nucleotide position 454 (G>A) of the *MICA* gene is of particular interest. The substitution of valine (Val) for methionine (Met) at position 129 in the α_2 domain of the MICA protein has been reported to affect NKG2D binding avidity (36–40). This dimorphism divides the *MICA* alleles into two groups (Table 2). In 2015, it has been observed that *MICA-129Met* alleles increased the risk of experiencing acute graft-versus-host disease. This effect could be the consequence on NKG2D signaling by *MICA-129Met* variant (40). In addition to this, it has been shown that the *MICA-129* dimorphism may directly affect plasma membrane expression and shedding of MICA, and these functional effects might contribute to the numerous disease associations (41).

MICA Molecule

MICA is a highly glycosylated membrane-anchored cell surface protein composed of 383 amino acids (12). Unglycosylated MICA appeared less stable than those incorporating glycosylated MICA

(36). Its expression has been reported on the surface of different cells and resembles the domain organization (Figure 4) of the α chain of MHC class I molecules (16, 42). MICA α chain does not bind $\beta 2$ -microglobulin and is independent of any transporter-associated protein. Attempts to identify peptides bound to MICA have been unsuccessful (10, 12). The crystal structure of MICA shows four distinct α helices arranged in an eight-stranded antiparallel β sheet. These helices in MICA roughly correspond to the two helices that define the peptide-binding groove in peptide-binding MHC class I proteins and homologs (42).

MICA is generally concentrated in lipid rafts and is S-acylated, similar to other lipid rafts-associated proteins. *In vitro* mutation of the S-acylation site, replacing a cysteine residue with a stop codon at aminoacid position 39, yields a truncated form of MICA, unable to activate NK cells (43).

The MICA molecule interacts with NK cells, $\gamma\delta$ T cells, and $\alpha\beta$ CD8 $^+$ T cells, which express NKG2D, a common activating NK cell receptor (4, 10, 44). NKG2D recognizes the human MICA protein in conjunction with a transmembrane signaling adaptor protein, DNAX-activation protein (DAP10) (4, 10).

It is noteworthy that the MICA molecule can also be recognized by $\gamma\delta$ T cells with the TCR variable region V $_{\delta}1$ (4, 45–47).

Both types of receptors, V $_{\delta}1$ TCR and NKG2D, can simultaneously recognize and bind to MICA on a V $_{\delta}1$ cell surface. There is close association between the tissue distribution of V $_{\delta}1$ cells and the physiological expression of MICA, as MICA affects V $_{\delta}1$ cell lineage development (46). In V $_{\delta}1$ $\gamma\delta$ T cells, the strength of the binding between TCR and MICA is weaker than that between NKG2D and MICA. Although weak, TCR:MICA complexes show unusual stability after they are formed, with long half-lives. TCR and NKG2D receptors compete for binding to MIC ligands, and it has been suggested that initial interactions at the point of contact may be dominated by NKG2D:MIC binding events, which then give way to longer-lived $\gamma\delta$ TCR:MIC complexes (47).

Conclusions on MICA

The *MICA* gene is polymorphic, and it is in linkage disequilibrium with *HLA-B* genes. The MICA protein is expressed on the cell surface, and it is possibly the proteolytic cleavage of the α_3 domain which in turn releases soluble MICA (sMICA). The MICA molecule does not present a peptide in its groove and can interact with the NKG2D receptor, which is the focus of the following paragraphs.

NKG2D OR KILLER CELL LECTIN-LIKE RECEPTOR K1 (KLRK1) GENE: STRUCTURE, POLYMORPHISMS, AND FUNCTION

NKG2D gene, also known as *KLRK1*, is located in the natural killer complex (NKC) on chromosome 12 (42, 48, 49). Human *NKG2D* (Figure 5) has 10 exons (50). Exons 2–4 encode the intracellular/transmembrane domain; exons 5–8 encode the ligand-binding ectodomain, which is a membrane-bound domain protruding into extracellular space (50, 51). *NKG2D* has a low number of nucleotide variations (48). *NKG2D* appears to be conserved

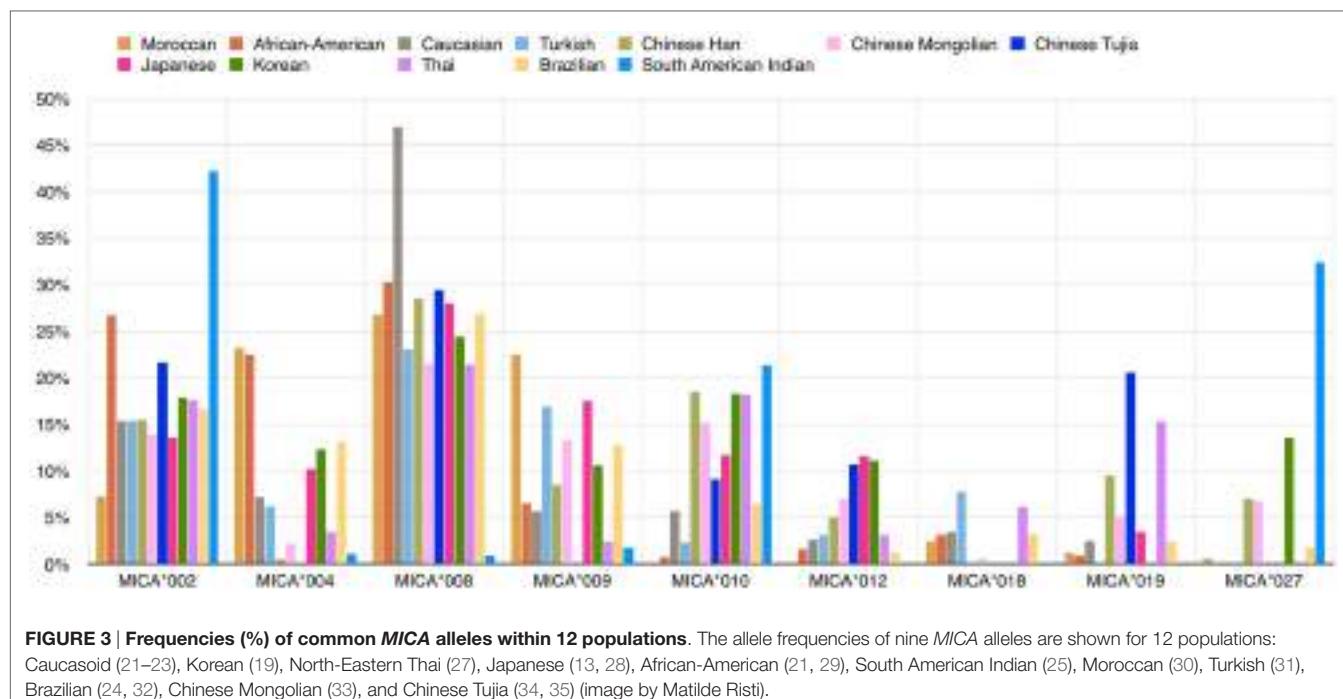


TABLE 2 | Dimorphism 129 Val/Met divides *MICA* alleles into two groups.

Dimorphism 129 val/met divides *MICA* alleles in two groups

ATG (Met)	GTG (Val)
MICA*001, *002, *007, *011, *012, *014, *015, *017, *018, *020, *023, *025, *026, *029, *030, *031, *032, *034, *035, *036, *037, *038, *039, *040, *041, *042, *043, *045, *046, *047, *050, *051, *052, *055, *059, *060, *061, *068, *072, *075, *078, *079, *081, *083, *084, *086	MICA*004, *005, *006, *008, *009, *010, *013, *016, *019, *022, *024, *027, *028, *033, *044, *048, *049, *053, *054, *056, *057, *058, *062, *063, *064, *065, *066, *067, *069, *070, *073, *074, *076, *077, *080, *082, *085, *087

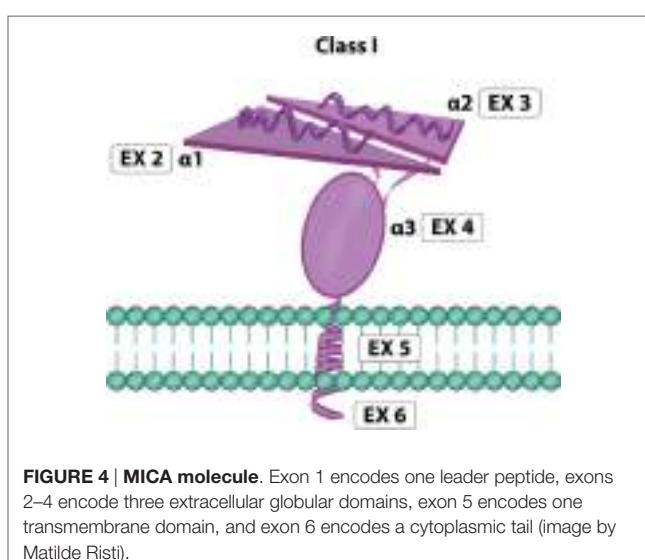
The most frequent alleles present in Figure 3 are shown in bold. The *MICA* alleles shown are from *MICA**001 to *MICA**087.⁴ “*MICA**003:01” label has never been assigned to any sequence. *MICA**021 sequence was renamed *MICA**012:03 in August 2007. The sequence originally labeled *MICA**071 was proven to contain errors and to be identical to *MICA**017 (March 2013) (see text footnote 3).

⁴<http://hla.alleles.org/alleles/classo.html>.

during evolution, with orthologs of *KLRK1* are present in the genome of all mammals, as well as in marsupials (4, 52).

Human *NKG2D* is expressed from at least three distinct alleles, and several gene transcription isoforms have been described, including an alternatively spliced variant that introduces a non-sense mutation resulting in a protein isoform that lacks the entire extracellular ligand-binding domain (53).

Hayashi et al. (54) evaluated the SNPs in the *NKG2D* gene region. They selected 20 SNPs with a >10% higher frequency in Caucasoid or Japanese populations (Table 3); these SNPs covered *CD94*, *NKG2D*, *NKG2F*, *NKG2E*, *NKG2A*, and *Ly49* genes. They selected 8 out of the 20 SNPs that were closely associated with



natural cytotoxic activity, having *P* values <0.001. All these SNPs are located in the *NKG2D* gene region, except for rs1983526 that is located in the promoter region of the *NKG2A* gene. These eight SNPs were split into two groups: group 1 (rs1049174, rs2617160, rs2617170, rs2617171, and rs1983526) and group 2 (rs2255336, rs2246809, and rs2617169). All the SNP combinations of group 1/group 1 and group 2/group 2 revealed a strong linkage disequilibrium, with r^2 values >0.9, whereas group 1/group 2 combinations showed much weaker linkage disequilibrium, with r^2 values <0.5. This indicates that the five group 1 and three group 2 SNPs belong to two different haplotype blocks (*NKG2D* hb-1 and hb-2), each of which generates two major haplotypes associated with low

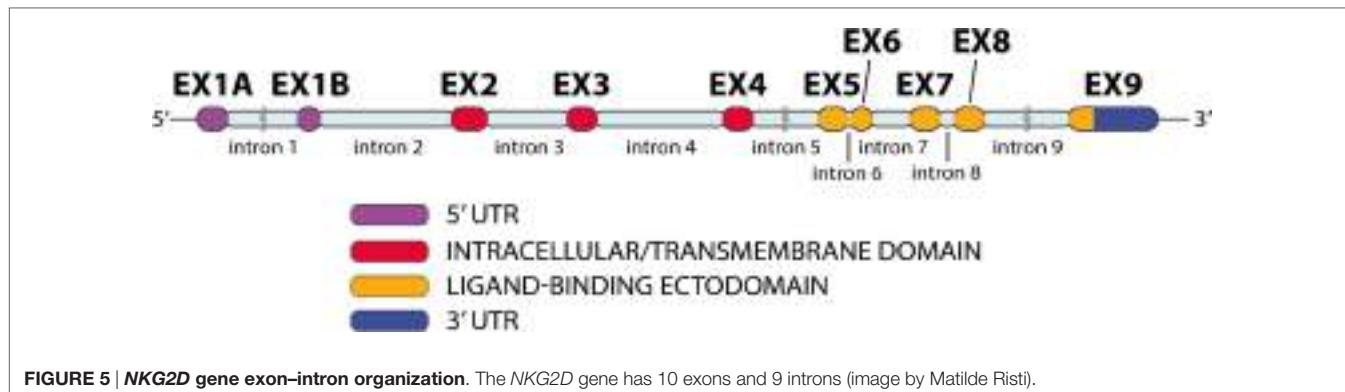


TABLE 3 | 20 SNPs selected by Hayashi et al. in their study (54).

SNP ID	Variation	SNP ID	Variation	NKG2D hb-1	Low	High
rs3759272	G>T	rs2617170	T>C	rs1049174	C	G
rs2537752	T>A	rs2617171	C>G	rs2617170	C	T
rs1049174	G>C	rs1971939	C>G	rs2617171	C	G
rs2255336	A>G	rs1915319	A>G	rs1983526	C	G
rs2294148	G>A	rs4763525	G>A	rs2617160	T	A
rs2049796	A>C	rs3003	C>T	NKG2D hb-2	Low	High
rs2617160	A>T	rs1983526	C>G	rs2255336	G	A
rs7972757	A>G	rs10772285	G>C	rs2246809	G	A
rs2246809	A>G	rs1915325	G>A	rs2617169	T	A
rs2617169	T>A	rs2607893	C>T			

Blue fields belong to group 1 and green ones represent group 2. Each of the different haplotype blocks (*NKG2D* hb-1 and hb-2) is split in low and high natural cytotoxic activity haplotypes. hb-1 and hb-2 may be successfully predicted knowing only rs1049174 (in bold).

(LNK) and high (HNK) natural cytotoxic activity phenotypes (Table 3) (54).

A separate study on a European population confirmed that the NKG2D region haplotype associated with increased cancer susceptibility in the Japanese population also exists in Europeans at similar frequency. Therefore, the conclusions of the original study may also be applicable to this population (55).

NKG2D: HNK1 and LNK1 Haplloblocks

Several studies have demonstrated that high and low natural cytotoxic activity allele (HNK1 or LNK1) belonging to NKG2D haplotype blocks 1 (hb-1) may be successfully predicted by only a single SNP (dbSNP: rs1049174) (54, 56, 57).

A study on Japanese individuals demonstrated that the *HNK1* haplotype is associated with a greater activity of NK cells in the peripheral blood and a lower prevalence of cancers originating from epithelial cells (58). Espinoza et al. showed an association between the *NKG2D-HNK1* haplotype (haplotype frequency, 61%) in bone marrow donors and a significantly reduced transplant-related mortality and better overall survival for unrelated donors of HLA-matched myeloablative bone marrow recipients with standard-risk disease (58).

The rs1049174 distribution for 25 populations (Figure 6) is reported on the 1,000 genomes website.⁵ HNK is reported to be associated with the rs1049174 (G) allele, and LNK with rs1049174 (C) (54, 56).

NKG2D Protein

The NKG2D is a member of a C-type lectin-like family receptor called CD94/NKG2 (42). Despite its inclusion in the NKG2 family, NKG2D displays only limited sequence similarity to other members of the NKG2 family of NK cell surface receptors (NCRs) and CD94 and forms homodimers, rather than heterodimers, with CD94, as do other NKG2 NCRs (42).

Natural killer group 2 member D is a transmembrane-anchored receptor expressed as a disulfide-linked homodimer on the cell surface, with a molecular weight of ~42 kDa (42).

In humans, each NKG2D homodimer (Figure 7) associates with two DAP10 homodimers to form a hexameric structure (59), which can signal by recruitment of phosphatidylinositol 3-kinase (36).

Human NKG2DLs are MICA and MICB, and a group of glycosylphosphatidylinositol-bound surface molecules including UL16 binding protein(ULBP)-1, -2, -3, and -4 (6), RAET1G (or ULBP5), and RAET1L (or ULBP6) (60), which share about 25% identical amino acids in their $\alpha_1\alpha_2$ domains that are variably scattered throughout the aligned sequences without discernible patterns of sequence conservation (36).

Signals triggered by the NKG2D receptor are transmitted through the associated DAP10 dimer (Figure 7) (59) because NKG2D lacks a tyrosine-based inhibitory motif in its cytoplasmic tail (4, 61).

Natural killer group 2 member D is expressed by all human NK cells, $\gamma\delta$ T lymphocytes, $\alpha\beta$ CD8 $^+$ T lymphocytes (6), interferon-producing killer DC (62), invariant NKT cells cells, and a small subset of effector or memory CD4 $^+$ T cells (4, 52, 63). Expression of NKG2D on NK cells and CD8 $^+$ T cells can be modulated by cytokines due to their effects on transcription and posttranscriptional processing of *NKG2D* and *DAP10*. In

⁵http://browser.1000genomes.org/Homo_sapiens/Variation/Population?db=cor&e;r=12:10524865-10525865;v=rs1049174;vdb=variation;vf=750969.

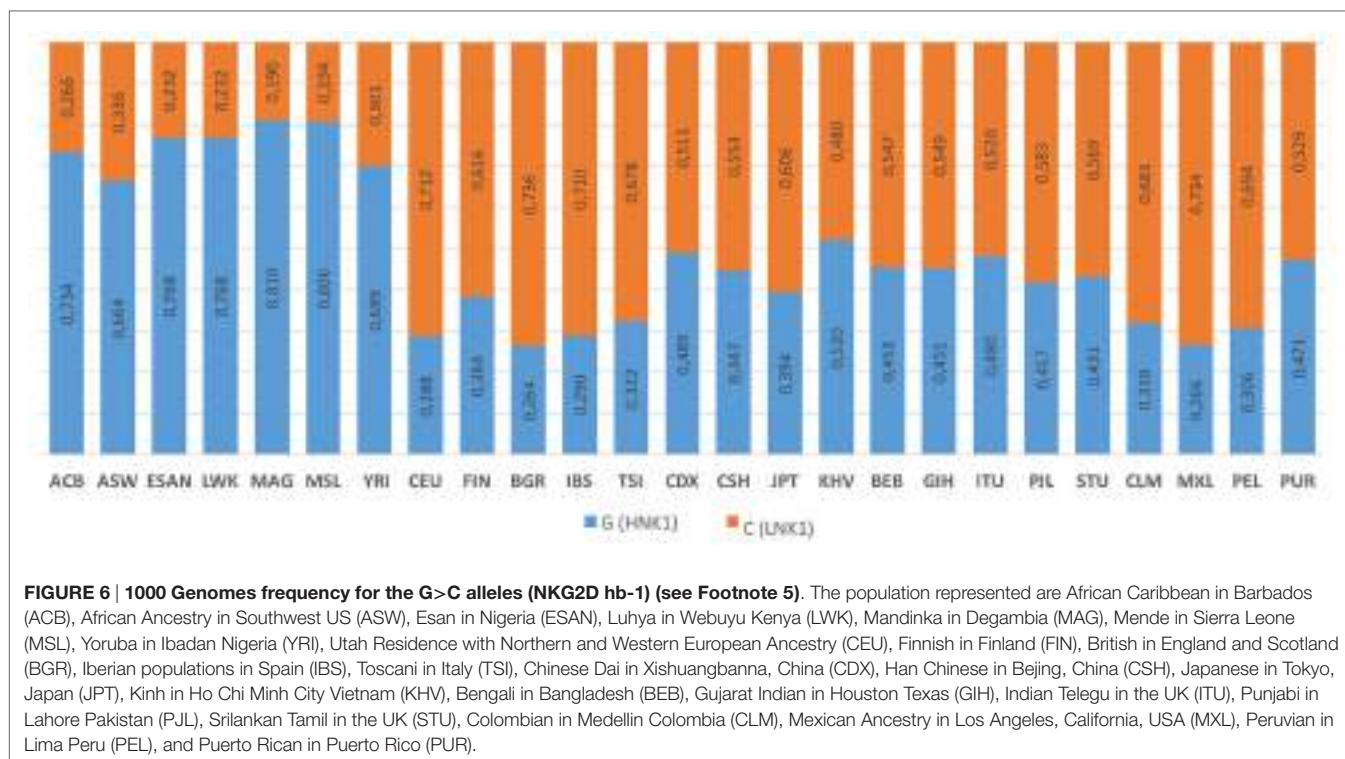


FIGURE 6 | 1000 Genomes frequency for the G>C alleles (NKG2D hb-1) (see Footnote 5). The population represented are African Caribbean in Barbados (ACB), African Ancestry in Southwest US (ASW), Esan in Nigeria (ESAN), Luhya in Webuye Kenya (LWK), Mandinka in Degambia (MAG), Mende in Sierra Leone (MSL), Yoruba in Ibadan Nigeria (YRI), Utah Residence with Northern and Western European Ancestry (CEU), Finnish in Finland (FIN), British in England and Scotland (BGR), Iberian populations in Spain (IBS), Toscani in Italy (TSI), Chinese Dai in Xishuangbanna, China (CDX), Han Chinese in Beijing, China (CSH), Japanese in Tokyo, Japan (JPT), Kinh in Ho Chi Minh City Vietnam (KHV), Bengali in Bangladesh (BEB), Gujarat Indian in Houston Texas (GIH), Indian Telegu in the UK (ITU), Punjabi in Lahore Pakistan (PIL), Srilankan Tamil in the UK (STU), Colombian in Medellin Colombia (CLM), Mexican Ancestry in Los Angeles, California, USA (MXL), Peruvian in Lima Peru (PEL), and Puerto Rican in Puerto Rico (PUR).

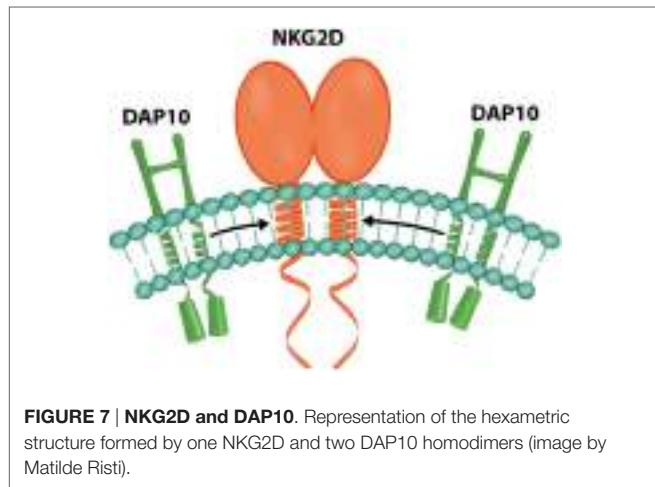


FIGURE 7 | NKG2D and DAP10. Representation of the hexameric structure formed by one NKG2D and two DAP10 homodimers (image by Matilde Risti).

humans, IL-2, IL-7, IL-12 (64), IL-15, and IFN- α (65) upregulate NKG2D expression, whereas TGF β (65–67), IFN β 1 (68), and IL-21 (69), IL-4, IL-12, and IFN γ (65) downmodulate NKG2D. This downregulation can also be attributed to the overexposure to soluble or membrane-bound NKG2DLs, which promote the internalization and subsequent degradation of the receptor or catabolites produced on macrophage activation [reactive oxygen species (ROS) and L-kyurenine] (65). This is a possible explanation of the mechanism of oxidative stress, which is a common feature of chronic renal failure. ROS trigger the upregulation of MICA and downregulation of NKG2D in NK cells *in vitro* (70). DAP10 availability is also a decisive factor in NKG2D surface

expression, and miRNAs can downregulate NKG2D expression in NK cells, reducing its cytotoxic effect (65).

Fernandez-Sanchez et al. (65) have shown for the first time that epigenetic mechanisms are involved in the regulation of NKG2D expression. They analyzed the region around the translation initiation site of the *NKG2D* gene (which included 11 CpG sites between -992 and +263 positions), and they found the greatest differences in DNA methylation patterns between the positions -992 and -255. These CpGs were highly methylated in Jurkat, HUT78 cell lines and CD4 $^{+}$ T cells, partially methylated in CD8 $^{+}$ T lymphocytes and NK cells, and fully demethylated in NK cells lines. They discovered that the acetylation of histone H3 lysine 9 (H3K9) is important for correct NKG2D expression in NK and CD8 $^{+}$ T cells, while DNA demethylation may be associated with an increased expression of NKG2D in CD4 $^{+}$ T cells. The DNA methylation profile of *DAP10* gene was also analyzed, but no differences were found. CD4 $^{+}$ T lymphocytes and T cell lines (Jurkat and HUT78) had a DNA methylation; instead NKG2D-positive cells (CD8 $^{+}$ T lymphocytes, NK cells, and NKL cell line) had an unmethylated *NKG2D* gene and high levels of histone H3 lysine 9 acetylation (H3K9Ac). It was observed that the histone acetyltransferase inhibitor, curcumin, reduced H3K9Ac levels in the *NKG2D* gene, downregulated NKG2D transcription, and led to a marked reduction in the NKG2D-mediated lytic capacity of NK cell lines (65).

Another interesting study by Karimi et al. (71) of human primary NK and CD8 $^{+}$ T cells discovered a novel splice variant of human NKG2D that encodes a truncated receptor lacking the ligand-binding ectodomain (NKG2D $^{\text{TR}}$). Overexpression of this truncated isoform severely attenuated cell killing and

IFN γ release mediated by full-length NKG2D (NKG2D^{FL}). A specific knockdown of an NKG2D^{TR} isoform enhanced NKG2D-mediated cytotoxicity, suggesting that NKG2D^{TR} is a negative regulator of NKG2D^{FL}. At the biochemical level, it was demonstrated that NKG2D^{TR} bound to DAP10 and interfered with the DAP10–NKG2D^{FL} interaction. In addition, NKG2D^{TR} formed heterodimers with NKG2D^{FL} and negatively modulated NKG2D^{FL} preventing its surface expression. Therefore, NKG2D^{TR} constitutes a mechanism for regulation of NKG2D-mediated function in human CD8 $^{+}$ T cells and NK cells (71).

Unlike CD8 $^{+}$ T cells, TCR-mediated activation is not sufficient to induce NKG2D expression on CD4 $^{+}$ T cells, and the factors responsible for induction of NKG2D on CD4 $^{+}$ T cells are still unknown (71).

Saez-Borderias et al. (63) provided the first evidence that a subset of human cytomegalovirus (HCMV)-specific CD4 $^{+}$ T cells displays NKG2D. Their data suggest that CD4 $^{+}$ NKG2D $^{+}$ cells expanding in HCMV-stimulated cultures correspond to virus-specific memory T cells that have acquired NKG2D while losing CD28 (63).

Conclusions on NKG2D

The NKG2D gene can be split into two haploblocks: *HNK1* and *LNK1* (high and low cytotoxic activity related). The NKG2D protein is a homodimer associated with two DAP10 molecules and can interact with MICA. In NK cells, the NKG2D protein is an activation receptor which is able by itself to trigger cytotoxicity. This is the main reason why it is interesting to study the relationship between MICA and NKG2D in depth in the following paragraphs.

MICA LIGAND AND ITS RECEPTOR NKG2D: FUNCTIONAL INTERACTIONS

The crystal structure of the MICA–NKG2D complex shows that NKG2D binds to one MICA molecule as a homodimer. One of the NKG2D molecules binds mostly to the $\alpha 1$ domain of MICA, while the other binds mostly to the $\alpha 2$ domain (6). The contact between these two molecules creates a small pocket (roughly 6 Å wide \times 6 Å thick \times 14 Å long) (42).

The NKG2D homodimer overlays MICA diagonally in way that resembles $\alpha\beta$ TCR overlaying MHC I molecules. The central section of the MICA $\alpha 2$ domain is disordered when MICA is crystallized in isolated form, but it becomes ordered when MICA is bound to NKG2D and forms part of the interface between the two molecules (6).

MICA glycosylation was not essential, but it enhanced complex formation with NKG2D. Likewise, the glycosylation state of NKG2D had no substantial effect on complex formation (36).

MICA–NKG2D is considered a versatile ligand–receptor pair. As a matter of fact, NKG2D can act as a primary receptor or costimulatory molecule during infections, autoimmunity, or antitumor immune responses (6). For example, it has been shown that endothelial MICA triggers an activating signal in allogeneic polyclonal NK cells through the immunoreceptor NKG2D, which may have account for a significant part in EC lysis by allogeneic NK cells. *In vitro* coculture assays show that interaction of

endothelial MICA with NKG2D provides an immune suppressive pathway by downregulating NKG2D on the NK cell surface (14).

Boukouaci et al. (72) suggested that endocytosis of the NKG2D receptor, upon binding to sMICA, is considerably more rapid than the replenishment of cell surface NKG2D by *de novo* synthesis. The same authors also found that sMICA down regulates NKG2D receptor expression on CD8 $^{+}$ T cells. sMICA upregulates the IFN γ production only by cytokines-activated NK cells, while it has no effect on non-activated cells. The researchers demonstrated that sMICA upregulates IFN γ expression by IL-12/IL-18-activated CD3 CD56 $^{+}$ NK cells, demonstrating the pro-inflammatory effect of sMICA (72). A study with a mouse model found that Lewis rat hearts transplanted into BALB/c mice developed typical acute rejection (AR) in 6 days. The severity of xenograft rejection increased with time, from 2 to 6 days. Also increasing over time, the MICA protein and MICA mRNA reached their highest value after 6 h. The prevalence of anti-MICA was significantly higher among mice with severe AR. However, sMICA was significantly increased during AR at 2 h, then gradually decreased, and reaching its lowest value after 6 h (73).

MICA–NKG2D AND KIDNEY TRANSPLANT

In the last few decades, the role of MICA and NKG2D in kidney transplants has emerged (Table 4). The involvement of NK cells was discovered in 1995 when some indirect evidence was reported during rejection of kidney transplants. Accumulation of CD56 $^{+}$ NK cells expressing granzyme in kidney biopsies of patients undergoing AR suggested a role of their cytolytic activity in kidney-allograft rejection (74). Over the years, the association between NK cells and the mechanisms of microcirculation injury during antibody-mediated rejection (AMR) in kidney transplants has become increasingly evident. The researchers proposed that donor-specific antibodies (DSA) were able to bind to the endothelium and to recruit NK cells that produce IFN γ and trigger antibody (Ab)-dependent cellular cytotoxicity (75).

NK Cells and Kidney Damage in Mice and Cell Lines

Natural killer group 2 member D-ligand engagement delivers a strong dominant activating signal that overrides the inhibitory signal delivered by self-MHC class I, thus activating NKG2D-expressing cells, resulting in innate and adaptive immunity activation (113).

Zhang et al. (116) reported a study on ischemia/reperfusion injury (IRI) on mice and discovered the capacity of NK cells to injure renal tubular epithelial cells *in vitro*. *In vivo* data supported the hypothesis that NK cells interact with tubular epithelia through NKG2D/Rae-1 interaction to mediate kidney damage following IRI.

Luo et al. (89) performed an *in vitro* study on human renal proximal tubular epithelial cell line (HK-2). They discovered that hypoxia-inducible factor-1- α (HIF-1 α) plays a very important role in upregulating MICA expression and enhancing NK cell cytotoxicity toward target cells during hypoxia/reoxygenation in HK-2 cells. HIF is a heterodimer consisting of an α -subunit (HIF-1 α) and a β -subunit (HIF-1 β), the HIF-1 β protein is constitutively

TABLE 4 | Relevant published work regarding NKG2D, MICA, and kidney transplants.

Reference	Summary	MICA biomarker
Relevant published works regarding MICA and transplants		
Zwirner et al. (76)	Several patients had specific antibodies (Abs) against MICA. Most of them were detected in serum samples collected at different times after organ rejection	Yes
Hankey et al. (77)	MHC class I chain-related expression was documented in allografted kidneys and pancreas. Expression of MICB was observed in epithelial cells in allografted kidney and pancreas that showed histologic evidence of rejection and/or cellular injury	Yes
Opelz (78)	This work showed that non-HLA immunity contributed substantially to long-term kidney transplant failure. The targets for Abs causing late rejections could be called minor histocompatibility antigens	Yes
Mizutani et al. (79)	Patients who rejected transplants had anti-HLA and anti-MICA Abs more frequently than those with functioning grafts. These Abs found in the peripheral circulation were not necessarily donor-specific, but their association with failure was consistent with a causality hypothesis	Yes
Amezaga et al. (80)	Anti-MICA Abs were not detected pretransplant nor posttransplant in patients receiving a compatible graft. Anti-MICA Abs were detected posttransplant acute antibody-mediated rejection in patients receiving an incompatible graft	Yes
Mizutani et al. (81)	Anti-HLA and anti-MICA Abs were present independently on a more frequent basis in patients with failed grafts than those with functioning grafts	Yes
Panigrahi et al. (82)	Patients who developed both anti-HLA and anti-MICA Abs rejected their grafts more frequently than those having either of these Abs	Yes
Zou et al. (83)	Pre-sensitization of kidney transplant recipients against MICA antigens had been associated with an increased frequency of graft loss and might contribute to allograft loss among recipients who were well matched for HLA	Yes
Seiler et al. (62)	Unlike previous reports, in this work the researchers could not detect elevated MICA mRNA levels in kidney biopsies derived from patients undergoing acute rejection (AR) or chronic allograft nephropathy. In contrast, they observed a strong NKG2D mRNA induction during renal-allograft rejection, which was verified by immunohistology in kidney biopsies	No
Suarez-Alvarez et al. (84)	Anti-MICA Abs were detected in 17.6% of the patients and correlated with the development of AR. The presence of anti-MICA Abs could be an important marker for diagnosis because of their contribution to the outcome of the graft, regardless of presence of anti-HLA Abs	Yes
Alvarez-Marquez et al. (85)	At the time of the biopsy, 21% patients had only anti-HLA I Abs, 15.8% had anti-GSTT1 Abs, 10.5% had anti-HLA II Abs, and 10.5% had anti-MICA Abs. Besides anti-HLA Abs, donor-specific Abs against MICA and GSTT1 antigens could be responsible for the occurrence of Ab-mediated kidney graft rejection	Yes
Racca et al. (86)	This work did not show a correlation between MICA expression and renal graft state. The state of kidney allograft could be measured by using HLA-G1 isoforms, but not MICA mRNA levels, as markers	No
Lemy et al. (87)	The comparison between anti-MICA Abs ⁺ and anti-MICA Abs ⁻ patients showed that the incidence of AR episodes during the first year was similar in both groups. MICA Abs did not adversely affect renal graft outcomes	No
Li et al. (88)	Anti-MICA Abs were detected in 11 of the 15 transplant patients, irrespective of interval acute graft rejection. Also, integrative genomics predicted localization of the MICA antigen on the glomerulus in the kidney. MICA localization may explain both immunoregulatory and pathogenic roles for MICA after transplantation	Yes
Luo et al. (89)	HIF-1 α plays a very important role in upregulating MICA expression and enhancing natural killer (NK) cell cytotoxicity toward target cells during hypoxia/reoxygenation in HK-2 cells. Their results demonstrated that hypoxia/reoxygenation-promoted MICA expression on HK-2 cells is through a HIF-1 pathway	Yes
Cox et al. (90)	Anti-MICA and anti-HLA Abs significantly associated with AR and anti-MICA donor-specific antibodies (DSA) and anti-HLA DSA correlated with decreased graft function by univariate and multivariate analysis. The researchers concluded that mismatching for MICA epitopes in renal transplantation is a mechanism leading to production of MICA Abs that associate with AR and graft dysfunction	Yes
Narayan et al. (91)	Case report: this case demonstrated that donor-specific anti-MICA Abs could be associated with both acute antibody-mediated rejection (AMR) and type IIA acute cellular rejection and emphasized the necessity of treating both humoral and cellular components of the rejection	Yes
Yao et al. (92)	The authors proved that Anti-MICA Abs ⁺ rate was significantly higher in sensitized recipients and it had significant effect on the recovery of allograft function in early postoperative period. Protein A immunoabsorption plays an important role in decreasing preexisting Abs, especially the anti-MICA Abs	Yes

(Continued)

TABLE 4 | Continued

Reference	Summary	MICA biomarker
Zhang et al. (93)	Anti-MICA Abs were present in 28.9% of patients and they were associated with renal-allograft deterioration. The researchers concluded that, besides anti-HLA Abs, the presence of posttransplant anti-MICA Abs was associated with poor graft outcome and increased the risk of graft failure	Yes
Lemy et al. (94)	Anti-MICA Abs ⁺ patients were more frequently anti-HLA Abs sensitized and regrafted. Four-year death-censored graft survival was not different between MICA ⁺ and MICA ⁻ patients. These data did not support an independent pathogenic role for MICA in long-term renal graft injury	No
Li et al. (95)	The levels of the peak mean fluorescence intensity of MICA Abs in patients with impaired renal function were significantly higher than those in normal renal function controls. They also concluded that some MICA Abs might be more important than others in mediating graft rejection	Yes
Seyhun et al. (96)	Anti-HLA class II and anti-MICA Abs ⁺ were only important predictors of graft failure when present together with anti-HLA I Abs ⁺ . Patients who developed anti-HLA Abs alone or both anti-HLA Abs and anti-MICA Abs rejected their grafts more frequently than Abs ⁻ recipients	Yes
Rodriguez Ferrero et al. (97)	They compared patients with versus without preformed circulating antibodies (circulating anti-MICA Abs and anti-HLA Abs), and they did not observe a significant difference in graft survival or renal function at 3-month follow-up	No
Solgi et al. (98)	This research supported the idea that monitoring of anti-HLA and anti-MICA Abs as well as soluble CD30 levels early after transplant had predictive value for early and late allograft dysfunctions and the presence of these factors was detrimental to graft function and survival	Yes
Akgul et al. (99)	In this study, the scientist observed the role of anti-HLA II Abs in the development of chronic active AMR and in long-term allograft survival. It is observed that anti-MICA and anti-GSTT1 Abs showed no effect on rejection mechanisms	No
Chaudhuri et al. (100)	Anti-MICA and anti-HLA Abs appeared in approximately 25% of unsensitized pediatric patients, placing them at greater risk for acute and chronic rejection with accelerated loss of graft function	Yes
Ding et al. (101)	When comparing patients with acute graft rejection against recipients with stable renal functions, the researchers highlighted a significantly higher positivity rate of anti-MICA Abs. The status of anti-MICA Abs can predict the occurrence and treatment outcomes of AR, and affect the long-term survival of the renal grafts	Yes
He et al. (102)	By following transplantation recipients during follow-ups, anti-HLA and anti-MICA Abs expression was proven to have a predictive value for early and late allograft dysfunction. The presence of donor-specific Ab is detrimental to graft function and graft survival	Yes
Jin et al. (103)	They observed the prevalence of panel-reactive antibody (PRA) and anti-MICA Abs to be increased among Ptc, albeit not significantly different from C4d AR. These results implied that Ptc could be an early indicator of AR	Yes
Li et al. (104)	CD19 ⁺ B cells and CD19 ⁺ CD27 ⁺ memory B-cell subsets were detected from peripheral blood mononuclear cells obtained from six anti-MICA-sensitized kidney recipients. Kidney recipients had a higher percentage of CD19 ⁺ CD27 ⁺ B cells compared with healthy controls. This study thus showed that B cells may be stimulated to secrete Abs	Yes
Sanchez-Zapardiel et al. (105)	The researchers detected that pretransplantation sensitization against anti-MICA and anti-HLA Abs were independent events. Preformed anti-MICA Abs independently increase risk for kidney rejection and enhance the deleterious effect of PRA ⁺ status early after transplantation	Yes
Tonnerre et al. (106)	The researcher found that individual carrying <i>MICA A5.1/MICA A5.1</i> had 10-fold higher levels of <i>MICA</i> mRNA and MICA proteins at the endothelial cell surface. They also demonstrate a significant association between D/R MICA A5.1 mismatch and anti-MICA alloimmunization, particularly when donors carry the A5.1 mutation. They concluded that A5.1 mutation is an immunodominant factor and a potential risk factor for transplant survival	Yes
Zhang et al. (107)	5 years after transplantation, the frequencies of <i>de novo</i> anti-HLA and anti-MICA Abs were 25.8 and 12%, while 26.5% of patients had proteinuria. All of these factors have been associated with poor graft survival	Yes
Sapak et al. (108)	The researchers did not prove a complete correlation between the recipient anti-MICA Abs specificities and MICA antigens of the donor. They assumed that anti-MICA Ab induction occurred not only due to the allogeneic stimulation itself but also due to other factors that needed to be elucidated	No
Ming et al. (109)	Case report: the patient's HLA alloantibodies were not specific to the first kidney donor, but the MICA alloantibodies were. This indicates the importance of MICA virtual crossmatch in the process of selection for the kidney donor if the recipient is sensitized.	Yes
Xu et al. (110)	Serum anti-HLA II Abs, anti-MICA Abs, and anti-HLA plus MICA Abs all statistically increased in renal-transplanted recipients	Yes

(Continued)

TABLE 4 | Continued

Reference	Summary	MICA biomarker
Cai et al. (111)	Transplant recipients had Abs against denatured HLA class I, II, and MICA antigens. However, only C1q-fixing Abs were associated with graft failure, which was related to AMR	Yes (only for c1q-fixing denatured MICA Abs)
Sanchez-Zapardiel et al. (112)	Occasionally, preformed anti-MICA Abs may be cytotoxic by activating and fixing complement. This could lead to a reduced function in early kidney grafts	Yes
Relevant published works regarding NKG2D and transplant		
Feng et al. (113)	Ischemia/reperfusion injury (IRI) caused mRNA expression of Rae-1 and protein expression of Rae-1 in ischemic kidneys. This study suggested that the expression of the NKG2D ligand, Rae-1, may play a potential role in innate immunity associated with IRI	
Zheng et al. (114)	The absence of enhancement of NKG2D expression in the kidney in AN in immunodeficient mice suggested that the populations expressing NKG2D were likely to be CD8 or $\gamma\delta$ T cells, which were not present in the immunodeficient mice, rather than macrophages, which were present and activated in both models of AN	
Seiler et al. (62)	Unlike previous reports, in this paper, the researchers could not detect elevated MICA mRNA levels in kidney biopsies derived from patients undergoing AR or chronic allograft nephropathy. In contrast, they observed a strong mRNA induction of NKG2D during renal-allograft rejection, which could be verified by immunohistology in kidney biopsies	
Hadaya et al. (115)	The results of this paper have shown an expansion of the NKG2D ⁺ NK cell population during acute cytomegalovirus (CMV) infection (after kidney transplantation), which decreased over time to a level very similar to that of the control group. This suggests that the NKG2D receptor could play a similar role in NK and CD4 ⁺ T cells	
Zhang et al. (116)	In this study, the researchers demonstrated for the first time that NK cells could induce kidney TEC death <i>in vitro</i> and that NKG2D and Rae-1 interactions played a critical role in this killing in mice	
Shabir et al. (117)	Cytotoxic CD4 ⁺ CD28 ^{null} cell is an important biomarker for and potential mediator of adverse events after kidney transplantation. NKG2D represents an integral component of CMV immuno surveillance and immuno evasion and was upregulated on CD4 ⁺ CD27 ⁻ CD28 ^{null} cells isolated from patients of this study. The researchers proposed it as an important component of the cytotoxic effects (either protective or pathogenic) of these cells	

"Yes" and "No" labels have been used if, in the studies analyzed, MICA has been valued as a possible biomarker ("Yes") or not ("No").

present, while HIF-1 α has a unique O₂-dependent degradation domain, which leads to its degradation under normoxia conditions. The authors speculate that HIF-1 α upregulates the surface expression of MICA on grafts during renal IRI, causing NK cells cytotoxicity against the organ (89).

Possible Causes of End-stage Renal Disease

A 2009 study of the possible causes of end-stage renal disease (ESRD) (70), while note directly related to kidney transplants, inevitably reported findings of consequences for kidney transplantation. Peraldi et al. evaluated seven patients with ESRD that were treated with peritoneal dialysis, and not with the hemodialysis procedure; NKG2D expression on NK cells was significantly decreased in these patients compared to healthy donors, indicating that reduction in NKG2D expression was independent of the dialysis procedure and linked with chronic renal failure. The authors also discovered that oxidative stress in presence of increased ROS production is one of the most significant consequences of chronic renal failure, alone or in concert with other mediators, and it seems to decrease the NKG2D levels on NK cells in ESRD and to favor the upregulation of MICA expression (70).

Anti-MICA Abs and Rejection

Some mechanisms have been proposed for MIC-mediated organ rejection. MICA antigens expressed in the allograft could induce

the generation of anti-MICA Abs, which in turn might injure cells in the presence of complement.

This section contains no works that focus solely on NKG2D since most of the manuscripts are almost exclusively conserved with anti-MICA Abs: NKG2D is often just a side note; its presence and the link with MICA are given.

MICA-Sensitized Kidney Recipients and Higher Percentage of CD19⁺CD27⁺B Cells

CD19⁺CD27⁺ B cells are the subset of memory B cells that have the potential ability to secrete Abs. Li et al. (104) assessed the serum from 68 long-term survival kidney recipients and found 11 subjects who were MICA positive. They analyzed six MICA-sensitized kidney transplant recipients and six healthy volunteers who did not receive a transplant (control group). Healthy controls had a higher percentage of CD19⁺CD27⁻ in PBMCs than transplant patients, while the percentage of CD19⁺CD27⁺ in B cells was higher in transplant patients. The MICA-sensitized transplant patients had a significantly lower average percentage of CD19⁺ B cells in PBMC than healthy controls (3.58 ± 0.80 versus $8.53 \pm 1.04\%$; $P < 0.01$). These results suggest that CD19⁺CD27⁺ B cells from sensitized patients have the potential ability to secrete Abs. In the same study, PBMC cells were isolated and cultured and stimulated with different molecules [toll-like receptor-9 ligand ODN-2006 CpG, PMA, B-cell activating factor (BAFF), CD40 ligand (CD40L), human recombinant IL-2 (rhuIL-2), rhuIL-10,

rhuIL-4, rhuIL-21, CD40L, and BAFF] including MICA antigens. After stimulation, B cells from healthy controls and transplant patients had a lower percentage of apoptosis than non-stimulated cells. The average percentage of apoptosis cells from transplant patients was significantly higher than from healthy controls, and the IgM production (the first Ab produced by B cells after antigen stimulation) was higher in stimulated B cells from transplant patients than from healthy controls. The authors speculate that the B-cell population may be compromised by the transplant because patients are under immunosuppressive regimens, which may alter the apoptosis of B stimulated cells compared with healthy controls. The same study also performed an *in vitro* study with drugs and found that bortezomib and mycophenolic acid could inhibit B-cell Ab secretion (104).

MICA Abs

Hankey et al. (77) first reported that MICA and MICB expression on epithelial cells in transplanted kidneys and pancreases with histological evidence of rejection and cellular injury played a role in allograft rejection. The study showed that in a healthy kidney there was no immunochemical evidence of MIC expression. In contrast, the majority of biopsies with histologic proof of rejection or acute tubular necrosis (ATN) showed MICA positive staining of the tubular epithelium in the proximal and distal tubules. For this reason, it was concluded that alloantibodies against MICA might play a role in allograft rejection.

Zwirner et al. (76) found that several patients who had undergone a kidney transplant had specific Abs against MICA, and most of them were detected in serum samples collected at different times after organ rejection. However, these Abs were not directed against the alleles expressed by the patients, and it was speculated that if the presence of MICA Abs was probably caused by multiple blood transfusions received by the patients while awaiting a transplant, or resulting from a pregnancy or a previous transplant (76).

Lemy et al. (87) analyzed the MICA Abs from 494 controls and 597 patients with chronic kidney disease. They found a three times higher prevalence of MICA Abs in patients with chronic kidney disease when compared with controls (14.9 versus 4.7%). Nevertheless, they speculated that even if the increase in MICA Abs prevalence among patients affected by chronic kidney disease was probably related to previous renal transplantation and transfusions. Logistic regression analysis and analysis of chronic kidney disease patients who have not been subjected to transfusions and renal transplantations suggest that the increase of urea (and other nitrogenous waste) in the blood is connected to an increase of MICA immunization. The authors also reported that MICA Abs were more frequent in men than in women, despite pregnancy being an independent risk factor for the development of MICA Abs (87). This finding is in sharp contrast with other published work. The fact that nearly one-third of MICA chronic kidney disease stage V patients have never experienced any identifiable immunizing event indicates that there must be other causes for MICA sensitization. At the same time, one-fifth of the same patients showed the presence of autoreactive MICA IgG Abs, distinctly rare with respect to HLA Abs. The authors showed that patients with MICA Abs had a somewhat better overall graft

survival than MICA Abs⁻ patients. Finally, Lemy et al. found in MICA Abs⁺ and MICA Abs⁻ patients a similar incidence of AR episodes during the first year (10.2 versus 12.8%), as well as similar levels of proteinuria and creatinine (87).

Another study of MICA Abs screened 147 recipients with end-stage renal disease; 82 of these patients were Abs⁺ (55.8%). Forty patients had both anti-HLA and anti-MICA, 33 had only anti-HLA, and 9 only anti-MICA Abs in the posttransplant period. The authors found that patients who developed HLA alone, or both HLA and MICA Abs, rejected their grafts more frequently than Abs⁻ recipients. The rates of HLA class I, class II, or both Abs⁺ were greater in the rejection patients than the non-rejection patients ($P = 0.011, 0.037$, and 0.0275 , respectively). So the authors speculated that HLA class II and MICA Abs⁺ were the only important predictors of graft failure when both of them were present with HLA class I Abs⁺ (96).

In a retrospective study, Solgi et al. (98) analyzed sera samples of 40 living unrelated donor kidney recipients, looking at anti-HLA and anti-MICA Abs and the levels of soluble CD30 (sCD30) and sMICA. They found that patients with pre- and posttransplant HLA Abs had a higher incidence of AR episodes ($P = 0.01$ and $P = 0.02$), more graft loss ($P = 0.001$), and lower graft survival during a mean follow-up of 3 years. This group of patients also had higher levels of sCD30 and serum creatinine and decreased contents of sMICA early after transplantation, as compared to the patients without HLA Abs. Anti-MICA Abs were observed in 8/40 (20%) and 5/40 (12.5%) of all patients pre- and posttransplant, respectively. HLA and MICA Abs were both found in two out of four cases with graft loss. In a comparison of transplant rejecting to functioning graft groups, sCD30 levels increased at day 14 ($P = 0.001$), while sMICA levels were insignificantly lower in the first group (98).

Chaudhuri et al. (100) studied the evolution of humoral immunity in low-risk pediatric patients during the first 2 years after renal transplantation. They correlated the presence of serum anti-HLA DSA and serum MICA Abs with clinical outcomes and histology (the biopsies were performed at 0, 6, 12, and 24 months). They found anti-HLA Abs in 22% of patients, 6% of which were donor-specific, while 6% developed anti-MICA Abs. Three percent of patients developed *de novo* Abs to both HLA and MICA. The presence of *de novo* Abs was associated with significantly higher risks for AR ($P = 0.02$), chronic graft injury ($P = 0.02$), and decline in graft function ($P = 0.02$). Graft function was monitored by the difference between creatinine clearances. Anti-MICA and -HLA Abs were found in 25% of unsensitized pediatric patients. This was correlated with a greater risk of acute and chronic rejection (100).

Zhang et al. (107) associated the presence of *de novo* MICA Abs and proteinuria with graft failure, after renal transplantation. They investigated 275 patients without preexisting anti-HLA and -MICA Abs. Five years after renal transplantation, 25.8% showed *de novo* anti-HLA Abs, 12% showed *de novo* anti-MICA Abs, and 26.5% proteinuria. *De novo* anti-HLA Abs were associated with increased proteinuria after transplantation (relative risk, 3.12). Anti-HLA Abs and proteinuria were both associated with poor 5-year graft survival ($P = 0.027$ and $P = 0.006$, respectively). Patients with *de novo* anti-MICA Abs were also apt to have

proteinuria. The authors concluded that *de novo* anti-HLA and -MICA Abs and proteinuria are all associated with poor graft survival (107).

Pretransplant Panel-Reactive Abs and Preexistent Circulating Abs

Opelz (78) studied the influence of pretransplant panel-reactive antibody (PRA) status on the long-term outcome of kidney grafts from HLA-A, -B, and -DR, identical sibling donors. In over 10 years of follow-up, he discovered that non-HLA-directed immunity and Abs against HLA had a similar influence for the long-term results for kidney recipients with PRA. Opelz suggested that the targets for Abs causing late rejections could be the so-called minor histocompatibility antigens (78).

Sanchez-Zapardiel et al. (105) studied 727 transplanted patients and showed that the effect of anti-MICA Abs occurs independently of the presence of anti-HLA Abs. Patients were categorized into four groups according to the presence (+) or absence (-) of anti-HLA and anti-MICA Abs: HLA⁺MICA⁺ ($n = 27$); HLA⁻MICA⁻ ($n = 510$); HLA⁺MICA⁻ ($n = 165$), and HLA⁻MICA⁺ ($n = 25$). A notable difference was observed 3 months after transplantation, when HLA⁻MICA⁺ patients had a graft rejection rate of 8% compared with 2% in HLA⁻MICA⁻ patients. The patients were also grouped according to the presence of preexisting anti-HLA Abs, as measured by % PRA (PRA⁺ or PRA⁻): PRA⁺MICA⁺ ($n = 7$), PRA⁻MICA⁻ ($n = 610$), PRA⁺MICA⁻ ($n = 65$), and PRA⁻MICA⁺ ($n = 45$). The incidence of rejection was found to be superior in PRA⁺MICA⁻ cohort versus PRA⁻MICA⁻ patients (24 months after transplantation), but allograft rejection rate was the highest when comparing PRA⁺MICA⁺ patients with PRA⁻MICA⁻ patients 3 months after transplantation, a finding which was repeated at 6 months (105). This work is of interest because it performed a comparative study on the effects of anti-MICA and anti-HLA Abs on kidney transplants.

The Rodriguez Ferrero et al.'s (97) study included 22 recipients of kidney transplantations from deceased donors, and no differences between patients that showed preexistent circulating antibodies (CA) and those that did not were reported. In regards to the incidence of AR episodes, the only factor associated with CA was re-transplantation. So the authors concluded that CA monitoring is important for highly sensitized renal transplants, but they did not observe a difference in graft survival or renal function in the first 3-month follow-up (97).

Cd4 Deposition and C1q-Fixing Abs

A study of patients with acute antibody-mediated rejection (AAMR), who had MICA*008 Ab, showed that the presence of anti-MICA Abs and the deposition of C4d in biopsies performed at the time of AAMR was associated with the detection of DSA or Abs against HLA (80). The observation that the control group of 30 patients with long-term functioning grafts did not have anti-MICA*008 Abs provided indirect evidence of the importance of anti-MICA Abs in chronic rejection. Furthermore, all patients receiving an allograft fully matched at MICA had functioning grafts (80). It is also important to mention that MICA Abs are able to activate complement in *in vitro* experiments (80).

Alvarez-Marquez et al. (85) selected 58 patients that underwent a kidney biopsy because of primary non-function, delayed graft function or acute dysfunction of a previously functional graft, suspected by oliguria, increase of serum creatinine levels, or proteinuria. At the time of the transplant, all patients showed negative complement-dependent cytotoxicity crossmatches. Researchers demonstrated that 80% of a group of 19 patients with clinically evident graft dysfunction and with C4d deposition in kidney biopsies had Abs directed against donor-specific HLA class I, class II, MICA, or GSTT1 (glutathione-S-transferase T1) antigens (85).

In the Li et al.'s (88) study, a human ProtoArray platform was used to study 37 serum samples from 15 renal transplant patients (pediatric and young adult) with ($n = 10$) and without ($n = 5$) AR, and seven normal controls. To test serum Abs, they used a ProtoArray containing 5,056 non-redundant human proteins expressed in a baculovirus system, purified from insect cells and printed in duplicate onto a nitrocellulose-coated glass slide. Moreover, all patients were primary transplant recipients, and the biopsies were graded by the Banff classification. The authors found that the mean immune response signal in posttransplant patient serum showed an increase in anti-MICA Abs when compared with healthy normal controls ($n = 7$), but anti-MICA Abs signal intensity was unrelated to the sampling time interval post-transplantation. Mean MICA Abs signal intensity was higher in transplant patients with C4d⁺AR (121.4) versus C4d⁻AR (4.3), so a correlation between high MICA Abs levels and C4d⁺ graft rejection $r = 0.54$ ($P = 0.039$) was observed. On ProtoArray, each gene on the cDNA platform was compared between a specific kidney compartment versus all other compartments, by a two-unpaired class comparison and a multi-class comparison. The signal intensity of anti-MICA Abs ranked in the top 15 for glomerulus, so the MICA antigen was found to have a 2.7-fold higher expression in the glomerulus when compared to the other 6 normal kidney compartments. Cytoplasmic granular staining for MICA in normal and stable transplanted kidneys was observed solely in podocytes within glomeruli. In AR, in addition to the persisting glomerular staining, the infiltrating mononuclear lymphocytes also showed strong positive staining for MICA. So the authors demonstrated that Ab responses in patients are modulated by MICA after transplantation in patients, irrespective of graft rejection (88).

Another study correlates Cd4 deposition and creatinine levels. Ding et al. (101) evaluated serum anti-MICA Abs before and after kidney transplant, and they also examined PRA, serum creatinine, urine, graft ultrasound, lymphocyte subsets, and the pathology of graft biopsy. The study was split into two parts. In the first part, patients with AR were grouped into MICA⁺, MICA⁻ ($P < 0.05$) and control groups. There were a significantly higher number of anti-MICA Abs positive patients with acute graft rejection compared with stable renal functions patients (control group).

Two to three days after the occurrence of AR, the anti-MICA Abs level increased gradually. Anti-rejection treatment had no effect on anti-MICA Abs but lowered serum creatinine to a normal level. In the second part, the authors analyzed chronic graft rejection patients. The number of anti-MICA Ab positive patients was significantly higher than those with stable renal

function ($P < 0.05$), and the serum creatinine levels were significantly higher in MICA⁺ than in MICA⁻ cases ($P < 0.05$). The authors also found that graft biopsy of all MICA⁺ cases showed C4d deposition (101).

Jin et al. (103) studied 53 cases of AR that showed C4d deposition in the peritubular capillaries, 50 cases of ARs without C4d deposition, 30 with peritubular capillaries alone, 28 with ATN, and 78 patients with surveillance biopsies (control group). The authors observed that the prevalence of PRA and anti-MICA Abs was increased among the peritubular capillaries alone group (30.0 and 43.3%, respectively), albeit not significantly different from the group with C4d⁺ AR (49.1 and 39.6%, respectively). They also observed that the immunophenotype of infiltrating T lymphocytes and serum Abs (85.9% of control biopsies presented) had a regulatory phenotype while in the peritubular capillaries cohort, 93.3% of biopsies showed the cytotoxic phenotype. These results showed that peritubular capillaries in biopsy specimens from patients with early renal-allograft dysfunction could be an indicator of AR, especially acute humoral rejection (103).

Cai et al. (111) collected samples from 975 kidney transplant recipients, and they tested for C1q-fixing Abs against denatured HLA class I, class II, and MICA antigens. Among 169 patients who lost renal grafts, 44% had c1q-fixing Abs against denatured HLA/MICA antigens, which was significantly higher in patients with functioning renal transplants (25%). They concluded that C1q-fixing Abs were significantly associated with graft failure caused by AMR (72.73%) and they affirmed that only c1q-fixing Abs were associated with graft failure and AMR (111).

MICA Allele Epitopes and Eplets

Regarding the anti-MICA Abs, Duquesnoy et al. (118) developed an eplet-based version of the HLA-Matchmaker algorithm as a tool to assess the epitope specificity of these Abs. A repertoire of 38 potentially immunogenic *MICA* eplets was selected (based on *MICA* structure molecular viewing and the amino acid sequence differences between *MICA* alleles). These eplets are based on a functional epitope structure (a configuration of amino acids within a 3 Å radius of an Ab accessible polymorphic residue on the molecular surface). In this study, the eplet frequencies were calculated from *MICA* allele frequencies in 1,245 European-Americans and 605 African-Americans. Many eplets are shared by very similar groups of *MICA* alleles. For instance, the combination of eplets called CMGWS “supereplet” is composed by 36C, 129M, 206GW, and 215S epitopes and shared by the same group of *MICA* alleles (A*001, A*002, A*007, A*011, A*012, A*015, A*017, A*018, A*021, A*030, A*041, A*043, A*045, A*046, A*047, A*014, A*020, A*023, A*026, A*029, A*036, A*040, A*050, A*052, and A*055). The random chance that these eplets are a mismatch is 20.1% in African-Americans and 24.0% in European-Americans. Alternatively, the combination of eplets named AYVE “supereplet” is composed by 25AY, 129V, and 173E and was shared by another group of *MICA* alleles (A*004, A*006, A*008, A*009, A*010, A*016, A*019, A*024, and A*044). The random chance of their being a mismatch is 28.2% in African-Americans and 20.1% in European-Americans (118).

Panigrahi et al. (82, 119) analyzed the presence of Abs against MICA*001, MICA*002, MICA*004, MICA*008, and MICA*009

in serum samples of 185 patients transplanted with live related donor kidneys. Sixteen percent of all recipients developed anti-MICA Abs during the posttransplant period, 83% of the patients whose grafts eventually failed had both anti-HLA and anti-MICA Abs as compared to 29% patients who had only anti-MICA Abs, and 11% of those without any of the Abs (HLA or MICA) (82, 119).

Analysis of anti-MICA*001, MICA*002, MICA*004, MICA*008, and MICA*009 Abs in serum samples from 1,910 kidney recipients showed that a correlation between the presence of anti-MICA Abs and the reduced in kidney-allograft survival was not influenced by the simultaneous presence of Abs against HLA (120). In this study, decreased renal-allograft survival is associated with anti-MICA Abs formed before transplantation. It was also found that patients with Abs against MICA before transplantation did not receive more transfusions than patients without such Abs, in contrast with the Zwirner et al.'s study (76). So the authors speculate that cross-reactivity with substances from the environment may play a role in priming the immune system, facilitating anti-MICA Ab production (120).

Suarez-Alvarez et al. (84) screened 284 kidney transplant sera for anti-MICA Abs and mapped the epitopes of MICA by screening a library of synthetic overlapping peptides from the extracellular domains of the protein against the sera from kidney transplant patients with anti-MICA Abs. Anti-MICA Abs were detected in 50 of 284 patients (17.6%), and they correlated with the development of AR. The authors found that nine regions were reactive with anti-MICA Abs. Five epitopes were located in constant regions (II, III, IV, VI, and IX) and were present in all *MICA* alleles, while the other four regions (I, V, VII, and VIII) mapped to variable sites of polymorphic amino acids among the different alleles products of MICA. In particular, regions V, VII, and VIII were the regions with the highest amino acid variability. Three polymorphic residues, 173 (E/K), 175 (S/G), and 181 (R/T), had determined allele-specific epitopes. The aminoacid 208Y and 213T, instead, contributed in the cross-reactivity among alleles (84).

Cox et al. (90) identified MICA IgG Abs directed against MICA*001, *002, *004, *007, *008, *009, *012, *017, *018, *019, and *027. Analysis of 116 healthy control subjects revealed only one subject with anti-MICA Abs (0.9%) and five subjects (4%) with anti-HLA class II Abs, while in a subgroup of 227 transplant recipients and their donors the coproduction of Abs to HLA and MICA significantly associated with acute cellular rejection (ACR). Analysis of patients with AAMR established strong associations with the presence of Abs against HLA class I and II, but not anti-MICA. By aligning *MICA* allele profiles present in the subgroup of 227 renal graft recipients and their respective donors, it was possible to establish the precise position of amino acid mismatches that correlate strongly with MICA Ab production. Mismatching at residues 36, 129, 173, 175, 213, and 251 showed the strongest association with anti-MICA Ab production in transplant recipients, while 91, 125, 156, and 221 residues were also mismatched between recipients and donors, but were not significantly associated with anti-MICA Ab production. There are two immunodominant motifs: MICA-G1 is characterized by residues 36 cysteine (C), 129 methionine (M), 173 lysine (K), 206

glycine (G), 210 tryptophan (W), and 215 serine (S). Alternatively MICA-G2 epitopes share residues 36 tyrosine (Y), 129 valine (V), 173 glutamic acid (E), 206 serine (S), 210 arginine (R), and 215 threonine (T). The majority of these recipients (10 out of 17 individuals, 59%) developed *de novo* donor-specific anti-MICA Abs posttransplantation, and there was a significant association of graft dysfunction with the presence of anti-MICA DSA alone after 2 years. In conclusion, it was discovered that mismatching *MICA* alleles lead to the development of anti-MICA Abs in some renal graft recipients, and the presence of anti-MICA DSA was independently associated with decreased glomerular filtration rate (eGFR) and poorer graft outcome (90).

Tonnerre et al. (106) went beyond the usual studies of anti-MICA Abs and focused on searching for a specific allele that could lead to a poorer outcome. The authors performed a study that showed that the *MICA**008 (A5.1) molecule is a major antigenic determinant and target for recipient sensitization of kidney transplant patients. *MICA* A5.1 is associated with four alleles: *023, *028, *053, and *008. The authors divided primary EC cultures from transplant donors in *MICA* A5.1 homozygous, heterozygous, and control. The MICA surface expression was significantly higher on ECs from A5.1/A5.1 donors than from controls. The *MICA* A5.1 allele also leads to a reduction of sMICA and an increase in the MICA level in exosomes in ECs. Anti-MICA (A5.1) Abs intensities in the sera of recipients with anti-MICA Abs were not higher than intensities observed for other anti-MICA (control) Abs. However, when tested on EC cultures expressing physiologic levels of membrane-bound MICA, the sera only bound to ECs from *MICA* A5.1 donors. This seemed to show that anti-MICA Abs bind ECs' targets in an allele-specific manner.

In fact, the combination of the donor carrying *MICA* A5.1 and the recipient having a non-*MICA* A5.1 allele was overrepresented in the group of MICA-sensitized patients compared with the group of non-immunized recipients (106).

Sapak et al. (108) concluded that anti-MICA Abs could not be responsible for the rejection if they were not directly detected in the transplanted graft. In the sera of 124 renal recipients, the authors found only 22 patients positive for anti-MICA Abs. The most frequent anti-MICA Abs were directed against *MICA**018 and *MICA**001. *MICA**008 had the highest gene frequency (31%), followed by *MICA**002 (14%). Comparing *MICA* allele profiles of donors and anti-MICA Ab epitopes of their respective recipients, Sapak et al. found a match in only in 9 donor-recipient pairs (41%) while the sera of the other 13 patients was negative for Abs against graft MICA molecules, but positive for Abs against other MICA antigens. The majority (59%) of anti-MICA Abs in patients were not donor-specific, so the authors suggested that anti-MICA Ab induction was not caused by renal graft allogeneic stimulation but was also probably stimulated by other still unknown immune mechanisms (108).

Sanchez-Zapardiel et al. (112) studied 727 kidney recipients. They found that PRA⁺MICA⁺ recipients exhibited a longer time to reach optimal serum creatinine level after transplantation ($P = 0.005$) had the lowest eGFR at 3 months and PRA⁺MICA⁺ status independently increased the risk for chronic kidney disease stage 5 at month 3. Pretransplant anti-MICA Abs were

poly-specific; anti-AYVE supereplet reactivity was higher in HLA⁺MICA⁺ versus HLA⁻MICA⁺ patients and superior than anti-CMGWS supereplet within HLA⁺MICA⁺ patients. The authors also found that some preformed anti-MICA Abs might bind complement, using the C1q Luminex assay. Sanchez-Zapardiel et al. analyzed 13 anti-MICA⁺ pretransplant sera that were positive for the C1q binding assay and one of them (serum 3) exclusively recognized the AYVE supereplet with a strong reactivity against MICA*027 antigen. The authors concluded that these preformed anti-MICA Abs are able to mediate cell death by fixing and activating the complement cascade. So they speculated that the anti-MICA Abs might contribute to worse early kidney graft function (112).

Correlation between Anti-MICA Abs and Creatinine Levels or Estimated Glomerular Filtration Rate (eGFR) or Death-Censored Graft Survival (DCGSs)

Yao et al. (92) included 29 sensitized recipient patients who had undergone living-related donor renal transplantation between 2007 and 2009. They found a statistical difference in postoperative serum creatinine levels within 1 week between anti-MICA Ab-positive (135.4 ± 21.4 mol/L) and anti-MICA Ab-negative groups (108.6 ± 31.6 mol/L), but no significant difference between the two groups at discharge. To decrease the preexisting Abs (mainly IgG, IgM, and IgE), all recipients were treated with protein A immunoabsorptions, and this therapy was effective in decreasing anti-MICA Abs (92).

Zhang et al. (93) studied patients receiving primary kidney transplants (all from deceased donors) between 2004 and 2007. No significant association was found between the presence of anti-MICA and -HLA Abs, nor between the presence of anti-MICA Abs and 1-year graft survival rate. However, during the follow-up period, eGFR decreased $24.0 \pm 3.4\%$ in the anti-MICA Abs positive group, while it decreased only $8.4 \pm 3.0\%$ in anti-MICA Abs negative patients. A strong correlation between the production of anti-MICA Abs and renal impairment was also found. For these reasons, the authors concluded that patients with anti-MICA Abs had a more rapid deterioration of graft function, compared to those without anti-MICA Abs (93).

In another study that did not recognize MICA as a biomarker, sera from 779 kidney transplant recipients was tested with two single-antigen flow bead assays 1 year after transplantation. Thirteen of the 779 patients were lost to follow-up, 50 had lost their graft, and 33 died with a functioning graft. The prevalence of anti-MICA Abs was 5.3% at 1-year posttransplantation, and that MICA⁺ patients were more frequently HLA sensitized and regrafted. However, 4-year DCGSs were not different between MICA⁺ and MICA⁻ patients (97 versus 94%, $P = 0.28$), and 4- and 8-year survival rates were similar in MICA⁺ and MICA⁻ patients. Thus, the hypothesis of an independent pathogenic role for MICA in long-term renal graft injury was not supported, and the authors questioned the utility of monitoring anti-MICA Abs posttransplant with single-antigen flow bead assays (94).

MICA Abs in Case Study

Narayan et al.'s (91) case study focused on a 14-year-old girl with branchiooto renal syndrome who underwent re-transplantation

with an HLA crossmatch-negative deceased donor kidney. She lost her first kidney transplant to chronic rejection at the age of 10 and underwent allograft nephrectomy. She was highly sensitized, and to improve her chances for transplantation, she underwent desensitization with high-dose IVIG and rituximab. When she received a deceased donor renal transplant, the pretransplant anti-HLA Ab testing showed no anti-donor HLA Abs. The patient maintained good allograft function until postoperative day 10 when she presented with fever and anuric renal failure. The only Ab found was donor-specific anti-MICA Ab, specifically directed against MICA*012 protein. Evaluation of the pretransplant serum revealed preformed anti-MICA*012 Abs with levels that were elevated both before transplant and at the time of rejection. Anti-MICA Abs levels declined with the initiation of plasmapheresis and IVIG and correlated well with normalization of renal function and resolution of ACR and AMR. The authors speculated that the sensitization to the MICA*012 protein was caused by prior sensitization from the first renal transplant or previous infections or transfusions. The conclusion of their research is that donor-specific anti-MICA Abs can be associated with both AMR and Banff type IIA ACR and may require treatment with plasmapheresis (91).

Ming et al. (109) studied a patient who suffered early aggressive AMR in the presence of DSA against MICA after her first renal transplant. The researchers found that anti-MICA-DSA in recipient serum could bind MICA-G1 antigens expressed in the cultured human umbilical cord vein endothelial cells (HUVECs). The recipient serum was cytotoxic to these HUVECs, but not against HUVECs that did not express MICA-G1 antigens in the presence of complement. The researchers discovered that the patient had been sensitized to MICA antigens and HLA, before transplantation, and the HLA alloantibodies were not specific to the first kidney donor, but the MICA alloantibodies were. In light of this discovery, the second renal transplant was with a negative MICA virtual crossmatch, and it was successful (109).

microRNA and mRNA's Analysis

Seiler et al. (62) showed that an elevated NKG2D mRNA expression in biopsy material was correlated with the severity of AR and detected NKG2D⁺ cells located in clusters around tubules in biopsies derived from patients diagnosed with acute and chronic rejection. The expression of *NKG2D* mRNA was also detected in urinary sediments obtained 2–3 days before the AR episode. However, significant levels of MICA mRNA were not detected in the patient groups analyzed (62). For the first time, the focus was on the importance of the role of the NKG2D molecule, which is responsible for MICA signal transduction.

Another controversial paper regarding the role of MICA is the Racca et al.'s study (86), in which the authors obtained peripheral blood samples from 29 renal-transplanted patients (19 men). They classified patients into three groups: AR group (9 patients with acute grade I/II allograft rejection), chronic rejection group (10 patients with chronic allograft rejection), and stable evolution group (10 patients with clinically stable allograft evolution). The authors observed that MICA mRNA levels in peripheral blood mononuclear cells showed similar expression levels in all groups evaluated and in the control group. They also found similar levels

of MICA expression in a comparison of biopsy specimens from AR and nephrotoxic ATN patients. They did not find a correlation between MICA expression and renal graft state (86). It is interesting to note that the MICA expression in biopsies did not have a healthy control group, while expression of MICA mRNA may be a posttranscriptional control that modules MICA expression on the cell surface. The Racca et al.'s (86) study still represents an interesting opportunity to discuss the role of MICA as a biomarker.

Xu et al. (110) studied miR-338-5p, a microRNA downregulated in AMR renal allografts, and negatively correlated with BAFF. This molecule plays an important role in the differentiation, development, and proliferation of B lymphocytes. BAFF could be released in a soluble form (sBAFF) after cleavage and would bind to BAFF receptor. The receptor-associated factor 3 is a sort of adaptor for the BAFF-BAFF-R connection, it is implicated in a signal transduction, and it appeared to be a candidate target for miR-338-5p. In the study, 49 follow-up renal-transplanted recipients and a healthy control group were examined, and it was found that anti-HLA II Ab, anti-MICA Ab, and anti-HLA + MICA mixed Abs were all statistically increased in recipients. Serum miR-338-5p was significantly downregulated in renal-transplanted recipients compared with healthy volunteers and was inversely correlated with sBAFF. The authors speculate that miR-338-5p may regulate the BAFF signal, and they suggested that sBAFF was significantly negatively correlated with anti-MICA Abs (110).

Cytomegalovirus (CMV) and Polyomavirus and Transplantation

Cytomegalovirus infection is the most common viral complication after renal transplantation and solid organ transplantation in general. One hundred ninety-six recipients who underwent kidney transplantation during the past 6 years were assessed with at follow-up of at least 12 months. In this study, it was shown that the activating receptor NKG2D was expressed in a significantly higher number of NK cells at day 0 and day 20 compared to day 180 ($P = 0.01$ and $P = 0.003$, respectively) and compared to the control group ($P = 0.0003$ and $P = 0.0004$, respectively) (121). This finding suggests a possible mechanism for the activation of NKG2D that goes beyond organ rejection, but it is closely related. In fact, in the Hadaya et al. (121) study, it was shown that an expansion of the NKG2D⁺ NK cell population occurred during acute CMV infection which decreased over time to a level very similar to that of the control group.

An interesting study that involved NKG2D, performed by Shabir et al. (117), demonstrated that CD4⁺CD28^{null} T cell expansion is driven by latent CMV infection inflammation. The immune surveillance of CMV may have an unwanted consequence in the development of endothelial injury, which was proven to be mediated by CD4⁺CD27⁻CD28^{null} cells in *in vitro* experiments. NKG2D was upregulated on CD4⁺CD27⁻CD28^{null} cells isolated from patients in this study and might have an important component of the cytotoxic effects of these cells. In fact, CD4⁺CD28^{null} cells were found predominantly in CMV-seropositive patients, and expanded in the posttransplantation period, and expressed markers of cytotoxicity (NKG2D and perforin) and endothelial homing (CX3CR1). Isolated CD4⁺CD27⁻CD28^{null} cells

previously exposed only to CMV-derived antigens showed signs of endothelial damage and apoptosis, and this effect was mitigated by NKG2D-blocking Ab. They concluded that the increase in CD4⁺CD28^{null} cell frequencies was associated with delayed graft function and lower eGFR at end follow-up, and this could be mediated by NKG2D (117).

Another study by Tonnerre et al. (122) investigated the implication of MICA in BK polyomavirus (BKPyV) reactivation in a cohort of 144 transplant donor/recipient pairs including recipients with no reactivation (control). BKPyV is frequently reactivated in kidney transplant recipients receiving an immunosuppressive regimen and is associated with nephropathy (BKPyVAN) and graft rejection. They investigated the impact of the *MICA A5.1* mutation on recipient BKPyV reactivation, and they found that recipients carrying a *non-MICA A5.1* (*nA5.1*) genotype transplanted with a kidney from a donor carrying the *A5.1 MICA* variant had a lower risk of BKPyV reactivation ($P = 0.0148$). So they speculated that *MICA A5.1* could be a protective allele toward BKPyV infection (122). Interestingly, these researchers also found that the donor (*A5.1*)-recipient (*nA5.1*) combination was overrepresented in the group of MICA-sensitized patients, but in the latter, MICA *A5.1* seemed to be a protective factor for a virus related to graft rejection (106).

CONCLUSION

Since the *MICA* gene was first described, it has been the subject of many studies aiming to comprehend its immunobiology and the role it plays in fine-tuning the innate and adaptive immune response. MICA appears to be involved in transplant rejection, immune response against viruses and intracellular bacteria, inflammation, homeostasis of epithelia, immune response against tumors, and tumor immune escape mechanisms. However, there remain a number of open issues to be addressed surrounding MICA's functions and roles. Developing and implementing typing strategies for *MICA* alleles may increase the chance for positive outcomes in solid organ transplantation by allowing better matching. MICA's biological function is achieved through its interaction with the NKG2D receptor. This activating receptor and its ligands are deeply involved in the outcomes of transplanted grafts, in fact, the overexpression of NKG2DLs

could be involved in rejection episodes and can contribute to graft loss (44).

Various studies have shown that anti-MICA Abs, binding to MICA molecules expressed at the endothelial allograft cell surface, may have relevance to kidney transplantation outcome (81, 106). However, it is important to note that some studies, such as that of Lemy et al. (87), where the presence of anti-MICA Abs do not show adverse effects in renal graft outcomes (87). Also, *MICA* mRNA level analysis in blood mononuclear cells did not show a correlation between *MICA* expression and renal graft state (86). Seiler et al. (62) did not find an enhancement of mRNA expression levels of MICA in kidney biopsies from patients undergoing AR or chronic allograft nephropathy, but they observed increased *NKG2D* expression. In an interesting study performed by Sapak et al. (108), 41% of the detected anti-MICA Abs were donor-specific, but an astonishing 36% were anti-MICA Abs against self-MICA antigens and several patients (27%) produced both (108).

Regarding NKG2D, there are studies that report that it is possible to prolong graft survival and to prevent CD28-independent rejection of cardiac allografts after blocking NKG2D (123).

We can conclude that the role of MICA and NKG2D in transplant outcome is not yet clear; MICA-mediated rejection probably is not just a reaction to the MICA non-self protein. The stress condition following a transplant causes a general inflammatory status in the recipient. This could increase MICA production, thus activating the response *via* the NKG2D receptor. The clinical impact of these interactions will remain unclear until further studies are performed.

AUTHOR CONTRIBUTIONS

MR: planning and organizing structure of the review; research and analysis of the papers; wrote the review; and planning and creation of figures and tables. MB: planning and organizing structure of the review and contributions to the sections writing/critical review of the manuscript.

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Elusive Role of the CD94/NKG2C NK Cell Receptor in the Response to Cytomegalovirus: Novel Experimental Observations in a Reporter Cell System

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Human cytomegalovirus (HCMV) infection promotes the differentiation and persistent expansion of a mature NK cell subset, which displays high surface levels of the activating CD94/NKG2C NK cell receptor, together with additional distinctive phenotypic and functional features. The mechanisms underlying the development of adaptive NK cells remain uncertain but some observations support the involvement of a cognate interaction of CD94/NKG2C with ligand(s) displayed by HCMV-infected cells. To approach this issue, the heterodimer and its adaptor (DAP12) were expressed in the human Jurkat leukemia T cell line; signaling was detected by transfection of a reporter plasmid encoding for Luciferase (Luc) under NFAT/AP1-dependent control. Engagement of the receptor by solid-phase bound CD94- or NKG2C-specific monoclonal antibodies (mAbs) triggered Luc expression. Moreover, reporter activation was detectable upon interaction with HLA-E+ 721.221 (.221-AEH) cells, as well as with 721.221 cells incubated with synthetic peptides, which stabilized surface expression of endogenous HLA-E; the response was specifically antagonized by soluble NKG2C- and HLA-E-specific mAbs. By contrast, activation of Jurkat-NKG2C+ was undetectable upon interaction with Human Fetal Foreskin Fibroblasts (HFFF) infected with HCMV laboratory strains (i.e., AD169, Towne), regardless of their differential ability to preserve surface HLA-E expression. On the other hand, infection with two clinical isolates or with the endotheliotropic TB40/E strain triggered Jurkat-NKG2C+ activation; yet, this response was not inhibited by blocking mAbs and was independent of CD94/NKG2C expression. The results are discussed in the framework of previous observations supporting the hypothetical existence of specific ligand(s) for CD94/NKG2C in HCMV-infected cells.

Keywords: human, natural killer cell, cytomegalovirus, CD94, NKG2C, HLA-E, UL40

INTRODUCTION

Inhibitory receptors specific for MHC class I molecules with immunoreceptor tyrosine-based inhibitory motifs play a key role in preventing NK cell responses against normal autologous cells. This function is mainly fulfilled by members of the human killer-cell immunoglobulin-like receptor (KIR) family, which recognize sets of classical HLA class I (HLA-I) molecules, and by the CD94/NKG2A lectin-like heterodimer specific for HLA-E. Conversely, other KIRs and CD94/NKG2C, which display a lower affinity for HLA-I ligands trigger protein tyrosine kinase pathways through DAP12, an adaptor with immunoreceptor tyrosine-based activation motifs. Similar inhibitory and activating NK cell receptors (NKR) have been identified among the murine Ly49 and NKG2 lectin-like receptor families (1, 2). The hypothesis that MHC-specific activating NKR may contribute to the innate response against pathogens was supported by the evidence that Ly49H specifically interacts with the MHC class I-related murine cytomegalovirus glycoprotein m157, triggering NK cell effector functions and the development of a memory-like response that confers resistance against the viral infection in some mice strains (3–5).

With this remarkable exception, no formal proof has been thus far obtained supporting the involvement of other activating KIR, NKG2, or Ly49 receptors in direct recognition of pathogen molecules (6). In this regard, human cytomegalovirus (HCMV) infection has been shown to promote the differentiation and persistent expansion of a mature NK cell subset, which displays high surface levels of the activating CD94/NKG2C NKR ($\text{NKG2C}^{\text{bright}}$), together with additional distinctive phenotypic and functional features (7–12). The magnitude of such adaptive NK cell subset redistribution appears variable in healthy blood donors, being undetectable in some HCMV+ individuals. This NK cell response pattern has been as well observed following active HCMV infection in newborns and immunocompromised patients (13–17). Although expansions of NKG2C+ NK cells have been reported in the context of other infections (18–21), the effect appears restricted to individuals coinfecting by HCMV, thus suggesting that it is specifically induced by this herpes virus, being potentially amplified along the immune response to other pathogens.

The mechanisms underlying differentiation and expansion of $\text{NKG2C}^{\text{bright}}$ NK cells remain uncertain. Engagement of CD94/NKG2C by specific monoclonal antibodies (mAbs) or HLA-E, expressed in the 721.221 (.221) HLA-I defective cell line, triggered NKG2C+ NK-cell effector functions and proliferation in response to IL-2 or IL-15, strongly suggesting that the receptor might play a direct role in the response to HCMV infection (22, 23). *In vitro* proliferation of NKG2C+ cells was observed coculturing PBMCs or purified NK cells from some HCMV+ donors with HCMV-infected fibroblasts. The response required the participation of cytokines (i.e., IL-12, IL-15) and was antagonized by anti-CD94 (22), -NKG2C, or -HLA-E mAbs (23). These observations supported the hypothesis of an instructive process driven by a cognate interaction of the CD94/NKG2C receptor with ligand(s) displayed by HCMV-infected cells (24). Paradoxically, no formal evidence has been obtained supporting

an active role of the CD94/NKG2C receptor in triggering *in vitro* NK cell effector functions against HCMV-infected cells, suggesting that NKG2C-mediated NK cell activation might be hampered by viral immune evasion mechanism(s) (25). By contrast, antibody-dependent stimulation *via* CD16 (Fc γ R-III) efficiently activates adaptive NKG2C+ NK cells to mediate specific cytotoxicity, cytokine production, and proliferation in response to HCMV- and other virus-infected cells (26–29). CD2 has been shown to play an important co-stimulatory role in antibody-dependent activation of NKG2C+ cells (30, 31). Recently, increased baseline proportions of adaptive NKG2C+ NK cells in kidney transplant recipients have been directly related with a reduced incidence of posttransplant HCMV infection (32), suggesting that they may play a role in antiviral defense, involving CD94/NKG2C and/or CD16-dependent activation (33).

Previous reports revealed that binding of HLA-E to a peptide from the HCMV UL40 leader sequence preserves its expression in infected cells, engaging the CD94/NKG2A inhibitory receptor (34, 35). On the other hand, viral MHC class I-modulating molecules (i.e., US2-US11) were shown to play a prevalent role in governing the response of NK cells against infected targets (36).

In the present study, we approached the identification of putative ligand(s) for CD94/NKG2C in HCMV-infected cells, reducing the complexity of NK cell-infected target interactions. To this end, both receptor subunits and DAP12 were stably expressed in the human Jurkat leukemia T cell line. Signaling was detected by transient transfection of a reporter plasmid encoding for Luciferase (Luc) under NFAT/AP1-dependent control. Our results are discussed in the hypothetical framework on the development of adaptive NKG2C+ cells in response to HCMV.

MATERIALS AND METHODS

mAbs and Flow Cytometry Analysis

Flow cytometry was performed using mAbs specific for the following surface molecules: anti-NKG2C-PE (clone 134591) R&D Systems (Minneapolis, MN, USA), anti-HLA-I-APC (clone HP-1F7) generated in our laboratory and conjugated by Immunostep (Salamanca, Spain). The following indirect antibodies were used as purified or culture supernatants: anti-HLA-E (clone 3D12) provided by Dr. D. E. Geraghty (Fred Hutchinson Cancer Research Centre, Seattle, WA, USA), anti-CD3 (clone SpvT3B); anti-NKG2A (clone Z199), anti-NKG2D (clone BAT221), anti-NKp46 (clone Bab281), anti-NKp30 (clone AZ20), anti-DNAM1 (clone F22), anti-CD16 (KD1) provided by Dr. A. Moretta (University of Genova), and Dr. D. Pende (National Institute for Cancer Research, Genova); anti-LFA1 (clone TS/18), anti-ICAM1 (clone HU5/3) provided by Dr. F. Sánchez-Madrid (Hospital Univ. de la Princesa, Madrid); anti-KIR3DL1 (clone DX9) provided by Dr. L. Lanier (University of California San Francisco, CA, USA); anti-KIR2DL2/S2/L3 (clone CH-L) provided by Dr. S. Ferrini (National Institute for Cancer Research, Genova, Italy); anti-KIR3DL1/3DL2/2DS4/2DS5/2DS2/3DS1 (clone 5.133), provided by Dr. M. Colonna (University of Saint

Louis, MO, USA). Anti-CD94(clone HP-3B1), anti-ILT2(LILRB1, LIR1) (clone HP-F1), anti-CD2 (clone MAR206), anti-KIR2DL1 (clone HP-DM1), anti-KIR2DL1/2DS1/2DS3/2DS5 (clone HP-MA4), anti-KIR2DL5 (clone UP-R1), and anti-KIR2DL1/S1/S4 (clone HP-3E4) were produced in our laboratory.

Briefly, cells were pretreated with human IgG (10 µg/ml) to block Fc receptors, incubated with individual NKR-specific mAbs for 30 min, washed, and further incubated with a secondary PE-tagged F(ab')2 rabbit anti-mouse Ig (The Jackson Immunoresearch, West Grove PA, USA); anti-myc mAb (9E10, IgG1) was used as negative control. Data were acquired on FACSCalibur flow cytometer (BD Biosciences) and processed using FlowJo software (TreeStar, OR, USA).

Cell Lines and Culture Conditions

The Jurkat leukemia T cell line and its transfectants were grown in RPMI-1640 medium (Gibco, Grand Island, New York, NY, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco), termed as complete medium. Jurkat-CD94+ cells were kindly provided by Dr. Lewis Lanier, obtained as previously described (37) and cultured in complete medium with G418 (1 mg/ml) (InvivoGen, San Diego, CA, USA).

The 721.221 (.221) HLA-I-deficient EBV-transformed B lymphoblastoid cell line and its transfectant .221-AEH (kindly provided by Dr. D. E. Geraghty, Fred Hutchinson Cancer Research Centre, Seattle, WA, USA) were cultured in RPMI-1640 complete medium .221-AEH cells were generated by stable transfection of .221 cells with a construct in which the leader sequence of the HLA-E*0101 allele was replaced by that of HLA-A2 and were selected in the presence of 300 µg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) (38).

Synthetic leader sequence peptides from HLA-G (VMAPRTLFL) or the AD169 UL40 viral protein (VMAPRTLIL) were purchased from CRG-UPF proteomic core facility (Parc de Recerca Biomèdica de Barcelona, Spain). As described (39), to stabilize HLA-E surface expression, HLA-Ia-defective 721.221 cells were incubated overnight with peptides (10 mM) at 26°C; HLA-E surface expression was monitored before and after incubation with peptides by flow cytometry.

Human Fetal Foreskin Fibroblast (HFFF) cells provided by Prof. John Trowsdale (University of Cambridge, UK), and the human lung fibroblast cell line MRC-5 provided by Dr. A. Angulo, were maintained in Dulbecco modified essential medium (DMEM) (Gibco) supplemented with 10% FBS, penicillin, and streptomycin.

Generation of Jurkat-NKG2C+ Reporter Cells

Jurkat-NKG2C+ cells were established using a retroviral expression system to stably express DAP12 and NKG2C proteins in Jurkat-CD94+ cells. DAP12 and NKG2C cDNA constructs were subcloned from pJFE14 expression vector to pBABE-puro retroviral vector using XbaI-EcoRI (DAP12) and BamHI-EcoRI (NKG2C) restriction sites. As described (40), the retroviral constructs were individually transfected using the non-modified

polyethyleneimine reagent (PEI, Sigma-Aldrich, St. Louis, MO, USA) into the helper-virus free amphotropic producer cell line Phoenix-A, a derivative of the human embryonic kidney cell line 293T (provided by Dr. Ramon Gimeno, IMIM, Barcelona, Spain). At 48, 72, and 96 h post-transfection, supernatants containing retroviral particles of DAP12 and NKG2C were collected, filtered with 45 µm filter (Millipore, Billerica, MA, USA), and centrifuged with Beckman SW28 rotor at 25,000 rpm for 90 min at 4°C. Pelleted virus were resuspended in 1 ml of RPMI medium and used to transduce Jurkat-CD94+ cells. To this end, 500,000 cells were plated (48 well/plates) in 1 ml mixed concentrated retroviral medium (0.5 ml DAP12 and 0.5 ml NKG2C) in the presence of 8 µg/ml polybrene (Sigma-Aldrich) and spun for 90 min at 930 g. Cells were supplemented with fresh medium at 6 h post-transduction and selected with 1.5 µg/ml puromycin (Sigma-Aldrich) for 48 h; subsequently, cells positive for NKG2C surface expression were sorted (Influx Cell sorter, BD Bioscience), cloned by limiting dilution and expanded.

HCMV Preparations and Infection of Human Fetal Foreskin Fibroblasts (HFFF)

This work was carried out in an authorized UPF p2-level biohazard facility, in compliance with the official requirements for CMV manipulation. Stocks of concentrated HCMV strains AD169 and Towne, provided by Dr. A. Angulo, TB40/E, provided by Dr. C. Sinzger (Institute for Medical Virology, University of Tübingen), and two HCMV clinical isolates: UL1271 (41) and #119, provided by Dr. H. Hengel, were prepared as follows. Almost confluent MRC-5 fibroblasts were infected at low multiplicity of infection (MOI) and supernatants were recovered when maximum cytopathic effect was reached (7–10 days) followed by clearing of cellular debris by centrifugation at 1,750 g for 10 min (42). Thereafter, the virus was concentrated for 3 h by centrifugation at 29,000 g at 15°C. Pelleted virus was resuspended in serum-free Dulbecco medium, stored at –80°C, and titrated by standard plaque assays.

Human fetal foreskin fibroblasts were seeded in 48-well plates 2 days prior infection at 4×10^4 cells/well. Confluent cells were incubated alone (mock) or with different viral strains at MOI of 10. After 2 h of absorption at 37°C, cells were washed twice with PBS and then fresh DMEM medium was added. Depending on the experimental design, HFFF cells were washed again at 24, 48, or 72 h postinfection. The infection rate was assessed by monitoring expression of the IE1 protein by indirect immunofluorescence with mAb MAB810R (clone 8B1.2) (Millipore) and Alexa Fluor 488-Labeled F(ab')₂ goat anti-mouse secondary (Invitrogen, Carlsbad, CA, USA). Alternatively, infected cells were indirectly identified assessing down-modulation of surface HLA-I expression at 72 h postinfection.

Preparation and Activation of CD94-NKG2C+ Reporter Cells

Human cytomegalovirus-infected HFFs were cocultured with Jurkat-NKG2C+ cells previously transfected with a reporter plasmid encoding Luciferase (Luc) under the control of NFAT/AP1 promoter (3X NFAT/AP1-Luc) generated as described (43)

and provided by Dr. Jose Aramburu (Universitat Pompeu Fabra, Barcelona, Spain). Transfection of Jurkat cells was carried out using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA) following the protocol provided by the manufacturer. Luc-transfected Jurkat cells were cocultured with the different targets for 18–24 h. After coculture, cells were collected, lysed, and Luc activity was measured using Promega Luciferase Assay system (Promega, Madison, WI, USA). The data were normalized referring the specific luminescence counts to those of non-treated (NT) Jurkat-NKG2C+ cells and are represented as fold-change induction. Stimulation of Jurkat with plate-bound anti-CD3, -CD94, -NKG2C, or co-culture with 721.221 and .221-AEH cells were used as controls. To verify the involvement of NFAT/AP1 in the reporter activation, experiments were carried out in the presence of 1 μ M FK506 calcineurin inhibitor (Sigma-Aldrich), pretreating Jurkat-NKG2C+ cells with the drug for 2 h. For antibody-blocking assays, Jurkat-NKG2C+ cells or infected HFFF cells were, respectively, preincubated for 2 h with anti-NKG2C (clone 134522, R&D Systems) or anti-HLA-E (clone 3D12) mAbs (5 μ g/ml) prior to coculture; anti-CD94 F(ab')₂ fragments obtained from the HP-3B1 clone (37) were employed in some experiments.

Statistical Analysis

Jurkat-NKG2C+ cell activation, assessed by induction of Luc activity in response to control stimuli (i.e., anti-NKG2C or .221-AEH cells) in different experiments ($n = 15$), was verified to follow a normal distribution applying the conventional Shapiro-Wilk test. Statistical analysis of the results was carried out applying the Student's *t*-test.

RESULTS

Generation and Phenotypic Characterization of a Human CD94/NKG2C+ Reporter T Cell Line

To study the role of the CD94/NKG2C NKR in recognition of HCMV-infected cells, a reporter cell system was developed expressing the receptor segregated from other NKR. For this purpose, NKG2C and DAP12 were stably transduced in an available CD94-transfected human Jurkat leukemia T cell line. Jurkat-NKG2C+ cells were sorted and cloned by limiting dilution assessing the expression of adhesion molecules and NKR. A clone (97), which expressed CD94/NKG2C was selected (Figure 1A). These cells had downregulated CD3, displayed adhesion/co-stimulatory molecules (i.e., LFA-1, CD2, ICAM-1, and DNAM-1), but lacked activating (i.e., NKG2D, NKp46, NKp44, NKp30, CD16, aKIR) and inhibitory (i.e., iKIR, NKG2A, TIGIT, ILT2) NKR (Figure 1B). The marginal expression of ILT2 was particularly important as this HLA-I specific inhibitory receptor, which interacts with the UL18 HCMV molecules, was detectable in the parental Jurkat-CD94+ cells (not shown).

Specific Recognition of HLA-E by Jurkat-NKG2C+ Reporter Cells

In order to detect signaling by the CD94/NKG2C-DAP12 complex, Jurkat-NKG2C+ cells were transiently transfected with

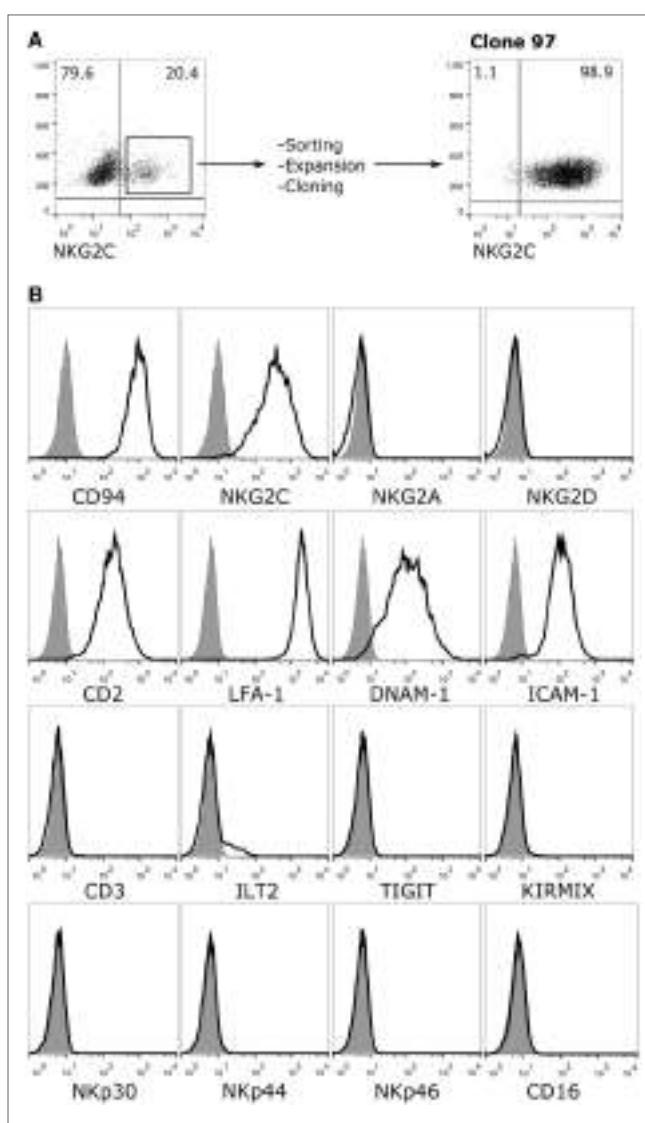


FIGURE 1 | Phenotypic characterization of Jurkat-NKG2C+ reporter cells. **(A)** Jurkat-CD94+ cells were transduced with retroviral particles carrying the sequences encoding for NKG2C and DAP12 as described in Section “Materials and Methods.” After sorting, expansion, and limiting dilution, clone 97, which expressed high levels of the CD94/NKG2C heterodimer was selected. **(B)** Jurkat-NKG2C+ cells were stained for the indicated receptors and analyzed by flow cytometry. KIR MIX corresponds to a mixture of the following monoclonal antibodies: HP-MA4 (KIR2DL1/2DS1/2DS3/2DS5), CHL (KIR2DL2/2DL3/2DS2), 5.133 (KIR3DL1/3DL2/2DS4/2DS5/2DS2/3DS1), DX9 (KIR3DL1), UP-R1 (KIR2DL5). Gray histograms correspond to the isotype control.

a plasmid encoding for Luciferase (Luc) under the control of NFAT/AP1-dependent promoter. Engagement of the receptor by solid-phase bound CD94- or NKG2C-specific mAbs triggered Luc expression (Figure 2A). Of note, a slightly higher background in Jurkat-NKG2C+ cells compared to the parental cell line suggested that the low constitutive HLA-E expression in Jurkat-NKG2C+ cells promoted a limited self-activation of the reporter (Figure S1 in Supplementary material). However, coculture of Jurkat-NKG2C+ cells with the .221-AEH cell line,

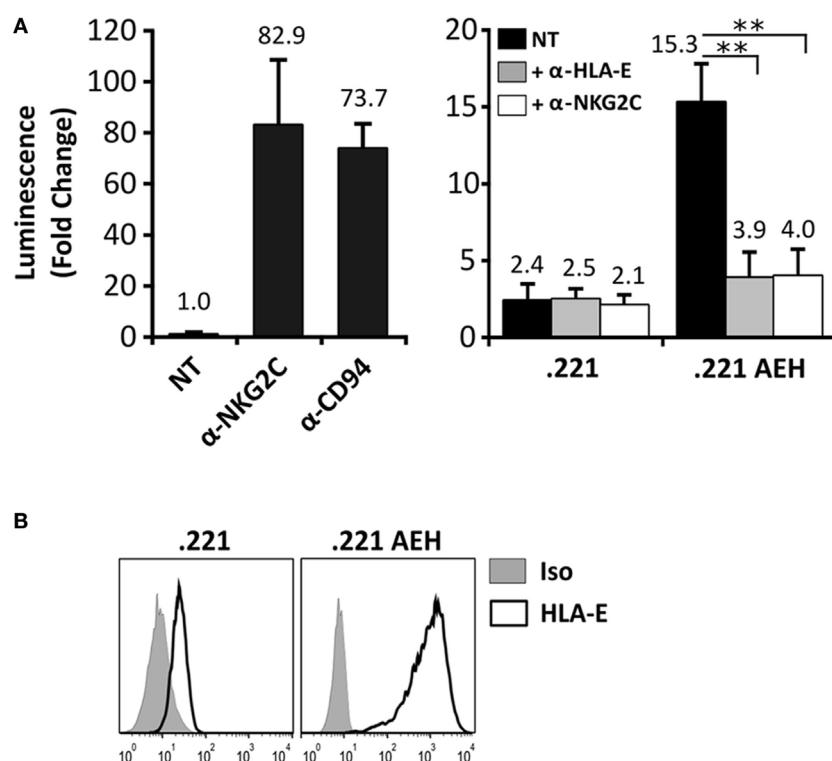


FIGURE 2 | Specific engagement of the CD94/NKG2C receptor by HLA-E activates the Jurkat-NKG2C+ reporter. **(A)** Jurkat-NKG2C+ cells were electroporated with 3x NFAT/AP1-Luc plasmid, followed by stimulation with: (a) anti-NKG2C or anti-CD94 monoclonal antibodies (mAbs) pre-adsorbed to culture plates or (b) .221 or .221-AEH cells. After 18–24 h, cells were collected, lysed, and Luc activity was measured. Blocking experiments were carried out pretreating .221 and .221-AEH cells with anti-HLA-E 3D12 mAb (gray bars) or Jurkat-NKG2C+ cells with anti-NKG2C mAb (white bars); NT, not treated. Data correspond to three independent experiments (mean \pm SD). **(B)** HLA-E expression by .221 and .221-AEH was assessed prior to coculture with Jurkat-NKG2C+ cells (gray histograms represent the isotype control). Statistically significant differences are indicated (** $p < 0.01$).

which displays HLA-E in the absence of classical HLA-I, induced Luc expression and the response was specifically antagonized by soluble NKG2C- and HLA-E-specific mAbs (Figures 2A,B). Similar results were obtained stimulating Jurkat-NKG2C+ cells with the .221 cell line preincubated with synthetic HLA-I leader sequence peptides, known to stabilize surface HLA-E expression (Figures 3A,B). These results validated the sensitivity and specificity of the reporter system to detect CD94/NKG2C receptor-ligand interaction.

Response of Jurkat-NKG2C+ Reporter Cells to Fibroblasts Infected by HCMV Laboratory Strains

On that ground, experiments were carried out incubating Jurkat-NKG2C+ cells with HFFF, infected at different time-points (24–72 h) with HCMV laboratory strains (AD169 and Towne). As compared to positive controls stimulated with anti-NKG2C mAb or .221-AEH cells, no differences were perceived comparing Luc expression in Jurkat-NKG2C+ cocultured either with mock- or HCMV-infected HFFF (Figure 4A); similar results were obtained incubating the reporter with MRC5 fibroblasts (not shown). Total HLA-I expression was downregulated in cells infected by both HCMV strains. By contrast, HLA-E was

preserved by AD169, which displays a canonical UL40 leader peptide (VMAPRTLIL) binding to the HLA class Ib molecule, but was lost in Towne-infected cells (Figure 4B). As reported (44) and according to the annotated GenBank sequences, this HCMV strain contains a deletion spanning the UL40 leader sequence. These results point out that the undetectable response of Jurkat-NKG2C+ to fibroblasts infected by laboratory strains was unrelated to their ability to sustain surface HLA-E expression.

Response of Jurkat-NKG2C+ Reporter Cells to Fibroblasts Infected by HCMV Clinical Isolates

TB40/E HCMV strain retains the ability to replicate in endothelial and myelomonocytic cells, differing from AD169 and Towne (45). Moreover, it is well established that HCMV clinical isolates substantially diverge from laboratory strains, rapidly undergoing important genomic changes upon *in vitro* passage, as reviewed in Ref. (46). Thus, additional experiments were carried out coculturing Jurkat-NKG2C+ cells with HFFF infected with TB40/E, which encodes for a UL40 leader peptide variant unable to preserve HLA-E surface expression (VVAPRTLIL) (25), or with two HCMV clinical isolates

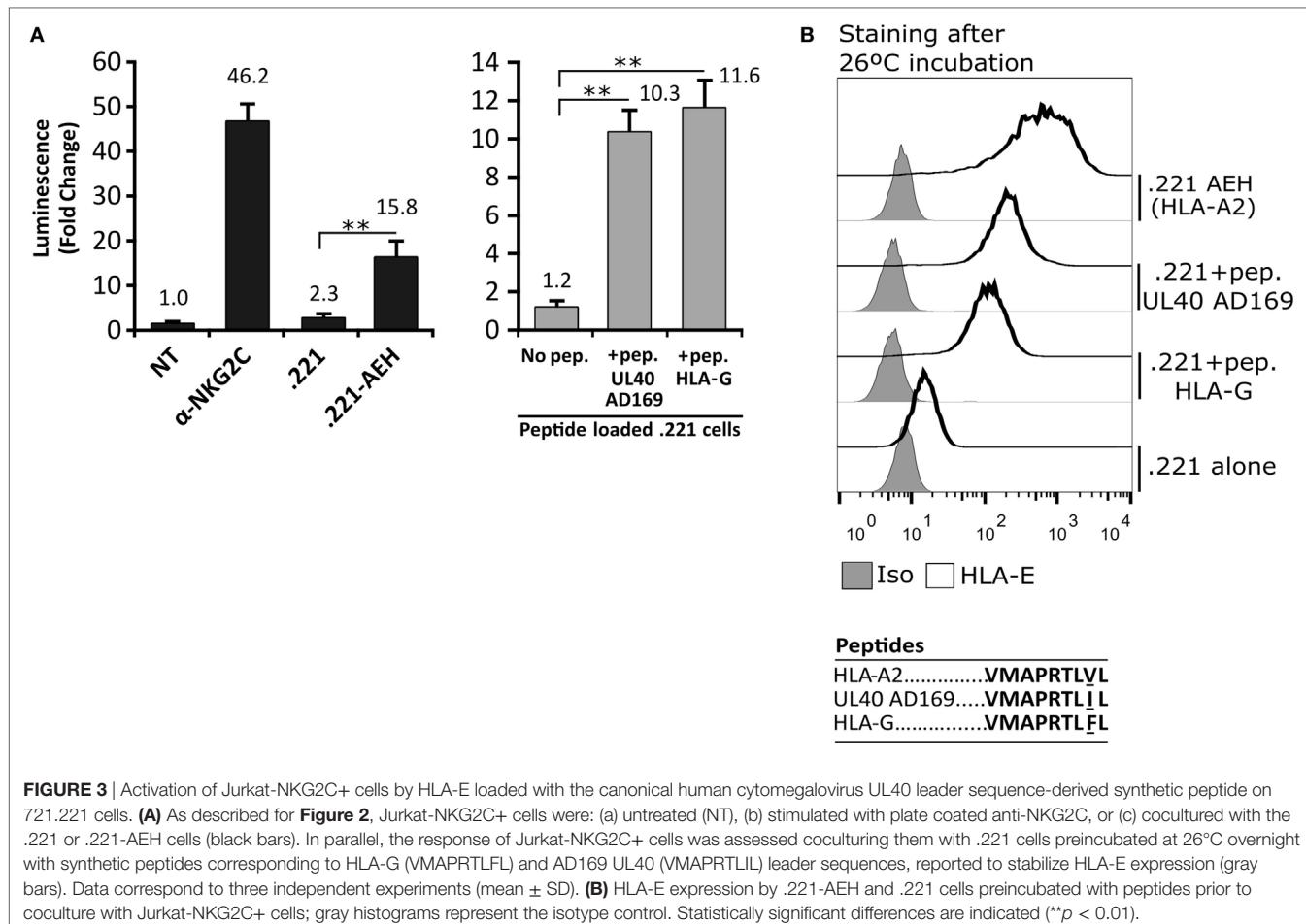


FIGURE 3 | Activation of Jurkat-NKG2C+ cells by HLA-E loaded with the canonical human cytomegalovirus UL40 leader sequence-derived synthetic peptide on .221 cells. **(A)** As described for **Figure 2**, Jurkat-NKG2C+ cells were: (a) untreated (NT), (b) stimulated with plate coated anti-NKG2C, or (c) cocultured with the .221 or .221-AEH cells (black bars). In parallel, the response of Jurkat-NKG2C+ cells was assessed coculturing them with .221 cells preincubated at 26°C overnight with synthetic peptides corresponding to HLA-G (VMAPRTLFL) and AD169 UL40 (VMAPRTLIL) leader sequences, reported to stabilize HLA-E expression (gray bars). Data correspond to three independent experiments (mean \pm SD). **(B)** HLA-E expression by .221-AEH and .221 cells preincubated with peptides prior to coculture with Jurkat-NKG2C+ cells; gray histograms represent the isotype control. Statistically significant differences are indicated (** $p < 0.01$).

(#UL1271 and #119), which share the canonical UL40-nanomer and preserved HLA-E expression (**Figures 5A,B**). Remarkably, a specific response of the reporter was detected upon infection with these viruses, which appeared greater for #119 and gradually increased along postinfection time. Yet, controls indicated that Luc expression in response to HFFF infected with HCMV clinical isolates was not inhibited by pretreatment with anti-NKG2C or -HLA-E mAbs (**Figures 6A,B**); similar results were obtained with anti-CD94 F(ab')₂ fragments (data not shown), ruling out the potential influence of an overlapping agonistic effect mediated by anti-NKG2C bound to virus-encoded FcR. Moreover, the effect was also induced in CD94+ and wild-type Jurkat cells, which lacked NKG2C, unequivocally revealing that reporter activation was independent of this NKR (**Figure 6C**); similar results were obtained with TB40/E-infected cells (data not shown). As this response was dominant, the possibility that it might eventually mask a subtle involvement of CD94/NKG2C cannot be entirely ruled out.

DISCUSSION

A number of observations indirectly support the hypothesis that a specific and direct interaction of CD94/NKG2C with infected cells contributes to drive the adaptive NK cell response; yet,

molecular evidence remains thus far elusive (10). To explore the presence of putative ligand(s) for CD94/NKG2C in HCMV-infected cells, the receptor was stably expressed along with DAP12 in the human Jurkat leukemia cell line, transiently transfected with an NFAT/AP1-dependent Luc-encoding reporter. The rationale for using a human parental cell line was based on our previous experience with heterologous CD94/NKG2C+ rat basophilic leukemia cells (RBL), which failed to respond to receptor engagement by HLA-E+ .221-AEH cells (not shown). A Jurkat-NKG2C+ clone that shared with NK cells key adhesion receptors (i.e., LFA-1, CD2, and DNAM1) but lacked inhibitory NKR, was selected.

The possibility that constitutive HLA-E expression by Jurkat-NKG2C+ cells might promote self-activation of the reporter, potentially impairing its responsiveness to external stimuli, was beforehand considered a potential drawback. Yet, Luc expression in Jurkat-NKG2C+ cells was induced following stimulation with CD94 or NKG2C-specific mAbs. Importantly, reporter activation was detectable upon interaction with HLA-E+ .221-AEH cells, as well as with .221 cells incubated with synthetic peptides which stabilized surface expression of endogenous HLA-E; in these settings, inhibition by anti-NKG2C and -HLA-E mAbs supported specific receptor-ligand engagement. By contrast, reporter activation was undetectable upon interaction of

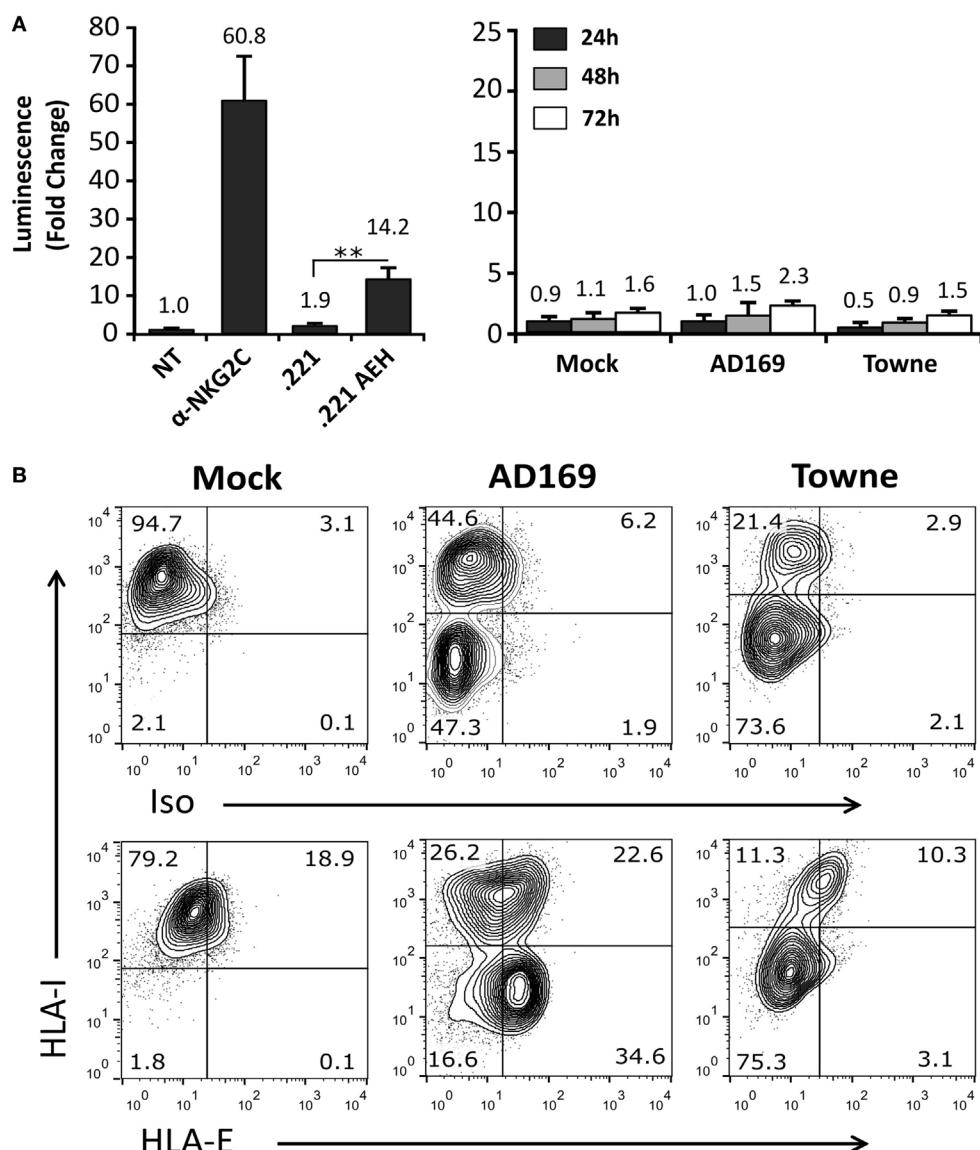


FIGURE 4 | Cells infected with AD169 or Towne human cytomegalovirus (HCMV) strains do not activate the Jurkat-NKG2C+ reporter independently of HLA-E expression. **(A)** HFFF cells infected with AD169 or Towne HCMV strains (multiplicity of infection = 10) were cocultured at 24, 48, and 72 h postinfection with Jurkat-NKG2C+ cells electroporated with the reporter plasmid. The average infection rate for each experiment was >50%. As a control, reporter activation following CD94/NKG2C receptor engagement by specific monoclonal antibodies or HLA-E was assessed in parallel, as described in **Figure 2**. Data correspond to three independent experiments (mean \pm SD). **(B)** Total HLA-I and HLA-E expression at 72 h postinfection in mock-treated and HCMV-infected HFFF cells. Statistically significant differences are indicated (** p < 0.01).

Jurkat-NKG2C+ cells with HFFF or MRC5 fibroblasts following infection with common HCMV laboratory strains (i.e., AD169, Towne), regardless of their ability to preserve surface HLA-E expression in infected cells.

Among variables that may potentially condition the expression of CD94/NKG2C ligand(s) by HCMV-infected cells, genomic differences between viral strains and changes associated to *in vitro* passage of the virus were considered potentially important. In fact, cells infected with two different clinical isolates and, to a lesser degree, with the endotheliotropic TB40/E strain, triggered Jurkat-NKG2C+ activation. Yet, this

response was not inhibited by NKG2C- nor HLA-E-specific blocking mAbs and, furthermore, was independent of CD94/NKG2C expression. Experiments were carried out to explore the molecular basis of this dominant effect, which might potentially mask CD94/NKG2C-specific signaling. Pretreatment of Jurkat-NKG2C+ with a calcineurin inhibitor (FK506) hampered Luc expression triggered by anti NKG2C mAb or .221-AEH cells, but did not prevent the response of Jurkat-NKG2C+ cells to HCMV clinical isolates (data not shown), pointing out that a non-conventional activation of the reporter plasmid was induced under these experimental conditions. Further studies are required

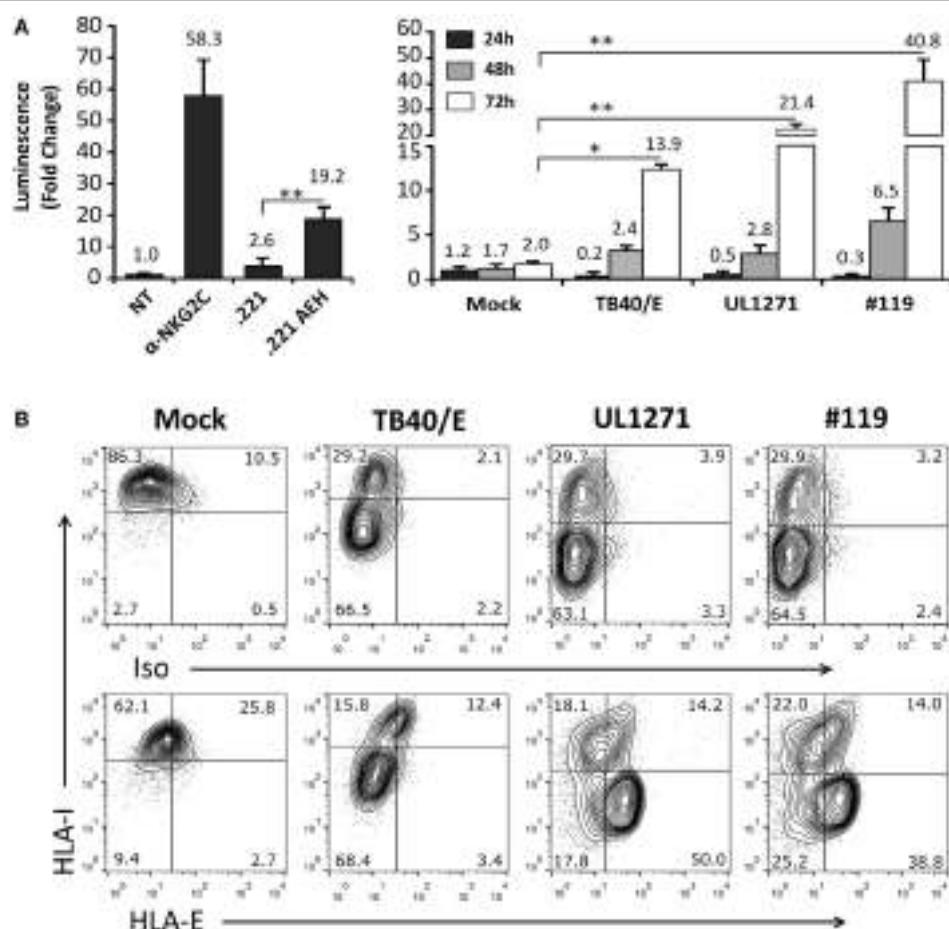


FIGURE 5 | Activation of Jurkat-NKG2C+ reporter cells by HFFF infected with human cytomegalovirus TB40/E or clinical isolates is independent of HLA-E expression. **(A)** The Jurkat-NKG2C+ reporter was cocultured with HFFF cells at different time-points (24–72 h) postinfection with TB40/E or two different clinical isolates (UL1271 and #119) (multiplicity of infection = 10). Data correspond to three independent experiments (mean \pm SD). Average infection rate for each experiment was >50%. **(B)** Total HLA-I and HLA-E surface expression was monitored at 72 h postinfection. Statistically significant differences are indicated (* p < 0.05; ** p < 0.01).

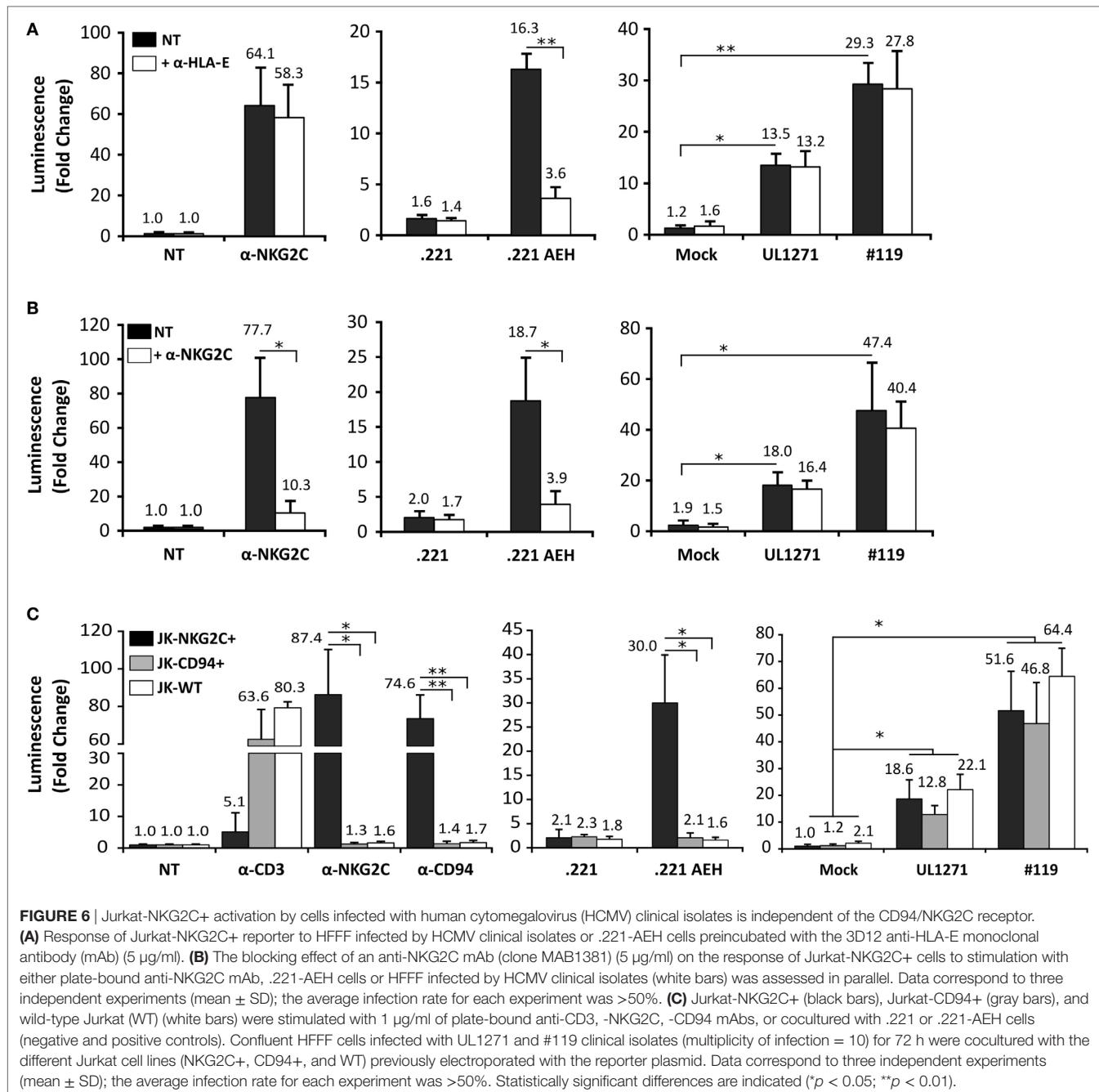
to assess the possibility of controlling this drawback, eventually re-designing the system.

NKG2C+ NK cells were reported to be activated upon interaction with primary aortic endothelial cells infected by the VHL/E HCMV strain (47); nevertheless, no data supporting an involvement of the receptor (e.g., blocking with anti-CD94 or -NKG2C mAbs) were provided. In our hands, Jurkat-NKG2C+ did not detectably respond to HCMV-infected MRC5 fibroblasts nor to human umbilical vein endothelial cells. Yet, the possibility that expression of putative CD94/NKG2C ligands might vary depending on the infected cell lineage is not ruled out.

Our observations should be discussed in the framework of previous indications supporting a CD94/NKG2C receptor interaction with infected cells. In this regard, NKG2C+ NK cells were shown to undergo *in vitro* expansion upon coculture with AD169 or Towne-infected fibroblasts (22, 23). The effect was dependent on cytokines (i.e., IL-12, IL-15) and was prevented by blocking anti-CD94, -NKG2C, or -HLA-E

mAbs. The response was observed only in samples from some HCMV+ blood donors displaying baseline NKG2C^{bright} NK cell expansions, indicating that these experimental conditions promoted their proliferation, but did not strictly reproduce the differentiation process induced *in vivo* by primary HCMV infection. Of note, *in vitro* expansion was prevented upon infection by an HCMV BACmid mutant lacking the whole set of viral genes (i.e., US2-US11), which target HLA-I molecules, thus preserving their expression in infected cells (22). These experiments were interpreted as an indication of a relatively weak stimulation via CD94/NKG2C that was overridden by inhibitory KIR-HLA-I interactions which, by contrast, did not impair CD16-triggered NK cell effector functions against cells infected by the same virus (28).

On the other hand, a relation of NKG2C gene copy number with the magnitude of NKG2C+ NK cell expansion has been reported in HCMV+ healthy blood donors, in children with congenital infection and in renal transplant recipients (9, 14, 32, 48). Moreover, CD94/NKG2C surface expression levels and



activation upon engagement by HLA-E were also greater in NKG2C^{bright} NK cells from NKG2C^{+/+} than NKG2C^{+/del} individuals (9). These data supported that subtle differences in CD94/NKG2C surface density may quantitatively influence adaptive NK cell differentiation/activation in response to HCMV, thus providing another indirect indication for a relatively low avidity of the hypothetical receptor–ligand interaction. The increased surface expression of CD94/NKG2C in differentiated adaptive NK cells (NKG2C^{bright}) from HCMV+ subjects as compared to the profile detected in non-infected individuals (NKG2C^{dim}) is also in line with this view. Of note, a limited affinity and/or reduced expression levels of the putative CD94/NKG2C ligand

in infected cells may presumably render this pathway particularly sensitive to viral immune evasion mechanisms, eventually accounting for the undetectable activation of NKG2C⁺ NK cell effector functions and of Jurkat-NKG2C⁺ cells, in that case, independently of HLA-specific inhibitory receptors.

Despite the negative results obtained with the reporter system, the existence of specific ligand(s) for CD94/NKG2C in HCMV-infected cells still remains a plausible hypothesis, compatible with the involvement of a viral glycoprotein or/and an HLA-E-peptide complex. The latter option is suggested by the blocking effect of an anti-HLA-E mAb in the expansion of NKG2C⁺ NK cells cocultured with infected fibroblasts (23). A

first candidate to be considered is the UL40 leader sequence-derived peptide reported to promote HLA-E expression in HCMV-infected cells by a mechanism refractory to the viral US6 TAP inhibitor (34) and favored by the intrinsic HLA-E resistance to other viral molecules targeting HLA-I (49). UL40-dependent HLA-E expression in infected cells was shown to inhibit NK cell activation by engagement of the CD94/NKG2A inhibitory receptor, presumably contributing to immune evasion (34). The canonical UL40-derived nonamer (VMAPRTLIL) binding to HLA-E is identical to that displayed by some HLA-I leader sequences, predictably promoting a low affinity interaction with CD94/NKG2C (50). Heatley et al. (51) compared the ability of a panel of UL40-nonamer variants to stabilize HLA-E in RMA-S cells, assessing the interaction of the corresponding HLA-E/peptide complexes with CD94/NKG2A or CD94/NKG2C by surface plasmon resonance assays, as well as their recognition by NK cell subsets, respectively, displaying these NKR. Of note, quantitative differences in the interaction of distinct HLA-E-peptide complexes were noticed, but none appeared preferentially recognized by CD94/NKG2C.

It is conceivable that HLA-E expression by HCMV-infected cells, in which HLA-I ligands for inhibitory KIRs are downregulated, might promote specific activation of NKG2C+ NKG2A-NK cells. Supporting this hypothesis, Jurkat-NKG2C+ cells were activated by .221 cells displaying HLA-E bound to the synthetic AD169 UL40-derived peptide (VMAPRTLIL), shared by some HLA-I alleles. Nevertheless, no reporter response was perceived following interaction with AD169-infected cells expressing HLA-E, pointing out to the influence of additional factors, and indicating that experimental approaches based on loading target cells with synthetic peptides provide valuable information but do not precisely reflect the complexity of HCMV infection. A putative role of UL40 in NKG2C+ NK cell expansion, detected following coculture with AD169-infected cells, was addressed employing a UL40 deletion mutant generated in an AD169 BACmid (HB5) (22, 23). In this setting, cells infected with Δ UL40 HB5 retained the ability to promote *in vitro* expansion of NKG2C+ NK cells; however, a caveat for interpreting these experiments is the gene deletion encompassing US2-US6 introduced for the generation of the HB5 BACmid. In fact, HCMV impairs surface expression of HLA-I molecules but not their biosynthesis and, therefore, endogenous HLA-I leader sequences in the absence of US6 may be presented by HLA-E in HB5-infected cells, eventually competing with the UL40-derived nonamer, or even replacing it in Δ UL40 HB5-infected HFFF. On the other hand, NKG2C+ NK cell expansions detected in cocultures with Towne-infected fibroblasts (22) may indirectly provide a case against the involvement of HLA-E expression, which was downregulated consistent with the existence of a genomic deletion spanning the UL40 leader sequence of this HCMV strain (44). Of note, no relation of the HLA-E dimorphism with the expansion of NKG2C+ cells was noticed in previous studies (7, 9).

In summary, molecular evidence supporting that a specific interaction of CD94/NKG2C with infected cells drives the adaptive NK cell response to HCMV remains elusive, leaving unanswered key interrelated questions, particularly, the cellular mechanisms underlying the stable expansion of NKG2C^{bright}

NK cells and the basis for the wide variability of this effect in HCMV+ individuals. Observations in immunocompromised patients suggested that this pattern of response may compensate an inefficient T-cell-mediated control of the primary infection, eventually determined in healthy individuals by viral/host genetic factors as well as by other circumstantial variables (e.g., age at infection, viral load, etc.).

We hypothesize that primary HCMV infection promotes the differentiation, proliferation, and survival of a pool of progenitors, possibly stemming from NKG2C^{dim} NKG2A-NK cells present in seronegative individuals. Increased surface expression of CD94/NKG2C presumably constitutes a key early event, facilitating activation of this NK cell subset upon interaction with low-avidity ligand(s) displayed by infected cells. Such NKG2C-driven selection is dependent on cytokines required for adaptive NK cell differentiation/expansion and may be tuned by specific KIR-HLA-I interactions, consistent with the oligoclonal KIR expression profile of adaptive NK cells (8, 52). Following a contraction phase after viral replication is controlled, a pool of long-lived NKG2C^{bright} NK cells with clonal expansion potential survive, and homeostatic proliferation contributes to their persistent increased numbers in the circulation. The process is reminiscent of the generation of memory cytotoxic T lymphocytes as proposed for the response of Ly49H in mice (53); in fact, responsiveness of differentiated NKG2C^{bright} NK cells to cytokines (e.g., IL-15, IL-2) becomes dependent on signaling by activating NKR (e.g., NKG2C or CD16). HCMV reactivation/reinfection as well as other infectious pathogens may boost antibody- and cytokine-dependent activation of adaptive NK cells, leading to their progressive acquisition of late differentiation features (e.g., FcR γ downregulation). Further efforts are warranted to understand how HCMV infection resets the NK-cell compartment homeostasis in some individuals, and the implications that such persistent reconfiguration of the NK cell compartment may have in the development of the immune response under different pathological conditions.

AUTHOR CONTRIBUTIONS

AP developed the reporter system, carried out the experimental work, and revised the manuscript. MC-G collaborated in the experimental development. DF and AA collaborated in the genetic analysis of HCMV as well as in critical discussion of the results. HH contributed with essential support for the analysis of HCMV clinical isolates as well as in critical discussion of the results. AM contributed to the design, follow-up, and interpretation of the results. ML-B proposed the experimental approach, contributed to the design, follow-up, and interpretation of results, and wrote the draft that was revised by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/article/10.3389/fimmu.2017.01317/full#supplementary-material>.

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Regulation of the Functions of Natural Cytotoxicity Receptors by Interactions with Diverse Ligands and Alterations in Splice Variant Expression

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The natural cytotoxicity receptor (NCR) family is constituted by NKp46, NKp44, and NKp30 in humans, which are expressed mainly on natural killer (NK) cells and are encoded by the *ncr1*, *ncr2*, and *ncr3* genes, respectively. NCRs have classically been defined as activating receptors that trigger cytotoxicity and cytokine responses by NK cells upon engaging with ligands on tumor cells. Several new findings, however, have challenged this model and identified alternative mechanisms regulating the function of NCRs. Recent reports indicate that ligand matters, since the interaction of NKp44 with distinct ligands on target cells can either activate or inhibit NK cells. Also, the NCRs have been found to interact with distinct specificities to various heparan sulfate glycosaminoglycans, which are complex polysaccharides found in extracellular matrix or on cell surface heparan sulfate proteoglycans (HSPGs). The NCRs can engage with HSPGs in trans as a co-ligand on the target cells or in cis on the NK cell surface to regulate receptor–ligand interactions and NK cell activation. A number of splice variants of *ncr2* and *ncr3* have also been identified, and a predominant expression of certain variants results in inhibitory signaling through NKp44 and NKp30. Several recent studies have found that the selective expression of some of these inhibitory splice variants can significantly influence outcome in the contexts of cancer, infection, and pregnancy. These findings establish that NCR functions are more diverse than originally thought, and better understanding of their splice variant expression profiles and ligand interactions are needed to establish their functional regulation in the context of human health.

Keywords: natural cytotoxicity receptors, natural killer cells, RNA splice variants, cytotoxicity, cancer immunology, virus immunity, human immunology, pregnancy

INTRODUCTION

It has become increasingly clear that human natural killer (NK) cells use an array of germline-encoded cell surface receptors to spontaneously recognize and respond to the abnormal status of tumor cells, virus-infected cells, and stressed cells (1). Different NK cell surface receptors transduce either activating or inhibitory signals, directly or through associated adaptor proteins, to dynamically regulate the activation state of NK cells (2–4). Inhibitory receptors, such as killer cell Ig-like receptors (KIRs) and CD94/NKG2A, provide NK cells with a dominant, tolerizing immune checkpoint through their recognition of the MHC class I (MHC-I) molecules ubiquitously expressed on the surfaces of most normal cells. The loss of MHC-I by many transformed cells, however, overcomes this inhibitory checkpoint to favor activation. The loss of inhibitory restraint allows activating receptor signals to predominate and triggers exocytosis of perforin and granzymes to induce targeted apoptosis of the MHC-I-deficient cell and localized secretion of pro-inflammatory cytokines [especially interferon (IFN)- γ , TNF- α , and several chemokines].

The activation signals transduced in NK cells are derived from adhesion molecules (especially LFA-1), co-stimulatory receptors (such as NKG2D, DNAM-1, and SLAM family receptors), and several activating receptors physically linked to the immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane proteins, DAP12, TCR- ζ , and/or Fc ϵ RI- γ (2–4). The key ITAM-coupled activating receptors on human NK cells, include CD16 (Fc γ RIIIa), an activating subfamily of KIR (KIR2DS or KIR3DS receptors), CD94/NKG2C, and the natural cytotoxicity receptors (NCRs).

The human NCRs consist of three receptors, named NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337). These NCR were classically defined as germline-encoded receptors that play important roles in the activation of human NK cells toward transformed target cells (5, 6). Recent work, however, has established that the NCRs can also generate inhibitory responses under certain circumstances. Here, we will review our current understanding of the expression and function of NCRs on NK cells, particularly in humans, although it is important to note that the NCRs are also expressed on other innate lymphoid cells (ILCs) and a subset of T cells, which has been previously reviewed elsewhere (7, 8).

NCRs AND THEIR STRUCTURES

The NCRs were initially discovered and characterized by the laboratories of Alessandro and Lorenzo Moretta in the late 1990s (9–13). They are type I transmembrane glycoproteins that were originally recognized as activating receptors and named in accordance with their molecular weight on SDS-PAGE (NKp30, NKp44, and NKp46). NKp46 is the only NCR also expressed in mice, although a receptor analogous to NKp30 has been shown to be expressed in 1 of 13 mouse strains examined (14) and in rats (15, 16). While the *ncr2* and *ncr3* genes encoding NKp44 and NKp30, respectively, are localized to human MHC class III locus on chromosome 6, the NKp46 encoding gene, *ncr1*, is found

near the leukocyte regulatory complex on human chromosome 19 (10, 12, 13).

NKp46 has been shown to be a highly selective marker of all NK cells in mouse and man, although surface expression can be low on some NK cells, particularly in humans, and the receptor is also expressed on some ILCs and a small subset of T cells (9, 17, 18). Importantly, NKp46 is not expressed by CD1d-restricted invariant NKT cells in mice and humans (17). NKp46 has been shown to provide NK cells with the capacity to recognize and kill a variety of tumor target cells (19–21). NKp46 ligands have been reported to be enriched in areas of high malignant potential and high proliferation within melanoma lesions, whereas surrounding normal melanocytes were found to lack NKp46 ligands (22). Evidence in mice also suggests that NKp46 also contributes to the development of type 1 diabetes by interacting with an uncharacterized ligand on pancreatic islet beta cells (21). As a tumor immunosuppressive mechanism, the surface expression of NKp46 on NK cells can be down-modulated by exposure to L-kynurenine, which is a tryptophan catabolism product generated by the indoleamine 2,3-dioxygenase (IDO) enzyme in tumor microenvironments (23).

NKp30, similar to NKp46, is expressed on nearly all human NK cells (13). This NCR has been shown to play important roles in crosstalk between NK cells and dendritic cells (DCs) through promoting both the maturation of and the cytotoxicity of immature DC (24, 25). Surface expression levels of NKp30 and NKp46 can be upregulated by IFN- α , IL-2, and prolactin and downregulated by cortisol and methylprednisolone (26–28). In addition, both receptors are also commonly downregulated in “adaptive” or “memory-like” NK cells that are found in some cytomegalovirus-infected individuals (29, 30). TGF- β has been shown to selectively down-modulate the expression of NKp30, but not NKp46 on NK cells (31).

NKp44 is distinct among NCRs, since it is unique to humans and only expressed constitutively on some CD56^{bright} NK cells in a subset of individuals, but expression can be upregulated on essentially all NK cells after culture with IL-2, IL-15, or IL-1 β (11, 32). Similarly, NKp44 can be upregulated on plasmacytoid DCs upon culture with IL-3 (33). Therefore, NKp44 may also be considered a marker of cytokine-activated NK cells in humans. IL-2-induced upregulation of NKp44 on NK cells can be inhibited by prostaglandin E2, which is readily produced by tumor-associated fibroblasts, especially when exposed to NK cells in culture (34). Similarly, prednisolone can suppress IL-2-mediated upregulation of NKp44 (26).

The extracellular domains of NCRs consist of one (NKp30 and NKp44) or two (NKp46) Ig-like domains that are responsible for ligand binding (10, 12, 13). Ligand binding and signaling function by NKp30 is highly dependent upon integrity of the membrane proximal stalk region (35). Crystal structures have revealed that NKp30 and NKp44 can form homodimeric structures with NKp30 dimerizing in a head-to-tail fashion to form an I-type Ig-like fold and two NKp44 V-type Ig-like domains form a saddle-shaped dimer with unique disulfide bridging (36, 37). On the other hand, the crystal structure of NKp46 demonstrates two C2-type Ig-like domains that are folded and oriented similar to the Ig-like domains of KIRs (38). Evidence for a homodimerization interface

within the membrane proximal Ig-like domain of NKp46 has also been reported, and disruption of this dimerization interaction prevented ligand binding and activating function of the receptor (39). In conclusion, the three NCRs have unrelated structures, and grouping these receptors together is based more on their shared functional properties than related structure or genetic evolution (13).

The transmembrane domains of all three NCRs contain a positively charged lysine (NKp44) or arginine (NKp30 and NKp46) residue that interacts with acidic aspartic acid residues found in the transmembrane regions of the adaptor proteins DAP12 (NKp44) or TCR- ζ and/or Fc ϵ RI- γ (NKp30 and NKp46) (10). Physical association with DAP12 via these transmembrane charged residues is essential for surface expression of NKp44 (40). The reductions in surface expression levels of NKp30 and NKp46 on “adaptive” or “memory-like” NK cells is associated with the lack of Fc ϵ RI- γ expression in these cells (29, 30), exemplifying the importance of associating with this specific adaptor to transport a functional receptor to the cell surface. In addition to promoting surface expression, physical association with these associated transmembrane adaptors provides potent activation signaling function to the NCRs, since the tyrosine phosphorylation of their cytoplasmic ITAM domains results in the recruitment and activation of the Syk and ZAP-70 protein tyrosine kinases (2, 41). A unique activation signaling crosstalk has been reported between the NCRs, in which engagement of one NCR appears to initiate signaling through the others (41). Curiously, while several mRNA splice variants encoding NKp44 have been described, the major protein product or isoform was found to also contain a cytoplasmic ITIM-like domain. Although early work suggested that this domain was incapable of providing inhibitory signaling function in an NK-like cell line (40), more recent work has demonstrated ITIM-mediated inhibitory function by NKp44 upon recognition of a specific ligand, proliferating cell nuclear antigen (PCNA), as detailed below (42).

LIGANDS OF THE NCRs

Despite a great deal of work by numerous research groups, our understanding of the ligands for NCRs is still not clearly established. A diverse array of molecules have been reported to interact with the extracellular domains of NCRs, including carbohydrate-based contacts, cell surface proteins, and surprisingly, several intracellular-localized proteins that appear to reach the surface of infected or transformed cells. While engagement with most of these reported ligands stimulates activation of NK cells, some have been found to inhibit their functions. Our current understanding of putative ligands for NCRs and their functions are described below and summarized in **Figure 1** and **Table 1**.

Viral Ligands

Several viral-derived NCR ligands have been reported. Early work showed that the viral hemagglutinin (HA) of influenza virus on the surface of infected cells can readily engage with branched α -2,3- and α -2,6-sialylated O-glycan sequences conjugated on NKp46, and influenza-infected target cells can be killed by human NK cells in a NKp46-dependent manner (65–68). In fact,

exposure of NK cells to influenza virions or free HA has been found to decrease NCR-mediated cytotoxicity, which was associated with loss of TCR- ζ protein expression (71). In addition, influenza virus-infected DCs stimulate IFN- γ production by NK cells in an NKp46- and HA-dependent manner (69). Furthermore, it has been demonstrated that NKp46-deficient mice are more susceptible to death after infection with influenza virus (72). These interactions are consistent with the known sialic acid-binding properties of viral HAs. HAs from influenza and Sendai viruses have further been shown to also interact with NKp44, but not NKp30, and NKp44 $^{+}$ NK cells can kill cells infected with these viruses (54, 55). Similarly, both NKp46 and NKp44 were found to interact with the HA from avian Newcastle disease virus and this interaction potentiates cytotoxicity of target cells infected with this paramyxovirus (56).

Hemagglutinin from the orthopox family viruses, human vaccinia virus, and murine ectromelia virus has been shown to interact with NKp46 and NKp30 (43). Late-stage vaccinia virus-infected target cells were further shown to be less susceptible to NK cell cytotoxicity compared to uninfected targets, and this reduced killing was dependent upon viral HA in the target cells and NKp30 in the NK cells (43). The results from this study suggest that the HA on the surface of vaccinia virus-infected cells interacts with NKp30 to either block its activating function or to mediate inhibitory signaling in NK cells, whereas NKp46 engagement with vaccinia virus-derived HA on target cell surfaces stimulates cytotoxicity responses.

NKp44 has been shown to recognize the envelope glycoproteins from West Nile and Dengue flaviviruses (57). In particular, NKp44 was found to directly bind domain III of WNV envelope protein, but does not appear to involve viral HA, since independent of sialylation of oligosaccharides on NKp44. Consistent with this finding, West Nile virus-infected cells more readily bind a soluble recombinant form of NKp44 and stimulate NK cells to degranulate and produce IFN- γ in an NKp44-dependent manner (57). It should be noted that expression of Dengue viral non-structural proteins in target cells reduces susceptibility to NK cell cytotoxicity through upregulating MHC-I expression (73).

In addition, NKp30 has been shown to directly interact with pp65, which is the main tegument protein of human cytomegalovirus (HCMV) (44). HCMV infected target cells were found to be less susceptible to NK cell-mediated killing, and this inhibition was lost if the target cells were infected with pp65-deficient HCMV or if anti-NKp30 blocking antibodies were added (44). The authors of this report further provided evidence that treating NK cells with a recombinant soluble form of pp65 resulted in the dissociation of the TCR- ζ signaling adaptor protein from NKp30 (44). In this way, pp65 appears to provide HCMV with a mechanism to avoid NK cell-mediated immunity by disrupting activation signaling through NKp30.

Other Ligands Expressed by Pathogens or Pathological Conditions

Natural cytotoxicity receptors have also been shown to directly recognize bacterial and parasite pathogens. It has been demonstrated that NKp30 (and to a lesser extent, NKp46) can interact with the Duffy binding-like (DBL)-1 α domain of *Plasmodium*

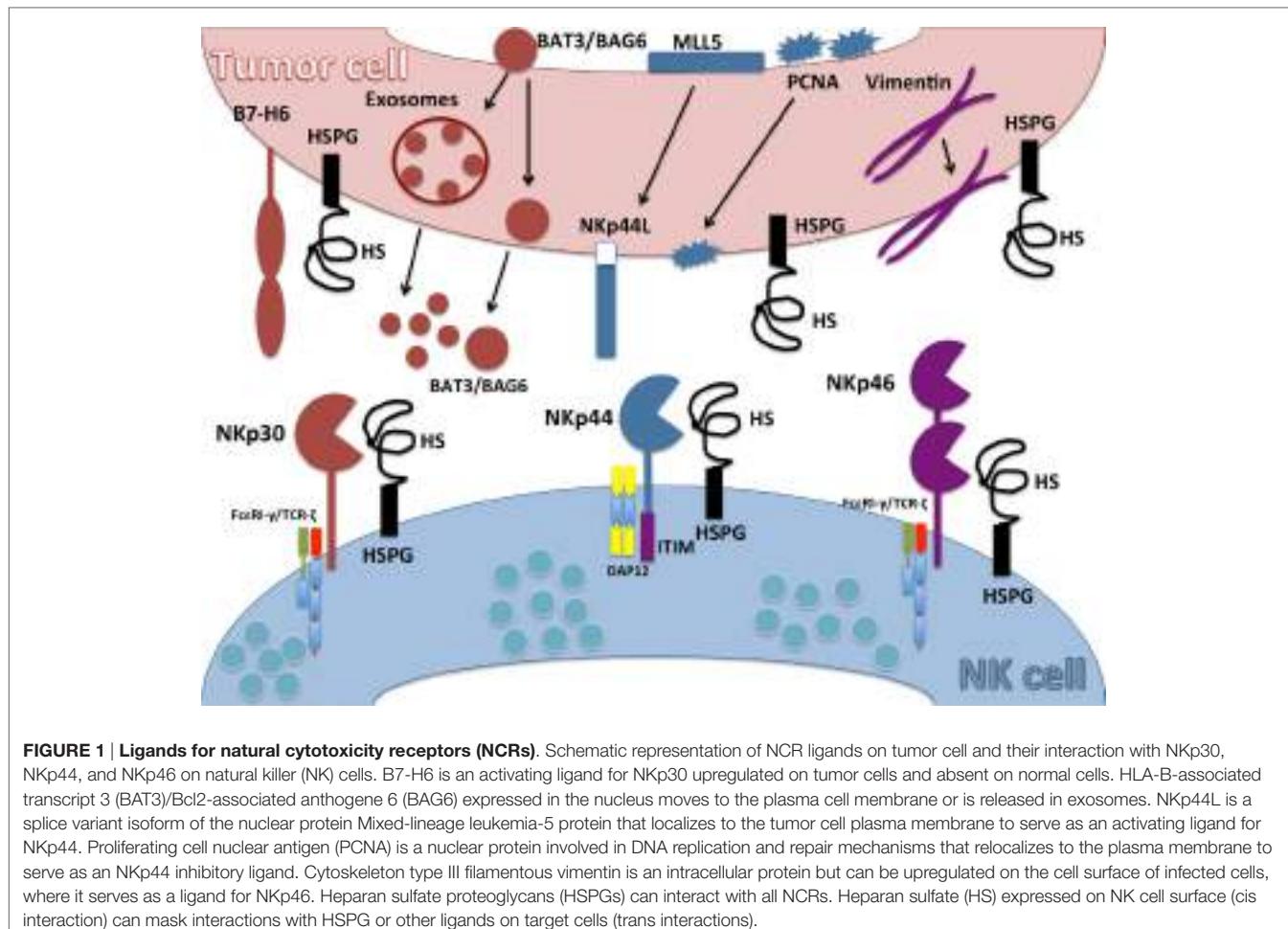


FIGURE 1 | Ligands for natural cytotoxicity receptors (NCRs). Schematic representation of NCR ligands on tumor cell and their interaction with NKp30, NKp44, and NKp46 on natural killer (NK) cells. B7-H6 is an activating ligand for NKp30 upregulated on tumor cells and absent on normal cells. HLA-B-associated transcript 3 (BAT3)/Bcl2-associated anthogene 6 (BAG6) expressed in the nucleus moves to the plasma cell membrane or is released in exosomes. NKp44L is a splice variant isoform of the nuclear protein Mixed-lineage leukemia-5 protein that localizes to the tumor cell plasma membrane to serve as an activating ligand for NKp44. Proliferating cell nuclear antigen (PCNA) is a nuclear protein involved in DNA replication and repair mechanisms that relocates to the plasma membrane to serve as an NKp44 inhibitory ligand. Cytoskeleton type III filamentous vimentin is an intracellular protein but can be upregulated on the cell surface of infected cells, where it serves as a ligand for NKp46. Heparan sulfate proteoglycans (HSPGs) can interact with all NCRs. Heparan sulfate (HS) expressed on NK cell surface (cis interaction) can mask interactions with HSPG or other ligands on target cells (trans interactions).

falciparum erythrocyte membrane protein-1 to mediate cytolysis of malaria-infected erythrocytes (45). The interaction appears to be direct, since the effect can be inhibited by the addition of recombinant soluble forms of these NCRs or peptides matching the sequence of DBL-1 α (45).

It has been reported that *Mycobacterium bovis bacillus Calmette-Guérin* (BCG) can directly interact with NKp44, and exposure to BCG can increase NKp44 expression on CD56^{bright} NK cells (58). This study also found that additional *Mycobacterium* family members can bind NKp44, such as *Nocardia farcinica* and *Pseudomonas aeruginosa*. Interactions of these bacteria with NKp44 did not activate NK cell functions, however, so the relevance of these interactions is currently unclear.

NKp44 has been reported to recognize an uncharacterized ligand on cartilage-derived chondrocytes, and cytotoxicity of primary chondrocytes by long-term IL-2 activated-NK cells was inhibited by an NKp44-blocking antibody (59). These results suggest that NKp44 activation signaling may promote NK cell-mediated autoimmunity in chronic inflammatory cartilaginous disease.

Heparan Sulfates

Heparan sulfate (HS) glycosaminoglycans (GAGs) have also been shown to interact with all of the NCRs, with different affinities

for the three receptors (46–48, 60). While these carbohydrate-directed interactions likely do not represent primary ligands for the NCRs, they appear to have the capacity to regulate NCR function or may play a supporting role as co-ligands (61). HS GAGs consist of long, unbranched, anionic polysaccharides that are found on cell surfaces and the extracellular matrix (74). The HS GAG polysaccharides are composed of repeating disaccharide units of uronic acid (iduronic or glucuronic acid) and glucosamine that are differentially sulfated at N, 2-O, 3-O, and 6-O positions to generate highly diverse structures with unique protein binding properties (75). HS GAG can be conjugated to a small subset of proteins to form HS proteoglycans, including syndecans and glycans, and their negative-charged configurations can provide docking sites for basic domains on chemokines, FGF, and wnt ligand family members, thereby “presenting” them to cell surface receptors (76–79).

Interestingly, HS GAG are highly diverse structures, and the three NCRs preferentially recognize highly sulfated HS structures via basic amino acid patches on the receptor surfaces, and each NCR demonstrates distinct HS binding specificity (47, 48, 60). Therefore, it is conceivable that each NCR has the capacity to distinguish particular configurations of HS GAG primary and tertiary structures that might be uniquely expressed in the

TABLE 1 | Ligands for natural cytotoxicity receptors.

Receptor	Ligands	Function	Reference
NKp30	Hemagglutinin (HA) of human vaccinia virus	Inhibition	(43)
	pp65, Main tegument protein of human cytomegalovirus	Inhibition	(44)
	(DBL)-1 α domain of <i>Plasmodium falciparum</i> erythrocyte membrane protein-1	Activation	(45)
	Heparan sulfate (HS) glycosaminoglycans (GAGs)	Activation/regulation	(46–48)
	BAT3/BAG6	Activation	(49–51)
	B7-H6	Activation	(52, 53)
	Redirected cytotoxicity and blockade of natural cytotoxicity with NKp44 antibody	Activation	(11)
NKp44	HA of influenza and Sendai viruses	Activation	(54, 55)
	HA from avian Newcastle disease	Activation	(56)
	Domain III of WNV envelope protein of West Nile and Dengue virus	Activation	(57)
	<i>Mycobacterium bovis bacillus Calmette-Guérin</i> (BCG)	Unclear	(58)
	Unknown ligand on cartilage-derived chondrocytes	Activation	(59)
	HS GAGs	Activation/regulation	(47, 60, 61)
	NKp44L	Activation	(62, 63)
NKp46	Proliferating cell nuclear antigen	Inhibition	(42, 64)
	HA of influenza virus	Activation	(65–69)
	HA of avian Newcastle disease	Activation	(56)
	HA of human vaccinia virus	Activation	(43)
	(DBL)-1 α domain of <i>Plasmodium falciparum</i> erythrocyte membrane protein-1	Activation	(45)
	HS GAGs	Activation/regulation	(47)
	Vimentin	Activation	(70)

contexts of tumor microenvironments or sites of infection or inflammation. We have also shown the HS GAGs can interact with another NK cell receptor, KIR2DL4, and the binding can modulate receptor function (80). In addition, an interaction of NKp44 with the heparan sulfate proteoglycans (HSPGs), syndecan-4, in cis on the NK cell surface can modulate the surface distribution and function of the receptor (61). Based on this and other reports (47), we have proposed that interactions with HSPGs in cis (on the surface of NK cells) may be impacting KIR2DL4 and NCR functions through masking interactions with HS GAG or other ligands on adjacent target cells (trans interactions) and/or may be affecting the trafficking of NCR to intracellular degradation and recycling pathways upon endocytosis (61, 81). In this way, cis interactions between NCR and HSPGs may provide an allosteric regulation mechanism. It is also intriguing to speculate that treatment of patients with structurally related heparin as a therapeutic agent could impact NK cell functions through binding to NCRs and other NK cell surface receptors, including KIR2DL4.

Intracellular Proteins As Cell Surface Ligands

The expression of a ligand for NKp44, named NKp44L, was first shown to be induced by the HIV-1 envelope protein gp41 on infected CD4 $^{+}$ T cells, and the expression increased in patients with increasing viral load (62). NKp44L is an activating ligand, since NK cell-mediated lysis of HIV-infected CD4 $^{+}$ T cells was inhibited by antibodies to NKp44 or NKp44L (62). The NKp44L was subsequently identified as a unique splice variant isoform of mixed-lineage leukemia-5 (MLL5) protein (63). While full-length MLL5 is a nuclear protein, the NKp44L splice variant is localized near the plasma membrane in the cytoplasm and expressed in several tumor tissues and transformed cell lines, but not in normal tissues (63).

It was reported that NKp46 is involved in NK cell-mediated cytolytic attack of monocytes infected with *Mycobacterium tuberculosis* (20). Subsequent work established that this is due to an interaction with vimentin, which is expressed at high levels in infected monocytes and appears on the cell surface (70). Since vimentin is a type III intermediate filament of the cytoskeleton, however, it is unexpected to find on the cell surface, but this follows an emerging theme of several traditionally intracellular proteins serving as putative cell surface ligands for NCRs. NK cells were more efficient at lysing target cells transfected to overexpress vimentin, and this cytotoxicity was inhibited by antibodies targeting NKp46 or vimentin (70).

In addition, NKp30 has been shown to interact with the HLA-B-associated transcript 3 (BAT3)/Bcl2-associated anthogene 6 (BAG6) protein to stimulate NK cytolytic responses (49). BAT3/BAG6 is predominantly expressed in the nucleus, but can move to the plasma membrane in cells exposed to heat shock and can be secreted in exosomes by tumors and stressed cells (49, 82). BAT3/BAG6-expressing exosomes can stimulate cytokine release from NK cells upon interaction with NKp30, and BAT3/BAG6 expression by DC is responsible for activation NK cells to mediate the crosstalk with DC (49, 50). Similarly, RIG-I stimulation of melanoma cell lines was shown to trigger the extracellular release of BAT3/BAG6-containing vesicles that can stimulate NK cell cytolytic responses (51). In contrast, a soluble form of BAT3/BAG6 has been found at high levels in the plasma of CLL patients and can suppress NK cytolytic responses, apparently by blocking recognition of this and other ligands on tumor cells (82, 83).

It was also reported that NKp44 can interact with PCNA, at target cell surfaces (42). PCNA is highly expressed in proliferating cancer cells, where it is usually tightly associated with DNA and involved in DNA replication and repair mechanisms (84). Surprisingly, PCNA was found to migrate to the plasma membrane of target cells within the immunological synapse with NKp44-expressing NK cells, and this interaction inhibited cytolytic function and IFN- γ production by the NK cells (42). A second report has also described the interaction of PCNA and NKp44 and the association of PCNA with MHC-I molecules at the plasma membrane of tumor cells as a potential surface transport mechanism (64). The PCNA-induced inhibition was found to be mediated through the ITIM-like sequence in the cytoplasmic domain of NKp44 (42), despite earlier work in

which the ITIM-containing NKp44 cytoplasmic domain was shown to lack inhibitory function in the context of a chimeric receptor construct (40). It appears that PCNA interaction with the full NKp44 receptor establishes a unique conformation that transduces an inhibitory signal. Inhibitory function was also previously reported for NKp44 expressed on ILCs and plasmacytoid DCs (33, 85).

B7-H6

NKp30 has been found to bind to a cell surface protein member of the B7 family, named B7-H6 (52). B7-H6 is not normally expressed on healthy cells, but can be upregulated on human tumor cells through a Myc-mediated mechanism (86) or upon stimulation of monocytes and neutrophils with TLR ligands or pro-inflammatory cytokines (87). Upon recognition by NKp30, B7-H6 triggers cytotoxicity and cytokine production by NK cells (52). The interaction of B7-H6 with NKp30 is the most rigorously characterized of NCR ligands, since it is the only NCR-ligand interaction so far confirmed in an X-ray crystallography structure (53). It has also been shown that some tumors can escape NKp30 recognition by shedding B7-H6 from their surfaces with the metalloproteases, ADAM-10, and ADAM-17 (88). Soluble and tumor-associated expression of B7-H6 in the peritoneum of ovarian cancer patients has also been shown to correlate with reduced surface expression of NKp30 on peritoneal NK cells, presumably due to chronic interaction with ligand (89).

SPLICE VARIANTS OF NCRs RESULTING IN DISTINCT RECEPTOR ISOFORMS

A variety of mRNA splice variants encoding different isoforms of NCRs have been recognized for many years, but only recent work has established that some of these variant NCR isoforms can facilitate inhibitory functions. Distinct splice variant expression patterns have also been shown to correlate with outcomes in cancer and infectious disease, suggesting potential prognostic value in patients. A summary diagram of current reported functions of distinct isoforms of NKp30 and NKp44 is presented in Figure 2.

Splice Variants of *ncr3*

The expression of distinct isoforms of NKp30 is of great interest, because this NCR is involved in DC-to-NK cell crosstalk (25), can facilitate tumor cell recognition (13, 49, 90), and can influence the prognosis of different infectious diseases (91). Six splice variant transcripts have been identified from the *ncr3* gene, which was originally called 1C7 (92, 93). The most highly expressed *ncr3* variants are designated a, b, and c that encode NKp30 proteins with an extracellular V-type Ig domain, while d, e, and f isoforms encode NKp30 receptors possessing a C2-type Ig domain that lacks 25 amino acids (93). The three members of each subgroup share three distinct cytoplasmic domains encoded by splice variations within exon 4. Although the d, e, and f protein isoforms of NKp30 have not been studied to date, several groups have examined the functions and interesting clinical outcomes associated with differential expression of the a, b, and c isoforms, as discussed below.

Alternative splicing of the *ncr3* gene impacts functions of NKp30 isoforms that can be immunosuppressive or immunostimulatory. Delahaye and colleagues expressed isoforms NKp30a, NKp30b, and NKp30c in the human NK cell line NKL to characterize their functions. It was shown that antibody- or B7-H6-mediated engagement of NKp30 on NKL transfected with either NKp30a or NKp30b isoforms stimulated production of large amounts of IFN- γ , degranulation, and cytotoxicity responses. In contrast, engagement of NKp30c on NKL cell transfectants did not result in degranulation or elicit cytotoxicity, but instead produced the inhibitory cytokine IL-10 and very little IFN- γ (94). Similar immunostimulatory functions for NKp30a and NKp30b isoforms were observed when NKp30 transfectants were cocultured with DCs, whereas NKL-NKp30c transfectants demonstrated minimal responsiveness in these assays. In all of these experiments, NKp30a stimulated the most potent activating responses, whereas NKp30c was inhibitory or non-responsive, and NKp30b induced intermediate activation (94).

It was also shown that NKp30a associated more tightly with TCR- ζ upon crosslinking, as compared to NKp30c (94). Surprisingly, p38 MAP kinase activation was more pronounced when NKp30 was engaged in NKL-NKp30c cells than for NKL cells transfected with NKp30a or NKp30b. Furthermore, treatment of NKL-NKp30c cells with a p38 inhibitor produced IFN- γ upon exposure to immature DCs (94). Therefore, NKp30b and especially NKp30a are stimulatory isoforms that can induce cytotoxicity and cytokine production, whereas NKp30c induces an immunosuppressive response that appears to involve activation of p38 and the production of IL-10.

NKp30 Isoforms and Cancer

In addition to characterizing different functions for distinct NKp30 isoforms, Delahaye performed a retrospective analysis of NKp30 expression profiles in 80 patients with gastrointestinal stromal tumors (GIST), which is a malignancy that expresses NKp30 ligands. In that analysis, predominant expression of the immunosuppressive NKp30c isoform over the immunostimulatory NKp30a/b isoforms was found to be associated with reduced overall survival in imatinib-treated patients (94). Moreover, a subset of GIST patients with predominant expression of the NKp30c isoform and a distinct haplotype involving two SNPs in the *ncr3* gene were found to be associated with particularly poor survival (94). For GI carcinomas and variety of other cancers, we observed that both cancerous and matched normal tissues manifested balanced NKp30c inhibitory and NKp30a/b activation profiles; yet, we found skewed NKp30 splice variant profiles in about 50% of a variety of tumor tissues compared to their matched normal tissues (95).

Neuroblastoma is another malignancy where NCRs are involved in the tumor cell recognition (96–98). Semeraro et al., found that neuroblastoma tumor samples express the NKp30 ligand, B7-H6, and some patients with metastatic neuroblastoma had high levels of soluble B7-H6 in their serum, which was associated with reduced expression of NKp30 on the surface of NK cells and higher degree of metastases (99). Furthermore, serum from patients with high soluble B7-H6 suppressed NK cell IFN- γ responses (99). Analysis of NKp30 isoform expression

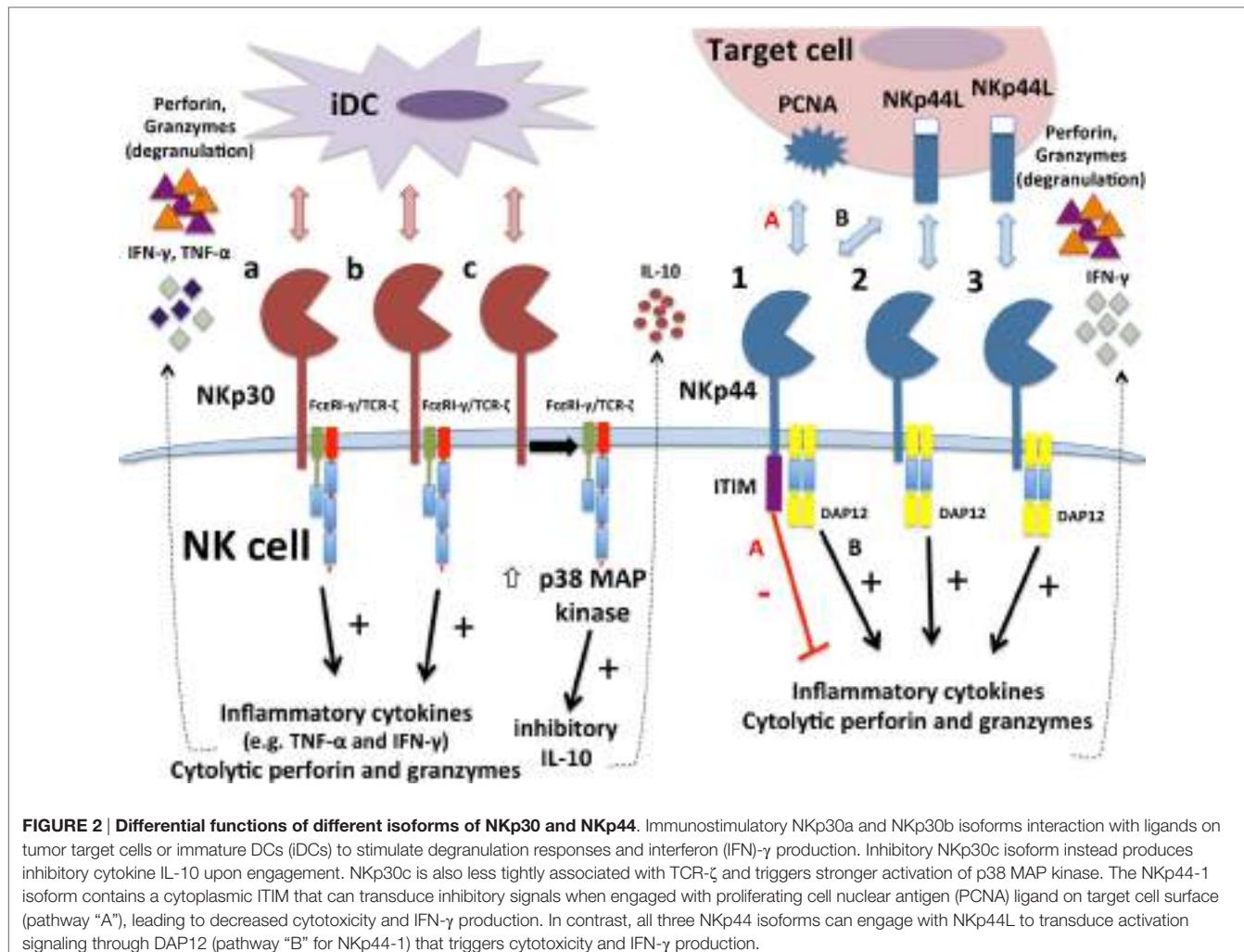


FIGURE 2 | Differential functions of different isoforms of NKp30 and NKp44. Immunostimulatory NKp30a and NKp30b isoforms interact with ligands on tumor target cells or immature DCs (iDCs) to stimulate degranulation responses and interferon (IFN)- γ production. Inhibitory NKp30c isoform instead produces inhibitory cytokine IL-10 upon engagement. NKp30c is also less tightly associated with TCR- ζ and triggers stronger activation of p38 MAP kinase. The NKp44-1 isoform contains a cytoplasmic ITIM that can transduce inhibitory signals when engaged with proliferating cell nuclear antigen (PCNA) ligand on target cell surface (pathway "A"), leading to decreased cytotoxicity and IFN- γ production. In contrast, all three NKp44 isoforms can engage with NKp44L to transduce activation signaling through DAP12 (pathway "B" for NKp44-1) that triggers cytotoxicity and IFN- γ production.

suggested lower expression of NKp30a/b isoforms in patients with metastatic neuroblastoma compared to patients with localized disease or healthy donors. Comparison of the ratios of expression of mRNA for individual NKp30 isoforms revealed that high expression of the suppressive NKp30c isoform over the activating NKp30b isoform was associated with shorter progression-free survival of patients with metastatic disease (99). Surprisingly, IFN- γ was found to suppress the expression of *B7-H6* and *BAT3* mRNA, whereas IL-10 increased *B7-H6* mRNA expression on neuroblastoma tumors. The results of this study suggest that high expression of IL-10 by NKp30c-expressing NK cells induces *B7-H6* expression on tumor, thereby potentiating further expression of this immunosuppressive cytokine.

The potential prognostic value of NKp30 isoforms was also recently investigated in melanoma patients (100). The overall mRNA expression levels of each of the NKp30 isoforms were found to be reduced compared to expression in healthy donors, but expression ratios between the isoforms did not differ. Levels of mRNA expression of the immunosuppressive NKp30c isoform were found to be higher on NK cells from stage IV melanoma patients, but relative expression levels of each NKp30 isoform did not predict overall survival of patients. Interestingly, long-term

surviving melanoma patients were found to express higher levels of stimulatory NKp30a transcript. In accordance with this finding, the long-term survivors were more likely to have SNPs associated with reduced expression of suppressive NKp30c and increased expression of NKp30a. Furthermore, NK cells from long-term surviving patients exhibited increased degranulation potential in response to NKp30 stimulation (100). Therefore, higher expression of NKp30a in melanoma patients appears to be beneficial.

NKp30 Isoforms and Infectious Diseases

Surface expression NKp30 on NK cells was found to be down-regulated in HIV-1 patients, but expression patterns of NKp30 isoforms do not affect disease progression or survival (101). NK cells from hepatitis C virus (HCV)-infected patients also showed reduced expression of NKp30 on the surface of NK cells, and expression of the immunosuppressive NKp30c transcript was found to be significantly decreased in infected patients compare to healthy controls (102). Also NKp30a/NKp30c ratio was significantly higher compared to healthy individuals suggesting an immunostimulatory profile in infected patients. If stratified according to mRNA expression levels, patients with low expression of all three isoforms had lower surface expression of NKp30

on NK cells, but higher ratio of NKp30a/NKp30c isoform transcripts. Accordingly, NK cells from these patients exhibited more potent degranulation and cytokine production responses upon engagement of the receptor, as compared to healthy controls (102). Interestingly, positive correlations were observed between NKp30a isoform mRNA levels and liver stiffness and between NKp30a/NKp30c ratio and a measure of liver fibrosis, suggesting that reduction of immunosuppressive NKp30c isoform and increase of immunostimulatory NKp30a isoform is associated with advanced liver disease in HCV-infected patients.

NKp30 in the Decidua of Pregnant Women

Natural killer cells from decidual tissue in pregnant uterus (dNK) have unique phenotypical and functional properties compared to NK cells found in peripheral blood (pNK) (103). Mouse studies originally demonstrated that dNK cells function to support embryo implantation by secretion of numerous factors, including IFN- γ , to promote angiogenesis and trophoblast invasion (104). Comparison of freshly isolated pNK and dNK cells from the same pregnant donors showed that pNK cells express high levels of NKp30a and NKp30b isoforms and low levels of NKp30c isoform, while dNK cells had high levels of NKp30c but significantly lower amounts of NKp30a/b (105). While crosslinking NKp30 on IL-15-stimulated pNK cells induced degranulation, freshly isolated dNK cells did not degranulate, and co-crosslinking NKp30 in dNK cells did not inhibit NKp46-mediated degranulation, consistent with the lack of activation by NKp30c in previously described studies by Delahaye et al. (94, 105). Coculturing pNK cells in presence of IL-15 and IL-18 induced increased expression of NKp30a/b isoforms, whereas further addition of TGF- β suppressed the induction of expression of all three isoforms, although expression of NKp30b and NKp30c isoforms was greater than NKp30a upon addition of TGF- β (105). Overall, the combination of IL-15, IL-18, and TGF- β , which are found together in the decidua stromal microenvironment, shifted pNK cells toward higher expression of inhibitory NKp30c isoform and expression of other markers characteristic of dNK cells (105).

Shemesh et al. found significantly increased mRNA encoding the activating NKp30a/b isoforms in the placenta of women who had experienced sporadic or recurrent miscarriage within the first trimester, whereas this shift toward activating isoforms was not evident in the peripheral blood (106). The increase in activating isoforms did not correlate with higher expression of TNF- α , IFN- γ , IL-10, and placental growth factor mRNAs in the placental tissue of those women who experienced sporadic miscarriage, as compared to those that had undergone elective abortions. These results suggest that increased expression of these activating isoforms of NKp30 on dNK cells may be in some way contributing to failed pregnancies through promoting dysregulated cytokine production in the placenta.

Splice Variants of *ncr2*

Three major mRNA splice variants of *ncr2* have been recognized, and one of these (NKp44-1) encodes the classic receptor that possesses a cytoplasmic ITIM, while the others (NKp44-2 and NKp44-3) have alternative sequences in the cytoplasmic region that lack ITIMs. It has long been known that IL-2-cultured

NK cells upregulate expression of an activating form of NKp44-1 associated with the transmembrane signaling protein, DAP12, which becomes tyrosine phosphorylated upon receptor engagement with an antibody (11, 40, 41). Recent analysis of the major NKp44 isoforms, however, has demonstrated that isolated human NK cells cultured in IL-2- or IL-15 express predominantly NKp44-1 mRNA and have reduced capacity to kill PCNA-transfected target cells in an NKp44-dependent manner (12, 107). Furthermore, NK-92 cells transduced to overexpress NKp44-1 show suppressed cytotoxicity and diminished immune synapse formation toward PCNA-transfected target cells, as compared to NK-92 transduced to overexpress the other isoforms. These results indicate that the NKp44-1 isoform is an inhibitory receptor when engaged with the PCNA ligand expressed by target cells, whereas NKp44-2 and NKp44-3 do not transduce inhibitory signals (107).

NKp44 Isoforms in Cancer

Shemesh et al. studied the impact of NKp44 isoforms on overall survival of patients with acute myeloid leukemia (AML) using TCGA RNA-Seq data (107). Whereas no survival advantage was found in newly diagnosed patients who expressed mRNA encoding NKp44, as compared to those lacking expression, survival was significantly diminished in patients who exclusively expressed NKp44-1, as compared to patients who also expressed at least some detectable level of NKp44-2 and/or NKp44-3 or lacked NKp44 expression altogether. These results imply that NKp44 plays a key role in NK cell responsiveness toward AML tumors, but exclusive expression of the ITIM-containing NKp44-1 isoform can stifle these beneficial responses, presumably through inhibitory signaling, thereby resulting in poor patient outcome.

The same group found higher incidence of NKp44 mRNA in various solid tumor tissues, as compared to surrounding normal tissues (95). Those tumor samples that expressed NKp44 mRNA were found to consist of predominantly NKp44-1 isoform. Thus, NK cells in the tumor microenvironment predominantly express the inhibitory NKp44 isoform.

NKp44 in the Decidua of Pregnant Women

Siewiera et al. found that while NKp44-2 mRNA is expressed more by freshly isolated pNK cells than NKp44-1 and NKp44-3, dNK cells from women undergoing elective first trimester abortions (healthy pregnancies) express all three isoforms of NKp44 at similar levels (105). While crosslinking NKp44 on IL-15-stimulated pNK cells induces degranulation, crosslinking on freshly isolated dNK cells did not result in degranulation and co-crosslinking suppressed degranulation in response to crosslinking NKp46, demonstrating predominant inhibitory function for NKp44 in decidua/placental NK cells (105).

Shemesh et al. also found that NKp44-2 and NKp44-3 isoforms predominated in decidua tissue obtained from the majority of first trimester spontaneous abortions, while a NKp44-1-dominant (inhibitory) profile was found in dNK cells from most elective abortions or term deliveries (healthy pregnancies), which is consistent with the inhibitory function of dNK cells in the study by Siewiera et al. (95).

Splice Variants of *ncr1*

Recent work by Shemer-Avni et al. has also provided new insights on differential splicing of *ncr1* (108). Five major splice variants of *ncr1* have been described, and three of these encode NKp46 protein isoforms containing both extracellular domains, while two lack the first Ig-like domain (D1, encoded by exon 3). Surprisingly, NK-92 cells transduced with a D1-negative *ncr1* cDNA degranulated significantly more efficiently toward HEK293T target cells, as compared to NK-92 cells transduced to express conventional NKp46 protein containing both Ig-like domains (108). Using NKp46-reactive antibodies, D1-negative NKp46 was not observed in fresh peripheral blood from healthy donors, but a subset expressing the NKp46 D1-negative receptor was found after long-time culturing of NK cells in IL-2 (108). This subset of NK cells containing D1-negative NKp46 degranulated more robustly in response to a combination of plate-bound anti-NKp46 and anti-NKp30 antibodies (108).

This group also studied NKp46 isoform expression in upper airway lavage samples from pediatric patients with respiratory tract viral infections. While most of these samples expressed both NKp30 and NKp46 mRNA, none contained mRNA encoding NKp44. When *ncr1* splice variants were analyzed, most of the lavage samples from virus-infected patients were found to contain D1-negative NKp46 isoform transcripts (108). Taken together, these results suggest that NK cells by IL-2 or viral infection can express isoforms of NKp46 lacking the D1 Ig-like domain, and NK cells expressing these domain-deficient receptors exhibit increased functional capacity.

CONCLUSION

Significant progress has been made in recent years to improve our understanding of the functions and ligand recognition capacities of NCRs. Clearly these receptors play important roles in NK cell recognition of tissue changes in cancer, viral infections, decidua tissues in pregnancy, and immature DC. This new knowledge is crucial for establishing the basis of molecular mechanisms controlling NK cell responses under these diverse conditions. In

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contrast to the original dogma that NCRs are exclusively activating receptors, several new findings have revealed inhibitory functions for these receptors.

Numerous ligands or co-ligands for NCRs have now been described. Several of these can surprisingly trigger inhibitory signals or induce production of inhibitory cytokines when engaged with NCRs. Furthermore, some of these putative ligands are classically nuclear or cytosolic proteins that appear to relocate to the cell surface in cancer cells, where they can engage with NCRs. Also, heparan sulfates seem to have capacity to interact with all three of the NCRs and may regulate their functions in trans and cis.

The study of differentially spliced isoforms of NCRs has revealed surprising insights, since some of these isoforms elicit inhibitory function. Furthermore, dominant expression of the inhibitory forms has been linked to poor outcome in the context of cancer, but healthy outcome in pregnancy. Nonetheless, our understanding of the complexities of NCR isoforms is still in its infancy and requires a great deal of additional study.

Future work is clearly needed to sort out true NCR ligands and functional mechanisms responsible for the functions of some NCR isoforms, and their complexities are growing. Importantly, a firmer foundation of understanding promises to provide potential opportunities as prognostic indicators of disease status or opportunities to develop therapeutic strategies to manipulate NCRs on NK cells that could be beneficial to treat a wide variety of human pathologies.

AUTHOR CONTRIBUTIONS

TP and KC authored and edited the manuscript. AS, MB, and AP provided critical input and editing.

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Shaping of Natural Killer Cell Antitumor Activity by *Ex Vivo* Cultivation

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Natural killer (NK) cells are a promising tool for the use in adoptive immunotherapy, since they efficiently recognize and kill tumor cells. In this context, *ex vivo* cultivation is an attractive option to increase NK cells in numbers and to improve their antitumor potential prior to clinical applications. Consequently, various strategies to generate NK cells for adoptive immunotherapy have been developed. Here, we give an overview of different NK cell cultivation approaches and their impact on shaping the NK cell antitumor activity. So far, the cytokines interleukin (IL)-2, IL-12, IL-15, IL-18, and IL-21 are used to culture and expand NK cells. The selection of the respective cytokine combination is an important factor that directly affects NK cell maturation, proliferation, survival, distribution of NK cell subpopulations, activation, and function in terms of cytokine production and cytotoxic potential. Importantly, cytokines can upregulate the expression of certain activating receptors on NK cells, thereby increasing their responsiveness against tumor cells that express the corresponding ligands. Apart from using cytokines, cocultivation with autologous accessory non-NK cells or addition of growth-inactivated feeder cells are approaches for NK cell cultivation with pronounced effects on NK cell activation and expansion. Furthermore, *ex vivo* cultivation was reported to prime NK cells for the killing of tumor cells that were previously resistant to NK cell attack. In general, NK cells become frequently dysfunctional in cancer patients, for instance, by downregulation of NK cell activating receptors, disabling them in their antitumor response. In such scenario, *ex vivo* cultivation can be helpful to arm NK cells with enhanced antitumor properties to overcome immunosuppression. In this review, we summarize the current knowledge on NK cell modulation by different *ex vivo* cultivation strategies focused on increasing NK cytotoxicity for clinical application in malignant diseases. Moreover, we critically discuss the technical and regulatory aspects and challenges underlying NK cell based therapeutic approaches in the clinics.

Keywords: natural killer cells, natural killer cell cultivation, natural killer cell expansion, natural killer cell therapy, natural killer cell cytotoxicity, *ex vivo* stimulation

INTRODUCTION

As an important part of the innate immune system, natural killer (NK) cells are deployed as first line of defense against aberrant cells caused by viral infections or malignancies. Human NK cells can be identified *via* their morphology as large granular lymphocytes, and *via* their surface marker profile, as they express by definition CD56, but not CD3. The NK cell compartment can be further divided into subpopulations. There are two main NK cell subsets that can be distinguished, the CD56^{high}CD16^{neg} subpopulation, which has mostly immune modulatory function, mainly accomplished by interferon (IFN)- γ secretion, and the CD56^{low}CD16^{pos} fraction with direct cytotoxic capacity (1–3). NK cell activation is based on a balanced system integrating signals from activating and inhibitory receptors. Inhibitory signals derive mainly from germ-line encoded inhibitory killer cell immunoglobulin-like receptors (KIRs). Ligands for inhibitory KIRs, in humans major histocompatibility complex (MHC) class I molecules, are highly expressed by healthy cells and thereby prevent NK cell activation. Malignant cells often downregulate MHC class I molecules on their surface to evade T cell attack (4). However, these so-called “missing-self” cells are recognized by NK cells through inhibitory receptors, and as signals from activating receptors prevail, NK cells become active and react against the encountered targets. Alternatively, NK cells can be activated by overexpression of stress-induced surface ligands on infected or abnormal cells, which are recognized by activating receptors, such as the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46, and the so-called C-type lectin-like receptors, such as NKG2D (1, 5–9). In this case, activating signals outbalance inhibitory self-signals and lead to NK cell activation. Furthermore, NK cells become activated upon encounter of antibody-coated targets by CD16, which binds to the Fc portion of the antibody and mediates a strong activating signal. By means of activating and inhibitory receptors, NK cells, unlike T and B-lymphocytes, can react immediately without prior priming or antigen presentation.

Activated NK cells execute effector functions through different mechanisms. NK cells mediate direct cytotoxicity *via* the exocytosis pathway with release of cytotoxic granules, which contain granzymes and perforin, resulting in lysis of the target cell (10). In addition, NK cells induce apoptosis of target cells by expression of death receptor ligands, such as Fas ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (11). Production and release of IFN- γ by NK cells after activation also has multiple functional consequences, with particular relevance in tumor surveillance, as IFN- γ inhibits tumor angiogenesis, has antimetastatic activity, and acts pro-apoptotic (12, 13).

The ability of tumor cells to bypass the immune response is a basic prerequisite for cancer formation and progression. Within immune editing, tumors undergo genetic, epigenetic, and phenotypic changes, thereby becoming a heterogeneous cell population that is hardly visible to or assailable by immune cells due to down-regulation of tumor antigens and NCR ligands (14). Additionally, malignant cells suppress NK cells by blocking the NKG2D receptor *via* shedding of NKG2D ligands (15–17) or upregulation of inhibitory MHC class I molecules (18, 19). Immunosuppressive

cytokines such as transforming growth factor- β , interleukin (IL)-10, or immunosuppressive enzymes, such as indoleamin 2,3-dioxigenase, further impair antitumor NK cell responses of cancer patients (20–22).

Ex vivo modulation of NK cell receptor expression is therefore an important tool to overcome immune response inhibition. A number of studies reported an upregulation of DNAM-1, NKG2D, and other NK cell-activating receptors under certain culture conditions, mostly involving stimulation by IL-2 (23–26). In addition, other ILs such as IL-12, IL15, IL-18, or IL-21 and Type I IFNs shape the NK cell receptor expression profile (27–31).

Natural killer cells can play an important role for cellular immunotherapy and the adoptive transfer of NK cells represents an attractive strategy to treat cancer patients (32, 33). In this context, *ex vivo* expansion of NK cells prior to their clinical application is not only required to increase the applicable cell doses but it is also reasonable to pre-activate and modify their antitumor features. For *ex vivo* cultivation, NK cells from different sources can be stimulated with different cytokines, and, to reach efficient expansion rates, NK cells are cultured among autologous accessory cells or together with different types of growth-inactivated autologous or allogeneic feeder cells (Figure 1). Of note, it is possible to genetically engineer NK cells *ex vivo* to further augment their antitumor activity, for example, to integrate chimeric antigen receptors against distinct tumor antigens (34, 35). In this review, we focus on the cultivation of NK cells without genetic modifications. Many different protocols exist for *ex vivo* expansion of NK cells, all with different features and capacities. Here, we give a comprehensive overview of strategies to obtain appropriate amounts of functional NK cells. We will discuss starting material and culture systems as well as the use of cytokines, feeder cells, and other additives.

STARTING MATERIAL FOR NK CELL EXPANSION AND ROLE OF NK CELL PURITY

Until recent, 92% of clinical studies used NK cells from peripheral blood, either donor (79% of recruiting trials) or patient derived (13% of recruiting trials) (36). Alternatives are the use of NK cell lines, or the differentiation of NK cells from umbilical cord blood or pluripotent stem cells (37–39). NK cell lines, such as NK-92, avoid the need for donor selection and enable the production of large cell doses to treat patients on a flexible schedule (40). Nevertheless, NK cell lines require growth inactivation mainly achieved by irradiation, possibly reducing their antitumor potential due to short *in vivo* persistence. Differentiation of NK cells from cord blood CD34⁺ cells is attractive because of the “off-the-shelf” availability from a cord blood bank. Similarly, NK cells from pluripotent stem cells are a promising concept for the future but still in early development (39, 41). In this overview, we focus on peripheral blood-derived NK cells, currently the main source for NK cells for clinical use.

The NK cell purity, meaning the frequency of NK cells among other cells, is an important factor for the intended therapeutic

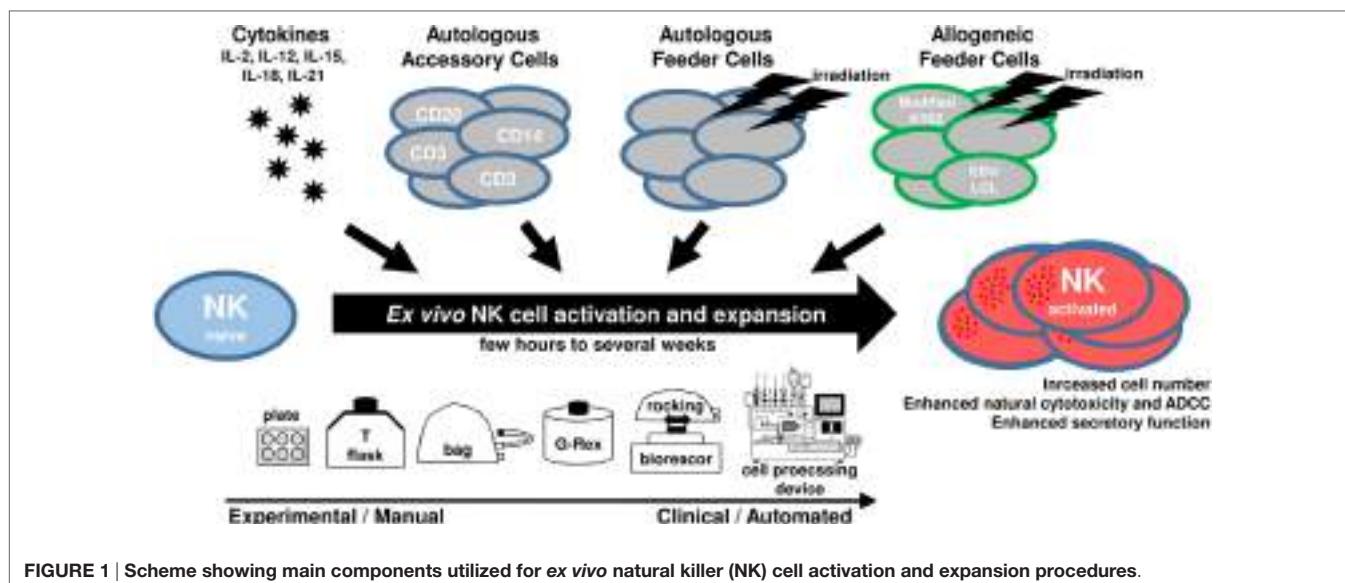


FIGURE 1 | Scheme showing main components utilized for ex vivo natural killer (NK) cell activation and expansion procedures.

application. For *ex vivo* expansion, NK cells are often cultured within a mixture of cells, such as PBMC, thereby avoiding further purification. Whereas the cultivation of NK cells among other accessory cells is a practical strategy for autologous therapeutic settings, it may be critical for allogeneic applications, since non-NK cells may induce unwanted side effects. Alloreactive T cells are a major risk factor for the patient, as they mediate “graft-versus-host disease” (GvHD), a severe complication following allogeneic hematopoietic stem cell transplantation (HSCT) (42). Furthermore, donor-derived B cells can lead to B cell lymphoproliferative disorder after reactivation of an Epstein–Barr virus (EBV) infection (43, 44), and they can cause the passenger lymphocyte syndrome (45), both critical side effects for the patient. Therefore, purification of NK cells might be required and is realized so far in most clinical settings by magnetic cell separation, for instance, by depletion of CD3-expressing cells and subsequent enrichment for CD56-expressing cells (26, 46–49). In addition, a first proof of concept is shown for good manufacturing practice (GMP)-compliant fluorescence-activated cell sorting to purify for NK cell subsets, such as NK cells expressing a single KIR (50).

CYTOKINE-INDUCED NK CELL EXPANSION

Aims of adoptive transfer of *ex vivo* expanded NK cells are the enhancement of natural cytotoxicity and homing to tumor sites under maintenance of “self” protection. Studies performed with cytokine-stimulated NK cells or PBMC have shown the safety of this approach and indicated some clinical responses upon adoptive NK cell transfer following HSCT. In the next paragraph, we summarize *ex vivo* NK cell expansion protocols starting with purified NK cells (Table 1) or PBMC (Table 2). Concepts administering cytokines in the presence of growth-inactivated feeder cells will be discussed in later sections of this article.

THE ROLE OF IL-2

Interleukin-2 plays an important role in activation of NK cells *via* binding to the IL-2 receptor (IL-2R), a heterotrimeric protein expressed on NK cells and other immune cells. This led to the interest in both (i) using IL-2 for stimulation of autologous NK cells in cancer patients and (ii) *ex vivo* activation and expansion of allogeneic donor NK cells for adaptive immunotherapy. At the beginning of the 1980s, researchers around Rosenberg and colleagues showed that IL-2 exposed lymphokine-activated killer (LAK) cells were able to attack autologous fresh tumor cells and that this effect could mainly be ascribed to NK cells (71, 72). Nevertheless, in first clinical trials using adoptive transfer of LAK cells and IL-2 therapy, the clinical response did not exceed the efficacy of IL-2 monotherapy (73).

Importantly, during the last 20 years, it has been elaborated that NK cells play a major role in the regulation of the balance between GvL and GvHD after allogeneic HSCT, especially haploid-identical HSCT (33, 74–78), demonstrating improved antitumor activity while avoiding GvHD. In order to make haploid-identical NK cells available for clinical use, large-scale GMP-conform manufacturing protocols were established. After starting with a leukapheresis product that was depleted for CD3⁺ cells and enriched for CD56⁺ cells, cultivation in medium containing IL-2 (1,000 U/mL) for up to 2 weeks yielded $0.1\text{--}3 \times 10^9$ CD56⁺CD3⁻ NK cells (Table 1), sometimes sufficient for multiple infusions to patients with hematological malignancies (26). Median NK cell expansion was fivefold and median NK cell purity was >94 with <0.1% T cell contamination (26). *Ex vivo* stimulation with IL-2 induced elevated cytokine secretion by NK cells, enhanced intracellular STAT3/AKT signaling, and upregulation of various NCRs and NKG2D receptors (52). Depletion of CD3 cells from leukapheresis products without subsequent CD56 enrichment and short-term activation with IL-2 overnight led to a final product containing 40% NK cells (Table 2). In all cases, IL-2-activated NK cells demonstrated a much higher cytotoxic activity against

TABLE 1 | *Ex vivo cultivation of pure natural killer (NK) cells with cytokines only.*

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
IL-2 + IL-15	PBMC, CD3 depleted and CD56 enriched or PBMC, CD3/CD19 depleted in flasks	>1 (5 days)	75–100% NK ≤0.1% T cells	Upregulated: CD69, Nkp30, and Nkp44	Cytolysis of leukemia cell lines and primary acute leukemic blasts	In vitro	(51)
IL-2	PBMC, CD3 depleted, and CD56 enriched in bags and flasks	4–5 (12–14 days)	~92–95% NK <0.1% T cells	Increased CD56/CD16 frequency; increased p-STAT3 and p-AKT; upregulation of CD69, NKG2D, and natural cytotoxicity receptors (NCRs); increasing amount of NK cells without killer cell immunoglobulin-like receptors	Improved cytotoxic activity against leukemia and tumors	Clinical	(26, 52, 53)
IL-15	Isolated CD3-CD56 ⁺ cells	N/A (2–5 weeks)	94–99% NK	Enhanced killing via NCRs, DNAM-1 and NKG2D	Enhanced cytolysis of lymphoma and rhabdomyosarcoma cell lines via NCRs	In vitro	(54)
IL-2 + IL-21	PBMC, sorted for CD3-CD56 ⁺	None with IL-21 only; strongly with IL-2 + IL-21	N/A	Upregulated: CD69, CD25; activation of STAT3	Enhanced cytotoxicity against K562	In vitro	(55)
IL-12 + IL-15 + IL-18	PBMC, sorted for CD3-CD56 ⁺ cells	N/A (12–16 h)	≥90% NK	Upregulated: CD94, NKG2A, Nkp30, Nkp44, NKG2D, Nkp46, CD69, and CD25 Downregulated: Nkp80	Memory; increased IFN-γ production upon stimulation that is preserved during cell division Responsive to picomolar concentrations of IL-2	In vitro	(56–59)

K562 target cells compared to unstimulated NK cells (26, 43, 52, 53, 60). In addition, after cryopreservation and thawing, NK cells showed a moderate to high viability when activated with IL-2, whereas the viability of unstimulated NK cells was low (26).

Transfer into the clinic in 2004 and 2005 with first patient studies using those IL-2-activated donor NK cells were performed in parallel in Europe and the US, for both, haploidentical HSCT (53), and in the non-transplant setting (43). In the latter one, Miller and coworkers used IL-2 expanded haploidentical NK to treat 43 patients with advanced cancer (43), with 19 of them suffering from acute myeloid leukemia, followed by studies in patient with ovarian and breast cancer and B-cell non-Hodgkin lymphoma (60, 79). Importantly, the authors reported *in vivo* persistence and even expansion of the alloreactive donor NK cells in patients pretreated with high dose preparative regimen, consisting of 5 days of 60 mg/kg intravenous cyclophosphamide and 25 mg/m² intravenous fludarabine (43). Of note, successful NK cell engraftment was dependent on the patients' pretreatment regimen, which was also responsible for the patients' elevated IL-15 plasma concentrations (43). In addition, it was demonstrated that *in vivo* persistence of donor NK cells at day 7 after infusion and successful *in vivo* expansion (more than 100 donor-derived NK cells per microliter of patient blood 14 days after transfer) correlated with leukemia clearance (60). Expansion of host regulatory T cells was associated with low numbers of NK cells (60). In parallel, Koehl et al. reported on three pediatric patients with multiply relapsed leukemia (still in blast persistence at HSCT) treated with repeated transfusions of IL-2-activated donor NK cells post-haploidentical HSCT (53), which led to complete remission remaining for several weeks up to some months. In the following clinical study, they also demonstrated a small clinical benefit in patients with various malignancies receiving IL-2-activated compared to patients receiving resting NK cells only (80). Interestingly, IL-2-stimulated NK cells but not unstimulated NK cells promoted NK cell trafficking and changes in the distribution of leukocyte subpopulations in the peripheral blood. In the meanwhile, safety and feasibility using IL-2-activated and -expanded NK cells for adaptive immunotherapy has been demonstrated in various clinical studies as summarized in a recent review by Koehl and others (33).

IMPACT OF IL-15 ON NK CELL EXPANSION

Carson et al. postulated that NK cells might be dependent on other cytokines than IL-2 such as IL-15 (81, 82). The trimeric IL-15 receptor on NK cells shares two subunits with the IL-2R, but not CD25 forming the high affinity IL-2R. Therefore, they also share some functions, e.g., maintenance of NK cell survival (82). Similarities and differences between IL-2 and IL-15 effects on NK cells have been extensively reviewed elsewhere, and IL-15 might be the preferable cytokine for cancer therapy as it inhibits activation-induced cell death and it is considered safe (83–85). In addition, compared to IL-2, IL-15 leads to more sustained antitumor capacity of NK cells *via* signaling through mammalian target of rapamycin and stress-activated gene expression (86). However,

TABLE 2 | *Ex vivo* cultivation of natural killer (NK) cells with accessory cells.

Protocol features	Starting material/ culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
IL-2	PBMC, CD3 depleted in bags and flasks	N/A (overnight)	33% NK 0.1% T cells	N/A	N/A <u>Enhanced cytotoxicity <i>In vitro</i></u> <u>Enhanced cytotoxicity <i>In vitro</i></u>	Clinical	(60) (61, 62) (43)
		N/A (14–16 h)	26.7%				
		N/A (overnight)	40% NK 0.9% T cells				
IL-15	PBMC, CD56 enriched	23 (20 days)	98% NK	Expression of NKp30, NKp44, NKp46, NKG2D, and 2B4	Cytotoxic <i>In vitro</i>	Clinical	(63)
IL-15 + IL-21	PBMC, CD3 depleted	3.7 CD56 ⁺ /CD122 ⁺ (2–3 weeks)	>90% CD56 ⁺ /CD122 ⁺ <0.3% CD3 ⁺ /CD56 ⁻ <3% CD3 ⁺ /CD56 ⁺	67% CD56 ⁺ CD16 ⁺	Cytotoxic against K562 and patient bone marrow blasts	Clinical	(64)
OKT-3 + IL-2	PBMC in plates	193 (21 days)	~55% NK ~22% T cells	N/A	Substantial cytotoxicity against K562	<i>In vitro</i>	(65)
	PBMC in flasks	1,625 (20 days)	~65% NK ~22% T cells	<i>Upregulated:</i> 2B4, CD8, CD16, CD27, CD226, NKG2C, NKG2D, NKp30, NKp44, NKp46, LIR-1, KIR2DL3, and CXCR3 <i>Downregulated:</i> CCR7	Increased cytotoxicity against tumor cell lines and primary MM cells <i>In vitro</i>	<i>In vitro</i>	(25)
	PBMC	1,036 (total cells) (19 days)	~30% NK ~40% T cells	<i>Upregulated:</i> NKG2A, LILR-B1, NKG2D, NKp30, NKp44, and NKp46	<i>In vitro</i> cytotoxicity increases during culture	Clinical	(66)
	PBMC in a bioreactor, flasks, and plates	77—bioreactor 530—bags 770—flasks (20 days)	38%—bioreactor 31%—bags 44%—flasks	Bioreactor compared to flasks: higher expression of CD11b, NKG2D, and NKp44	Bioreactor compared to flasks: higher cytotoxicity	<i>In vitro</i>	(67)
OKT-3 + IL-2 + Alemtuzumab	PBMC in plates, flasks, and bags	646 (14 days) 1,537 (18 days)	60% NK 37% T cells <0.1% B cells	<i>Upregulated:</i> 2B4, NKG2D, NKp30, NKp44, KIR2DL1, LIR-1, and CD16 <i>Downregulated:</i> CCR7	Increased cytotoxicity <i>In vitro</i> and <i>in vivo</i>	Clinical	(68)
OKT-3 + IL-2 + IL-15	PBMC or CD56 ⁺ + CD56 ⁻ (1:1) in flasks and bioreactor (Cellbag)	PBMC: 112 1:1 Mix: 89 (21 days)	With PBMC: 34% With “1:1 Mix”: 92%	<i>Upregulated:</i> NKp30, NKp44, DNAM-1, NKG2D, and CD11a	Increased activity against neuroblastoma cell lines <i>In vitro</i> and <i>in vivo</i>	Preclinical model	(69)
aCD16 mAb + OK432 + IL-2	PBMC in flasks and bags	637–5,712 (day 21)	79% NK 8.4% T cells (day 21)	<i>Upregulated:</i> NKG2D, NKp44, and CD69 <i>Downregulated:</i> CD16 (transient)	Increased cytotoxicity against tumor cell lines and primary cancer cells <i>In vitro</i> ADCC activity	<i>In vitro</i>	(70)

recent data revealed that continuous IL-15 signaling causes functional exhaustion of NK cells by decreased fatty acid oxidation, resulting in lower cytotoxicity *in vitro* and decreased tumor control *in vivo* (87). Thus, optimal dosing and timing of IL-15 is critical for *ex vivo* NK cell activation. Purified NK cells expanded using IL-15 exhibit upregulation of NCRs and CD69 and cytolysis of leukemia and primary ALL blasts (51). Enhanced cytotoxicity of IL-15-stimulated NK cells against leukemia and rhabdomyosarcoma cell lines could be attributed to NCRs, DNAM-1 and NKG2D (54). Using IL-15 to expand NK cells from CD56-enriched PBMC for 20 days resulted in a 23-fold expansion of CD3-CD56⁺ NK cells with a final purity of about 98% (63). NK cells generated with the latter protocol were transferred to 15 non-small lung cancer patients in a phase I clinical trial in two to four doses of $0.2\text{--}29 \times 10^6$ NK cells/kg, showing the safety of the approach (63).

IL-21 ENHANCES NK CELL EFFECTOR FUNCTIONS

The cytokine IL-21, in combination with IL-2 or IL-15, is utilized in some protocols for NK cell stimulation (55, 64). IL-21 belongs to the IL-2 family and signals through a heterodimer consisting of the common γ -chain and the IL-21 receptor α -chain. Activated CD4⁺ T cells are the main producers of IL-21 and IL-21 affects many different cell types expressing the IL-21 receptor, including NK cells (88). IL-21 plays a role in the development of NK cells from bone marrow progenitors (89), and, in mice, it dampens the expansion of NK cells but is required for functional NK cell maturation (90, 91). Recently, expansion of “memory-like” NK cells has been shown to be IL-21 dependent in the context of tuberculosis infection (92). Wendt et al. observed increased proliferation of CD56^{bright} human NK cells (55), but another group reported no effect of IL-21 on the proliferation of NK cells from healthy human donors and from HIV patients (93). Moreover, IL-21 is known to trigger apoptosis, resulting in a shorter lifespan of NK cells *in vitro* (90, 94). Thus, the time span NK cells are exposed to IL-21 appears critical (95, 96). Besides its effect on NK cell proliferation, IL-21 enhances the effector functions of NK cells, including secretory and cytotoxic functions as well as enhanced ADCC responses (93, 97, 98). Culturing CD3-depleted PBMC for 13–20 days with IL-21 and IL-15 without additional feeder cells yields activated NK cells with a purity of >90%, which were applied in a clinical trial with 41 leukemia patients receiving infusions of donor-derived NK cells 2–3 weeks after HSCT (64). Although the NK cells expanded weakly under this condition (3.7-fold), they possessed potent cytotoxic activity against primary bone marrow blasts prior to transplantation, and infusions with a median dose of 2×10^8 NK cells/kg were well tolerated and correlated with a reduction in leukemia progression compared to historical controls (64).

IL-12/15/18 INDUCED MEMORY NK CELLS

Interleukin-12 was originally discovered as NK cell-stimulating factor, inducing proliferation, enhanced cytotoxicity, and

production of IFN- γ by NK cells when added to PBMC (99, 100). IL-12 is produced by DCs, macrophages, and B cells, and its receptor consists of two subunits (α and β), which mediate signaling through members of the JAK-STAT family (101). IL-2 enhances the response of NK cells to IL-12 by increasing the expression of the IL-12 receptor and STAT4, a relevant factor for IL-12 signaling (102). Furthermore, it was revealed that IL-12-mediated IFN- γ production of NK cells requires priming with IL-18, a cytokine also known to enhance IL-15-induced NK cell proliferation (103, 104). Due to the synergistic effects, it seems reasonable to combine the different cytokines for *ex vivo* stimulation of NK cells. In this context, the combination of IL-12, IL-15, and IL-18 raised special interest, as it leads to the so-called “cytokine-induced memory-like” (CIML) NK cells in mice and humans, which exhibit an increased capacity to produce IFN- γ upon re-stimulation at later time points (56, 105). Importantly, this memory response is a cell intrinsic effect that is passed on to offspring cells and is maintained up to several months (56). In mice, the intrinsic ability for mediated IFN- γ production coincided with demethylation of the conserved non-coding sequence 1 in the IFN- γ locus (106). Furthermore, adoptive transfer of CIML NK cells had a clear antitumor activity against established melanoma or lymphoma *in vivo*, which required IL-2 from CD4⁺ T cells (57, 106). For both, murine and human NK cells, IL-12, IL-15, and IL-18 together induce an increased expression of CD25, making CIML NK cells responsive to low concentrations of IL-2 *in vitro* and *in vivo* (57, 58). Thus, there is a clear rationale to apply adoptive transfer of *ex vivo*-generated CIML NK cells together with IL-2 injections as a combination therapy. Recently, CIML NK cells together with low dose IL-2 therapy were evaluated in a first-in-human phase I clinical trial with promising results, as clinical response was observed in five of nine treated patients (107).

AUTOLOGOUS ACCESSORY CELLS AND AUTOLOGOUS FEEDER CELLS FOR NK CELL EXPANSION

Although cytokines efficiently activate NK cells and result in cell products with advanced effector functions, cytokines alone do not allow pronounced *ex vivo* expansion (Table 1). Consequently, in addition to the activation with cytokines, stimuli from autologous accessory cells can be used to further enhance the expansion of NK cells to overcome the hurdle of limited NK cell doses for adoptive NK cell therapy (Table 2). Outgrowth of NK cells from the whole PBMC fraction is more effective than cultivation of pure NK cells, because other cell types provide additional factors for NK cell proliferation. CD14⁺ cells, for instance, enhance the *ex vivo* NK cell proliferation *via* direct cell contact and soluble factors (108, 109). After activation, for instance by concanavalin A, T cells also trigger NK cell proliferation (110).

Stimulation of PBMC with IL-2 and the clinically approved anti-CD3 antibody OKT-3 leads to a profound outgrowth of NK cells (25, 65–67, 111), probably by activation of T cells and this is utilized by several clinical protocols for NK cell cultivation. Nevertheless, starting the culture from PBMC goes along with

extensive coexpansion of unwanted CD3⁺/CD56⁻ T cells and CD3⁺/CD56⁺ NK-like T (NKT) cells, accounting for the majority of cells in the final cellular product. Surprisingly, infusion of this heterogeneous cell product without removal of potentially alloreactive T cells did not cause side effects, such as GvHD, in a safety trial with five cancer patients, evaluating the cultivated cellular product in an allogeneic setting (66). This can be explained by the fact that T cells may lose their alloreactivity during extended *ex vivo* expansion (112). Thus, low NK cell purities may be less critical for long-term cultivated cellular products compared to NK cells directly obtained from a donor, but more clinical data are required to prove this hypothesis. Of note, the approach also allows efficient expansion of functional patient-derived NK cells, as shown for B cell chronic lymphocytic leukemia and multiple myeloma patients, enabling therapy with autologous NK cells and further circumventing possible safety risks of therapy with donor-derived cells (25, 111).

Starting with PBMC enriched for CD56 cells together with the corresponding non-CD56 PBMC in a 1:1 mixture favors a 89-fold NK cell expansion with a final product consisting of 92% NK cells after 21 days (69). Alternatively, adding irradiated autologous PBMC to the culture is a strategy to benefit from these “feeder cells” for NK cell activation and expansion but to avoid their coexpansion (**Table 3**). Of note, to make a clear difference, we use the term “feeder cells” for all inactivated cells that are added to the culture, whereas cocultured non-NK cells that are not inactivated are defined as “accessory cells.” Besides its growth inactivating function, irradiation can induce upregulation of stress-regulated surface molecules on PBMC, such as ULBP1–3, that further trigger NK cell activation, e.g., through NKG2D (113). Still, irradiated autologous PBMC induce only weak NK cell proliferation without additional activation of the feeder cells (e.g., only 16-fold expansion within 2 weeks) (24). Whereas irradiated autologous PBMC previously activated with IL-2, OKT-3 and RetroNectin allow a median 4,720-fold NK cell expansion after 3 weeks with a NK cell purity of 91% starting from PBMC (114). To obtain a more pure final product with 98% NK cells, it is possible to start the culture with already CD3-depleted PBMC and add irradiated autologous PBMC as feeder cells together with IL-2 and OKT-3 (23). The highest purity can be achieved by cell sorting, representing also the method of choice to expand defined NK cell subpopulations. As demonstrated by Siegler et al., GMP-sorted and highly pure single KIR⁺ NK cells can be expanded 160- to 390-fold in 19 days with IL-2, IL-15, OKT-3, and irradiated autologous PBMC (50).

NK CELL EXPANSION WITH ALLOGENEIC FEEDER CELLS

Using irradiated allogeneic cells as feeder cells is another option to stimulate NK cell expansion *ex vivo* (118) (**Table 4**). Compared to autologous PBMC, allogeneic PBMC may be even more efficient as feeder cells for NK stimulation. Accordingly, in a study testing the expansion of NK cells from patients with advanced lymphomas or terminal solid tumors, 300-fold NK expansion was obtained with irradiated allogeneic PBMC feeder cells from healthy donors, whereas only 169-fold expansion was achieved with irradiated autologous PBMC feeder cells from the patients

(115). Furthermore, whereas the availability of autologous feeder cells is limited, as they have to be obtained directly from the patient, for allogeneic feeder cells it is possible to utilize established cell lines. Cell lines can be grown easily to sufficient numbers and different cell lines in fact trigger NK cell proliferation, such as HFWT, K562, RPMI 1866, Daudi, KL-1, MM-170, and different EBV-transformed lymphoblastoid cell lines (EBV-LCL) (99, 119–122).

Culturing PBMC together with the Wilms tumor cell line HFWT and IL-2 leads to significant NK cell expansion (124, 145), and interestingly under this condition NK cells not only arise from mature CD3⁻CD56⁺ NK cells but also from CD3⁻CD14⁻C D19⁻CD56⁻ NK cell precursors expressing CD122 (146). In 2004, early clinical data showed that adoptive transfer of autologous NK cells generated by coculture with irradiated HFWT is safe and patients with recurrent malignant glioma partially responded to the treatment (125).

Another advantage of cell lines is that it is relatively easy to genetically modify them and to integrate additional factors for NK cell stimulation. In recent years, modified K562 cells have been utilized, such as K562 expressing membrane-bound IL-15 and 41BBL (K562-mb15-41BBL) (126). While unmodified K562 only induce a weak NK cell proliferation (2.5-fold NK cell expansion in 1 week), with K562-mb15-41BBL the NK cell number can be significantly increased by 20- or 1,000-fold in 1 or 3 weeks (126). In addition, stimulation of NK cells with K562-mb15-41BBL demonstrated that NK cells actually have a substantial proliferative potential *ex vivo*, with up to 30 population doublings and 5.9×10^4 -fold NK cell expansion (147). NK cells expanded with K562-mb15-41BBL exhibit enhanced natural cytotoxicity against several allogeneic and autologous tumors *in vitro*, efficiently mediate ADCC and showed antitumor efficacy in mouse xenograft models for the treatment of sarcoma and myeloma (128, 148, 149). Of note, in a clinical trial assessing adoptive transfer of K562-mb15-41BBL following HSCT, acute GvHD occurred in five of nine patients, although the donors were completely HLA matched and the doses of injected NK cells and cotransferred T cells were low ($1-10 \times 10^5$ and $\leq 2 \times 10^4/\text{kg}$) (131). These observations suggested that the acute GvHD was T cell mediated, but NK cells apparently may promote this severe side effect indirectly (150). Importantly, another group utilized NK cells expanded with a similar K562 variant expressing 41BBL and IL-15 in another treatment setting and did not observe GvHD, although up to 1×10^8 NK cells/kg were administered (129).

Furthermore, Denman and colleagues revealed that K562 expressing 41BBL and membrane-bound IL-21 instead of IL-15 are even more effective for *ex vivo* expansion of NK cells, and weekly restimulation with this cell line supports a sustained NK cell proliferation over several weeks (134). In coculture with K562 expressing membrane-bound IL-21 and 41BBL, NK cells show an increased telomere length and enhanced activation of the STAT-3 signaling pathway, explaining the positive effect for sustained expansion of NK cells over long time (134, 151). Adoptive transfer of NK cells expanded with K562 expressing membrane-bound IL-21 and 41BBL into tumor-bearing mice improved the survival of the animals, indicating a therapeutic effect of these NK cells (135).

TABLE 3 | *Ex vivo* cultivation of natural killer (NK) cells with autologous feeder cells.

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
Irr. autologous PBMC (depleted for CD3-/CD56+ cells) + IL-2 + IL-15	PBMC, CD3 depleted, and CD56 enriched in flasks	16 (14 days)	97% NK 0.2% T cells	Upregulated: NKG2D, DNAM-1, NKp30, NKp44, CD158a, and CD158e	Efficient degranulation and lysis of K562 <i>In vitro</i>	<i>In vitro</i>	(24)
Irr. autologous PBMC activated with OK432, FN-CH296 and OKT-3 + IL-2	PBMC in flasks and bags	4,720 (21–22 days)	91% NK ~12% NK-like T and T	Strong expression of NKG2D and CD16	Elevated cytotoxicity that is maintained for up to 4 weeks after infusion to patients	Clinical	(114)
Irr. autologous PBMC + OKT-3 + IL-2	PBMC, CD3 depleted, and CD56 enriched in plates	169 (14 days)	84% NK	Upregulated: CD16, CD56, NKG2D, NKp30, and NKp44	Increased cytotoxicity against tumor cell lines <i>in vitro</i>	<i>In vitro</i>	(115)
	PBMC, CD3 depleted in flasks and bags	278–1,097 (21–26 days)	91–98% NK	Most cells express NKG2D, CD16, CD94, NKp46, KIR2DL1, KIR3DL1, and KIR2DL2/3	Efficient lysis of tumor cell lines <i>in vitro</i> ; persistence in patients up to several months; cytotoxic potential is lost <i>in vivo</i> , while ability for ADCC is maintained	Clinical	(116)
	PBMC, CD3 depleted in bags	691 (14 days)	98% NK 0.06% T cells	Upregulated: NKG2C, NKp30, NK44, CXCR4, CD25, CD62L, and CD69	Increased cytotoxicity against tumor cell lines <i>in vitro</i> ; antitumor effect and ADCC activity in a leukemia xenograft mouse model; up to 4 days persistence in patients	Preclinical model	(23)
Irr. autologous PBMC (depleted for CD3-/CD56+ cells) + OKT-3 + IL-2	PBMC, CD3 depleted, and CD56 enriched in plates and flasks	546 (14 days)	94.9% NK 2.2% T cells	Upregulated: NKG2D, NKp30, NKp44, tumor necrosis factor-related apoptosis-inducing ligand, and DNAM-1 <i>Downregulated</i> : NKp80	Increased cytotoxicity against tumor cell lines <i>in vitro</i>	<i>In vitro</i>	(113)
Irr. autologous PBMC + OKT-3 + IL-2 ± IL-15	PBMC, CD3 depleted, and CD56 enriched in plates and bags	117/63 in bags (\pm IL-15)	Bags: 45% NK 0.6% T cells	Upregulated: NKG2D, NKp44	High cytotoxicity against K562 and high productivity of IFN- γ	<i>In vitro</i>	(50)
	Good manufacturing practice killer cell immunoglobulin-like receptor (KIR) sorted NK cells in bags	993 in plates (19 days)	~100% NK >0.01% T cells	Single KIR + NK cells	Anti-leukemic activity against primary acute myeloid leukemia cells <i>in vitro</i> and <i>in vivo</i>	Preclinical model	(50)

TABLE 4 | *Ex vivo* cultivation of natural killer (NK) cells with allogeneic feeder cells.

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
Irr. allogeneic PBMC activated with ConA + IL-2	<i>In vivo</i> IL-2 primed PBMC depleted for non-NK cells in flasks	1–148 (14 days)	64–98% NK	N/A	Cytotoxic activity against leukemic cell lines	Clinical	(123)
Irr. allogeneic PBMC activated with ConA, PHA and ionomycin + IL-2 + IL-15	PBMC, depleted for CD3, CD4, CD19, and CD33 in bags	80–200 (15 days)	91% CD56 0.3% CD3 (day 12)	Upregulated: CD16, CD25	Increased cytotoxicity against tumor cell lines <i>in vitro</i> ; decreased frequency of INF- γ producing cells	<i>In vitro</i>	(118)
Irr. allogeneic PBMC + OKT-3 + IL-2	PBMC, CD3 depleted, and CD56 enriched in plates	300 (14 days)	94% NK	Upregulated: CD16, CD56, NKG2D, NKp30, and NKp44	Increased cytotoxicity against tumor cell lines <i>in vitro</i>	<i>In vitro</i>	(115)
Irr. HFWT + IL-2	PBMC in flasks	113 (2 weeks)	86% CD56 ⁺ /CD16 ⁺	N/A	Cytotoxic against tumor cell lines <i>in vitro</i>	Clinical	(124, 125)
Irr. Jurkat/KL-1 + IL-2	PBMC in flasks	~130 (2 weeks)	40–90% NK	Upregulated: CD54, CD11a, CD48, CD2, CD49d, CD58, NKp30, NKp44, 2B4, DNAM-1, NKG2D, CD25, and CD69 Downregulated: CD16	Increased cytotoxicity against tumor cell lines <i>in vitro</i> and antitumor activity <i>in vivo</i>	Preclinical model	(121)
Irr. K562 expressing membrane-bound IL-15 and 41BBL + IL-2	PBMC in plates	1,089 (3 weeks)	“Virtually pure”	N/A	N/A	<i>In vitro</i>	(126)
	PBMC in bags	23, 152, and 277 after 7, 14, and 21 days	96.8% NK 3.1% T cells (day 21)	Marked differences of gene expression profile compared to unstimulated or IL-2-stimulated NK cells	Increased cytotoxicity against tumor cell lines <i>in vitro</i> and antitumor activity <i>in vivo</i>	Preclinical model	(127)
	PBMC	447 (days 10–14)	88% NK 2.2% T cells (day 14)	Upregulated genes for cytolytic activity, cytokines, chemokines, activating receptors, adhesion molecules, cell cycle regulators, and multiple pathways	Increased cytotoxicity against primary MM cells <i>in vitro</i> and <i>in vivo</i> ; high productivity of IFN- γ	Preclinical model	(128)
	PBMC in G-Rex, bags	442—G-Rex 227—bags (10 days)	70% NK 5–35% T cells	Upregulated: NKp30, NKp44, NKG2D, CD26, CD70, and CXCR3 Downregulated: CD16, CD62L	Increased cytotoxicity and ADCC against primary tumor cells <i>in vitro</i> ; robust <i>in vivo</i> proliferation post-infusion	Clinical	(129, 130)
Irr. K562 expressing membrane-bound IL-15 and 41BBL + IL-15	PBMC, CD3 depleted, and CD56 enriched	1,000 (21 days)	N/A	Upregulated: CD56, NKG2D, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), CD158a, CD158b, and CD158e1	Increased cytotoxicity <i>in vitro</i> independent of killer cell immunoglobulin-like receptor mismatch; NK infusion contributed to acute graft-versus-host disease in first clinical trial	Clinical	(131, 132)
Plasma membrane particles of K562 expressing IL-15 and 41BBL + IL-2	PBMC in plates and flasks	1,265 (17 days)	86% NK cells 9% T cells 2% NK-like T	Upregulated: NKp30, NKp44, NKp46, NKG2D, 2B4, NKG2A, TRAIL, and Fas ligand (FasL) Downregulated: CD16	Increased cytotoxicity against leukemic cell lines and primary acute myeloid leukemia (AML) cells <i>in vitro</i>	<i>In vitro</i>	(133)
Irr. K562 expressing membrane-bound IL-21, 41BBL, CD64, CD86, and CD19 + IL-2	PBMC in flasks	4.8 × 10 ⁴ (21 days)	21.7% T cells	High expression of natural cytotoxicity receptors, CD16, and NKG2D	Cytotoxic against tumor cell lines <i>in vitro</i> ; capable of ADCC; increased telomere length	<i>In vitro</i>	(134)
		2,363 (14 days)	83% NK 9.1% T cells	Upregulated: DNAM-1, NKG2D, CD16, and CD56	Cytotoxic and capable of ADCC against neuroblastoma cell lines <i>in vitro</i> and <i>in vivo</i>	Preclinical model	(135)

(Continued)

TABLE 4 | Continued

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
Plasma membrane particles of K562 expressing membrane-bound IL-21 and 41BBL + IL-2	PBMC	825 (14 days) >10 ⁵ (28 days)	>90% NK (day 14)	N/A	Increased cytotoxicity against leukemic cell lines and primary AML cells <i>in vitro</i> ; enhanced proliferation <i>in vivo</i>	Preclinical model	(136)
Irr. allogeneic PBMC; irr. EBV transformed lymphoblastoid cell lines (EBV-LCL) (LAZ 388 cells) + PHA + IL-2	PBMC depleted for CD3 and monocytes in bags and plates	~43 (31–21 days)	90% NK <5% T cells	N/A	Increased cytotoxicity against tumor cell lines <i>in vitro</i>	Clinical	(137, 138)
Irr. EBV-LCL (TM-LCL) + IL-2	PBMC, CD3 depleted, and CD56 enriched in bags	800–1,000 (2 weeks)	98% NK	Upregulated: TRAIL, FasL, NKG2D, NKp30, NKp44, NKp46, CD48, CD25, LTB, MX1, and BAX	Increased cytotoxicity against tumor cell lines <i>in vitro</i>	<i>In vitro</i>	(139, 140)
Irr. EBV-LCL (SMI-LCL) + IL-2	PBMC, CD3 depleted, and CD56 enriched in bags	3,637 (24–27 days)	99.7% NK	Upregulated: TRAIL, FasL, NKG2D, NKp30, NKp44, and DNAM-1	Increased cytotoxicity and ADCC against tumor cell lines <i>in vitro</i>	Clinical	(141)
	PBMC, CD3 depleted, and CD56 enriched in CliniMACS Prodigy	850 (14 days)	>99% NK			<i>In vitro</i>	(142)
Irr. EBV-LCL (SMI-LCL) + IL-2 + IL-21	PBMC depleted for non-NK cells (research kit) in plates and flasks	2,900 (14 days) 2.7 × 10 ¹¹ (46 days)	>99% NK	Upregulated: TRAIL, NKG2D, and DNAM-1	Cytotoxic against tumor cell lines <i>in vitro</i> and <i>in vivo</i> ; enhanced and sustained production of IFN-γ and TNF-α	Preclinical model	(96)
Lysate of CTV-1	PBMC, CD3 depleted, and CD56 enriched	N/A (overnight)	97–98% NK	Upregulated: CD69 Downregulated: CD16	Cytotoxic against NK-resistant leukemia cell lines and primary tumors <i>in vitro</i>	Clinical	(143, 144)

The stimulatory effect of EBV-LCL on NK cell proliferation was discovered more than 30 years ago (152). In 1994, an early clinical trial already evaluated the adoptive transfer of autologous NK cells expanded with the LAZ 388 cell line to treat 10 patients with metastatic renal cell adenocarcinoma (137). More recently, the cell lines TM-LCL and SMI-LCL were reported for NK cell expansion, allowing around 800-fold expansion of highly pure NK cells within 2 weeks (139–142). NK cells generated with these EBV-LCL feeder cells are currently applied in a study testing them for adoptive transfer in an autologous setting with intended doses up to 1×10^9 NK cells/kg (141). Recently, it was reported that repeated stimulation with SMI-LCL in IL-2-containing medium and adding IL-21 only at start of cultivation enables 10¹¹-fold NK cell expansion after 6 weeks, to our knowledge representing the most efficient protocol to expand NK cells at the moment (96). NK cells generated with the latter method are highly cytotoxic *in vitro*, show a sustained high productivity of IFN- γ and TNF- α , similar to CIML NK cells, and they efficiently controlled melanoma in a xenograft mouse model (96).

Although feeder cells, and allogeneic feeder cell lines in particular, make it possible to generate substantial numbers of NK cells for adoptive therapy, from a regulatory point of view this strategy has drawbacks as feeder cell lines must be qualified as safe for human use. The cell line qualification of modified K562 cells, for instance, includes costly viral testing and assays to prove absence of bacterial and *Mycoplasma* contamination (153). In this context, lysates from cell lines containing the NK cell-stimulating factors could be an alternative to the intact feeder cells to minimize regulatory concerns. It was demonstrated that short cultivation of NK cells with lysate of the leukemia cell line CTV-1 primes NK cells to specifically lyse cell lines that are resistant to resting NK cells (143). Interestingly, the priming effect of CTV-1 on NK cells is KIR independent and does not require supplementation of cytokines, such as IL-2 or IL-15, making this an unique approach for NK cell activation (154). NK cells primed with CTV-1 were evaluated in the first UK clinical trial of a cell therapy regulated as a medicine, with an anti-leukemia effect in four of seven treated patients and no evidence of NK cell infusion-related toxicities (144). Another step forward from a regulatory standpoint could be to add only specific fragments of feeder cells to the culture that are responsible for the desired NK cell activation, instead of using intact feeder cells or their lysates. Of note, NK cells can be expanded *ex vivo* with IL-2 and plasma membrane particles prepared from K562-expressing membrane-bound IL-15 and 41BBL with a rate of expansion that is comparable to stimulation with intact feeder cells and far better than stimulation with soluble IL-15, 41BBL, and IL-2 (133). Plasma membrane particles from K562 expressing membrane-bound IL-21 and 41BBL work for *ex vivo* NK cell expansion as well and may be an option for *in vivo* NK cell expansion, as demonstrated in a first proof of concept using a mouse model (136).

TECHNICAL ASPECTS OF NK CELL EXPANSION

In general, one encounters technical challenges and opportunities when manufacturing NK cells as medicinal products, as reviewed recently (155). In this section, we focus on technical options for

NK cell culture, ranging from simple cell culture plates for small scale experiments to highly standardized and automated systems for clinical scale. The selection of the adequate culture system is based on the intended application of the cells. Most preclinical experimental studies grow NK cells in cell culture plates or tissue culture (T) flasks. These are commonly used and very convenient to test and compare different culture additives in parallel, e.g., different cytokine concentrations. However, for clinical applications in large scale, cultivation in plates and flasks is rather inappropriate for different reasons. First, due to the small volume of T flasks, numerous T flasks have to be handled at the same time, with for instance 51 T flasks for the treatment of a single patient (116). In addition, T flasks have to be opened from time to time for medium exchange or harvesting of cells, bearing the risk of contaminating the cellular product. Although the likelihood of contamination for each T flask is reduced to a minimum by sterile workflows in safety cabinets, the remaining risk potentiates by the number of flasks.

To overcome the drawbacks of small cell culture vessels, clinical NK cell cultivation is often done in cell culture bags, which make it possible to culture high volumes in a closed system, as all required steps can be done by sterile welding of tubing connections for the transfer of media, harvesting of cells, etc. Unfortunately, different reports describe that the NK cell expansion performance is reduced after transition of a protocol from T flasks to larger scale in cell culture bags (50, 67). In addition, bag systems still require several labor-intensive interventions during the culture, especially when different cultures are set up in parallel.

The G-Rex vessel is another system avoiding frequent processing steps for exchange of medium during the culture. In contrast to normal cell culture flasks, the bottom of the G-Rex is highly gas permeable, ensuring optimal CO₂ exchange and O₂ supply for the cells. Thus, by its design, G-Rex flasks can be filled directly with a high level of cell culture medium and exchange of medium is not necessary for long time. For NK cell culture, G-Rex were used for example for 10 days of culture without any cell manipulation or feeding, and resulted in higher fold expansion of NK cells compared to cell culture bags (130). Unfortunately, although G-Rex are scalable in general, multiple G-Rex flasks are still required to achieve high cell numbers for clinical trials, which can be cumbersome and costly, and G-Rex flasks are still an open system and may require adaption to a closed system (156).

Automated systems combine the need for reduced interventions during the culture with a closed system. Automation of the cell manufacturing ensures constant product quality without the need for highly skilled experts, is finally cost saving, and may be required for cellular therapy to become available beyond specialized academic centers (157). Although early integration of automation is associated with higher capital costs in the development phase, it allows a smooth transition at later stages of clinical development (158). A first feasibility study of automated NK cell cultivation with a stirred bioreactor was already published in 1996, showing advantages of the bioreactor culture over manually handled controls (159). More recently, different investigators report automated NK cell expansion procedures with a rocking motion bioreactor (67, 69, 156, 160), yielding 2–10 × 10⁹ NK cells

under GMP-compliant conditions. However, the latter system still needs preceding manual cultivation, because relatively high cell numbers are required as inoculum for the automated culture (67, 69, 156, 160). Alternatively, fully automated NK cell expansion with an automated cell processing device can be performed for clinical use, with as little as 10^6 NK cells being sufficient to initiate the automated culture that can yield up to 2.7×10^9 NK cells after stimulation with clinical grade feeder cells (142). Of note, in addition to the culture process, the cell processing device is designed for GMP-compliant cell separation, concentration, and washing applications, so that combined NK cell purification, cultivation, and final formulation of the cellular product is possible fully automated (161). Thus, the whole processing, from the starting material, such as a leukapheresis product, to the finally expanded NK cells, readily prepared for infusion, can be covered by a single instrument.

Centralized processing of NK cell products probably will be carried out mainly in specialized centers for manufacturing of cellular products. Consequently, after *ex vivo* cultivation, storage of the NK cell product and shipment to the location of use will be needed. Compared to naive NK cells, IL-2-activated NK cells are less sensitive to freezing, as they show higher recovery and viability after thawing (26). Still, different groups state that cryopreservation of cultivated NK cells goes along with a drop in cell viability and cytotoxicity, whereas the latter can be restored by a short re-stimulation, e.g., by a short resting in IL-2-containing medium (139, 156). Poor survival of the NK cells can be an issue during further *in vitro* culture post thawing, so that shipping of freshly formulated cells for direct infusion may be advantageous (129). Interestingly, some groups recently claim that freezing and thawing does not influence the cytotoxicity or the proliferative ability of cultivated NK cells in their hands (24, 68). These divergent observations possibly result from different cultivation methods and different protocols for freezing and thawing, which should be investigated further. Without freezing, transport of the readily prepared cells in an appropriate time frame is challenging, and any delay during the shipment affects the quality of the cellular product with critical consequences for the patient. Alternatively, automated and closed systems for cell processing open the way for scale out strategies and de-centralized NK cell manufacturing directly at the location of intended use, avoiding the freezing and shipment process (142). But, although de-centralized manufacturing in the clinics seems promising, cellular therapeutics are very complex and still in early development, so that manufacturing by well-trained specialists in specific facilities is reasonable at that state.

REGULATORY ASPECTS OF NK CELL CULTIVATION FOR CLINICAL USE

Apart from technical difficulties, one has to consider regulatory aspects for the use of *ex vivo*-generated NK cells with regulations varying in time and geographical policies (153). In Europe, for instance, cytokine-activated and -expanded NK cells are currently classified as advanced therapy medicinal products and will be regulated accordingly either centralized or under the hospital exemption by the member states [Regulation (EC)

No 1394/2007; Directive 2001/83/EC and Regulation (EC) No 726/2004]. Quality aspects related to somatic cell therapy medicinal product as defined in guidelines (CPMP/BWP/3088/99; EMEA/CHMP/410869/2006; Ph. Eur. 0784: Ph. Eur. 5.14) will apply to the identity, potency, and activity. The establishment of correspondingly adequate in process and quality controls as well as of process target values and product specifications will have to take into account the variability of the primary effector cell as the starting material (162).

CONCLUSION AND OUTLOOK

Comparing different protocols for NK cell cultivation in detail is challenging as these are extremely heterogeneous. The duration of *ex vivo* NK cell cultivation ranges from a few hours for short NK cell activation up to several weeks for long-term expansion, different starting materials are in use with varying NK cell purities, different cytokines are combined at different doses, and NK cells often are cocultured with different feeder cells at different NK-to-feeder ratios. Nevertheless, overall differently *ex vivo* expanded NK cells exhibit some common characteristics.

In general, *ex vivo* cultivated NK cells show an increased cytotoxicity and may become even responsive against tumor targets previously appearing resistant to NK cell lysis. This explains the use of IL-2 or IL-15 in virtually every protocol, as it is known since a long time that both cytokines amplify NK cell activity (81, 163). However, upon NK cell activation with different stimuli, including IL-2 and IL-15, downregulation of CD16 surface levels occurs by metalloproteases-mediated shedding of CD16 (164–166). The Fc receptor CD16 is crucial for NK cells to perform ADCC and would be of particular importance for potential combination therapies using NK cells together with therapeutic antibodies. Of note, although reduced levels of CD16 on NK cells are observed for several NK cell cultivation protocols the NK cells still mediate ADCC (70, 129, 142). Nevertheless, inhibition of the relevant metalloproteases to maintain CD16 on NK cells could be an option to further increase the ADCC function of *ex vivo* activated NK cells (164, 167).

Another clinically highly relevant aspect is the tumor-induced immunosuppression as important challenge for all cell therapeutic strategies. Remarkably, it ruled out from most preclinical and clinical NK cell studies that NK cells may gain the capability to overcome tumor immunosuppression. Different research groups have reported signs of NK cell suppression in cancer patients such as a lower expression of NK cell receptors, e.g., NCRs, NKG2D, DNAM-1, and 2B4 (22, 25, 168–170), the shedding of tumor cell ligands, such as NKp30 and NKG2D (171–174), or the release of blocking NKG2D ligands, such as MICA and ULBP3, via tumor-derived exosomes (175, 176). Notably, *ex vivo* cultivation of patient-derived NK cells is often possible with same efficacy as for donor-derived NK cells (25, 111) and can normalize the NK cell phenotype and activation (25). Additionally, elevated levels of NKG2D on *ex vivo*-activated NK cells can scavenge shed NKG2D ligands and counter their inhibitory effect (177). Furthermore, the high cytotoxicity of *ex vivo* expanded NK cells has been shown to be independent of KIR inhibition for some protocols (107, 132).

In comparison to other cell therapeutic approaches using, e.g., T cells, donor-derived allogeneic NK cells mediate GVL without an elevated risk for GVHD or even with a GVHD-reducing effect, as reported in mice and men (74, 75, 77, 78). However, contradictory results regarding GVHD induction have been reported in clinical trials assessing adoptive transfer of NK cells expanded with K562 feeder cell variants expressing 41BBL and IL-15 (129, 131). These reports show that there are still open questions that have to be unraveled to better understand the complex role of NK cells and their specific subsets in the bidirectional regulation of GVL and GVHD.

In conclusion, many different protocols are in use to expand NK cells *in vitro*, each with its specific advantages and disadvantages in regard of cell numbers, function, and handling efforts. The data summarized in this review underline the complexity related to the design of an optimal NK cell therapeutic protocol that should be not only reliable and safe in use but also highly efficient in targeting different forms of malignancies. With this in mind, additional studies need to be envisioned that not only further address *ex vivo* NK cell purification, expansion, and activation strategies but also the final clinical setting including pre-conditioning, dosing, and timing of the NK cell application. Efforts for harmonization of protocols at the European and worldwide level should be undertaken to ensure highest quality

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AUTHOR CONTRIBUTIONS

MG, JW, and EU extensively reviewed the current literature on *ex vivo* NK cell cultivation and expansion and prepared a comprehensive overview that is listed in the tables. MG, JW, UK, AC, VH, and EU wrote and critically reviewed the manuscript.

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In Vitro Culture with Interleukin-15 Leads to Expression of Activating Receptors and Recovery of Natural Killer Cell Function in Acute Myeloid Leukemia Patients

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Despite recent progress in the therapeutic approach of malignant hemopathies, their prognoses remain frequently poor. Immunotherapy could open a new window of great interest in this setting. Natural killer (NK) cells constitute an important area of research for hematologic malignancies, because this subpopulation is able to kill target cells spontaneously without previous sensitization, representing a novel tool in the treatment of them. Abnormal NK cytolytic function is observed in several hematological malignancies, including acute myeloid leukemia (AML) and myelodysplastic syndromes. Several mechanisms are involved in this abnormal function, such as decreased expression of activating receptors, increased expression of inhibitory receptors or defective expression of NK cell ligands on target cells. New immunotherapies are focused in identifying factors that could increase the expression of these activating receptors, to counteract inhibitory receptors expression, and therefore, to improve the NK cell cytotoxic capacities against tumor cells. In this work, we analyze the effect of interleukin (IL)-15 on the expression of NK cell-activating receptors that play a crucial role in the lysis of blasts from AML patients. Our results showed that IL-15 increased the surface expression of NKp30 on NK cells from healthy donors and AML patients with the consequent improvement of NK cell cytotoxicity. Besides, the upregulation of NKp30 induced by IL-15 is associated with an improvement of NK-mediated myeloid dendritic cells (DCs) maturation. NK cells cultured with IL-15 showed an upregulation of NKp30, which is associated with an increase anti-tumor activity and with an improved maturation of immature DCs. In our *in vitro* model, IL-15 exerted a great activating stimulus that could be used as novel immunotherapy in AML patients.

Keywords: natural killer cell, acute myeloid leukemia, interleukin-15, NKp30, dendritic cells, natural killer cell degranulation

INTRODUCTION

Natural killer (NK) cells constitutively possess lytic machinery and are able to spontaneously lyse virally infected or malignant cells without any prior sensitization to antigen. Their function is tightly controlled through a balance of signals from activating and inhibitory receptors, by cytokines and chemokines (1–3), as well as by cross talk with other immune cells, such as dendritic cells (DCs) (4), effector CD4⁺ T cells (5), and regulatory T cells (Tregs) (6, 7).

There are several major histocompatibility complex (MHC) class I-specific inhibitory receptors with different molecular structures and specificities for different alleles of human leukocyte antigen (HLA) class I molecules. These receptors act as negative regulators of NK cytotoxicity ensuring that normal autologous cells are preserved (4, 8, 9). The two main groups of inhibitory receptors are the killer Ig-like receptors (KIR) (8), which recognize polymorphic determinants of HLA-A, -B, or -C molecules and the heterodimeric receptors CD94-NKG2A/B, which recognize peptides derived from the leader sequences of different HLA class I molecules presented by the non-classical MHC class I molecule HLA-E (10–13). Normal autologous cells that express HLA class I molecules are protected from NK-mediated attack, but cells that have reduced expression because of malignant transformation become susceptible to NK cell attack. Loss or downregulation of a single HLA-I allele, a relatively frequent event in cancer, can be sufficient to make tumor cells susceptible to NK cell cytotoxicity (8, 9).

In the absence of inhibitory signals, NK cell cytotoxicity requires signaling through activating receptors upon interaction with their ligands on target cells. An ample set of activating receptors have been described including, among others, members of the C lectin-like family as NKG2D and members of the immunoglobulin superfamily as natural cytotoxicity receptors (NCRs) and DNAM-1 (also known as CD226) (4, 8, 9, 14). NKG2D ligands are a group of MHC class I-like molecules, the expression of which is induced by cellular stress. NKG2D ligands included MHC-I polypeptide-related sequences A (MICΑ) and B (MICΒ), and UL16-binding proteins (ULBP1–6) (15, 16). DNAM-1 specifically recognizes CD155 and CD112, two members of the nectin family, that are also expressed on different types of tumors (17–20).

Natural cytotoxicity receptors are major activating receptors involved in tumor cell detection and lysis. NCRs include NKp46 (21–23), NKp30 (24, 25), and NKp44 (26), which mediate cell lysis of many cancer cells. NKp30 and NKp46 are expressed both in resting and activated NK cells, whereas NKp44 expression is restricted to activated NK cells (4, 9, 14, 21, 27). NCR ligands include pathogen-associated as well as stress-related molecules. The identity of NCR ligands on tumors remains in part elusive. The nuclear factor HLA-B-associated transcript 3 (BAT3) and B7H6 have been described as NKp30 ligands (24, 28, 29). NKp46 and NKp44 were shown to interact with the viral hemagglutinin protein (24, 30–32). A truncated isoform of mixed lineage leukemia 5 is an activating ligand for NKp44 (33). By contrast, proliferating cell nuclear antigen is an inhibitory tumor ligand for

NKp44 (34). No tumor ligands for NKp46 have been identified so far.

We have recently demonstrated that acute myeloid leukemia (AML) patients have depressed NK cell function as well as altered cytokine production (35–37). NK cell-mediated rejection of leukemic blasts may be limited by the reduced expression of NK cell-activating receptors such as DNAM-1, NKp46, and NKp30 observed in AML patients (36, 37). Besides, NKp46 downregulation has been associated with decreased survival in AML patients (38).

In the last years, several cytokines have been extensively studied as potential therapeutic agents to manipulate the immune response against malignant cells due to their capacity of stimulate cell growth and survival as well as increase the cytotoxicity or cytokine production to boost immune reactivity (39–42). So far, only a small number of cytokines have reached clinical use probably due to the complexity of cytokine network. Among these cytokines tested in different *in vitro* and *in vivo* settings, interleukin (IL)-2 and IL-15 should be highlighted (40, 41, 43, 44). IL-2, initially described as a T cell growth factor, promotes CD8⁺ T cell and NK cell cytolytic activity and modulates T cell differentiation in response to antigen. Moreover, IL-2 is essential for the development and maintenance of Tregs that may represent a limitation for its use in patients with cancer. The major disadvantage of IL-2 is its toxicity, including severe capillary leak syndrome that can accompany this treatment (43). Recently, IL-15 has emerged as a potential immunotherapeutic candidate for the treatment of cancer. IL-2 and IL-15 are structurally related and have overlapping functions including their role in T cell proliferation, promotion of cytotoxic T cell differentiation, production of immunoglobulin by B cells, and generation, proliferation, and activation of NK cells. In contrast to IL-2, IL-15 is not required for the maintenance of Tregs and, based on preclinical studies, IL-15 causes less vascular capillary leak (42). These factors support the role of IL-15 for cancer immunotherapy, boosting both innate and adaptive immunity against tumors (45). However, so far, few clinical trials have analyzed the security and efficacy of IL-15 in cancer patients. Thus, a Phase I study (NCT01572493) assessing the safety and efficacy of IL-15 in adults with advanced malignancies has been suspended for undisclosed reasons. IL-15 is being tested as an immunological adjuvant to haploidentical NK cell transfer in AML patients (NCT02395822).

Due to the ability of NK cells to spontaneously kill tumor cells, this population represents an attractive tool for cancer immunotherapy (46–48). However, NK cell defective function in AML patients may limit tumor control. The possibility of manipulating NK cells by cytokines for therapeutic purposes open new area of research in cancer.

The biological effects of IL-15 on NK cells will depend on the direct effect on NK cells as well as by indirect consequences mediated by other cells stimulated by IL-15. Thus, in order to analyze the whole figure we have used peripheral blood mononuclear cell (PBMC) cultures stimulated with IL-15.

In this study, we aimed to assess (i) whether IL-15 induces and expansion of NK cells from healthy donors (HDs) and AML patients and its effect on the expression of activating receptors after short-term culture *in vitro*; (ii) whether IL-15 increases the

cytotoxic activity of NK cells; and (iii) whether the maturation of immature DCs (iDCs) is enhanced by culture with IL-15-stimulated PBMCs.

MATERIALS AND METHODS

Patients and HDs

Peripheral blood mononuclear cells were obtained from 14 newly diagnosed AML patients (ranged 18–89 years) at the Hospital San Pedro de Alcántara (Cáceres, Spain) prior to any treatment and from 20 HD volunteers (ranged 20–60 years). The study was approved by the local Ethics Committee and samples collected after written informed consent in accordance with the Declaration of Helsinki. Diagnosis was established by cytological criteria based on the French-American-British classification.

The collected blood was drawn into heparinized tubes and processed using Ficoll-Hypaque gradients. PBMCs were recovered and cell count and viability analysis were performed. PBMCs were immediately used for experiments. For co-culture experiments, we selected those patients with lower percentage of leukemic blasts and to eliminate leukemic blasts an adherence step was included in the protocol prior to co-culture with DCs.

Plasma was obtained after centrifugation and stored at -80°C for measurement of cytokine levels. Plasma samples from HDs and from AML patients used in the study had not been previously thawed.

Monoclonal Antibodies (mAbs)

Natural killer cell percentage and phenotype were evaluated in PBMC obtained from AML patients and HDs. The following anti-human mAbs were used for flow cytometry: CD56-FITC (NCAM16.2), CD56-PE (MY31), CD14-PE (MØP9), CD3-PerCP (SK7) all from BD Biosciences (San Jose, CA, USA); CD56-PECy7 (B159), CD16-APC Cy7 (3G8), NKG2D-PE (1D11), CD226-PE (DX11), CD107a-FITC (H4A3), CD107b-FITC (H4B4), CD86-FITC (2331(FUN-1)), CD1a-FITC (HI149) all from BD Pharmingen (San Diego, CA, USA); and NKp30-PE (AF29-4D12), NKp30-APC (AF29-4D12), NKp46-PE (9E2), NKp46-APC (9E2), CD3-VioBlue (BW264/56) all from Miltenyi Biotec (Bergisch Gladbach, Germany). Prior to use, mAbs were titrated to establish optimal staining dilutions. Isotype-matched immunoglobulins were included in all experiments as negative controls. Mean relative fluorescence intensity was calculated by dividing the mean fluorescent intensity (MFI) of the relevant mAb by the MFI of its isotype control.

Cell Culture and Flow Cytometry Analyses of NK Cells

Peripheral blood mononuclear cells were cultured in complete medium (RPMI-1640 supplemented with 10% FCS, L-glutamine, sodium pyruvate, non-essential amino acids and penicillin/streptomycin, all from BioWhittaker, Verviers, Belgium) and were stimulated with 100 ng/mL of recombinant human (rh)IL-15 from Peprotech (Rocky Hill, NJ, USA) or 750 U/mL of rhIL-2 (National Cancer Institute, Frederick, MD, USA) (49). Cells were harvested after 48 h, and the frequency and NK receptor

repertoire were assessed by multi-parameter flow cytometry using a FACScan cytometer and the CellQuest software (BD Biosciences) or MACSQuant cytometer and the MACSQuantify software (Miltenyi Biotec). NK cells were defined as CD3 $^{-}$ CD56 $^{+}$ cells within the lymphocyte gate and the expression of activating receptors analyzed was referred to this population. Analysis of NK cells at various time points after cytokine activation was performed in order to select the best timing for functional analysis (data not shown).

Analysis of IL-15 in Plasma

Interleukin-15 concentrations in the plasma of patients and healthy controls were determined by enzyme-linked immunosorbent assay (ELISA) using a Human IL-15 ELISA Ready-SET-Go! Kit (eBioscience) according to the manufacturer's instructions. The minimum detectable levels were 8 pg/mL, and the standard curve range was 8–1,000 pg/mL.

Two independent sets of experiments were performed. No significant variations were observed among the experiments. Plate was read in an Infinite® 200 (Tecan, Switzerland) plate reader.

NK Cell Degranulation Assay

Cytokine-stimulated NK cells were tested in a degranulation assay against the NK cell-susceptible target cell line K562. The analysis of NK cell degranulation was performed by measuring the expression of CD107a/b after activation with target cells at ratio 1:1 in the presence of BD GolgiStop (BD Biosciences) and a mixture of FITC-labeled anti-CD107a and anti-CD107b mAbs. After 4 h, cells were stained with PE-labeled anti-CD56 and PercP-labeled anti-CD3 from BD Biosciences and analyzed by flow cytometry by measuring the frequency of CD107a/b expression on CD3 $^{-}$ CD56 $^{+}$ NK cells. Spontaneous basal NK cell degranulation was always below 10%. Background expression of CD107a/b (CD107a $^{+}$ NK cells in medium only) was subtracted from expression with target cells.

Generation of DCs

Monocyte-derived iDCs were obtained by adherence of monocytes to plastic. Thus, PBMCs from HDs were resuspended at 5×10^6 cells/mL in complete medium and allowed to adhere for 2 h at 37°C in culture flasks. Then, the non-adherent cells were removed and the adherent cells, predominantly monocytes, were cultivated in complete medium supplemented with 50 ng/mL of recombinant human granulocyte-macrophage colony-stimulating factor (Peprotech) and 20 ng/mL of rhIL-4 (Peprotech). After 6 days, the percentage of iDCs was analyzed by flow cytometry. iDCs were defined as CD14 $^{-}$ CD1a $^{+}$ CD83 $^{-}$ CD86 $^{-}$ cells.

To generate mDCs, iDCs were plated either in the absence or in the presence of allogeneic PBMCs cells stimulated or not with rhIL15 or rhIL-2 at ratio 1:5. After 2 days, DCs were assessed for the expression of CD86. As positive control, optimal DC maturation was induced by *Escherichia coli* lipopolysaccharide (LPS) 1 $\mu\text{g}/\text{mL}$ (serotype 055:B5, Sigma-Aldrich, St Louis, MO, USA).

Statistical Analysis

Statistical analysis was performed using SPSS Statistics version 19. Because the cell population counts were generally not normally

distributed, we report medians and ranges for parameter values. Non-parametric statistical methods were used to analyze the data. Paired differences between NK cells from the same patient with or without stimulation by cytokines were tested using the Wilcoxon signed-rank test. For comparison of NK cells between HDs and AML patients, the exact Wilcoxon rank sum test was used.

RESULTS

Effect of IL-2 and IL-15 on NK Cell Expansion *In Vitro*

Natural killer cells were evaluated in HDs and AML patients at the time of diagnosis and prior to any treatment. In order to study the modulatory effect of IL-2 and IL-15 on NK cell proliferation, we cultured PBMCs from HDs and AML patients in presence of these cytokines. Our results showed that IL-15 but not IL-2 induced a significant increase ($p = 0.016$) in the percentage of NK cells from HDs after 48 h of culture (Figures 1A,B). By contrast, none of the cytokines had an effect on the percentage of NK cells from AML patients (Figures 1C,D).

Upregulation of NK Cell-Activating Receptors by IL-2 and IL-15 *In Vitro*

In order to assess the effect of IL-2 and IL-15 on the NK cell receptor expression, we cultured PBMCs from HDs and AML patients in presence of these cytokines.

In HDs (Figure 2, left panels), we observed a statistically significant upregulation of the NK cell-activating receptors NKp30 and NKG2D after culture of PBMCs with IL-2 ($p = 0.004$ and $p = 0.013$, respectively) (Figures 2A,D). IL-15 also induced a significant increase in the expression of NKp30 on NK cells surface ($p = 0.0044$) (Figure 2A), but the upregulation of NKG2D induced by IL-15 was not statistically significant ($p = 0.08$) (Figure 2D). No significant changes in the expression of NKp46 and DNAM-1 were observed (Figures 2B,C).

The analysis of the effect of IL-2 on NK cells from AML patients (Figure 2, right panels) only showed a statistically significant upregulation of DNAM-1 expression ($p = 0.046$) (Figure 2C), without significantly affecting the expression of the other receptors considered. By contrast, the incubation with IL-15 induced a significant increase of NKp30 and NKG2D ($p=0.001$ and $p=0.028$) (Figures 2A,D). A representative example of the effect of IL-15 is shown in Figure S1 in Supplementary Material.

Cytotoxic Activity of Cytokine-Stimulated PBMCs

Once we observed the effect of the different cytokines on NK cell receptor expression, we analyzed if these phenotypes correlated with the cytotoxic capacity of NK cells against K562, a susceptible target cell line. A representative example is shown in Figure 3A. Our results demonstrated that concomitant with the upregulation of the activating receptors following 48 h of

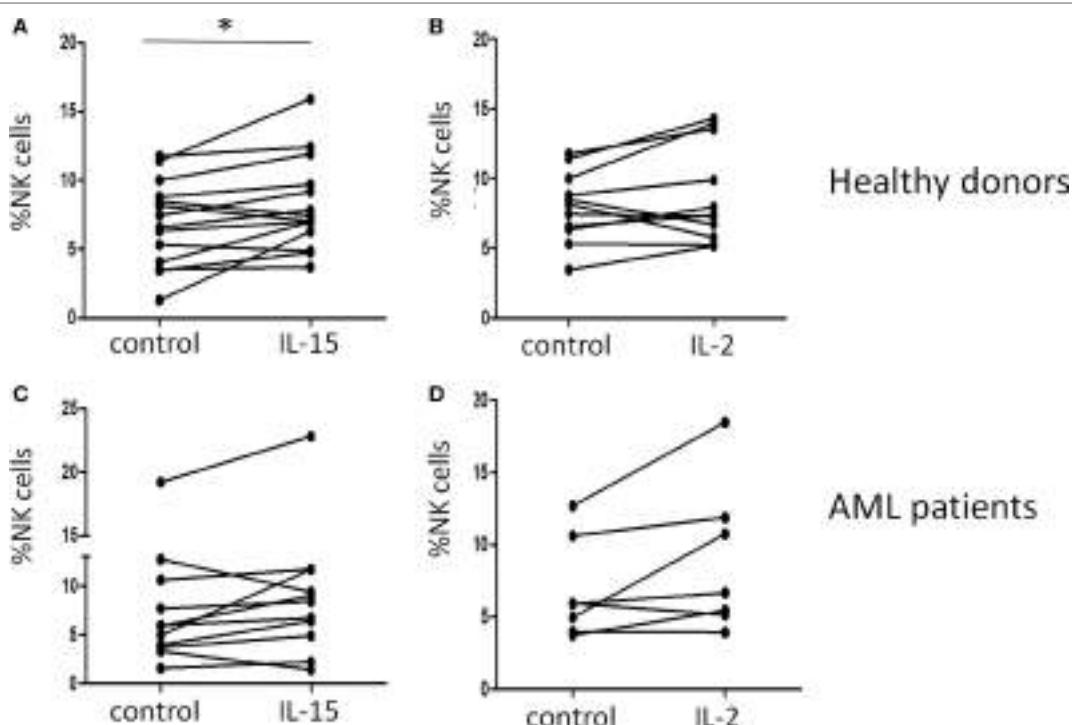


FIGURE 1 | Effect of interleukin (IL)-15 and IL-2 on natural killer (NK) cell expansion *in vitro*. The increase in the percentage of NK cells after incubation for 48 h with cytokines was analyzed by flow cytometry in healthy donors (A,B) and acute myeloid leukemia (AML) patients (C,D) in the presence of IL-15 (A,C) or IL-2 (B,D).

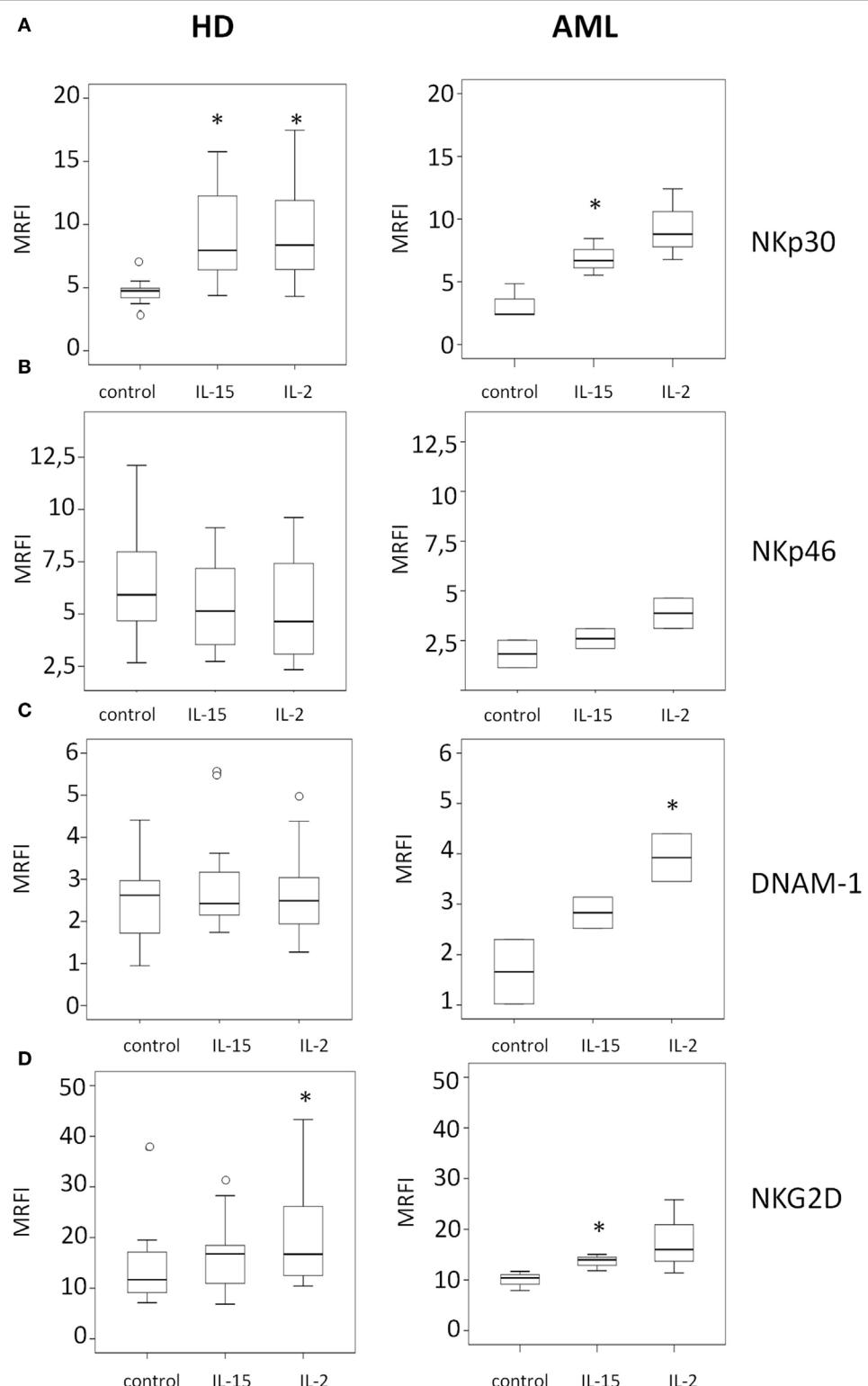


FIGURE 2 | Modulatory effect of interleukin (IL)-15 and IL-2 on natural killer cell activating receptor expression in healthy donors and acute myeloid leukemia patients. The expression of NKp30 (A), NKp46 (B), DNAM-1 (C), and NKG2D (D) was analyzed by flow cytometry. NK cell receptor expression was analyzed after 48 h of culture with medium alone (control), IL-15, or IL-2. Left panels represent HDs and right panels represent AML patients. Mean relative fluorescence intensity (MRFI) was calculated by dividing the mean fluorescent intensity (MFI) of the relevant monoclonal antibody by the MFI of its isotype control. The lower boundary of the box indicates the 25th percentile and the upper boundary the 75th percentile. Bars above and below the box indicate the 90th and 10th percentiles. The line within the box marks the median. Circles (○) represent outliers values. (* $p < 0.05$, compared to control).

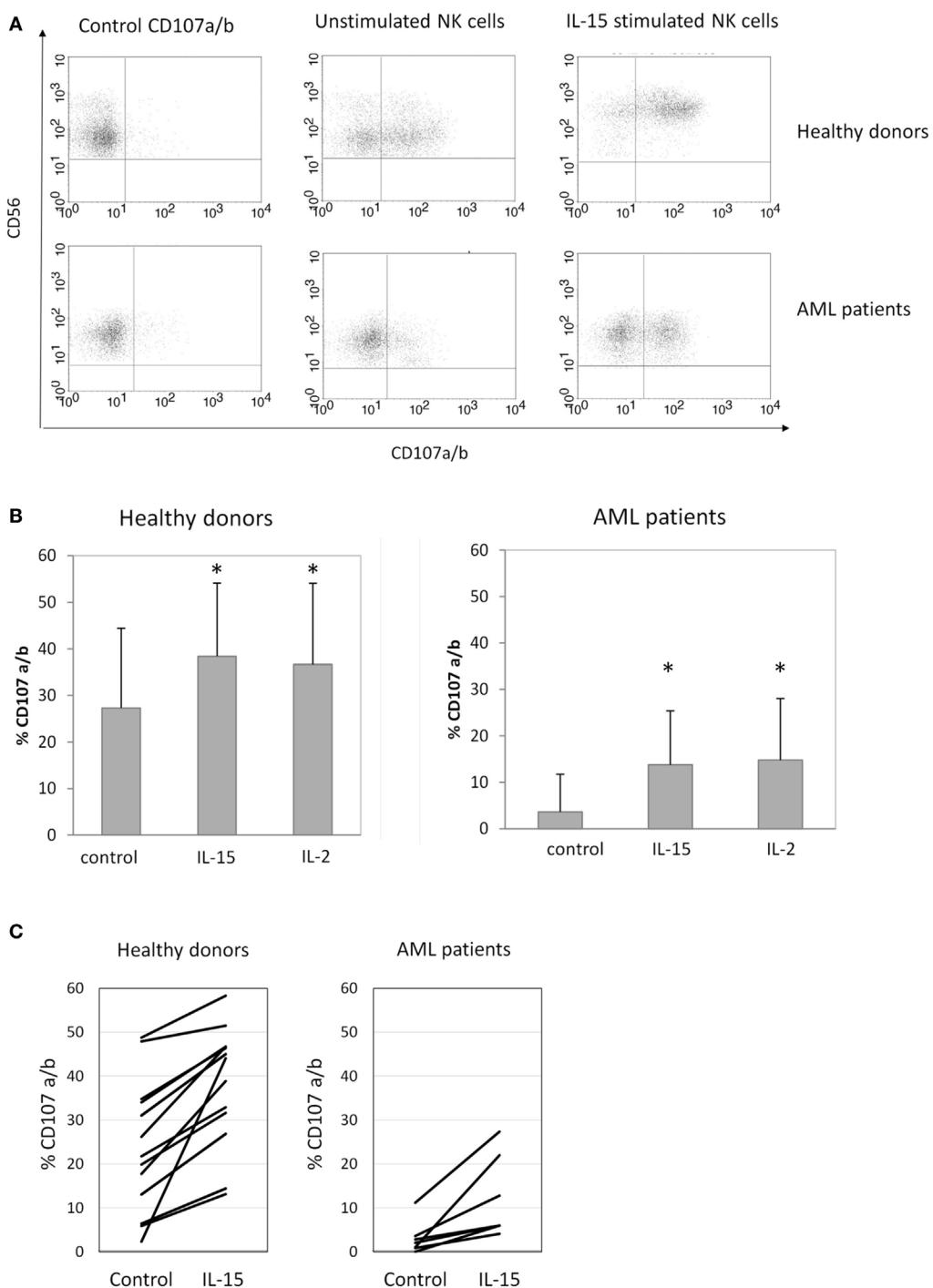


FIGURE 3 | Natural killer (NK) cell degranulation after cytokine stimulation. **(A)** Representative flow cytometry analysis of NK cell degranulation against K562 cells. **(B)** Column bars show the percentage of CD107a/b expression on NK cells from healthy donors (HDs) (left graph, $n = 16$) or acute myeloid leukemia (AML) patients (right graph, $n = 8$) in response to K562 cells. Error bars represent SD. **(C)** Individual representation of NK cell degranulation against K562 cells in HDs (right graph) and AML patients (right graph). NK cells were used after 48 h of culture with medium alone (control), interleukin (IL)-15, or IL-2 (* $p \leq 0.05$, compared to control).

IL-15 and IL-2 stimulation, there was a significant increase in NK cell cytotoxicity against K562 targets in both HDs and AML patients (Figure 3B). Although a high variability was observed, a

positive effect of IL-15 on NK cell degranulation was found in all individuals analyzed but with different increases in degranulation (Figure 3C).

Effect of IL-15 and IL-2-Stimulated PBMCs on Maturation of iDCs

Recent studies demonstrated that human NK cells display the ability to kill or induce maturation of both autologous and allogeneic monocyte-derived iDCs. This function depends on the engagement of NKp30 with its cellular ligands expressed by DCs. Considering that IL-15 and IL-2 have a potent effect in the expression of NKp30 on NK cell surface, we decided to study if these modifications affect iDC maturation mediated by NK cells. iDCs were generated as described in Section “Materials and Methods,” harvested, counted and analyzed by FACS. After culture with cytokine-stimulated PBMCs, DC maturation was evaluated by the expression of CD86. Unstimulated PBMCs were used as control to compare the effect of IL-15 and IL-2 stimulation on PBMCs capacity to induce DC maturation. LPS-stimulated iDCs were used as positive control.

Our result showed that PBMCs from HDs and AML patients stimulated with IL-15 induced higher maturation of iDCs than unstimulated PBMCs although due to the high variability observed the differences were not statistically significant (**Figure 4A**; Figure S2). In response to cytokine-stimulated PBMCs, DCs displayed a onefold increase in the expression of CD86 compared to their response to unstimulated PBMCs (**Figure 4B**).

Presence of IL-15 in Plasma of HDs and AML Patients

Finally, we characterized the concentration of IL-15 in the plasma of HDs and AML patients. Our results showed that IL-15 was significantly higher in AML serum than in HDs ($p < 0.001$) (**Figure 5**). No correlation was observed between IL-15 levels in plasma and NKp30 expression on NK cells probably due to the high variability observed among donors (data not shown).

DISCUSSION

Acute myeloid leukemia is a heterogeneous disease that presents with different phenotypic and genotypic alterations in hematopoietic progenitors with the subsequent accumulation of immature hematopoietic stem cells that prevent the production of adequate amount of healthy hematopoietic cells. AML is more common in the elderly and co-morbidities and frailty often impact on patient tolerance to intensive treatment regimens. Thus, the prognosis of elderly AML patients remains poor, despite recent advances in the management and treatment options of AML patients including novel immunotherapies (50).

The role of NK cells against leukemia is supported by the discovery of NK cell spontaneous cytotoxicity against leukemia cell lines *in vitro* and the clinical benefits observed in KIR ligand mismatched allogeneic stem cell transplantation. Evidence for graft-versus-leukemia effect mediated by NK cells is observed in clinical studies with haploidentical donor transplants where the presence of alloreactivity due to KIR ligand mismatch was correlated with higher survival rates (51). NK cell-mediated cytotoxicity preserves healthy cells, and consequently, tumor control can be achieved in the absence of graft-versus-host disease (52). In addition, donor-derived NK cells have demonstrated to play a relevant role after hematopoietic stem cell transplantation (53).

Natural killer cells in AML patients habitually present defects in their cytotoxicity against autologous leukemic blasts probably as consequence of a reduced expression of activating receptors such as NCRs or DNAM-1 (36–38). Besides, the low expression of NKp46 and the NCR^{dull} (NKp46^{dull}NKp30^{dull}) phenotype have been associated with decreased survival in AML patients (38). NK cell phenotype and function at diagnosis of AML associate with clinical outcome. Thus, AML patients with low expression of

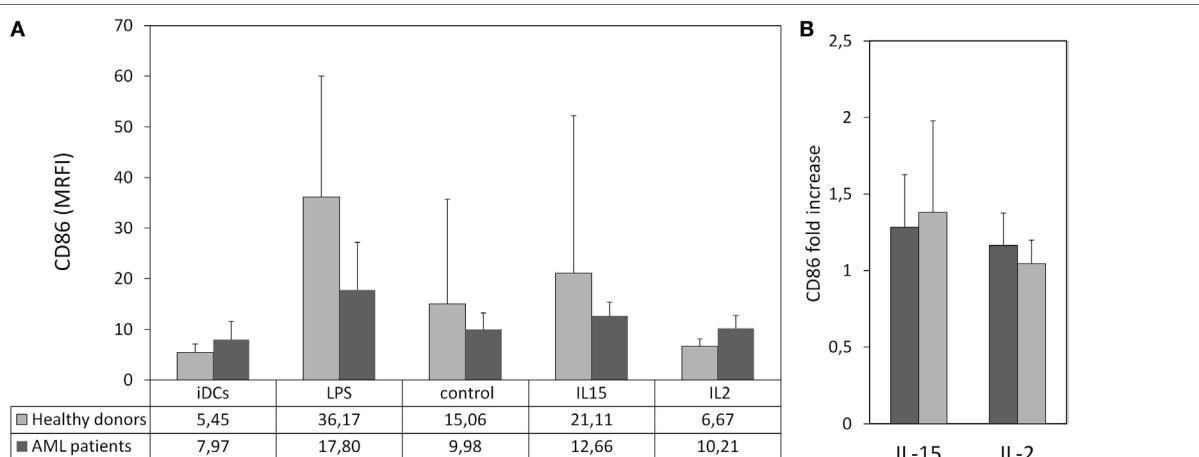


FIGURE 4 | Maturation of immature DCs (iDCs) co-cultured with cytokine-stimulated peripheral blood mononuclear cell (PBMC). Allogeneic iDCs were cultured in the presence cytokine-stimulated PBMC from healthy donors (HDs) or acute myeloid leukemia (AML) patients. Lipopolysaccharide (LPS) was used as positive control. Dendritic cell (DC) maturation was characterized by the expression of CD86. **(A)** Column bars show the MRFI of CD86 expression on DCs from HDs after culture in the presence of LPS- or cytokine-stimulated PBMC from HDs (light gray, $n = 6$) or AML patients (dark gray, $n = 4$). PBMCs were used after 48 h of culture with medium alone (control), interleukin (IL)-15 or IL-2. Error bars represent SD. **(B)** Comparative analysis of the effect of cytokines on PBMC-mediated DC maturation. Bars represent fold increase of CD86 expression on DCs cultured with PBMC treated with IL-15 or IL-2 relative to the expression in DCs co-cultured with unstimulated PBMCs.

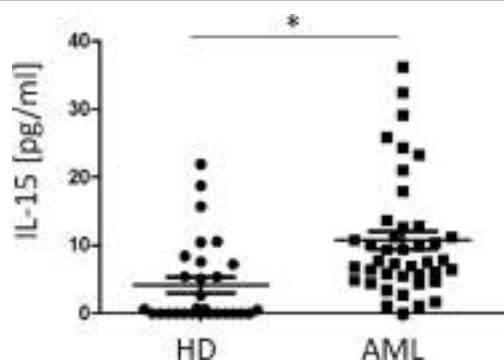


FIGURE 5 | Comparison of plasma IL-15 concentration in healthy donors (HDs) and acute myeloid leukemia (AML) patients. The horizontal bars represent the mean values. * $p \leq 0.05$.

activating receptors and decreased IFN- γ production had higher risk of relapse (54).

Previous reports demonstrated that IL-2 and IL-15 play an important role in the development, homeostasis, and function of T and NK cells. The administration of IL-2 as well as the adoptive transfer of IL-2-activated lymphocytes represented the first effective cancer immunotherapy for solid tumors such as melanoma. However, high doses of IL-2 were required to obtain therapeutic effect but a dose-related increase in toxicity was noted. Thus, IL-2 treatment was associated with adverse events including severe capillary leak syndrome that represent a major concern in IL-2 treatment. Besides IL-2 promotes the maintenance of Tregs that can reduce antitumor response (55). Since recombinant IL-2 was introduced, several clinical trials examining the role of IL-2 in preventing AML relapse have been developed. However, its use as monotherapy is not effective in terms of leukemia-free survival and overall survival (56). In a phase IV trial, AML patients in first complete remission received cycles of immunotherapy with histamine dihydrochloride and low dose of rIL-2 for 18 months to prevent leukemic relapse. The presence of high CD56^{bright} NK cell counts and high expression of NKp30 or NKp46 on CD16⁺CD56⁺ NK cells independently predicted leukemia-free survival and overall survival in this clinical trial (57).

Other cytokines have been proposed for cancer treatment, and, in the last years, IL-15 has emerged as a potential immunotherapeutic candidate (43). Preclinical studies suggest that IL-15 may represent a more efficacious cytokine for cancer immunotherapy with less toxicity obtained with intermittent administration of IL-15 compared with daily administration (45, 58).

We have analyzed *in vitro* the effect of IL-15 and IL-2 on NK cell phenotype and function including NK cell cross talk with DCs. In our study, IL-2 was included as positive control since its role on NK cell activation both *in vitro* and *in vivo* has been extensively reported. Recently, IL-15 has been proposed as an ideal candidate for the expansion of NK cells *in vivo* since it does not promote expansion of Tregs (59). In our study, IL-15 but not IL-2 induced a significant expansion of NK cells in HDs after

48 h of culture. By contrast, NK cell expansion was very limited in AML patients showing higher variability.

Our results also show that short time culture with IL-15 induces upregulation of NKp30 and NKG2D on NK cells from AML patients in concordance with previous reports (60). Other studies have also shown an increment of DNAM-1 and NKp46 expression after culture with IL-15 (40, 60). We have also observed an increment of DNAM-1 and NKp46 but this increase did not reach statistical significance. The upregulation of activating receptor expression induced by IL-15 is related to the increased NK cell degranulation against K562 cell line in concordance with previous reports (60, 61). Altogether, these results suggest that AML-induced defective function of NK cells could be overcome by IL-15. Besides, we detect a significant increased production of IFN γ and granzymes A and B after culture of NK cells with IL-15 (data not shown). These observations are consistent with previous reports supporting the pivotal role of IL-15 in NK cell antitumor activity (40, 60, 61).

Natural killer cell-mediated induction of DCs maturation was mediated by NKp30 and cytokines released after NK cell activation such as TNF- α and IFN- γ (62, 63). We have also found that IL-15-activated PBMCs from AML patients and HDs have a higher capacity to support maturation of DCs than untreated or IL-2 treated PBMCs, suggesting that the increased expression of NKp30 after IL-15 culture improves the capacity of NK cells to collaborate in the maturation of DC.

These results suggest that IL-15 in addition to enhance NK cell cytotoxicity could also collaborate with the development of adaptive immunity by promoting NK cell-mediated maturation of DCs. Further analysis will be required to confirm this function.

Cytokines present in the tumor microenvironment modulate antitumor responses. In AML patients, cytokine profile at diagnosis is frequently aberrant and associates with pathogenesis, disease progression, and survival. We have previously shown that plasma levels of TNF- α , IL-6, and IL-10 are increased in AML patients. Low levels of IL-6 and high levels of IL-10 were associated with longer event-free survival and patient survival (35). In the present work, since IL-15 has demonstrated a role in NK cell activation, we analyzed IL-15 plasma levels in AML patients. We found higher plasma IL-15 levels in AML patients compared to HDs confirming previous results (64). In lymphoid leukemia, it has been described an increase of IL-15 and IL-5R (65). It has been also shown that IL-15 could act as a growth factor for a minor fraction of AML cell lines expressing IL-2R β/γ promoting their survival and proliferation (66). In addition, the finding that AML patients have higher levels of IL-15 in plasma than HDs together with the possibility that IL-15 may promote blast growth has to be considered in protocols using IL-15 as adjuvant. We were surprised by the contradictory results showing high levels of plasma IL-15 in AML patients, whereas NKp30 expression on NK cells is diminished. It has recently been described that maintained levels of IL-15 may induce NK cell exhaustion (67), and it has been suggested that for *ex vivo* expansion optimal dosing and timing of IL-15 is critical to get adequate NK cell activation (68). We can hypothesize that maintained levels of IL-15 induce changes on NK cell phenotype

(e.g., activating receptor expression) that further contribute to the diminished NK cell activity observed in AML patients.

Thus, in spite of the encouraging data of IL-15 immunotherapy in murine models, together with its low toxicity in mice and primates that has led to the design of clinical trials in AML patients, the use of IL-15 *in vivo* as monotherapy or combined therapy in AML patients requires special caution.

A better understanding of IL-15/IL15R axis will allow the identification of novel therapeutic strategies directed to increase IL-15 immunomodulatory effect contributing to antitumor immune response but avoiding the promotion of leukemic cells survival. Our *in vitro* results support the relevance of IL-15 to induce functional active NK cells in AML patients with enhanced capacity to destroy leukemic cells and induce DCs maturation.

ETHICS STATEMENT

The study was approved by the local Ethics Committee and samples collected after written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

BS-C performed experiments. BS-C, CC, and AP analyzed data. JB, MJA, and HB selected the patients. BS-C, ED, RS, and RT

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designed the project and discussed data. BS-C and RT wrote the manuscript with support of all other co-authors.

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SUPPLEMENTARY MATERIAL

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A Two-Phase Expansion Protocol Combining Interleukin (IL)-15 and IL-21 Improves Natural Killer Cell Proliferation and Cytotoxicity against Rhabdomyosarcoma

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Rhabdomyosarcoma (RMS) is the most common soft tissue malignancy in children. Despite intensive research in recent decades the prognosis for patients with metastatic or relapsed diseases has hardly improved. New therapeutic concepts in anti-tumor therapy aim to modulate the patient's immune system to increase its aggressiveness or targeted effects toward tumor cells. Besides surgery, radiotherapy and chemotherapy, immune activation by direct application of cytokines, antibodies or adoptive cell therapy are promising approaches. In the last years, adoptive transfer of natural killer (NK) cells came into the focus of translational medicine, because of their high cytotoxic potential against transformed malignant cells. A main challenge of NK cell therapy is that it requires a high amount of functional NK cells. Therefore, ex vivo NK cell expansion protocols are currently being developed. Many culturing strategies are based on the addition of feeder or accessory cells, which need to be removed prior to the clinical application of the final NK cell product. In this study, we addressed feeder cell-free expansion methods using common γ -chain cytokines, especially IL-15 and IL-21. Our results demonstrated high potential of IL-15 for NK cell expansion, while IL-21 triggered NK cell maturation and functionality. Hence, we established a two-phase expansion protocol with IL-15 to induce an early NK cell expansion, followed by short exposure to IL-21 that boosted the cytotoxic activity of NK cells against RMS cells. Further functional analyses revealed enhanced degranulation and secretion of pro-inflammatory cytokines such as interferon- γ and tumor necrosis factor- α . In a proof of concept *in vivo* study, we also observed a therapeutic effect of adoptively transferred IL-15 expanded and IL-21 boosted NK cells in combination with image guided high precision radiation therapy.

using a luciferase-transduced RMS xenograft model. In summary, this two-phased feeder cell-free *ex vivo* culturing protocol combined efficient expansion and high cytolytic functionality of NK cells for treatment of radiation-resistant RMS.

Keywords: natural killer cells, radiotherapy, rhabdomyosarcoma, RH30 cells, RD cells, interleukin-15, interleukin-21

INTRODUCTION

With their ability to detect and directly destroy virally infected or malignant cells, natural killer (NK) cells form an important part of the first line defense of the immune system. They can be activated rapidly *via* germ-line encoded receptors that recognize the presence of stress ligands or absence of self-antigens on target cells (1–5).

In vivo development and survival of NK cells require cytokines (6–8). In this context, cytokines have been shown to activate NK cells potently during *ex vivo* expansion (9–12). The group of common γ -chain receptor cytokines encompassing interleukin (IL)-2, IL-4, IL-9, IL-15, and IL-21 has been studied intensively over the recent years. IL-2 and IL-15 have similar impacts on NK cells (13, 14). However, direct injection of IL-2 has been shown to be accompanied by severe side effects, such as vascular leak syndrome, activation-induced cell death, and strong induction of regulatory CD4^{pos} T cells, which did not occur after IL-15 administration (15, 16).

More recently, research has been focusing on IL-21 biology, but its effects on NK cell development are controversially discussed. IL-21 is known to be involved in the development and proliferation of NK cells from progenitor cells (17) and to induce receptor expression (18), interferon (IFN)- γ secretion and cytotoxicity (19). Conversely, IL-21 has also been reported to trigger apoptosis and to diminish IL-15-based benefits (20–22). These less favorable effects may be ascribed to the variability of experimental designs such as timing, cytokine concentration, additives, or accessory cells in culture as well as the developmental or maturation state and origin of NK cells. Of note, positive effects have been reported mostly upon cultivation of NK cells in the presence of auxiliary cells such as other peripheral blood mononuclear cells (PBMCs) (23), genetically modified feeder cells equipped with membrane-bound IL-21 (24, 25), or feeder-cell particles (26). The downside of these protocols is the necessity of elimination of hazardous cells, such as possibly graft-versus-host-disease (GvHD)-triggering cells or tumor-derived feeder cells, that might induce harmful side-effects *in vivo*. On the contrary, safer expansion strategies based on the exclusive application of cytokines, result in much lower absolute NK cell numbers (27). Thus, risk-free protocols for efficient expansion of functional NK cells are urgently needed.

Immune cell therapy is an effective anti-cancer strategy and hematopoietic stem cell transplantation (HSCT) has been shown to positively influence the outcome of patients with different hematologic diseases (28, 29). However, studies using HSCT did not achieve satisfactory improvement against high-risk rhabdomyosarcoma (RMS) (30–34). RMS is a rare malignant disease but the most common soft tissue cancer in children. The outcome of treatment for patients with stage IV RMS, relapsed or metastatic diseases arising from RMS, has scarcely improved during recent

decades and, in general, is unfortunately considered to be poor even upon combination of surgery, chemotherapy, radiotherapy (RT), and HSCT (35, 36).

Natural killer cells are considered to potently initiate graft-versus-tumor (GvT) effects without provoking, but even preventing GvHD (37–41), a possible risk of HSCT (42–44).

Here, we present a two-phase protocol that combines IL-15-triggered NK cell expansion with an IL-21 boost to exert the stimulatory effects of both cytokines. To avoid contamination by other cell types, we employed enriched NK cells and circumvented the addition of any accessory or feeder cells. The NK cell product was characterized intensively in terms of proliferation, phenotype, and functionality. Finally, IL-15-expanded and IL-21-boosted NK cell products from different human donors were used for combined adoptive immune cell and radiation therapy in a xenograft model of RMS.

MATERIALS AND METHODS

Purification of Primary Human NK Cells

This study was approved (approval no. 329/10) by the Ethics Committee of the Goethe University Frankfurt (Frankfurt, Germany) and was performed in accordance with the Declaration of Helsinki with written informed consent given by every participant. NK cells were isolated from freshly generated donor buffy coats provided by the German Red Cross Blood Donation Service (DRK-Blutspendedienst Baden-Württemberg-Hessen, Frankfurt, Germany), using immunomagnetic negative selection (EasySepTM Human NK Cell Enrichment kit, StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer instructions.

Briefly, PBMCs obtained from buffy coats by density gradient centrifugation were diluted to a final concentration of 100×10^6 cells/mL. Then, 50 μ L of enrichment cocktail were applied per milliliter cell suspension and incubated for 10 min. Subsequently, 100 μ L of microbead suspension were added and incubated for another 5 min. After incubation for 2.5 min in “The Big Easy” EasySepTM Magnet, the NK cell-enriched suspension was decanted into a new tube.

Cultivation of Primary Cells and Cell Lines

Purified primary NK cells were cultured at a concentration of 2×10^6 cells/mL in X-VIVO 10 medium (Lonza Group Ltd., Basel, CH) supplemented with 5% heat inactivated human fresh frozen plasma (FFP; provided by DRK-Blutspendedienst Baden-Württemberg-Hessen, Frankfurt, Germany) and 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco, New York, NY, USA). Correlating to the batch, the cells were provided with IL-2 (ProlleukinS, Novartis Pharmaceuticals, Horsham, UK), 100

U/mL (IL-2¹⁰⁰) or 1,000 U/mL (IL-2¹⁰⁰⁰), 10 ng/mL IL-15 (IL-15), 25 ng/mL IL-21 (both PeproTech, Rocky Hill, CT, USA) (IL-21) or combinations of those (IL-2¹⁰⁰ + 15, IL-2¹⁰⁰ + 15 + 21, IL-15 + 21). Every 3–4 days, half of the medium was replaced by fresh medium containing the corresponding cytokines or combinations. One batch was treated with IL-15 and boosted with IL-21 only 3–4 days before harvest and analysis (IL-15 + 21_{boost}).

Chronic myogenous erythroleukemia cell line K562 and RMS cell lines RH30 and RD (45) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat inactivated fetal calf serum (Invitrogen, Paisley, UK), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were splitted twice a week.

GFP/luciferase-expressing RD cells (RD^{GFP/Luc}) were generated via lentiviral transduction using vector particles pseudotyped with vesicular stomatitis virus G protein that were produced using the transfer plasmid pSEW-luc2, which encodes firefly luciferase and enhanced green fluorescent protein linked via a 2A peptide (46). GFP positive cells were enriched by fluorescence activated cell sorting (FACS) using a FACSaria II™ device (BD Biosciences, San Jose, CA, USA). Culture conditions for transduced cells were the same as for non-transduced cells.

Flow Cytometry

In order to check the quality of enriched NK cells and to monitor the phenotype of *ex vivo* expanded NK cells, samples were analyzed with a FACSCanto 10c™ system (BD Biosciences). Post-harvesting cells were resuspended in FACS buffer containing CellWASH (BD Biosciences), 0.5% bovine serum albumin (Sigma Aldrich, Taufkirchen, Germany) and 0.01% NaN₃ (0.1 M, Sigma Aldrich).

Intracellular staining was accomplished using formaldehyde (AppliChem GmbH, Darmstadt, Germany) for fixation and 90% methanol for membrane perforation.

The following antibodies were used: CD3-APC (#UCHT1), TRAIL-R-APC [#DJR2-4(7-8)], FAS-BV421 (#DX2), CD56-FITC (clone #HCD56), FAS-L-PE (#NOK-1), TRAIL-PE (#RIK-2), CD19-PerCP (#HIB19), CD16-PE/Cy7 (#3G8) all from Biolegend (San Diego, CA, USA); CD3-V450 (#UCHT1), CD19-V450 (#HIB19), CD14-V450 (#MφP9), CD45-BV510 (#HI30), NKp30-AF488 (#P30-15), DNAM-1-FITC (#DX11), NKp44-PE (#P44-8.1) CD45-APC (#2D1), CD137/4-1BB-APC (#4B4-1), CD107a-APC/H7 (#H4A3), IFN-γ-FITC (#B27), pAKT-AF647 (#F29-763), pERK1/2-AF647 (#20A), from BD Biosciences; CD56-APC/AF700, NKG2D-APC (#ON72), CD11a/LFA-1-FITC (#25.3) from Beckman Coulter Immunotech (Brea, CA, USA); CD45-PE (#HI30) from Invitrogen (Carlsbad, CA, USA); and NKp46-APC (#9E2), KIR2D-FITC (#NKVFS1), CD158e/k-PE (#5.133), NKG2A-APC (#Z199) from Miltenyi Biotec (Bergisch-Gladbach, Germany). Depending on the panel Zombie Violet Fixable Viability Kit (BioLegend), 7AAD (BD Biosciences) or strongly diluted DAPI were used for live/death discrimination.

Data were acquired on a FACSCanto 10c™ instrument (BD Biosciences, San Jose, CA, USA) and analyzed using Flowjo (Tree Star Inc., Ashland, OR, USA).

Cytotoxicity Assay

To investigate the killing capacity of *ex vivo* expanded NK cells a FACS-based cytotoxicity assay was employed. NK effector cells were harvested after 6 days of cytokine stimulation. Target cells were harvested and stained for 5 min with Celltrace CFSE (Molecular Probes, Eugene, OR, USA) in a final concentration of 5 µM. After all cells had been washed with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco), they were resuspended in X-VIVO 10 medium supplemented with 5% heat inactivated human FFP, 100 U/mL penicillin and 100 µg/mL streptomycin. NK cells and target cells were combined in a U-bottom 96-well plate at effector to target (E:T) ratios of 1:1, 5:1, and 10:1, adjusted to 25,000 target cells per well in a total volume of 200 µL. After 5 h of co-incubation the supernatant was removed, cells were harvested and resuspended in a 1:6,000 dilution of DAPI for live/death discrimination. From each well, the same amount of target cells was acquired using a FACSCanto 10c™ device. Samples exclusively containing target cells served as spontaneous lysis controls. Spontaneous lysis was subtracted from each sample to obtain specific lysis values. All experiments were conducted in triplicates for each NK cell donor.

To address additional antibody-dependent cellular cytotoxicity (ADCC) by NK cells, in a separate experiment, 10 µg/mL of anti-ErbB2 antibody Trastuzumab (Herceptin, ROCHE, Mannheim, Germany) were added and cytotoxicity compared to NK cell cytotoxicity in the absence of Trastuzumab.

Conjugation capacity of stimulated NK cells was addressed by staining NK cells with Celltrace CFSE, while target cells were stained with Celltrace Calcein Violet AM for 20 min at 4°C. After intensive washing, effector and target cells were co-incubated for 0–90 min, then shortly vortexed and fixed with 1–2% formaldehyde. Flow cytometry data were acquired on a FACSCanto 10c™ instrument.

Due to limited availability of NK cell numbers, cytotoxicity and conjugation assays were performed with cells from other donors than were used for proliferation assays.

Functional Activity and Degranulation Assay

Degranulation potential of cytokine stimulated NK cells was assessed as described (47), with cells harvested on day 6 of cultivation. Cells were washed and resuspended in fresh X-VIVO 10 medium supplemented with 5% heat-inactivated human FFP, 100 U/mL penicillin, and 100 µg/mL streptomycin. After 1 h, cells were incubated with anti-human CD107a, followed by an additional hour of incubation with GolgiStop™ (BD Biosciences). Cells were washed, blocked with human IgG and stained with Zombie Violet™ Fixable Viability Kit for live/death discrimination. Post washing, cells were stained for CD45, CD56, and CD16, fixed with formaldehyde solution (2% final concentration) and permeabilized with saponin buffer [0.2% saponin, 1% bovine serum albumin (both Sigma Aldrich) in DPBS]. In the end, cells were stained intracellularly with anti-human IFN-γ, washed, and measured by flow cytometry.

For Phosflow analysis freshly harvested cells were stained on the surface for CD45, CD3, and CD56, fixed and permeabilized with 90% methanol and intracellularly stained for pAKT,

pERK1/2, and pMAPK. Flow cytometry data acquisition was performed on a FACSCanto 10cTM instrument.

Due to limited availability of NK cells numbers, degranulation assays were performed with cell preparations from different donors than proliferation or cytotoxicity assays.

Immunoblot Analyses

Western blotting was deployed for the assessment of production and release of apoptosis-mediating perforin and granzyme B. NK cells and culture supernatants were harvested on day 6 of cytokine stimulation. Cells were lysed using RIPA buffer supplemented with cOmpleteTM Protease Inhibitor Cocktail (ROCHE) followed by sonification. Protein concentrations were determined *via* Bradford assay (Protein Assay Dye Reagent Concentrate, Bio-Rad, Munich, Germany). Separation of proteins was accomplished by SDS-PAGE followed by semi-dry blotting onto polyvinylidenefluoride membranes. Before antibody application, membranes were blocked with 5% skim milk powder in DPBS. Mouse monoclonal antibodies against human perforin (1:1,000, LifeSpan BioSciences, Seattle, WA, USA), granzyme B (1:200, #2C5, Santa Cruz, Heidelberg, Germany) and a rabbit antibody against human γ -tubulin (1:2,000, Sigma Aldrich) were used as primary antibodies. HRP conjugated rabbit anti-mouse IgG (1:15,000, Sigma Aldrich) and goat-anti rabbit (1:16,000, Sigma Aldrich) served as secondary antibodies. The antigen–antibody complexes were detected using an ECL-chemiluminescence system (PierceTM ECL, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions, and visualized using X-ray films (Fujifilm, Tokyo, Japan).

Due to limited availability of NK cell numbers, immunoblot assays were performed with cells from other donors than used for proliferation or functional assays.

Cytometric Bead Array

Cytokine secretion was examined by cytometric bead array analyses (CBA) on supernatants of stimulated NK cells on the sixth day of cultivation using BD CBA Flex Sets for IFN- γ , tumor necrosis factor (TNF)- α , macrophage inflammatory protein (MIP)-1 α , monocyte chemoattractant protein (MCP)-1, IL-8, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (BD Biosciences). The tests were performed according to the manufacturer's instructions using a mixture of PE-conjugated antibodies against the cytokines listed above. Data were acquired with the BD FACSVerseTM Bioanalyzer and were quantitated using the FCAP ArrayTM software (v3.0.1; BD Biosciences).

Murine RMS Xenograft Model and Treatment Protocol

The *in vivo* experiments were approved by the government committee (Regierungspräsidium Darmstadt, Darmstadt, Germany) and were conducted in accordance to the requirements of the German Animal Welfare Act.

Female, 12- to 16-week-old NOD/SCID/IL-2R $\gamma^{-/-}$ mice (NSG) mice were injected subcutaneously with 10^5 luciferase expressing RD^{GFP/Luc} cells. After visual detection of tumor nodes about 3 weeks post-cell injection, mice were imaged by performing a Cone-Beam CT (CBCT) operating at 65 kV, 0.5 mA and

irradiated while immobilized with 2.5% isoflurane anesthesia (AbbVie, Wiesbaden, Germany) using a Small Animal Radiation Research Platform (SARRP, Xstrahl Ltd., Camberley, UK). CBCT images were transferred to MuriPlanTM Software and individual isocenters were selected for targeted radiation therapy applying a two-field geometry. Fractionated single doses of 2.5 Gy using a 1–10-mm collimated beam operating at 175 kV, 15 mA were applied four times a week to reach a total dose of 27.5 Gy. Post-termination of RT, adoptive transfer of IL-15 expanded and IL-21 boosted NK cells was accomplished in three injections, once a week. NK cells were purified from 11 buffy coats as described above and maintained separately during cultivation in 25 cm² suspension cell culture flasks (Cellstar, Frickenhausen, Germany) using the IL-15 + 21_{boost} protocol as described before. After 10–11, 17–18, and 24–25 days of *ex vivo* expansion and stimulation, NK cells were harvested, washed, and pooled. The NK cells were injected intravenously *via* the tail vein with 10^7 NK cells in a total volume of 100 μ L per mouse. Due to limited availability of NK cell numbers, *in vivo* application was performed with cells from other donors than used for proliferation or functional assays.

Tumor growth was monitored by caliper measurements and bioluminescence imaging (BLI) using an IVIS Lumina II system (Perkin Elmer, Waltham, MA, USA). For the latter method, mice were anesthetized by isoflurane inhalation and subcutaneously injected with 150 μ g of *in vivo* grade VivoGloTM luciferin (Promega, Madison, WI, USA) dissolved in 100 μ L DPBS per mouse. Images were acquired after an incubation time of 15 min. BLI data analysis was performed using Living Image[®] software (Perkin Elmer).

Statistical Analyses

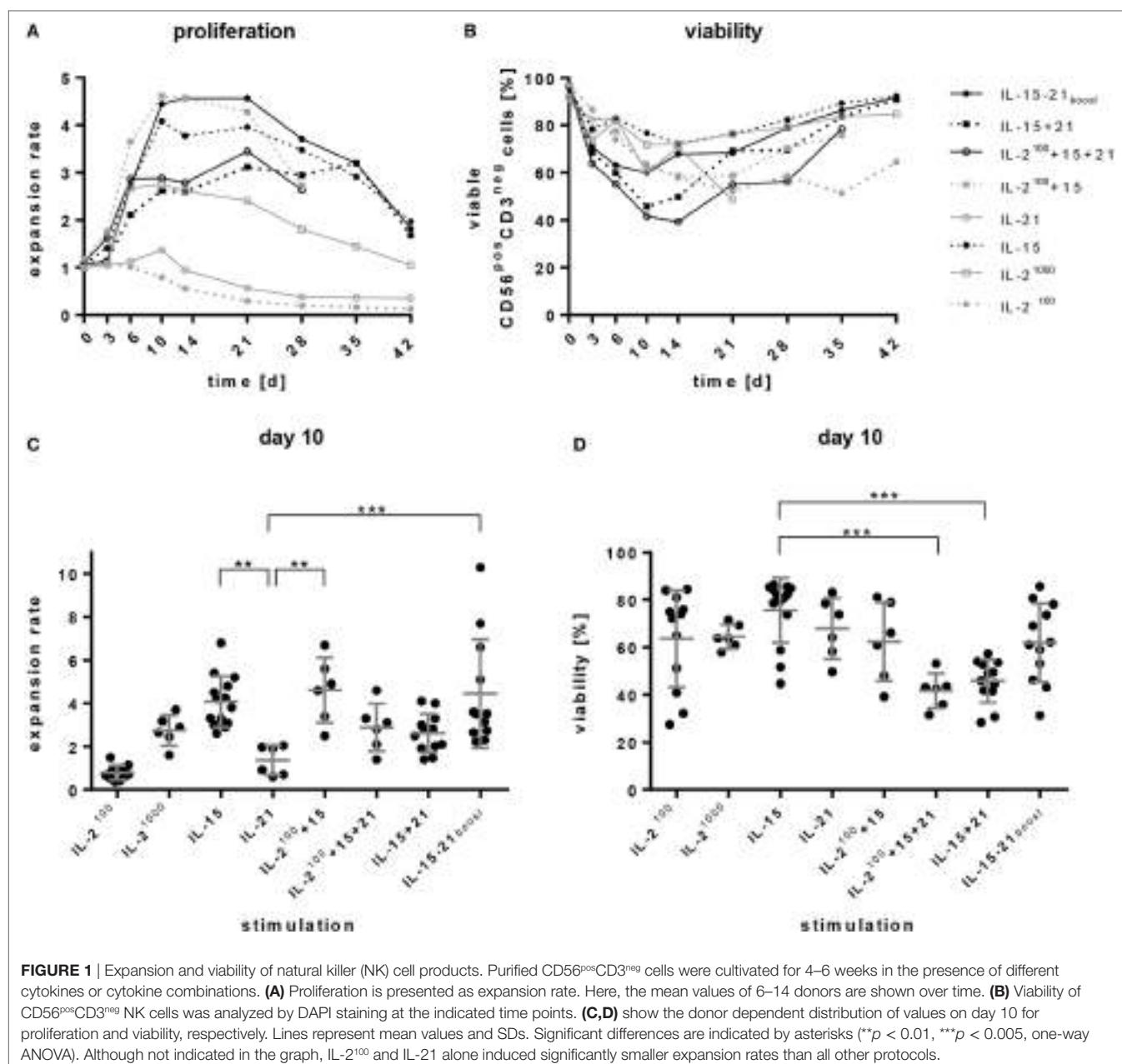
Results were analyzed using repeated measures one-way ANOVA with the Geisser–Greenhouse correction. For *in vivo* experiments, two-way ANOVA was used to compare the tumor-growth curves of different treatment groups. Statistical calculations were performed using GraphPad Prism v6 (GraphPad, La Jolla, CA, USA), and *p* values <0.05 were considered statistically significant.

RESULTS

IL-15-Driven NK Cell Expansion Is Not Impaired by a Short-term Boost with IL-21

The primary goal of *ex vivo* expansion of NK cells is to yield high cell numbers for adoptive transfer with an appropriate quality and optimal cytotoxic functionality of the NK cell product. To this end, NK cells were isolated by an immunomagnetic negative selection (as described in Section “Materials and Methods”) and expanded *ex vivo* under feeder-cell free cultivation conditions addressing the impact of different cytokines. NK cells were cultured with IL-2¹⁰⁰ (100 U/mL), IL-2¹⁰⁰⁰ (1000 U/mL), IL-15 (10 ng/mL), IL-21 (25 ng/mL), combinations of these cytokines or IL-15 and a 3-day IL-21 boost (IL-15 + 21_{boost}). Purity of the enriched NK cells was determined and cell numbers as well as viability were monitored over a period of 4–6 weeks (**Figures 1A–D**).

The average content of starting material after NK cell purification consisted of 89% NK cells. Purity of the CD3^{neg}CD56^{pos} NK cells decreased slightly on day 3, but increased afterward during



the culturing process. With protocols using IL-15, IL-15 + 21, and IL-15 + 21_{boost}, the purity reached over 95% from day 10 on. In cultures containing exclusively IL-2 also CD3^{pos}, cells expanded and diminished NK cell purity especially at late culture time points after 2–3 weeks (data not shown).

Remarkably, all protocols involving the addition of IL-15, such as IL-15 alone, IL-2¹⁰⁰ + 15, and IL-15 + 21_{boost}, led to a high increase in the number of NK cells over the first 10 days reaching a plateau until day 21. Permanent stimulation with IL-21 (IL-2¹⁰⁰ + 15 + 21 and IL-15 + 21) also provoked rapid but less distinct expansion. In general, all expansion rates decreased slowly after 3 weeks of *ex vivo* cultivation (Figure 1A).

High levels of IL-2 (IL-2¹⁰⁰) evoked proliferation of NK cells during the first 6 days, but average expansion rates declined

subsequently. Cultivation in the presence of low IL-2 levels (IL-2¹⁰⁰) or IL-21 alone did not induce proliferation, instead NK cells died (Figures 1A–D).

Irrespective of donor-dependent differences, stimulation with IL-15, IL-2¹⁰⁰ + 15, and IL-15 + 21_{boost} performed better than all other stimulation protocols. Of note, permanent exposure to IL-21 dampened IL-15-driven expansion, while a short boost with IL-21 did not disturb proliferation of NK cells, but, in some cases, even increased the expansion rate. With the IL-15 + 21_{boost} protocol, expansion rates ranged between 2- and 10-fold depending on the donor, exhibiting an average 4.5-fold increase on day 10 of culture (Figure 1C).

Percentages of viable cells decreased during the first days, but then recovered upon further stimulation (Figure 1B).

Comparisons on day 10 of cultivation indicated significantly higher frequencies of viable cells in the product of IL-15-stimulated cells than in products obtained from expansion protocols with permanent addition of IL-21. Nevertheless, a short boost with IL-21 did not significantly affect cell viability compared to IL-15 mono treatment (**Figure 1D**).

IL-21 Triggers a Mature Phenotype of NK Cells

Next, we investigated changes in the phenotype and subset composition of NK cells triggered by cytokine-induced *ex vivo* expansion. To monitor their maturation state, NK cells were analyzed for the distribution of the CD56^{high}CD16^{neg} and CD56^{dim}CD16^{pos} subset over 4–6 weeks (**Figures 2A,B**). As all stimulation protocols induced an upregulation of the NK cell marker CD56, only a discrimination of CD16^{pos} and CD16^{neg} NK cells was implemented for further analysis of the maturation state.

Depending on the expansion protocol, the frequency of CD16^{pos} NK cells was reduced. Only protocols with permanent IL-21 exposure (IL-21, IL-15 + 21, and IL-21¹⁰⁰ + 15 + 21) maintained a mature phenotype (**Figure 2A**). This effect became more pronounced the longer the *ex vivo* expansion endured (**Figures 2A,B**). In parallel with the decrease in mature phenotype, the proportion of less mature CD16^{neg} NK cells was increased (**Figure 2B**).

Furthermore, cytokine stimulation induced upregulation of activating and inhibitory receptors on the NK cell surface (**Figure 2C**). Interestingly, different cytokine combinations led to a significant increase of apoptosis inducing TRAIL or FAS ligand (FAS-L) in line with enhanced expression of TRAIL-R and FAS. We further observed that the presence of IL-21 had an additive effect with IL-15 regarding an enhanced surface expression of TRAIL, TRAIL-R, DNAM-1, FAS, FAS-L, NKp46, but also inhibitory NKG2A. For NKG2D, NKp30, NKp44 and inhibitory KIR2D and CD158 e/k (KIR3DL1/DL2), IL-21 counteracted the up-regulating effect of IL-15.

TRAIL, DNAM-1, NKp46, and NKG2A were rapidly upregulated upon short exposure to IL-21 if cells were pre-stimulated with IL-15 (IL-15 + 21_{boost}) and reached expression levels similar to those of NK cells stimulated permanently with IL-15 and IL-21 (IL-15 + 21). By contrast, changes in the levels of other surface markers were less rapid, showing significant differences for cells stimulated following the different protocols.

Almost all CD16^{neg} NK cells expressed two of the three activating receptors, NKG2D, DNAM-1, and NKp44, only a small fraction of these cells expressed only one (NKG2D) and an even smaller fraction none. After 6 days of NK cell expansion, almost all CD16^{neg} NK cells co-expressed all three receptors (Figure S1 in Supplementary Material, left panels). Among the CD16^{pos} fraction, half of it expressed only NKG2D and half co-expressed NKG2D and DNAM-1. After expansion with IL-15 alone or an additional short-term IL-21 boost, on day 6 of culturing, half of the CD16^{pos} fraction expressed all three receptors, and half co-expressed two. Permanent exposure to IL-21 further increased the fraction of CD16^{pos} NK cells that co-expressed all three receptors. Altogether, no prominent difference in the co-expression of NKG2D, NKp44, and DNAM-1 was observed upon expansion

with the three different protocols (Figure S1 in Supplementary Material, right panel).

To analyze the kinetics of the receptor expression induced by the different expansion protocols based on the use of IL-15 and/or additional IL-21, flow cytometry data were acquired at three time points, before stimulation, early at day 6, and late at day 18 (Figure S2 in Supplementary Material). Permanent presence of IL-21 diminished the expression of NKG2D, NKp30, and NKp44 on the NK cell surface. In contrast, it had hardly any influence, when applied after an IL-15-induced expansion phase. Expression of NKp46 was only slightly reduced by long-term IL-21 treatment. The higher expression level of DNAM-1, aroused by permanent presence of IL-21, stayed stable over time (Figure S2A in Supplementary Material).

Inhibitory receptors of the KIR family (KIR2D and CD158e/k/KIR3D) did not show any relevant changes during NK cell expansion, while NKG2A was strongly upregulated during NK cell expansion, independent from the cytokines used (Figure S2B in Supplementary Material).

Besides DNAM-1, expression of the adhesion molecules LFA-1 (CD11a) and 4-1BB (CD137) was addressed. Expression of LFA-1 was early increased under all tested cytokine conditions. In contrast, 4-1BB (CD137) was upregulated only after a longer culture period (Figure S2C in Supplementary Material). Accordingly, no prominent differences in conjugate formation were observed comparing the NK cells of all three tested cytokine expansion protocols (Figure S2D in Supplementary Material).

Short Stimulation with IL-21 Increases NK Cell Cytotoxicity

Besides an increase in the yield of NK cells, another aim of the *ex vivo* expansion is to obtain a therapeutic cell product that mediates optimal anti-tumor activity. In case of NK cell immunotherapy, cytotoxicity is a valuable indicator of functionality. Accordingly, cytotoxicity of cytokine-expanded NK cells was tested against targets such as the erythroleukemia cell line K562 and the RMS cell lines RD and RH30. The RD cell line represents the embryonal subtype of RMS while RH30 represents the alveolar subtype, which is more difficult to treat.

A short boost with IL-21 increased the cytotoxic effect of NK cells toward all three cell lines compared to stimulation with IL-15 alone or permanent exposure to IL-21 (**Figure 3**). An increase in specific lysis of approximately 10% was achieved by an IL-21 boost for all three cell lines as compared to sole IL-15 stimulation.

Cytotoxicity of expanded NK cells decreased with longer culture periods and reduced from 60% on day 6 to 40% on day 10 and 14 (E:T = 10:1). Still cytotoxicity of continuously expanded cells was superior to cytotoxicity of NK cells that were cryoconserved after 6 days of cytokine expansion (Figure S3A in Supplementary Material).

Despite the decrease in CD16^{pos} NK cell numbers, no decrease of ADCC was observed with NK cells expanded with the IL-15 or IL-15 + 21_{boost} protocols compared to NK cells expanded with the IL-15 + 21 protocol in an assay against ErbB2^{pos} RD cells supplemented with anti-ErbB2 antibody Trastuzumab. All expansion protocols resulted in an additional lysis of about 5 to 10%

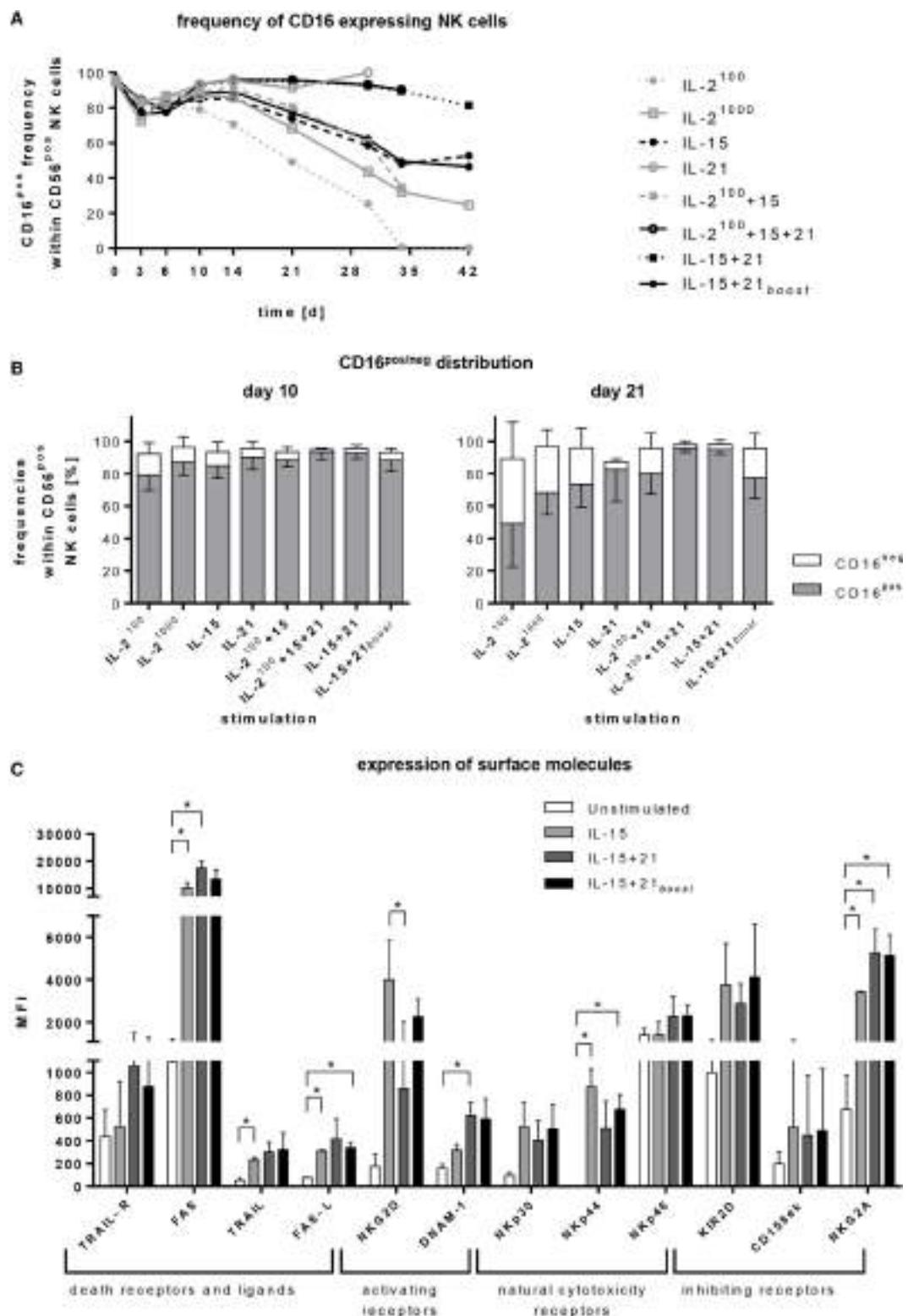


FIGURE 2 | Regulation of surface markers. Purified CD56^{pos}CD3^{neg} cells were cultivated for 4–6 weeks with different cytokines or cytokine combinations. **(A)** At indicated time points, frequencies of CD16^{pos} natural killer (NK) cells were assessed by flow cytometry. The graph represents means of 5–11 independent donors. **(B)** The graphs show the distribution of CD16^{pos} cells and their CD16^{neg} counterpart within CD56^{pos} NK cells on days 10 and 21. Bars represent mean values of 5–11 independent donors, lines indicate SDs. **(C)** Expression of various activating and inhibitory receptors after 6 days of cultivation is shown for selected protocols. Bars represent mean values of three independent donors, lines indicate SDs. Significant differences are indicated by asterisks (* $p < 0.05$, one-way ANOVA).

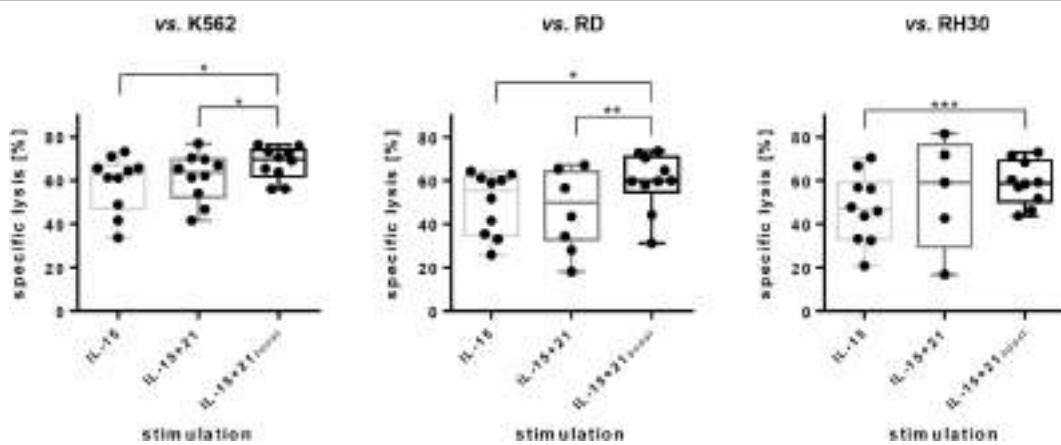


FIGURE 3 | Cytotoxic capacity of *ex vivo* expanded natural killer (NK) cells against erythroleukemia cell line K562 and RMS cell lines RD and RH30. NK cells were expanded for 6 days utilizing the stimulation protocols indicated. Data are given as mean values and SDs obtained with E:T ratios of 10:1 after 5 h of co-incubation. Specific lysis was calculated by subtracting spontaneous lysis values from frequencies of dead target cells. Spontaneous lysis was determined from target cells cultured without NK cells. Here, combined results from independent donors are shown ($n = 10$; $n = 5$ for NK cells expanded with IL-15 + 21 vs. RH30). Assays were performed in triplicates for each donor. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; vs. K562 and vs. RD: one-way ANOVA; vs. RH30: Student's *t*-test).

by ADCC at an E:T ratio of 10:1 (Figure S3B in Supplementary Material).

The IL-21-Dependent Increase in NK Cell Cytotoxicity Is Based on Elevated Degranulation

To execute cytotoxicity upon activation by target cell recognition, one basic mechanism of NK cells is to degranulate and release cytolytic enzymes. In order to assess the impact of IL-21 to the cytolytic capacity and the underpinning mechanism, we stained for CD107a (lysosomal-associated membrane protein 1), which is located in the membranes of lytic granules and is expressed on the NK cell surface upon degranulation when lytic granules fuse with the outer membrane (47, 48).

Exposure to IL-21 during cultivation significantly increased the number of degranulating cells compared to IL-15 mono-treatment. This effect was rapidly induced after a short boost with IL-21. Permanent stimulation with IL-21 evoked similar results, suggesting that degranulation activity is maintained in the continuous presence of IL-21 (Figure 4A).

To further investigate if granule exocytosis is associated with cytolytic functionality, the expression of the pore-forming protein perforin and the serine protease granzyme B was addressed by immunoblotting. Indeed, expression and release of both proteins were induced strongly upon continuous stimulation with IL-21, but not subsequent to a short boost with IL-21 (Figures 4B–D). In particular, permanent presence of IL-21 in the culture provoked roughly a doubling of the relative expression of granzyme B and perforin in comparison to short-term or no IL-21 (Figures 4C,D).

In accordance with the slower induction of *de novo* production of granzyme B and perforin after short-term compared with continuous exposure to IL-21, apoptosis-inducing enzymes were

released to a lesser extent despite similar degranulation levels were observed under both conditions (Figures 4A,B).

To further evaluate if this increase of degranulation was correlated with an increase of PI3K pathway signaling, we measured the phosphorylation of AKT and ERK1/2. Both were upregulated using IL-15 for expansion and even more with additional IL-21 (Figure S4 in Supplementary Material).

IL-21 Exposure Increases Cytokine Release by NK Cells

Natural killer cells can mediate direct cytotoxicity but also have an immunoregulatory function. The latter is achieved by secretion of cytokines. Hence, we analyzed the release of different cytokines after *ex vivo* expansion of NK cells using cytometric bead arrays (Figure 5).

Secretion of TNF- α was induced slightly by IL-15 and increased significantly upon stimulation with IL-21. Remarkably, after short-term exposure to IL-21, the TNF- α level was even higher in comparison with a continuous exposure to IL-21. A similar effect was observed for GM-CSF, although to a lesser extent. IFN- γ concentrations were significantly elevated with both protocols that utilized IL-21 causing a 10-fold increase in IFN- γ levels compared to IL-15 alone. Similarly, MIP-1 α was secreted to a higher extent in the presence of IL-21 compared with IL-15 alone, independent of the duration of administration (Figure 5).

Levels of MCP-1 were similar for all three stimulation protocols, while IL-8 release was gradually more induced by IL-21 as compared to IL-15 alone and was further increased depending on the duration of IL-21 exposure. Also the levels of anti-inflammatory IL-10 were increased depending on the duration of IL-21 presence. In contrast, IL-15 application alone almost prevented secretion of IL-10 by NK cells (Figure 5).

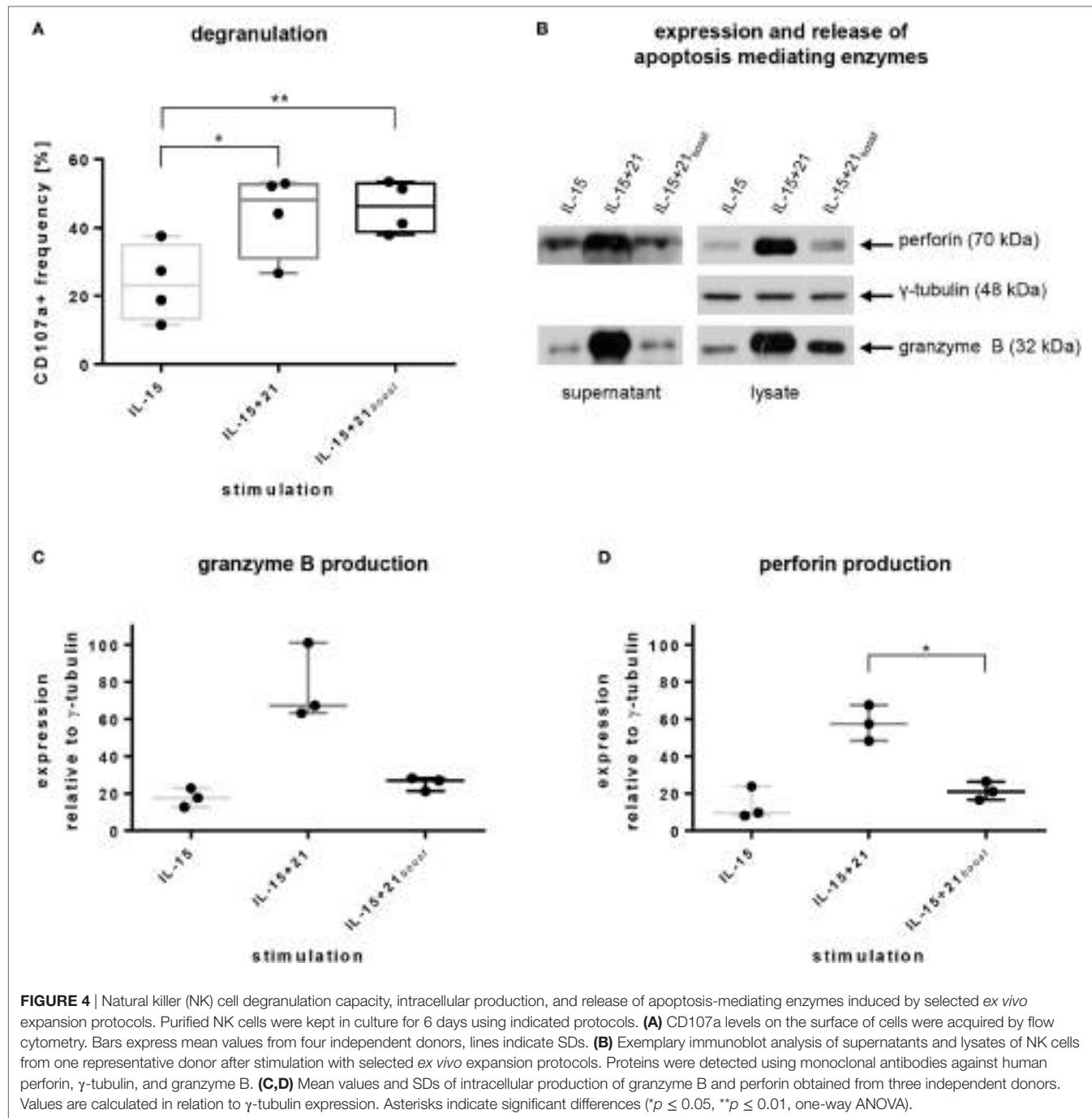
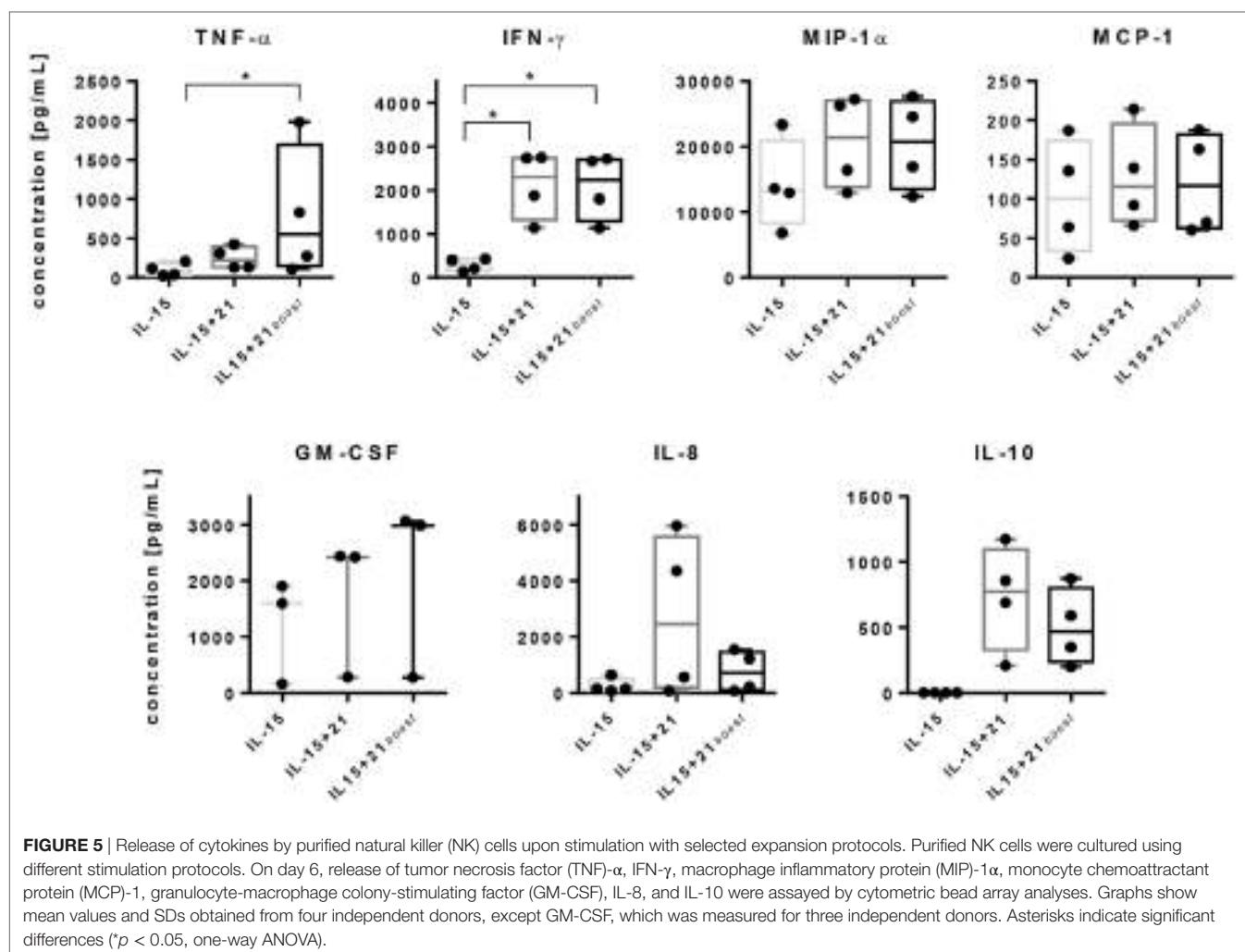


FIGURE 4 | Natural killer (NK) cell degranulation capacity, intracellular production, and release of apoptosis-mediating enzymes induced by selected ex vivo expansion protocols. Purified NK cells were kept in culture for 6 days using indicated protocols. **(A)** CD107a levels on the surface of cells were acquired by flow cytometry. Bars express mean values from four independent donors, lines indicate SDs. **(B)** Exemplary immunoblot analysis of supernatants and lysates of NK cells from one representative donor after stimulation with selected ex vivo expansion protocols. Proteins were detected using monoclonal antibodies against human perforin, γ-tubulin, and granzyme B. **(C,D)** Mean values and SDs of intracellular production of granzyme B and perforin obtained from three independent donors. Values are calculated in relation to γ-tubulin expression. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01, one-way ANOVA).

IL-21-Boosted Stimulation Enables *In Vivo* Cytotoxicity of NK Cells following Adoptive Transfer in a Xenograft Model

Given that NK cells cultured with the IL-15 + 21_{boost} protocol exhibited the highest expansion rate and cytotoxic activity toward different target cells, we finally aimed to validate the *in vivo* effectiveness of NK cells expanded with the two-phase protocol in a RMS xenograft model. To this end, NOD/SCID/IL-2Rγc⁻ (NSG) mice were subcutaneously injected with the RD^{GFP/Luc} cell line and after establishing tumors, mice were locally irradiated by an image

guided high precision local RT (Figure 6A). Subsequently, adoptive transfer of IL-15 + 21_{boost} continuously expanded NK cells was performed by three consecutive injections of 10⁷ NK cells. Tumor burden was monitored by caliper measurement (not shown) or BLI analysis of eight mice per group over 79 days (Figure 6B). Animals treated with RT monotherapy displayed a significant decrease in luciferase intensity indicating a growth retardation (Figures 6C,D). Following combined RT and NK cell therapy, the cytostatic effect was even more pronounced as compared to untreated or irradiated tumor-bearing controls (Figures 6B–E).



DISCUSSION

Immunotherapy represents a promising approach against malignant diseases. In this context, NK cells have been explored for several years because they exhibit prevailing anti-tumor activity (9, 49–54). A major advantage of NK cell-based immunotherapy is the possibility to employ these cells in an allogeneic or haploid-identical setting (6, 55, 56) without causing, or even preventing, GvHD (37–41). Numerous attempts have been made to expand NK cells efficiently for adoptive cell transfer focusing on different aspects such as high yields, efficient activation, cytotoxic potential, and/or good manufacturing practice (GMP) adequacy (27). Unfortunately, there are only limited publications available that state expansion rates after stimulating NK cells with cytokines only. Especially protocols using IL-21 on purified NK cells are quite rare. Wendt et al. did not mention absolute NK cell numbers but found an increased proliferation upon an IL-2 + 21 expansion over 72 h as shown by [3 H] thymidine incorporation. Researchers around Koehl et al. repeatedly reported expansion rates between four and five times after 12–14 days of culturing with high dose IL-2 100 . These results are similar to ours obtained with IL-15 or

IL-15 + 21 100 , although our IL-2 100 expansion did not exceed a three times increase in our tests. Compared to that, protocols allowing additional accessory PBMCs resulted in an expansion rate of 23-fold by IL-15 (57), while under addition of IL-21 only a 3.7-fold expansion rate was reached (23). Addition of gene modified feeder cells resulted in several hundreds of multiplications. When IL-21 was added to such culturing conditions, an expansion of up to 2.7×10^{11} was reached after 46 days (24). The use of membrane bound IL-21 and 4-1BB ligand still led to an expansion of more than 10 5 -fold (26).

In order to avoid contamination by possible GvHD causing cell subsets or genetically modified, tumor-derived feeder cells, we used purified NK cells for defining efficient expansion protocols. The major goal of the present study was to establish a feeder-cell free protocol for efficient expansion of primary NK cells and the improvement of their cytolytic capability to target highly aggressive and radiation-resistant RMS.

Natural killer cells were purified from buffy coats and stimulated with selected common γ -chain cytokines in different combinations. In the case of IL-2 stimulation, this study confirmed that the number of NK cells decreased under a low level of IL-2

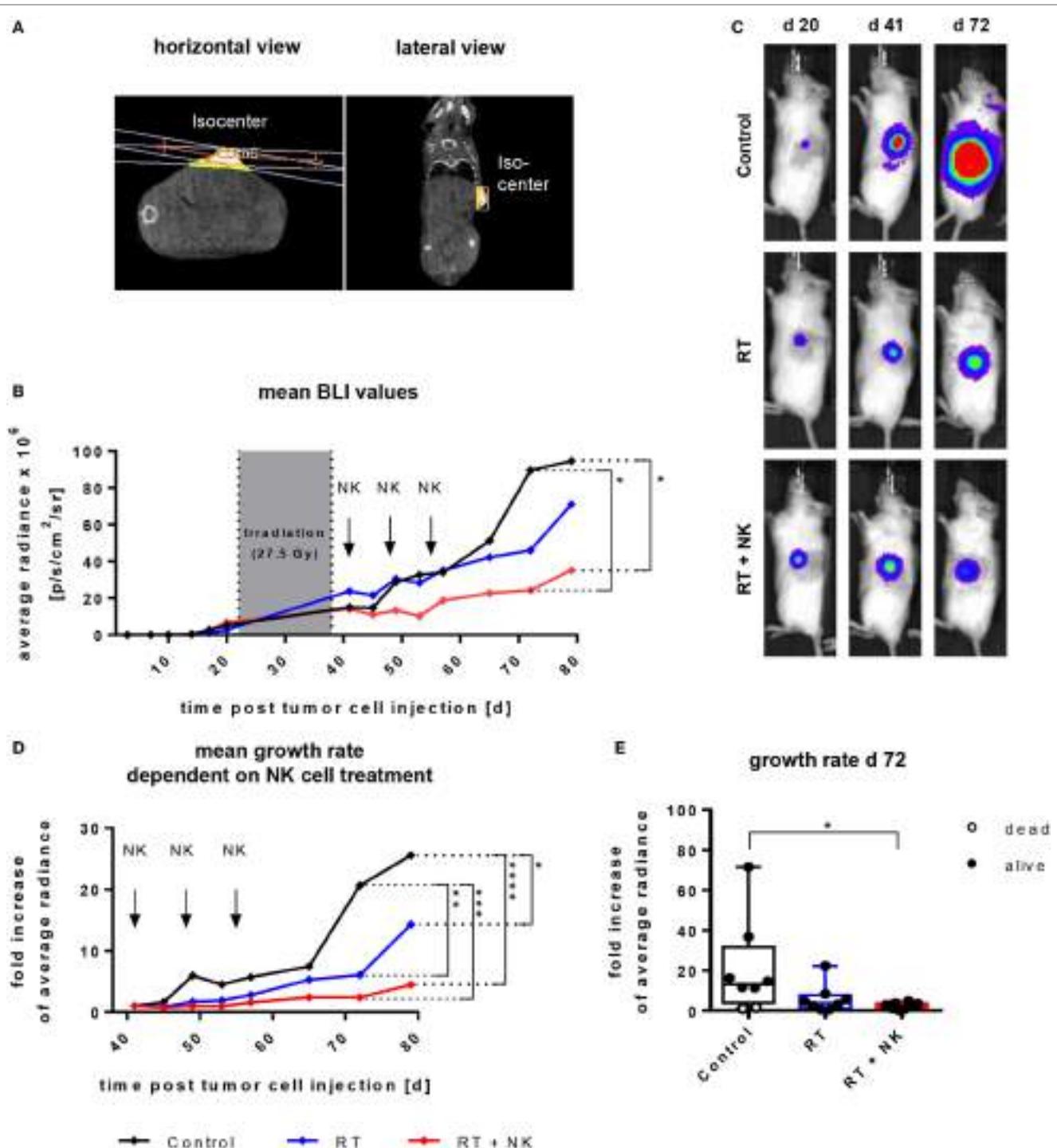


FIGURE 6 | Adoptive transfer of ex vivo IL-15-expanded and IL-21 boosted natural killer (NK) cells into RMS-bearing NSG mice subsequent to radiotherapy (RT). Mice were subcutaneously injected with 10^5 RD cells and 3 weeks later, tumor-bearing mice underwent local RT, followed by adoptive transfer of 10^7 NK cells by three weekly injections. NK cells were generated using the IL-15 + 21_{boost} protocol. **(A)** Exemplary CT-image guided planning of high precision tumor irradiation with a two-field geometry and RT isocenter presented in a horizontal and lateral view. **(B)** Time course of tumor growth, showing mean average radiance values from eight mice per group. The gray area indicates the period of RT, arrows indicate time points of NK cell administration. Significant differences are given as asterisks (* $p \leq 0.05$). **(C)** Pictures from bioluminescence imaging (BLI) of exemplary mice from each treatment group obtained before starting treatment (day 20), after termination of RT (day 41), and at the end of the experiment (day 72). **(D)** Kinetics of relative tumor growth rates normalized to tumor size at onset of NK cell treatment and **(E)** at day 72 after tumor inoculation. Displayed are mean values from eight mice per group and single values for each mouse **(E)**. Dead mice are shown as empty circles, asterisks indicate significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, two-way ANOVA).

(100 U/mL) and increased only slightly upon administration of a high concentration of IL-2 (1000 U/mL) (**Figure 1A**) (14). However, as low IL-2 levels were shown to reduce CD3^{pos} T cell outgrowth from contaminating cells remaining after NK cell purification (58), combinations of a low IL-2-dosis with other cytokine stimulants more specifically improve NK cell expansion (59, 60). Of note, all IL-15-based expansion protocols, which were tested in this study, induced an increase in the amount of NK cells in the early expansion phase. In contrast, long-term presence of IL-21 negatively influenced NK cell proliferation capacity, while a short-time IL-21 boost up to 3 days enhanced proliferation and NK cell cytotoxicity (**Figures 1A,C** and 3). In fact, the effects of IL-21 in NK cell proliferation are controversial. Immobilized or membrane-bound IL-21 has a positive effect on NK cell expansion (25, 61). Also, the longer survival of human NK cells following combined IL-21 and IL-2 stimulation has been reported (18). Conversely, an IL-21-induced apoptosis resulting in limited life span of NK cells has been described (20, 62). Overall, IL-21 appears to play an essential role in NK cell activation and cytotoxicity (21, 62).

Here, we observed a decline in the viability of NK cells after long-term exposure to IL-21 when added to other cytokines. Based on this observation, we developed a two-phase protocol first using IL-15 for the optimal expansion of NK cells, complemented by short-term addition of IL-21 to support proliferation and enhance the cytotoxic potential (IL-15 + 21_{boost}; **Figures 1A,B** and 3). Regarding the cytokine-induced expansion of NK cells, it is important to note that most published and also our own results demonstrate a strong donor-dependent variability. It will be of high interest and potential clinical relevance to address and better understand underlying mechanisms of a donor-dependent variation in cytokine-induced NK cell response.

Cytokine stimulation and *ex vivo* expansion modulate the phenotype and function of NK cells (52). CD56 surface expression has been reported to be reduced during the purification process, down to complete absence of CD56 from the cell surface, but to be upregulated upon *in vitro* stimulation (63). Accordingly, we also found an increased expression of NK cell activating receptors upon cytokine stimulation (**Figure 2**). The CD16-expressing NK cell population was diminished during stimulation with IL-2 or IL-15 (**Figure 2A**), as previously reported to be mediated by metalloproteinase ADAM17 (64–66). In contrast, IL-21 maintained surface expression of CD16, hence, increasing the frequency of the more mature CD16^{pos} NK cell population (**Figure 2B**).

In parallel with the changes in CD16 expression, apoptosis-mediated receptors and ligands such as TRAIL, FAS, and FAS-L were upregulated upon cytokine-induced expansion. In this respect, we observed a marked increase in FAS expression triggered by all protocols (**Figure 2C**), confirming recent reports (67, 68). Usually, this mechanism is employed for the killing of tumor cells but also plays a part in the achievement of lymphocyte homeostasis following immune responses against infections (69). Herein, we observed TRAIL, FAS-L, DNAM-1, and NKp46 to be expressed strongly on the NK cell surface under IL-21 stimulation when compared with IL-15 stimulation alone (**Figure 2C**). In contrast, the expression of NKG2D, NKp30, and NKp44 was

reduced under the influence of IL-21. It has been reported that NKp44 is downregulated by IL-21 treatment post-IL-15 stimulation (70, 71). Also, a decreased expression of NKp44 and NKG2D was attributed to IL-21-mediated inhibition of DAP10 and DAP12 (71, 72). In contrast, our results showed that the downstream signaling of DAP10 via the PI3K pathway was activated by IL-15, and even increased upon usage of IL-21 for NK cell expansion (Figure S4 in Supplementary Material). Upregulation of AKT phosphorylation is known to correlate with proliferation and cell survival. The even more activated ERK1/2 is associated with NK cell cytotoxicity, mediated by perforin and granzyme B mobilization (73, 74).

The maturation and activation state of NK cells in the final product is important, but the ability to carry out cytolytic functions is crucial. In line with that, we analyzed specific killing of the K562 erythroleukemia cell line as well as rhabdomyosarcoma cell lines RD and RH30. We observed an increase of cytotoxicity upon using the IL-15 + 21_{boost} protocol compared to IL-15 mono-treatment (**Figure 3**). For repeated NK cell applications, we tested continuously expanded cells and compared their cytotoxicity to that of cryopreserved cells. In line with reports on IL-15-stimulated NK cells (75), in our study, we observed a reduced cytotoxic capacity of cryopreserved cytokine-stimulated compared to continuously expanded NK cells (Figure S3A in Supplementary Material) and therefore performed analysis with the latter if several rounds of NK cell application were necessary. Another advantage of NK cell expansion using a feeder cell free IL-15 + 21_{boost} protocol will be that it easily fulfills the GMP criteria required for clinical application. Previous reports showed that IL-15-stimulated NK cells may attack various RMS cell lines more efficiently than unstimulated NK cells. Moreover, these investigations indicated that DNAM-1 and NKG2D may comprise initiators of cytotoxicity for resting NK cells, while killing by IL-15-stimulated NK cells involves additional factors including NKp30 and NKp46 (76). In our study, IL-21 enhanced NK cell cytotoxicity compared to IL-15 mono-treatment, increased DNAM-1 but reduced NKG2D expression. Although, LFA-1, among others, has been shown to be involved in the degranulation process (77) and we observed an increase in degranulating cells after prolonged IL-21 exposure or an IL-21 boost, we did not notice any difference in the expression levels of CD11a between the different stimulation protocols and also no prominent differences in the ability to form target cell conjugates (Figures S2C,D in Supplementary Material).

As previous reports showed that IL-21 induced killing can be independent from death receptor expression (62), but mediated by perforin (78), we investigated the release of lytic granules as one main mechanism mediating NK cell cytotoxicity. Indeed, IL-15 + 21_{boost}-stimulated NK cells showed an increased exposure of CD107a (**Figure 4A**), which is known to be an indicator of NK cell activity and degranulation (48). We further found that IL-21 strongly induced synthesis and release of granzyme B and perforin, but only after prolonged exposure. This is in accordance with reports on an increased production of perforin and IFN- γ in cytotoxic CD8^{pos} T cells from HIV patients upon IL-21 stimulation (79). Moreover, upregulation of intracellular perforin was reported for CD56^{pos} cells from HIV-infected individuals (80),

from stimulated PBMCs (18) and from patients with malignant melanoma upon IL-21 stimulation (81). Moreover, the increased activation of AKT and ERK signaling that we observed with IL-21 has been reported to be accompanied by intracellular perforin and granzyme B redirection (73).

Although cytokine release follows pathways distinct from lytic granule exocytosis, both mechanisms often are regulated similarly (82). In parallel to the release of lytic granules, secretion of TNF- α and IFN- γ was elevated after IL-21 stimulation (Figure 5). In the context of an immunocompetent host, NK cells not only contribute to the immune response by direct cytotoxicity but also as mediators at an intersection between innate and adaptive immunity (83, 84). Surprisingly, secretion of IL-10 was strongly induced by IL-21, but not by IL-15. As IL-10 is reported to play a crucial role in immune suppression, its function following IL-21 stimulation remains to be evaluated. Nevertheless, opposing effects were reported for IL-12, which was shown to activate NK cells and trigger pro-inflammatory T cell responses (85, 86), but also to induce IL-10 secretion (87).

Finally, we addressed the *in vivo* anti-tumor efficacy of IL-15 + 21_{boost} expanded NK cells if combined with ionizing radiation in a RMS xenograft model. Adoptive NK cell immunotherapy in combination with RT has been addressed only in a few studies so far. Ames et al. recently reported that NK cell inoculation was effective at targeting cancer cells with a stem cell phenotype from a variety of solid malignancies, which was most effective when combined with RT before application (88). It has further been shown that a combined transfer of IL-12 + 15 + 18 activated NK cells and high dose (5 Gy) synchronous irradiation resulted in a growth retardation of RMA-S lymphoma and B16-RAE-1e tumor cells (84). Consequently, in our proof of principle approach, we performed a multimodal treatment with a preceding local RT followed by adoptive transfer of IL-15 + 21_{boost} expanded NK cells. Here, we confirmed that combined NK cell immunotherapy and RT was superior to RT monotherapy in terms of growth retardation, which reaches a level of significance at 7.5 weeks. Moreover, for that purpose, a mouse CT-image guided and individually planned local irradiation procedure was applied using a SARRP with a fractionated daily 2.5 Gy RT protocol that to higher extent reflects the clinical situation where patients were treated with single 1.8 to 2 Gy fractions. Notably, combined modality treatment failed to cure the tumor burden (Figures 6B,D) indicating the necessity for advanced protocols with several repeated NK transfers or additional *in vivo* cytokine injections to further increase therapeutic efficacy. This will be addressed in future investigations.

In conclusion, we presented an optimized protocol for *ex vivo* NK cell expansion that combines the positive effects of both IL-15 and IL-21 on proliferation and activity of NK cells and that offers an ideal expansion protocol also under GMP conditions due to the absence of feeder cells. Additionally, our findings demonstrate

the high *in vitro* and *in vivo* antitumor efficacy of IL-15 + 21_{boost} expanded NK cells, which may become useful for the development of innovative combined modality treatment strategies for radiation-resistant RMS.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of the Goethe University Frankfurt (Frankfurt, Germany). All subjects gave written informed consent in accordance with the Declaration of Helsinki. This study was carried out in accordance with the recommendations of German Animal Welfare Act. The protocol was approved by the government committee (Regierungspräsidium Darmstadt, Darmstadt, Germany).

AUTHOR CONTRIBUTIONS

JW, EU, FR, and PB designed the project. JW, VP, JB, AW, SH, and BB performed experiments and analyzed data. JW, EU, WW, FR, PB, CB, TK, and SH discussed data. JW and EU wrote the manuscript with support from all other co-authors. All authors agreed to be accountable for the content of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00676/full#supplementary-material>.

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Influence of Irradiated Peripheral Blood Mononuclear Cells on Both Ex Vivo Proliferation of Human Natural Killer Cells and Change in Cellular Property

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Clinical studies with adoptive immunotherapy using allogeneic natural killer (NK) cells showed feasibility, but also limitation regarding the transfused absolute cell numbers. First promising results with peripheral blood mononuclear cells (PBMCs) as feeder cells to improve the final cell number need further optimization and investigation of the unknown controlling mechanism in the cross-talk to NK cells. We investigated the influence of irradiated autologous PBMCs to boost NK cell proliferation in the presence of OKT3 and IL-2. Our findings demonstrate a requirement for receptor–ligand interactions between feeders and NK cells to produce soluble factors that can sustain NK cell proliferation. Thus, both physical contact between feeder and NK cells, and soluble factors produced in consequence, are required to fully enhance NK cell ex vivo proliferation. This occurred with an indispensable role of the cross-talk between T cells, monocytes, and NK cells, while B cells had no further influence in supporting NK cell proliferation under these co-culture conditions. Moreover, gene expression analysis of highly proliferating and non-proliferating NK cells revealed important phenotypic changes on 5-day cultured NK cells. Actively proliferating NK cells have reduced Siglec-7 and -9 expression compared with non-proliferating and resting NK cells (day 0), independently of the presence of feeder cells. Interestingly, proliferating NK cells cultured with feeder cells contained increased frequencies of cells expressing RANKL, B7-H3, and HLA class II molecules, particularly HLA-DR, compared with resting NK cells or expanded with IL-2 only. A subset of HLA-DR expressing NK cells, co-expressing RANKL, and B7-H3 corresponded to the most proliferative population under the established co-culture conditions. Our results highlight the importance of the crosstalk between T cells, monocytes, and NK cells in autologous feeder cell-based ex vivo NK cell expansion protocols, and reveal the appearance of a highly proliferative subpopulation of NK cells (HLA-DR⁺RANKL⁺B7-H3⁺) with promising characteristics to extend the therapeutic potential of NK cells.

Keywords: natural killer cells, ex vivo expansion, immunotherapy, HLA-DR, RANKL, B7-H3

INTRODUCTION

Among the different approaches of immunotherapy to treat cancer, natural killer (NK) cells are very promising cell types with impressive outcomes in clinical studies. NK cells are innate lymphoid cells (1). They are characterized by their potent cytotoxic responses against virus-infected and malignantly transformed cells, without the need of prior immune sensitization, and in a major histocompatibility complex-unrestricted manner (2, 3). In addition, NK cells produce cytokines such as TNF- α and IFN- γ , which enhance immune responses, and engage in reciprocal interactions with other immune cells that contribute to different immune responses including anti-tumor effects (4).

To date, allogeneic NK cells for adoptive immunotherapy have already entered clinical studies successfully for both applications, post stem cell transplantation (5, 6), and in non-transplant settings to treat cancer patients (7–9). However, manufacturing of NK cells directly isolated from apheresis products can result in varying quantity (10, 11) and yield not always sufficient amounts to carry out multiple applications (12–14). An increase in the number of functional NK cells by *ex vivo* expansion methods is therefore of high interest and has recently been summarized (13).

Natural killer cells require multiple signals for their *ex vivo* survival, proliferation, and activation, involving soluble factors and the necessity of physical interactions with other cells. All of these components can be conveniently supplied by feeder cells (14, 15). Different types of feeder cells have been tested for their potential in supporting NK cell *ex vivo* expansion from both, autologous or allogeneic origin. Typically, they are irradiated prior to use and supplemented with survival and activating factors such as the cytokines IL-2 and IL-15 and/or the anti-CD3 monoclonal antibody (mAb) OKT3. Several approaches using autologous peripheral blood mononuclear cells (PBMCs) as feeder cells have demonstrated their utility to generate sufficient NK cell numbers for clinical applications (16–19). In terms of clinical manufacturing, autologous PBMCs are the preferable choice to avoid safety issues that allogeneic feeder cells may rise. Despite these advantages, little is known about the positive effect of autologous feeder cells on NK cell proliferation and activation. A beneficial role of monocytes in promoting NK cell *ex vivo* proliferation has been proposed (20). However, the underlying cellular and molecular changes that NK cells undergo during active proliferation yet need to be unraveled.

In this study, we established a co-culture system with autologous PBMCs to examine which components have a significant influence concerning the enhancement of NK cell proliferation. We further characterized the cellular and molecular changes occurring in actively proliferating NK cells. Our data provide a better understanding of mechanisms influencing and modulating *ex vivo* NK cell proliferation and might be the base to improve harmonized manufacturing protocols for future clinical NK cell studies.

MATERIALS AND METHODS

Cells and Cell Lines

Buffy coats from healthy donors (Klinikum Dortmund) were used for PBMC isolation. Daudi JP cells were a kind gift of

Dr. R. Seggewiss-Bernhardt, University Hospital of Wuerzburg, who obtained them from Prof. P. Fisch, University of Freiburg (21), and K562 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Both cell lines were maintained in complete RPMI medium, RPMI 1640 (Biowest) supplemented with 10% fetal bovine serum (Biochrom), and 2 mmol/L L-glutamine (PAA), in a humidified atmosphere with 5% CO₂ at 37°C.

NK Cell Isolation and Co-Culture with Autologous PBMCs

Peripheral blood mononuclear cells were isolated from buffy coats by density gradient centrifugation using Pancoll (PAN-Biotec). To obtain different feeder cell fractions, PBMCs were depleted first of CD56⁺ cells by MACS sort using anti-CD56 microbeads (Miltenyi Biotec), and when indicated they were further depleted of CD19⁺ and CD3⁺ or CD14⁺ cells using the corresponding microbeads (Miltenyi Biotec). The differently depleted PBMCs fractions were X-ray irradiated (20 Gy) (RS 2000 Biological Research Irradiator, Radsource) and used as autologous feeder cells for co-culture with NK cells within 1–1.5 h post-irradiation. The irradiated autologous CD56-depleted PBMCs ("IAPs") were used as the major feeder cells. NK cells were purified from PBMCs using the human NK cell isolation kit (Miltenyi Biotec), and expanded in 24-well plates, either in co-culture with autologous feeder cells at a 20:1 feeder-NK cell ratio, based on protocol from Ahn et al. (16), or without feeder cells, in complete culture medium; TexMACS medium (Miltenyi Biotec) supplemented with 5% human AB serum (Life Technologies) and 1,000 U/mL of Proleukin S (rhIL-2) (Novartis). Co-cultures were additionally supplemented with 10 ng/mL of anti-CD3 mAb (functional grade OKT3, Miltenyi Biotec). When indicated, NK cells were labeled prior cultivation with CellTrace™ Violet Cell Proliferation Kit [cell trace violet ("CTV") dye] (Life Technologies). Initial total cell densities of cultures were 1 × 10⁶ cells/mL. Culture plates were incubated in a humidified atmosphere with 5% CO₂ at 37°C. To determine NK cell fold expansions, NK cell densities were checked at different indicated time points by volumetric counting and detection of viable CD3⁻CD56⁺ cells using MACSQuant Analyzer 10 (Miltenyi Biotec). For 12-day expansions, NK cells were harvested from 24-well plates on day 7 and transferred to 25 or 75 T-flasks with replenishment of fresh medium without OKT3. Fresh medium was additionally replenished on day 9 or 10.

Co-Cultures in Transwell® Plates

Purified NK cells were cultured for up to 5 days with or without IAPs in 12-well polystyrene plates equipped with Transwell® inserts (Costar). The insert system consisted of a 500 µL upper well (12 mm diameter), separated from the bottom well (1.5 mL) by a 0.4 µm microporous tissue culture-treated polycarbonated membrane. All cells were resuspended in complete culture medium and IAPs suspensions were supplemented with OKT3 as described. Four different culture conditions were established, all of them containing 1 mL of NK cells (5 × 10⁵ cells/mL) at the bottom wells. In two conditions, either 1 mL of complete medium or

1 mL of IAP suspension were added to the NK cells on the bottom wells, and upper wells were left empty. In two other conditions, either 1 mL of IAP suspension (10×10^6 cells/mL) was inoculated through the insert, or a combination of 0.5 mL of IAPs (10×10^6 cells/mL) and 0.5 mL of purified NK cells (5×10^5 cells/mL) was inoculated, remaining the inoculated cells in both situations on the upper well of the insert. Transwell plates were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Flow Cytometry

Natural killer cell frequencies were determined using the following panel of mAbs from Miltenyi Biotec: CD45-VioGreen (5B1), CD3-FITC (BW264/56), BDCA1-PE (AD5-8E7), CD14-PE (TÜK4), CD19-PEVio770 (LT19), CD16-APC (VEP13), and CD56-APCVio770 (REA196). In experiments with feeder cell fractions containing additional depletions of CD19⁺ and CD3⁺ or CD14⁺ cells, the mAb BDCA-2-PE (AC144) was included instead of CD14-PE, and CD16-APC replaced by CD14-APC. The human FcR Blocking Reagent from Miltenyi was also used to block unspecific antibody binding to Fc receptors on CD14⁺ enriched feeder cell fractions. Propidium iodide (Miltenyi Biotec) was used at a final concentration of 1 µg/mL to exclude dead cells from the analysis.

To study the phenotypic differences between resting, proliferating, and non-proliferating NK cells, cells were labeled prior cultivation with or without IAPs, with the cell trace proliferation dye eFluor®670 (eBioscience), in order to differentiate proliferating and non-proliferating cells. Different candidate molecules were analyzed using several antibody panels designed based on previous work (22). These panels shared a backbone of mAbs: CD45-VioGreen, CD3-VioBlue, TCRγδ-VioBlue (11F2), CD14-VioBlue, CD19-VioBlue, and CD56-APCVio770, and SYTOX® Blue (Life Technologies) was used to exclude dead cells from the analysis. The backbone was combined with groups of the following mAbs (Miltenyi Biotec unless otherwise indicated): KLRLB1-FITC (191B8), CTLA-4-PE (BNI3), Siglec-9-PEVio770 (REA492), Siglec-7-PerCP700 (REA214), LILRB1-FITC (GHI/75), KLRG1-PE (REA261), CD16-PerCP700 (VEP13), RANKL-PE (DN254), B7-H3-PEVio770 (FM276), NKp44-PEVio770 (2.29), NKp80-FITC (4A4.D10), 4-1BB-PE (4b4-1), 2B4-PEVio770 (REA112), NKG2D-PerCP Cy5.5 (1D11) (Biologen), HLA-DP/DQ/DR-FITC (REA332), ALCAM-PEVio770 (REA442), HLA-A/B/C-PerCPVio700 (REA230), and HLA-DR-FITC (AC122). The corresponding mouse immunoglobulin (Ig) G1, IgG2A, IgG2B, IgM, or REAs conjugated with the respective dyes were used as isotype controls. Cells were acquired using MACSQuant Analyzer 10 and analyzed using MACSQuantify 2.8 software (Miltenyi Biotec).

Cytokine Detection

Supernatants from co-cultures of NK cells with IAPs further depleted of CD19⁺, and CD3⁺ or CD14⁺ cells were collected after 5 days and cytokine production was detected using the flow cytometry bead-based array MACSPlex Cytokine 12 kit, human according to manufacturer's instructions (Miltenyi Biotec). The MACSPlex Cytokine 12 kit allows for the detection of human GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10,

IL-12p70, IL-17A, and TNF-α. Samples were acquired using a MACSQuant Analyzer 10 and analyzed using the Express Mode option of the MACSQuantify 2.8 software (Miltenyi Biotec).

Cytotoxicity Assays

Target cell killing of K562 and Daudi JP cell lines was analyzed using a flow cytometry-based assay (23). Briefly, target cells were labeled with CTV dye and seeded in 96 well U-bottom plates at a cell density of 2×10^5 cells/mL, and incubated alone, or with expanded NK cells (2×10^6 cells/mL) at different effector-to-target (E:T) ratios, for 4 h in a humidified atmosphere with 5% CO₂ and 37°C. Antibody-dependent cellular cytotoxicity (ADCC) of expanded NK cells was further analyzed against the CD20⁺ Daudi cells, by adding 5 µg/mL of the anti-CD20 mAb rituximab (Hoffman-La Roche). After 4 h incubation, plates were transferred to 4°C for at least 30 min to stop cell killing before quantifying viable CTV-positive target cells using the MACSQuant Analyzer 10. The frequency of killed target cells was calculated by the difference between the number of viable target cells in samples with effector NK cells and samples with targets cells containing no effector cells. Representative flow cytometry data are included in Figure S1 in Supplementary Material.

Preparation of NK Cells for Sorting

Natural killer cells from five different donors were labeled with the cell trace proliferation dye eFluor®670 (eBioscience) and co-cultured for 5 days with IAPs. On day 5, cells were harvested and proliferating and non-proliferating NK cells were sorted according to the brightness of the cell trace dye in a FACSAria III cytometer (BD Bioscience, cell sorting facility of the Center for Molecular Medicine of Cologne, Cologne, Germany). 7-AAD (BD Pharmigen™) was used to exclude dead cells. To ease the sorting process, prior to sort, samples were enriched on NK cell content using the human NK cell isolation kit from Miltenyi. Sorted fractions containing the proliferating and non-proliferating ($\approx 0.8 \times 10^5$ cell in each fraction) were lysed in RA1 buffer (Macherey-Nagel) for total RNA isolation, and stored at -20°C until use. For comparison, resting NK cells (1×10^6 cells) were lysed and stored under identical conditions.

RNA Microarrays

Total RNA from sorted samples was isolated using the NucleoSpin® RNA kit (Macherey-Nagel), amplified and labeled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies), prior to hybridization to Agilent Whole Human Genome Oligo Microarrays 8 × 60K V2 chips (Agilent Technologies) according to manufacturer instructions. A detailed description of microarray processing can be found in Supplementary Material.

Pre-Processing of Microarray Data

Raw intensity data from feature extraction output files (FES 10.7.3.1, Agilent Technologies) were analyzed using the Rosetta Resolver® software (Rosetta Biosoftware). The following calculations were performed with software packages within R/Bioconductor (24, 25). Intensity values were corrected with

background subtraction and normalized by quantile normalization (26). Reliable signal intensities were considered significant when $p \leq 0.01$, using the Rosetta error model (27). Subsequent statistical analysis was performed on normalized Log2-transformed intensity values. The data set can be found at the NCBI GEO public database with the accession number GSE92512.

Statistical Analysis

Conventional statistics including parametric un-paired Student's *t*-test and one-way ANOVA with Tukey's *post hoc* test, or the non-parametric Wilcoxon test and Kruskal-Wallis test with *post hoc* Dunn's test were performed with Graph Pad Prism 7 software (GraphPad). All statistical analysis were two-sided and $p < 0.05$ considered statistically significant, and indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Only statistical differences are shown.

Description of the statistical analysis of the microarray data can be found in Supplementary Material.

RESULTS

Enhancement of *Ex Vivo* NK Cell Proliferation and Preservation of Function Using Irradiated Autologous CD56-Depleted PBMCs

We first established an NK cell expansion method using irradiated autologous CD56-depleted PBMCs (IAPs) as feeder cells to achieve high expansion rates compared with culture with rhIL-2 only. We compared the proliferation kinetics over 7 days of NK cells cultured with IAPs or with rhIL-2 only. NK cells cultured with IAPs showed significantly higher cell counts visible already at day 5 (mean fold expansion: 3.2, range: 1.4–5.7) compared with cultivation with rhIL-2 only (mean fold expansion: 1.3, range: 0.4–1.9). This differential NK cell expansion rate between the two cultivation methods became much more pronounced after prolonged cultivation, as observed in a set of NK cell expansions over 12 days. Here, cells cultured with IAPs reached particularly high cell numbers (mean fold expansion: 212, range: 80–419) compared with rhIL-2 cultivation only (mean fold expansion: 22.5, range: 6.2–57.3) (Figure 1A). The killing capacity of the differently expanded NK cells was tested against two different leukemia tumor cell lines, the K562 cell line to analyze natural cytotoxicity, and the CD20⁺ Daudi cells to also determine ADCC after opsonization of these cells with anti-CD20 mAb rituximab. The killing capacity of the differently expanded cells was preserved in all tested settings. NK cells displayed comparable natural killing rates toward K562 and Daudi targets (Figures 1B,C; Figure S1 in Supplementary Material), as well as comparable ADCC responses upon rituximab opsonization of Daudi cells. *In vitro* ADCC responses can be monitored best at low E:T ratios, where read-out of natural killing activity is lower. Independent of the expansion method, NK cells showed a clearer ADCC effect of rituximab-coated target cells at low E:T ratios (Figure 1C; Figure S1 in Supplementary Material). Taken together, co-culture with IAPs significantly enhanced *ex vivo* NK cell proliferation preserving their cytotoxic capacity.

Cross-Talk of NK Cells with Both T Cells and Monocytes Is Necessary to Produce Soluble Factors that Enhance *Ex Vivo* NK Cell Proliferation

Next, we sought to determine whether the enhancement of NK cell *ex vivo* proliferation with our established protocol is a consequence of direct cell-to-cell interactions between NK and feeder cells, or of exposure to soluble factors released by feeder cells. To address this question, we cultured NK cells for 5 days at the bottom of Transwell® plates, alone or with IAPs either in close contact or separated through a permeable membrane, allowing only traffic of soluble factors. NK cell fold expansions were significantly decreased when IAPs were separated through the membrane, reaching similar fold expansions as NK cells cultured alone. On the other hand, the addition of NK cells to IAPs separated by the membrane, rescued the expansion of the NK cells at the bottom of the Transwells (Figure 2A). These data indicate that cell-to-cell interactions between IAPs and NK cells are necessary to produce soluble factors beneficial for NK cell proliferation.

IAPs are composed of different immune cells, mainly T cells, B cells, monocytes, and dendritic cells. In this respect, we speculated whether any of these cells could have a predominant role in stimulating NK cell proliferation. NK cell fold expansions were compared after co-culture with a standard IAP feeder cell fraction and three other fractions that were depleted from CD19⁺ cells (B cells and a small subset of dendritic cells), or additionally further depleted from CD3⁺ (T cells) or CD14⁺ cells (monocytes), respectively (Figure 2B). Depletion of CD19⁺ cells from the standard IAP fraction, had no effect on NK cell proliferation, whereas further depletion of T cells or monocytes significantly reduced NK cell expansion, with a more dramatic effect upon the absence of monocytes (Figure 2C). Differences in NK cell fold expansions correlated with differences in number of dividing cells as observed with dilution of CTV dye labeling (Figure 2D).

We hypothesized that the differences in stimulating NK cell proliferation could be mirrored in the cytokine composition released by the different feeder cell fractions. GM-CSF, IFN- γ , IL-6, TNF- α , IL-5, and IL-9 could be detected at high levels (>100 pg/mL) and were produced at a similar extent in co-cultures of NK cells with IAPs or with CD19-depleted IAPs. A remarkable reduction in cytokine production was observed in co-cultures with further depletion of T cells or monocytes, respectively (Figure 2E). This, indeed, supported the differences observed in NK cell proliferation kinetics due to the different cellular composition of the feeder cell fractions. The presence of NK cells in co-culture with the different feeder cell fractions influenced the cytokine production, as observed when feeders were maintained in culture without NK cells (Figure S2 in Supplementary Material). Low detectable levels (<100 pg/mL) of IL-4, IL-10, IL-17A, IFN- α , and IL-12 were also found in all co-culture conditions, without prominent changes upon differential cell subsets depletions from IAPs (data not shown). IL-2 was detected at levels exceeding the maximum standard value (>10.000 pg/mL) in any co-culture condition, as a result of its supplementation at the beginning of the culture (data not shown); which was sufficient to support

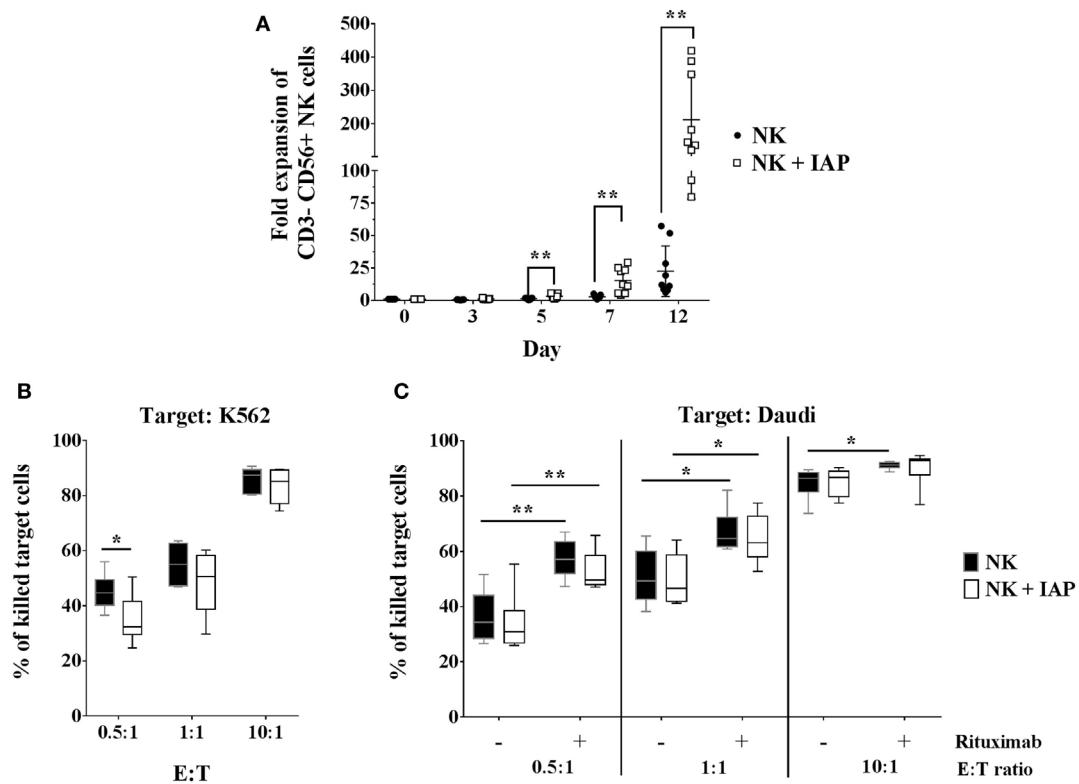


FIGURE 1 | Natural killer (NK) cell expansion using autologous CD56-depleted peripheral blood mononuclear cells (PBMCs) and cytotoxic responses compared with standard culture with rhIL-2 only. **(A)** Proliferation kinetics of NK cells in expansion with irradiated autologous CD56-depleted PBMCs (IAPs) or NK cells from the same donors but cultured with rhIL-2 only were determined by flow cytometry ($n = 9$). A separate set of samples was cultured using both methods to compare expansions up to 12 days (also $n = 9$). Fold expansion of cultured relative to resting CD56⁺ NK cells are shown for each group per donor including mean \pm SD. The non-parametric Wilcoxon test was used for statistical analysis. Natural cytotoxicity of 12-day expanded NK cells with and without IAPs was assessed against K562 **(B)** and Daudi cell lines, including antibody-dependent cellular cytotoxicity triggered by rituximab **(C)**. Frequencies of killed target cells were determined by flow cytometry and shown as mean, minimum to maximum, and SD. Statistical analysis was performed using un-paired Student's *t*-test. Figure S1 in Supplementary Material shows representative raw flow cytometry data of cytotoxicity assays.

NK cell growth independently of the presence or absence of T cells and monocytes. Taken together, our data indicate that the cross-talk between NK cells, T cells, and monocytes, is crucial to enhance *ex vivo* NK cell proliferation when using IAPs, not only due to occurrence of receptor-ligand interactions among these cells but also possibly due to the production of soluble factors as consequence of this cross-talk.

Transcriptional Analysis of Expanded NK Cells Co-Cultured with IAPs Helps to Unravel Characteristics of Highly Proliferating NK Cells

The multitude of signals occurring during the co-culture of NK cells with IAPs helped to boost the *ex vivo* proliferation of NK cells. However, at early time points during culture (day 5) not all NK cells are actively proliferating, but some remain quiescent. We aimed at identifying molecules or pathways responsible for the boost of *ex vivo* NK cell proliferation under the co-culture conditions. For this purpose, we performed a whole genome microarray analysis, with five different donors, to study the transcriptome of

resting (R0) NK cells, isolated on day 0, and compare it to proliferating (P), and non-proliferating (NP) NK cells co-cultured for 5 days with IAPs (Figure S3 in Supplementary Material). Principal components analysis (PCA) of the unfiltered transcriptomes separated the samples in three groups corresponding to resting, proliferating, and non-proliferating NK cells, respectively (Figure 3A). The expression differences between R0 and 5-day cultured samples appear to contribute most to the variation (principal component 1, 63.1%), while differences between P and NP can be ascribed to the second principal component (principal component 2, 11.5%). A total of 10,299 transcripts were differentially expressed among the three groups, only 2,902 of these transcripts were unique candidates (Figure 3B). Hierarchical clustering of the differentially expressed transcripts confirmed the PCA results of the unfiltered data by the formation of two main clusters according to the samples of resting and 5-day expanded NK cells, the latter separated in sub-clusters corresponding to P and NP (Figure 3C). A functional annotation analysis of all differentially expressed transcripts revealed to which biological processes and pathways the transcripts were related to. In addition to the anticipated associations with IL-2 pathway, the great

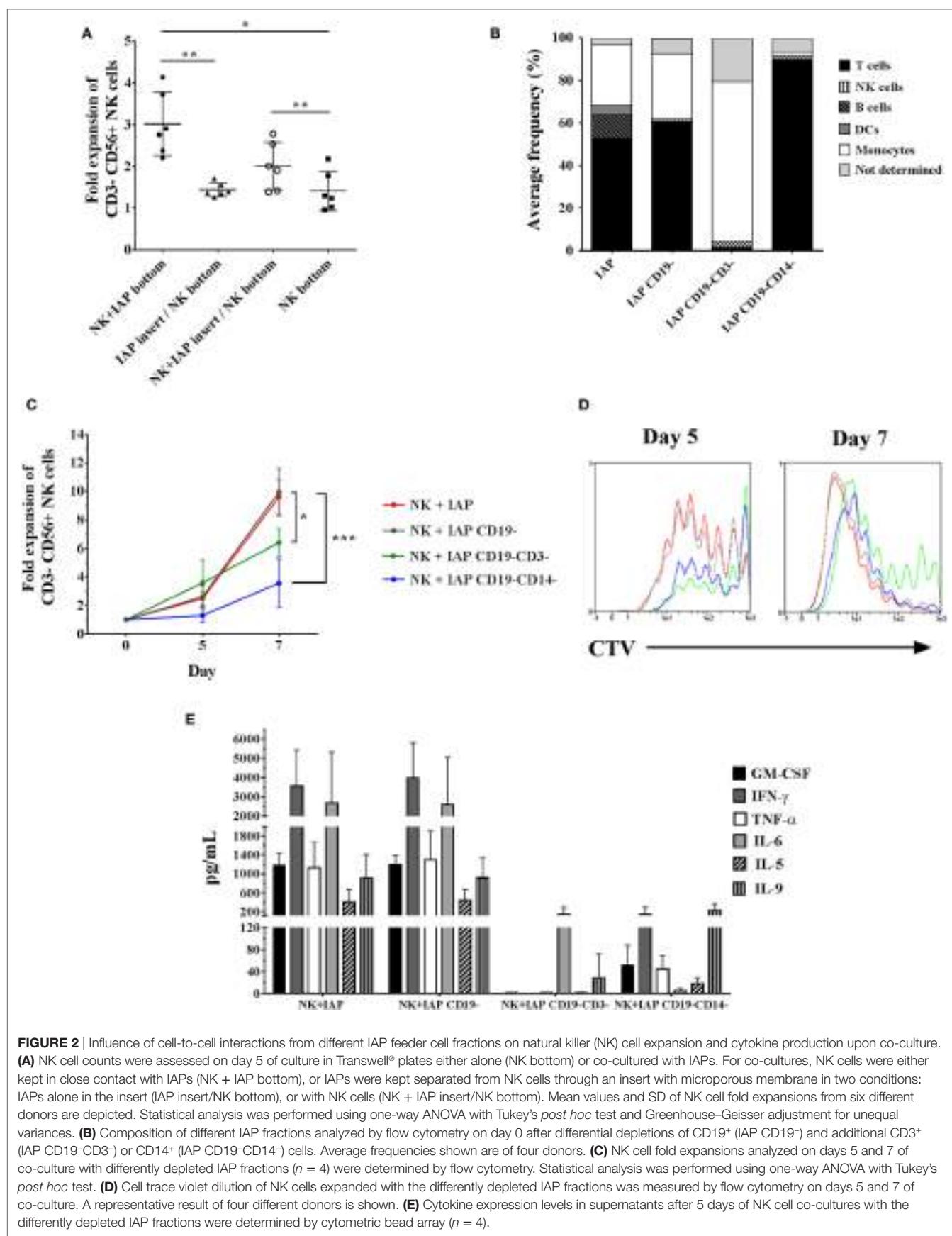


FIGURE 2 | Influence of cell-to-cell interactions from different IAP feeder cell fractions on natural killer (NK) cell expansion and cytokine production upon co-culture. **(A)** NK cell counts were assessed on day 5 of culture in Transwell® plates either alone (NK bottom) or co-cultured with IAPs. For co-cultures, NK cells were either kept in close contact with IAPs (NK + IAP bottom), or IAPs were kept separated from NK cells through an insert with microporous membrane in two conditions: IAPs alone in the insert (IAP insert/NK bottom), or with NK cells (NK + IAP insert/NK bottom). Mean values and SD of NK cell fold expansions from six different donors are depicted. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test and Greenhouse–Geisser adjustment for unequal variances. **(B)** Composition of different IAP fractions analyzed by flow cytometry on day 0 after differential depletions of CD19⁺ (IAP CD19⁻) and additional CD3⁻ (IAP CD19-CD3⁻) or CD14⁺ (IAP CD19-CD14⁻) cells. Average frequencies shown are of four donors. **(C)** NK cell fold expansions analyzed on days 5 and 7 of co-culture with differently depleted IAP fractions ($n = 4$) were determined by flow cytometry. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. **(D)** Cell trace violet dilution of NK cells expanded with the differently depleted IAP fractions was measured by flow cytometry on days 5 and 7 of co-culture. A representative result of four different donors is shown. **(E)** Cytokine expression levels in supernatants after 5 days of NK cell co-cultures with the differently depleted IAP fractions were determined by cytometric bead array ($n = 4$).

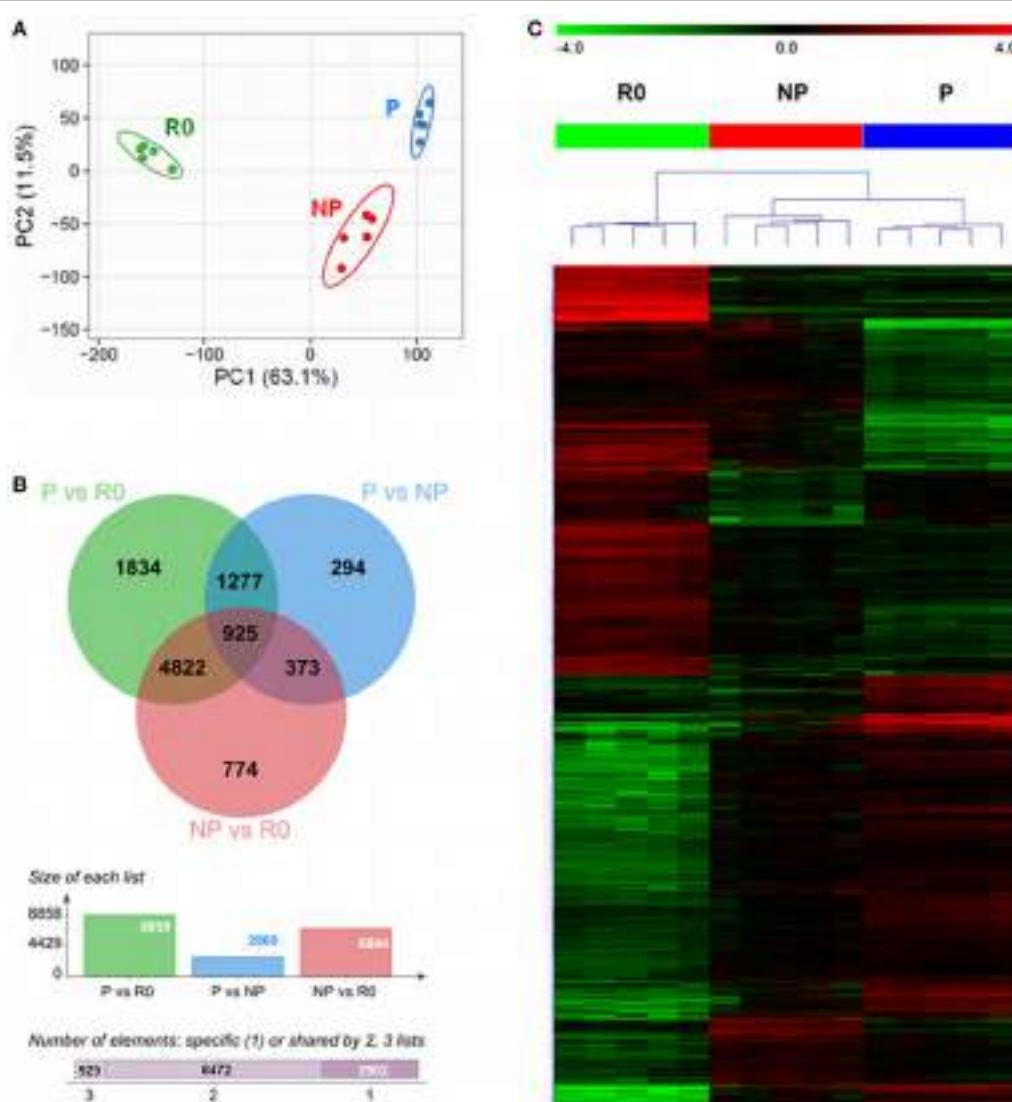


FIGURE 3 | Differential transcriptome signatures between resting, proliferating, and non-proliferating natural killer (NK) cells expanded with IAPs.

(A) Unsupervised principal component analysis of the transcriptomes of all samples ($n = 5$) for resting (R0); proliferating (P), and non-proliferating (NP) NK cells, displays the samples in a scatterplot of principal component 1 and principal component 2. The prediction ellipses are indicative of that with a 95% probability a new observation from the same group will fall inside the ellipse. **(B)** Venn diagram (top) displaying the overlap between the numbers of differentially expressed transcripts of each pairwise comparison, PvsR0, PvsNP, and NPvsR0. The columns diagram (middle) indicate the total number of differentially expressed reporters per pairwise comparison, and the horizontal bar (bottom) summarizes the number of transcripts specific or shared between two or three groups. **(C)** Heat map of all differentially expressed transcripts after hierarchical clustering (Euclidean distance, complete linkage method). Fold-change differences (row-wise centered to the median) are displayed within color saturation limits -4 (green) to +4 (red).

majority of the identified transcripts were associated with nucleotide metabolism and categories principally related to regulation of cell proliferation and activation (Figure S4 in Supplementary Material). This demonstrates that the sorted fractions indeed represented proliferating and non-proliferating cells.

In this regard, among the top 20 most highly expressed transcripts in proliferating NK cells compared with resting cells, were several positive regulators of the cell cycle and mitosis, such as cyclin A2 (CCNA2) and centrosomal protein of 55 kDa (CEP55), or the zinc finger proteins ZBED2 and ZBTB32 (Figure 4A). Zinc finger proteins are structurally

heterogeneous group of molecules which regulate gene expression by binding to DNA and RNA (28). Particularly, Tramtrack bric à brac zinc finger proteins (BTB-ZF) have important roles in controlling development and functional activity of lymphocytes (29, 30). Our data show that in proliferating NK cells, ZBED2 was the zinc finger protein with highest transcript levels, and the transcription factor ZBTB32 was the family member of BTB-ZF proteins with highest transcript levels (Figure S5A in Supplementary Material). These results point to a potential role of zinc finger proteins in modulating human *ex vivo* NK cell proliferation.

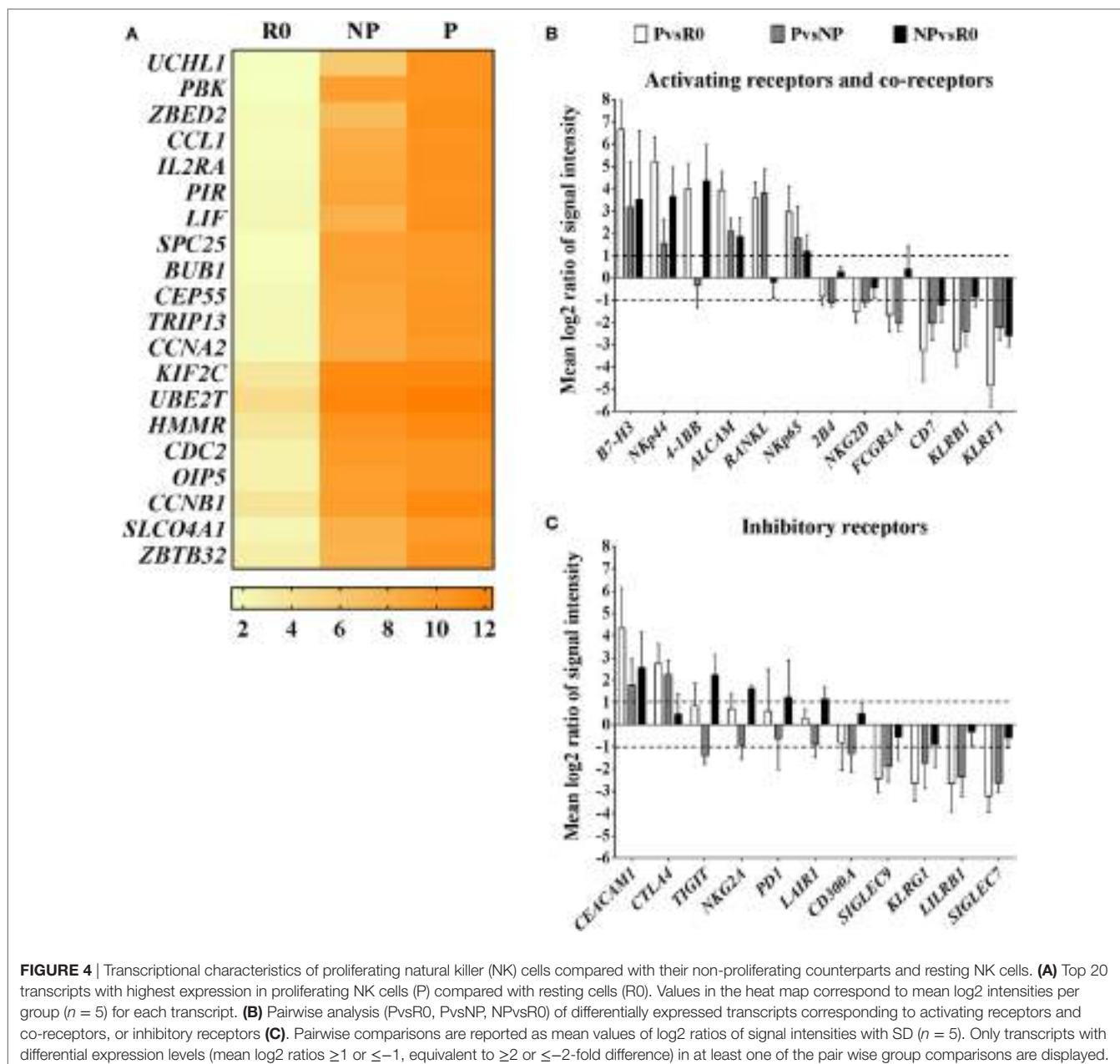


FIGURE 4 | Transcriptional characteristics of proliferating natural killer (NK) cells compared with their non-proliferating counterparts and resting NK cells. **(A)** Top 20 transcripts with highest expression in proliferating NK cells (P) compared with resting cells (R0). Values in the heat map correspond to mean log₂ intensities per group ($n = 5$) for each transcript. **(B)** Pairwise analysis (PvsR0, PvsNP, NPvsR0) of differentially expressed transcripts corresponding to activating receptors and co-receptors, or inhibitory receptors **(C)**. Pairwise comparisons are reported as mean values of log₂ ratios of signal intensities with SD ($n = 5$). Only transcripts with differential expression levels (mean log₂ ratios ≥ 1 or ≤ -1 , equivalent to ≥ 2 or ≤ -2 -fold difference) in at least one of the pair wise group comparisons are displayed.

Natural killer cell receptors and co-receptors play key roles in controlling NK cell activation. We additionally screened for the most well-known activating and inhibitory receptors and co-receptors differentially expressed among the groups. In general, transcript levels of activating receptors were increased in proliferating cells, meanwhile transcripts of many inhibitory receptors, were decreased (Figures 4B,C). Interestingly, among activating receptors and co-receptors, we found that proliferating NK cells compared with resting or non-proliferating contained increased transcript levels of receptor activator of the nuclear factor kappa-B ligand, RANKL, and CD276 molecule known as B7-H3. On the contrary, they had decreased transcript levels of the activating receptors FCGR3A (CD16), KLRF1 (NKp80), or KLRL1 (CD161) compared with resting and non-proliferating

cells (Figure 4B). Furthermore, we noticed that transcriptional changes in proliferating NK cells extended also to differences in expression of HLA class I and II molecules. Remarkably, proliferating NK cells expressed high transcript levels of HLA class II, that correlates with their activated status, whereas transcripts corresponding to HLA class I molecules were decreased in comparison with resting and non-proliferating NK cells, representing an unexpected finding (Figure S5B in Supplementary Material). Altogether these results suggest the existence of differential activation responses of NK cells toward the stimuli provided by IAPs, which may translate into a rapid proliferation of certain subsets of NK cells compared with others with delayed or no proliferative response to the same stimuli.

Phenotypic Hallmarks of Proliferating NK Cells

The changes observed in transcript levels of activating and inhibitory receptors and co-receptors are likely to extend at the protein level, and may reveal phenotypic characteristics of proliferating and non-proliferating NK cells. In addition, the detected phenotypic differences may depend on the stimuli used to promote *ex vivo* NK cell expansion. Therefore, we analyzed by flow cytometry, the expression of several selected molecules (Table S1 in Supplementary Material) in resting, proliferating, and non-proliferating NK cells expanded for 5 days with IAPs or rhIL-2 only. Most of the observed changes in protein content confirmed the results obtained by transcriptional profiling, with significant differences especially between resting and proliferating cells (Figure 5; Figure S6 in Supplementary Material). Among them, we identified that proliferating NK cells contained significantly decreased frequencies of cells expressing the inhibitory receptors Siglec-7 and -9, independently of the expansion protocol, compared with both non-proliferating and resting cells. Also, independently of the expansion method was a significant reduced expression of the activating receptors KLRB1, NKp80, CD16, and the inhibitory receptor KLRG1 within proliferating cells compared with resting cells (Figure S6 in Supplementary Material). All other analyzed activating receptors and co-receptors were expressed at higher levels in proliferating NK cells, except the NKG2D co-receptor 2B4. Despite almost all resting NK cells expressed 2B4 at the beginning of the culture, this could not be detected on day 5 of expansion with none of the expansion methods used (data not shown). This suggests an oscillating expression of 2B4 on NK cells during expansion, since expression of 2B4 has been reported after longer culture periods (23). Moreover, we could confirm RANKL and B7-H3 expression, absent in resting cells, appeared after 5 days of expansion. Their expression was mainly restricted to proliferating NK cells and reached higher levels when NK cells were expanded with IAPs instead of with rhIL-2 only (Figures 5A,B). Similarly, expression of HLA class II molecules and particularly HLA-DR, were increased after 5 days of expansion with IAPs compared with rhIL-2 only. Expression of HLA-DR persisted after longer term culture (data not shown). Interestingly, the reduction observed earlier in the expression of HLA class I molecules after 5 days of culture was verified here too, independently of the expansion protocol used.

Co-Expression of HLA-DR, RANKL, and B7-H3 Define a Subset of Highly Proliferating NK Cells

Given that co-culture with IAPs for 5 days produced an enrichment of HLA-DR expressing cells and induced expression of RANKL and B7-H3, we investigated the correlation of the co-expression of these three molecules with the proliferative status of NK cells by flow cytometry. HLA-DR expressing NK cells contained a subpopulation of double positive RANKL and B7-H3 NK cells that corresponded to the most highly proliferating NK cells (Figure 6; Figure S7 in Supplementary Material). This was substantiated by NK cells co-cultured with IAPs in contrast to cultures with rhIL-2

only. The HLA-DR⁺RANKL⁺B7-H3⁺ NK cell population was minimally represented in cultures with rhIL-2 only, but enlarged in co-cultures with IAPs. In summary, we identified a subset of NK cells that preferentially expand in response to the stimuli provided by IAPs and is characterized by expression of HLA-DR, RANKL, and B7-H3.

DISCUSSION

Recent developments in *ex vivo* NK cell expansion protocols for clinical applications have exploited the use of autologous feeder cells in combination with cytokines to boost NK cell proliferation and their activation status (16–19). However, the components responsible for this enhancement remain somewhat elusive, and little is known regarding the phenotypic and intracellular changes occurring when NK cells start to actively proliferate under these conditions. In this study, we established a co-culture protocol using irradiated autologous CD56-depleted PBMCs (IAPs) as feeder cells, to use it as a tool for the analysis of factors influencing *ex vivo* proliferation of NK cells. After ascertaining the achievement of higher numbers of functional NK cells by co-culture with IAPs compared with standard culture with rhIL-2 only, we subsequently analyzed the influence of cell-to-cell interactions, soluble factors, and specific PBMCs subpopulations on NK cell proliferation. The need of homotypic and heterotypic cell-to-cell interactions for NK cell survival, activation, and proliferation has been previously reported (20, 31, 32). Consistent with this, disruption of cell-to-cell interactions between NK cells and IAPs by using Transwell® plates results in lower NK cell proliferation than when feeder cells and NK cells are in close contact. Additionally, here we report that NK cell interactions with IAPs induce changes in production of soluble factors that improve NK cell *ex vivo* proliferation. The role of certain soluble factors, mostly of the cytokines IL-2, IL-15, and IL-21, in inducing mature human NK cell *ex vivo* proliferation has been well acknowledged (33, 34). In our set-up, sufficient IL-2 levels were achieved by supplementation of high concentrations of rhIL-2 at the beginning of the expansions, and also possibly by additional IL-2 production by T cells within the IAPs upon stimulation in culture. Concerning IL-15 and IL-21, we assessed their production using commercial enzyme-linked immunosorbent assays during a preliminary test on supernatants from 5-day expanded NK cells with and without IAPs. Levels of soluble IL-15 were undetected, and only background levels of IL-21 could be detected, without differences upon the presence or absence of IAPs. With this, we concluded that there was no significant production of neither IL-15 nor IL-21 in our co-culture system. Thus, our findings point to additional cytokines and other soluble factors produced during IAPs-NK cell co-cultures that should be further investigated, and may be used to improve *ex vivo* NK cell expansion methods without the use of feeder cells.

Considering the heterogeneous composition of IAPs, we analyzed the influence of subpopulations (mainly T cells, B cells, and monocytes) in promoting NK cell proliferation. In our co-culture method, the presence of both T cells and monocytes is sufficient to completely enhance *ex vivo* NK cell proliferation, whereas the presence of B cells is dispensable. We observed that monocyte depletion substantially reduces the production of cytokines from

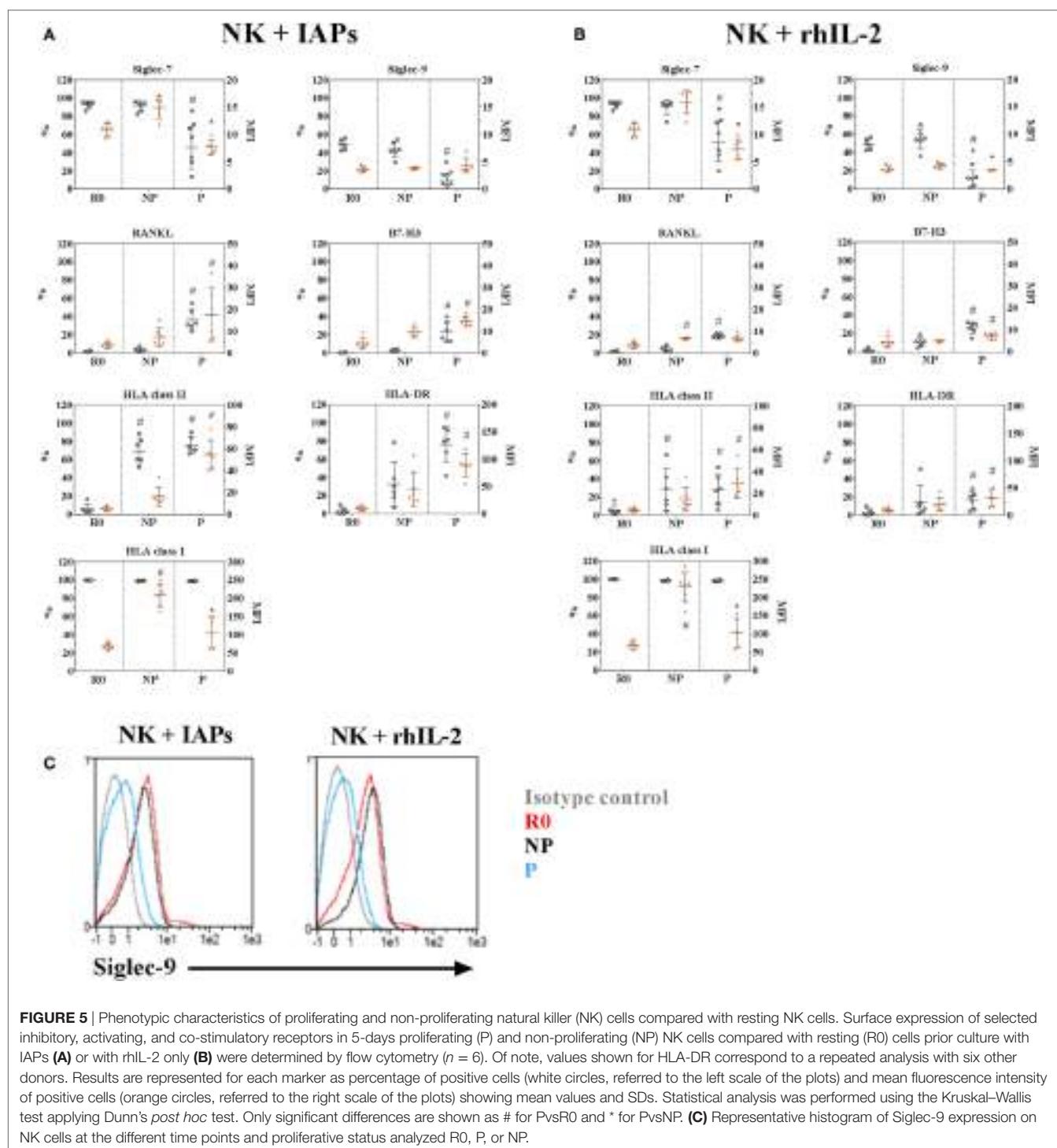
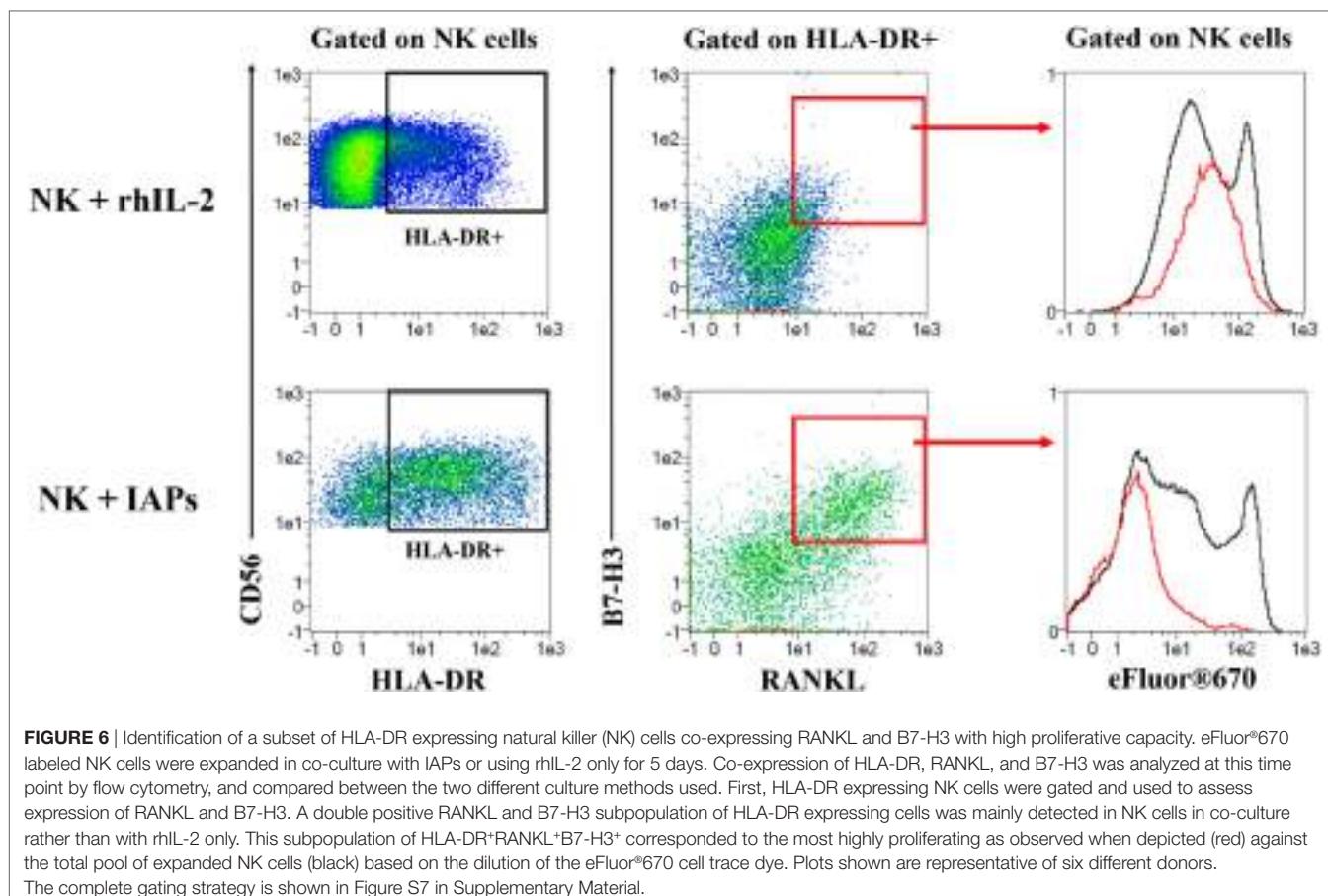


FIGURE 5 | Phenotypic characteristics of proliferating and non-proliferating natural killer (NK) cells compared with resting NK cells. Surface expression of selected inhibitory, activating, and co-stimulatory receptors in 5-days proliferating (P) and non-proliferating (NP) NK cells compared with resting (R0) cells prior culture with IAPs (**A**) or with rhIL-2 only (**B**) were determined by flow cytometry ($n = 6$). Of note, values shown for HLA-DR correspond to a repeated analysis with six other donors. Results are represented for each marker as percentage of positive cells (white circles, referred to the left scale of the plots) and mean fluorescence intensity of positive cells (orange circles, referred to the right scale of the plots) showing mean values and SDs. Statistical analysis was performed using the Kruskal-Wallis test applying Dunn's post hoc test. Only significant differences are shown as # for PvsR0 and * for PvsNP. (**C**) Representative histogram of Siglec-9 expression on NK cells at the different time points and proliferative status analyzed R0, P, or NP.

remaining feeder cells, which correlates with the lowest NK cell expansions. On the other hand, the presence of monocytes without T cells almost completely hindered cytokine production, although this caused a less prominent reduction of NK cell expansion. This indicates that the irradiated monocytes besides participating in promoting cytokine production from T cells, may also produce other soluble factors, or express certain surface molecules that result in promoting *ex vivo* NK cell proliferation. To a certain

extent, this correlates with previous observations showing the potential of monocytes to boost *ex vivo* NK cell expansion (20, 35), but it is in contrast with the exclusion of a beneficial effect of T cells shown in one of the studies (20). The reason of this difference may be explained by the use of non-irradiated accessory cells and only rhIL-2 supplemented in this former study, while in our set-up the T cells are irradiated and OKT3 is additionally supplemented together rhIL-2. It is important to remark that full



activation of peripheral blood T cells by OKT3 requires additional signals from accessory cells and the presence of IL-2 (36). These accessory cells are mainly monocytes that through their Fc receptors, bind OKT3, and induce crosslinking of the CD3 molecule on T cells leading to a proper stimulation (36–38). This may explain that upon the absence of monocytes, despite the presence of OKT3 and IL-2, cytokine production by T cells is severely hampered. Moreover, the effect of OKT3, murine IgG2a mAb, on NK cell activation through the Fc receptor CD16 would be minimal, as CD16 has no or low binding affinity to IgG2a isotype (37, 39). In summary, in our co-culture system, the interaction of irradiated T cells and monocytes through bidirectional binding of OKT3, together the presence of IL-2 seems sufficient to induce production of soluble factors at the beginning of the co-culture with clear further beneficial effects on NK cell *ex vivo* growth.

Several studies have performed transcriptional and microRNA expression analysis in human NK cells to determine global changes after short activation or long-term expansion (23, 40, 41). Using also whole genome expression analysis and functional annotation, we focused on transcriptional differences between proliferating NK cells and their remaining non-proliferating counterparts after 5-days in co-culture with IAPs. By choosing this time point for analysis, we assured the emergence of pro-survival and proliferation pathways, absent at earlier time points, or extinguished at later ones. Certainly, we identified among

the most highly expressed transcripts in proliferating NK cells many molecules involved in regulation of cell cycle and mitosis. Interestingly also transcripts corresponding to the zinc finger proteins, ZBED2 and ZBTB32 were upregulated. While only little is known about ZBED2 (42), the transcription factor ZBTB32 recently has been described to play a role in controlling proliferation of mouse cytomegalovirus (MCMV)-specific NK cells (43). Since in that study ZBTB32 was predominantly involved in the context of infection and inflammation, but was not required for homeostatic proliferation of mouse MCMV-specific NK cells, it was surprising to find increased levels of a transcript corresponding to ZBTB32 in proliferating human NK cells in our co-culture system. Therefore, it will be interesting to analyze which role zinc finger proteins, particularly, ZBED2 and ZBTB32 play in modulating the *ex vivo* expansion of mature human NK cells.

We describe also several phenotypic characteristics of highly proliferating NK cells compared with non-proliferating and resting ones. Actively proliferating NK cells lose expression of the inhibitory receptors Siglec-7 and -9 compared with both resting and non-proliferating counterparts. Several studies have shown that blocking of Siglec-7 and -9 receptors enhances NK cell cytotoxicity against target tumors, allowing for a better activation (44, 45). The reduced expression of Siglec-7 and -9 in proliferating NK cells may reflect a differential activation status related to high proliferation. Also in this regard, we detected reduced expression

of the activating receptors NKp80 and CD16 preferentially in the population of proliferating NK cells. Down-regulation of NKp80 and CD16 is known to occur early after NK cell activation (46–48). Therefore, decreased Siglec-7/-9, NKp80, and CD16 expression evidence that differential activation responses within the original pool of NK cells result in either active *ex vivo* proliferation or quiescence. Moreover, the expression of the molecules RANKL and B7-H3 was significantly induced in NK cells particularly proliferating in co-culture with IAPs, as opposed to cultures with rhIL-2 only. Although up-regulation of RANKL has been previously reported to occur in CD56^{bright} NK cells (49) little is known about its function in NK cells. On the other hand, B7-H3, a member of the B7/CD28 superfamily of costimulatory molecules is expressed in many human cancers and induced in several immune cells such as activated monocytes and dendritic cells with a controversial role in modulating T cell activity (50, 51). It would be interesting to determine whether B7-H3 is also a marker of activated NK cells *in vivo* and learn about its functional role.

Co-culture with IAPs also increased expression of particularly HLA-DR molecules in proliferating NK cells compared with culture with rhIL-2 only. Further analysis of the expression of RANKL and B7-H3 in HLA-DR expressing NK cells, revealed that a subpopulation of double positive RANKL and B7-H3 HLA-DR expressing NK cells, corresponds to the most actively expanded upon co-culture with IAPs. Increased frequencies of HLA-DR expressing NK cells have been observed already after few days of culture with IL-2, possibly due to clonal expansion of an original HLA-DR expressing NK cell subpopulation (52). Recently, the expression of HLA-DR molecules was described as a surrogate marker for NK cell clonality in chronic lymphoproliferative disorders (53). Our data support the hypothesis of a preferential clonal expansion of HLA-DR expressing NK cells possibly due to differential activation upon mitogens provided by IAPs as opposed to other NK cell subsets un-responsive to the same stimuli. Identification of the corresponding receptor(s) and pathway(s) responsive to pro-proliferative signaling will be key to optimize NK cell expansion protocols. Additionally, we describe a subset of HLA-DR⁺RANKL⁺B7-H3⁺ NK cells as the subset with the highest proliferative potential emerging under co-culture with IAPs. Subsequent studies may help to elucidate a functional role of RANKL and B7-H3 molecules in these highly proliferating NK cells. In addition, it will be interesting to verify the occurrence of a preferential expansion of HLA-DR expressing NK cells with other *ex vivo* expansion protocols, and whether this confers antigen presenting characteristics to NK cells, as it has been previously shown (54). Confirmation of antigen presenting

properties of expanded NK cells may be further considered to extend the therapeutic potential of NK cells.

In summary, our findings uncover molecular and phenotypic characteristics of *ex vivo* proliferating NK cells and roles of autologous feeder cells that may have an impact in the development of new expansion methods and analysis strategies of expanded NK cells. In addition, we propose that characterization of the newly described subpopulation of HLA-DR⁺RANKL⁺B7-H3⁺ NK cells will be of interest for the further optimization of NK cell expansion protocols, and improved immunotherapeutic applications.

ETHICS STATEMENT

This study was carried out using samples from healthy donors obtained from Klinikum Dortmund (Dortmund, Germany) with given consent from the Klinikum Dortmund to use those samples for research purposes.

AUTHOR CONTRIBUTIONS

MD-V designed and performed experiments, analyzed data, and wrote the manuscript; JK performed processing and statistical analysis of gene expression data and contributed to write the manuscript; UK provided advice and contributed to write the manuscript; and VH supervised research work, provided advice, and wrote the manuscript. All authors have discussed and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00854/full#supplementary-material>.

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The Smac Mimetic BV6 Improves NK Cell-Mediated Killing of Rhabdomyosarcoma Cells by Simultaneously Targeting Tumor and Effector Cells

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with transcriptional upregulation of NF- κ B target genes such as I κ B α and RelB. Taken together, our findings implicate that SM represent a novel double-hit strategy, sensitizing tumor and activating NK cells with one single drug.

Keywords: natural killer cells, second mitochondria-derived activator of caspases mimetic, rhabdomyosarcoma, RH30 cells, RD cells, BV6

INTRODUCTION

Cancer is the second leading cause of death in children after accidents (1). The most common type of cancer is leukemia, followed by cancer of the brain and nervous system, and soft tissue sarcomas including rhabdomyosarcomas (RMS) (2). The survival of pediatric cancer has improved through progress in treatment (1, 3). However, the outcome greatly depends on the type of cancer. RMS ranks second to last in the 5-year survival rate, four out of five high-risk patients are defeated by their disease (4, 5). Especially the histological subgroup referred to as alveolar RMS (ARMS) is known for its aggressive growth, while embryonal sarcomas (ERMS) are correlated to a slightly better outcome (3). The low overall survival rate is due to resistance against established therapy regimens comprising chemo- and radiotherapy in first place or relapse after initial therapy, underlining the necessity of new treatment approaches. Here, we address two possibilities of improving anticancer therapy for ARMS with one single drug: sensitizing tumor cells on the one hand, and activating immune effector cells on the other.

The essential idea of the first approach is to overcome one of the key features of malignant cells: the resistance to apoptosis (6). This resistance is frequently caused by an imbalance between pro- and antiapoptotic proteins, resulting in a dysfunctional intrinsic cell death program. Since anticancer therapies such as chemo- and radiotherapy function by triggering cell death pathways, the intrinsic defect within the cell leads to treatment failure, associated with tumor progression and poor survival (7). Proteins known to play a role in the regulation of apoptosis are the IAP proteins and their physiological antagonists: second mitochondria-derived activator of caspases (Smac). Overexpression of IAP proteins (8–11), as well as the reduced expression of Smac (12, 13) in human malignancies have been correlated with treatment resistance and tumor growth. This has led to the development of small-molecule compounds that antagonize IAP proteins, i.e., Smac mimetics (SM). SM assume the function of the endogenous Smac by neutralizing IAP proteins, thereby rendering cancer cells more susceptible to the induction of apoptosis (14). While X-linked IAP (XIAP) proteins directly bind and inhibit caspases (15–17), cellular IAP (cIAP) proteins play an important role in regulating signaling pathways within a cell through an intrinsic ubiquitin ligase activity in their C-terminal RING domain (18). One signaling pathway known to be regulated by cIAP proteins is the nuclear factor “kappa-light-chain-enhancer” of activated B-cells (NF- κ B) pathway. The NF- κ B family comprises several transcription factors that regulate genes that are involved in cell survival, as well as innate and adaptive immune response (19) such as the

tumor necrosis factor α (TNF α), TRAIL, interferon γ (IFN- γ), and MHC class I (20).

Research over the last years revealed the broad field of immune therapy as a promising anticancer strategy (21, 22). Especially NK cells, immune effector cells that physiologically recognize and eliminate cancer cells, are of great interest for adoptive cell therapy. NK cells are equipped with a broad range of receptors on their surface, allowing the discrimination between healthy and malignantly transformed or virus-infected cells. Once activated, NK cells have different strategies to kill their targets such as exocytosis of secretory lysosomes containing cytolytic proteins (perforin, granzymes, Fas ligand) (23, 24), induction of apoptosis in the death receptor-bearing target cell through ligation with FasL or TRAIL expressed on the NK cell surface (25, 26), or further immune activation through the secretion of cytokines such as IFN- γ and TNF α (27). Considering the current state of knowledge and understanding, NK cell infusions can be referred to as safe in autologous and allogeneic settings (28, 29). Some antitumor effects have been shown for hematological as well as several solid tumors (30–32). Current limitations of NK cell therapy are the exhaustion of cellular cytotoxicity (33) and the resistance of tumor cells to the induction of apoptosis *per se*. In this work, we investigate the potential of the small-molecule SM BV6 to influence both effector and target cells simultaneously, enhancing the susceptibility of tumor cells to NK cell-mediated killing on the one hand and increasing the cytotoxic activity of NK cells on the other.

MATERIALS AND METHODS

Cell Culture

All work with cell lines was performed under sterile conditions, using sterile media, buffer, and material. Both RMS cell lines used, RH30 as an alveolar and RD as an embryonal RMS cell line, were cultured in Roswell Park Memorial Institute 1640 GlutaMAX medium (RPMI 1640) with 10% fetal bovine serum and 1% penicillin/streptomycin (PenStrep). The cells were split approximately twice a week depending on the growth rate and stored in an incubator at 37°C, 5% CO₂, and 90% relative humidity.

This study was approved by the Ethics Committee of the Goethe University Frankfurt, Germany and carried out in accordance with the Declaration of Helsinki. All subjects gave written informed consent in accordance with the Declaration of Helsinki. NK cells were isolated out of freshly generated male donor buffy coats provided by the GRC-Blood Donor Service (DRK-Blutspendedienst) in Frankfurt, using immunomagnetic negative selection (EasySep Human NK Cell Enrichment Kit,

StemCell Technologies, Canada) according to manufacturer's instructions. The purity of the resulting cell suspension was analyzed through flow cytometry, using fluorochrome-conjugated antibodies against CD56 (clone: HCD56), CD19 (clone: HIB19), CD14 (clone: HCD14), CD16 (clone: 3G8), CD3 (clone: UCHT1) (Biolegend, USA), and CD45 (clone: HI30) (Invitrogen, UK). Only cell suspensions that showed more than 85% NK cells were taken into culture and used for further experiments. The freshly isolated cells were cultured at a concentration of 2×10^6 cells/ml of the hematopoietic medium xVivo (LonzaGroup, CH), which was enriched with 5% heat-inactivated human plasma, 1% PenStrep, 100 U/ml IL-2, and the Smac mimetic BV6 if necessary. The Smac mimetic BV6, which neutralizes XIAP, cIAP1, and cIAP2 (34), was kindly provided by Genentech, Inc. (South San Francisco, CA, USA). NK cells were fed approximately every 3 days through discarding half of the medium and adding the equivalent amount of fresh medium, containing the doubled amount of additives.

Determination of Cell Viability and Proliferation

To determine at which concentrations SM become toxic to RH30 and NK cells, the cells were challenged with increasing doses of SM, harvested, and stained with 4',6-diamidino-2-phenylindole (DAPI; BioLegend, USA) after 24 and 48 h. The cell suspensions were measured by flow cytometry using a BD FACSCanto10c™ instrument (BD Bioscience, San Diego, CA, USA) and data were analyzed using FlowJo (FlowJo LLC, Ashland, Oregon, USA), first gating on single cells, then defining the DAPI negative population as viable cells. To analyze the effect of SM on the proliferation of NK cells, the cells were isolated and taken into culture as described above with increasing doses of SM in addition to IL-2. On days 0, 3, 6, and 10, NK cells were harvested and counted using the COULTER® Ac-T diff™ Analyzer (Beckman Coulter, Germany), an automatic cell counter.

Surface Marker Profile Analysis

In search of SM-induced alterations in the expression of surface markers, unstimulated and SM-stimulated RH30, RD, and NK cells were stained with a panel of fluorochrome-conjugated antibodies and analyzed using flow cytometry. NK cells cultured with IL-2 alone, as well as cells that were additionally stimulated with 5 and 10 μ M SM were harvested on day 7 of culture, which complies with the timepoint of harvest for cytotoxicity assays. First, the harvested cells were incubated with 50 μ g/ml human IgG (Kiovig, Baxter, Germany) in order to saturate the Fc receptors of NK cells prior to the staining with fluorochrome-conjugated antibodies. This procedure ensured that the antibodies added afterwards marked their specific antigen, instead of being captured by their Fc fragment through the Fc receptors, which would cause a false-positive rate of the particular antigen. Then, a complex staining pattern was established including following antibodies: CD3/19/14-V450, CD45-BV510 (clone: HI30), CXCR4-PE-Cy7 (clone: 12G5), DNAX accessory molecule (DNAM)-1-FITC (clone: DX11), NKp44-PE (clone: p44-8.1), CD25-PE (clone: 2A3), CD62L-APC (clone: DREG-56), CD69-BV605 (clone:

FN50), and CD107a-APC-H7 (clone: H4A3) from BD Biosciences, San Diego, CA, USA; CD16-PE-Cy7, CD16-PE, and CD253(TRAIL)-PE (clone: RIK-2) from Biolegend, USA; NKp30-AlexaFluor488 (clone: 210845), and CCR7-FITC (clone: 150505) from R&D Systems; KIR2D-FITC (clone: NKVFS1), CD158e/k-PE (clone: 5.133), and NKp46-APC (clone: 9E2) from Miltenyi, Germany; NKG2A-APC (clone: 1D11) from Beckman Coulter, Germany; CX3CR1-PerCPeFluor710 (clone: 2A91) from eBiosciences, USA, and DAPI (Biolegend, USA), serving as a life-dead stain.

The data were analyzed using FlowJo (FlowJo LLC, Ashland, Oregon, USA), analyzing the antigen of interest in the population defined as NK cells (DAPI⁻/CD3⁻/CD19⁻/CD45⁺/CD56⁺).

For the analysis of the surface marker profile of RH30 and RD cells, cells were pretreated according to the conditions used as targets in cytotoxicity assays: 0, 5, or 10 μ M SM for 24 h. Afterwards, these cells were harvested and stained with a staining panel comprising the following antibodies: MICA (clone: AMO1), MICB (clone: BMO2), UL16-binding protein (ULBP)-1 (clone: AUMO3), -2 (clone: BUMO1), -3 (clone: CUMO3), and -4 (clone: 3B6) according to Ref. (35), B7-H6 (kind gift of Prof. Adelheid Cerwenka DKFZ, Germany (36)), followed by the secondary antibody GAM-APC from Jackson Immunoresearch, USA; intercellular adhesion molecule (ICAM)-1-PE (clone: HA58), ICAM-2-FITC (clone: CBR-IC2/2), ICAM-3-APC (clone: CBR-IC3/1), CD262 (TRAIL-R)-PE, and Nectin-2-PE (clone: TX31) from Biolegend, USA; poliovirus receptor (PVR)-FITC (clone: 300907) from R&D Systems, as well as DAPI (Biolegend, USA). The established data were also analyzed using FlowJo (FlowJo LLC, Ashland, Oregon, USA).

Cytotoxicity Assays

All cytotoxicity assays were performed using a flow cytometry-based method and an effector to target (E: T) ratio of 10:1. Herein, the target cells were stained with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, USA) for 5 minutes at room temperature, then washed three times prior to coculture with unstained effector cells. Coculture was completed in a round-bottom 96-well plate for four (pretreated tumor cells) or 16 h (pretreated NK cells). The RMS target cells were pretreated with 5 or 10 μ M SM for 3 or 24 h, harvested, and the cell suspension was adjusted to a concentration of 0.25×10^6 /ml. The NK cells used as effectors were cultured with 100 U/ml IL-2 alone or with 5 or 10 μ M SM in addition to IL-2, harvested on day 6 or 7 of culture and the cell suspension was adjusted to a concentration of 2.5×10^6 /ml. For the analysis of NK cell mediated cytotoxicity, 100 μ l of the target and 100 μ l of the effector cell suspension were pipetted into one well. For each combination, three wells were filled, representing technical replicates. In addition, control wells for all tumor conditions used in the experiment were added, containing target cells only in order to determine the spontaneous lysis. In the final evaluation of the experiment, the specific lysis was calculated as the percentage of dead cells in the wells containing target and effector cells minus the specific lysis of the respective tumor cell condition. Through this calculation of the specific lysis, the percentage of killed tumor cells was attributed completely to the NK cells. In

addition, three wells were added containing tumor cells and ethanol, serving as a positive control for the live-dead stain with DAPI. Once the coculture time was over, 100 μ l supernatant was taken from each well for further analyses (cytometric bead assay) and immediately frozen at -80°C . Then, each well was harvested using 50 μl Trypsin (Gibco Invitrogen, Germany), resuspended in 400 μl of a 1:6,000 DAPI dilution, measured at the flow cytometer, and analyzed with FlowJo.

The cytotoxicity assays as described above were repeated with different additives, with the aim to elucidate mechanisms behind the SM-induced increase in NK cell-mediated killing. The role of TNF α was analyzed through the addition of 0.25 mg/ml Enbrel (Pfizer, Germany); the role of caspases was analyzed through the addition of zVAD.fmk (Sigma, Germany) at a concentration of 40 μM ; and the role of TRAIL was analyzed through blocking TRAIL on NK cells with an antibody from BD Biosciences, USA (cat. 550515) at a concentration of 50 $\mu\text{g}/\text{ml}$ for 2 hours prior to coculture. The efficiency of the block was tested by staining the blocked cells with an antibody of the same clone.

Determination of mRNA Levels of TNF α , TRAIL, RelB, I κ B α , TRAIL-R1 (DR4), and TRAIL-R2 (DR5)

TRAIL mRNA levels were determined by quantitative real-time PCR analysis. Total RNA extraction and cDNA synthesis were performed as previously described (37). TRAIL and TNF α mRNA levels were assessed by Taqman Gene Expression Assay purchased from Life Technologies (TRAIL: Hs00921974_m1; TNF α : Hs01113624_g1) and the levels of RelB, I κ B α , TRAIL-R1 (DR4), TRAIL-R2 (DR5), and 28S rRNA by SYBR[®]Green qPCR assay from Applied Biosystems (Darmstadt, Germany) according to the manufacturer's instructions using the 7900HT fast real-time PCR system from Applied Biosystems (Darmstadt, Germany); RelB forward primer: GCTCTACTTGCTCTGCAGACA; reverse primer: GGCTGGGAGAAGTCAGC; I κ B α forward primer: GTCAAGGAGCTGCAGGAGAT; reverse primer: ATGG CCAAGTGCAGGAAC; DR4 forward primer: GGGTCCACA AGACCTCAAGT; reverse primer: TGCAGCTGAGCTAGGT-ACGA; DR5 forward primer: AGACCCTTGTGCTCGTTGTC; reverse primer: TTGTTGGGTGATCAGAGCAG; 28S rRNA forward primer: TTGAAAATCCGGGGAGAG; reverse primer: ACATTGTTCCAACATGCCAG.

The relative expression of the target gene transcript and reference gene transcript was calculated as $\Delta\Delta\text{C}_t$. 28S rRNA was used as reference gene.

Cytometric Bead Array

In order to screen supernatants of NK and RMS cells in culture as well as after coculture in cytotoxicity assays for changes in secreted cytokines through treatment with SM, supernatants were frozen at -80°C . Cytokine concentrations in these culture supernatants were determined by flow cytometry using the BDTM CBA Flex Set System (BD Bioscience, San Diego, CA, USA). Tests were performed according to the manufacturer's instructions using a mixture of PE-conjugated antibodies against

the cytokines listed above. Data were acquired with the BD FACSVerseTM Bioanalyzer and analysis was carried out by using the FCAP ArrayTM software (v3.0.1).

Statistical Analysis

Prior to analyses, the Shapiro-Wilk normality test was performed in order to prove normal distribution of the analyzed data. The statistical analyses of cytotoxicity assays as well as SM-induced changes in NK cell surface markers and viability experiments were performed using the repeated measures one-way ANOVA with Dunnett's multiple comparison in GraphPad Prism 6 (Inc. LA JOLLA, CALIFORNIA, USA). In case of the blocking experiments, which require the comparison of each group with every other group rather than the comparison to a control column, Turkey's multiple comparisons test was added to the repeated measures one-way ANOVA. Changes in mRNA levels and amounts of secreted cytokines upon stimulation with SM were analyzed through paired *t*-tests. All significant differences with a *p*-value less than 0.1 are marked in the results.

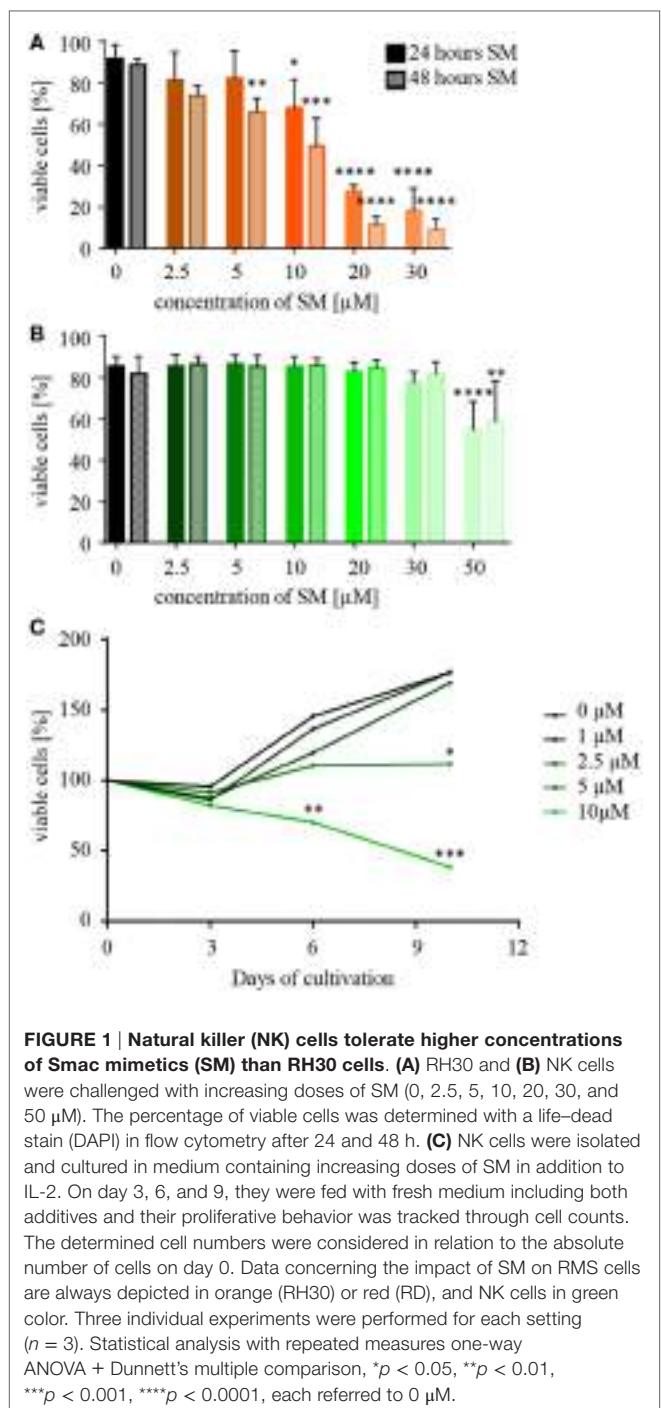
RESULTS

NK Cells Tolerate Higher SM Concentrations than RMS Cells

When testing a substance for its therapeutic potential, such as SM for the sensitization of tumor and the stimulation of NK cells, a first important piece of information is the optimal dose and the concentration of SM that becomes toxic to the respective cells. In order to investigate this, NK and RMS cells were incubated with increasing concentrations of SM and cell viability was analyzed after 24 and 48 h using flow cytometry. RH30 cells already reacted to 10 μM SM with a significantly decreased viability after 24 h (IC₅₀ 24 h: 15 μM), RD cells showed similar results (data not shown). NK cells, however, tolerated doses of up to 30 μM (IC₅₀ 24 h: 68 μM) (Figures 1A,B). Considering the objective of using SM to optimize NK cell therapy, the impact of SM on the proliferative behavior of NK cells is also of interest. Up to a concentration of 2.5 μM , SM did not affect the proliferation of NK cells (Figure 1C). Increasing the dose to 5 μM SM led to a balance between cell death and proliferation, while the presence of 10 μM resulted in a significantly decreased cell number (Figure 1C).

Pretreatment of either RMS or NK Cells with SM Increases NK Cell-Mediated Killing of RMS Cells

Following the hypothesis, that SM can restore the defective apoptotic machinery in tumor cells and therefore sensitize them toward NK cell-mediated killing, cytotoxicity assays were performed using targets that were pretreated with SM prior to coculture. Indeed, pretreating the ARMS cell line RH30 cells for 24 h with subtoxic concentrations of SM that had no or little effects on cell viability significantly increased the percentage of lysed cells from 24 to 54% regarding pretreatment with 10 μM SM (Figure 2A). Also, embryonal RD cells were rendered more susceptible to NK cell-mediated killing by SM as



pretreatment with 10 μM SM increased the specific lysis from 24 to 45% (Figure S1A in Supplementary Data). To ensure that the effectors, IL-2-stimulated NK cells, were responsible for the killing, specific lysis was calculated as the difference between the percentage of all dead cells (absolute lysis) and the percentage of RMS cells that simply died due to pretreatment in absence of effectors during the coculture period (spontaneous lysis). Furthermore, we observed that a 3-h pretreatment is insufficient to sensitize RH30 cells toward NK cell-mediated killing

(Figure S1B in Supplementary Data), similar observations were made regarding RD cells (data not shown).

In a next step, we investigated whether SM alter the cytotoxic potential of NK cells. For this purpose, cytotoxicity assays were performed using NK cells which were stimulated with SM in addition to IL-2 during their expansion period as effectors, and untreated RH30 cells as targets. The presence of SM during the 7 days of culture significantly increased the NK cell-mediated killing of targets compared to the killing through NK cells stimulated with IL-2 alone (Figure 2B). On average, NK cells that were stimulated with 10 μM SM in addition to IL-2 killed 67% of the untreated targets, while IL-2-stimulated NK cells only killed 43% (Figure 2B). This SM-stimulated increase in the cytotoxic potential of NK cells was seen in every tested donor, although the extent of the effect differed. The most impressive increase was observed during the experiment with donor four, where pretreated cells killed more than twice as much tumor cells as the untreated counterpart. We further observed that the increase in the cytotoxic potential is limited to the late phase killing. Evaluating the same cytotoxicity assays after a coculture time of only 4 instead of 16 h did not show comparable results (Figure S1C in Supplementary Data). Interestingly, the positive effect of pretreating NK cells was restricted to the more aggressive histological subtype of RMS tested: the ARMS cell line RH30. Testing the pretreated NK cells against the untreated ERMS cell line RD did not show a comparable result (Figure S1D in Supplementary Data).

TRAIL Signaling Contributes to SM-Induced Sensitization of RMS Cells to NK Cell Killing

Next, we aimed at unveiling the mechanisms that are responsible for the SM-conferred sensitization of RMS cells toward NK cell-mediated killing. Motivated by the hypothesis, that SM induce changes within the surface marker profile of RMS cells, untreated as well as pretreated cells were stained with fluorochrome-conjugated antibodies against a broad range of ligands for NK cell receptors: ULBP-1, -2, -3, -4, MHC class I polypeptide-related sequence (MIC)A, and -B as ligands for natural killer group2, member D (NKG2D); ICAM-1, -2, and -3 as ligands for lymphocyte function-associated antigen-1; Nectin-2 and PVR as ligands for DNAM-1, as well as B7-H6 as a ligand for NKp30. The markers were screened for their surface expression and changes in their mean fluorescence intensity normalized to the untreated control. However, none of the tested surface markers showed a significant change in their expression level upon treatment with SM (Figure S2 in Supplementary Data). The analyses were performed with RH30 (Figure S2A in Supplementary Data) as well as RD cells (Figure S2B in Supplementary Data), leading to similar results.

Since SM have been described to stimulate TNF α production, which contributes to SM-induced cell death in an autocrine/paracrine manner (34), we tested whether the increased susceptibility of RH30 cells is dependent on TNF α by adding Enbrel, the inhibiting soluble TNF receptor (TNFR2) fused with an IgG1 Fc part, to the coculture. However, the addition of Enbrel did not protect RH30 cells from NK cell-induced killing (Figure 3A),

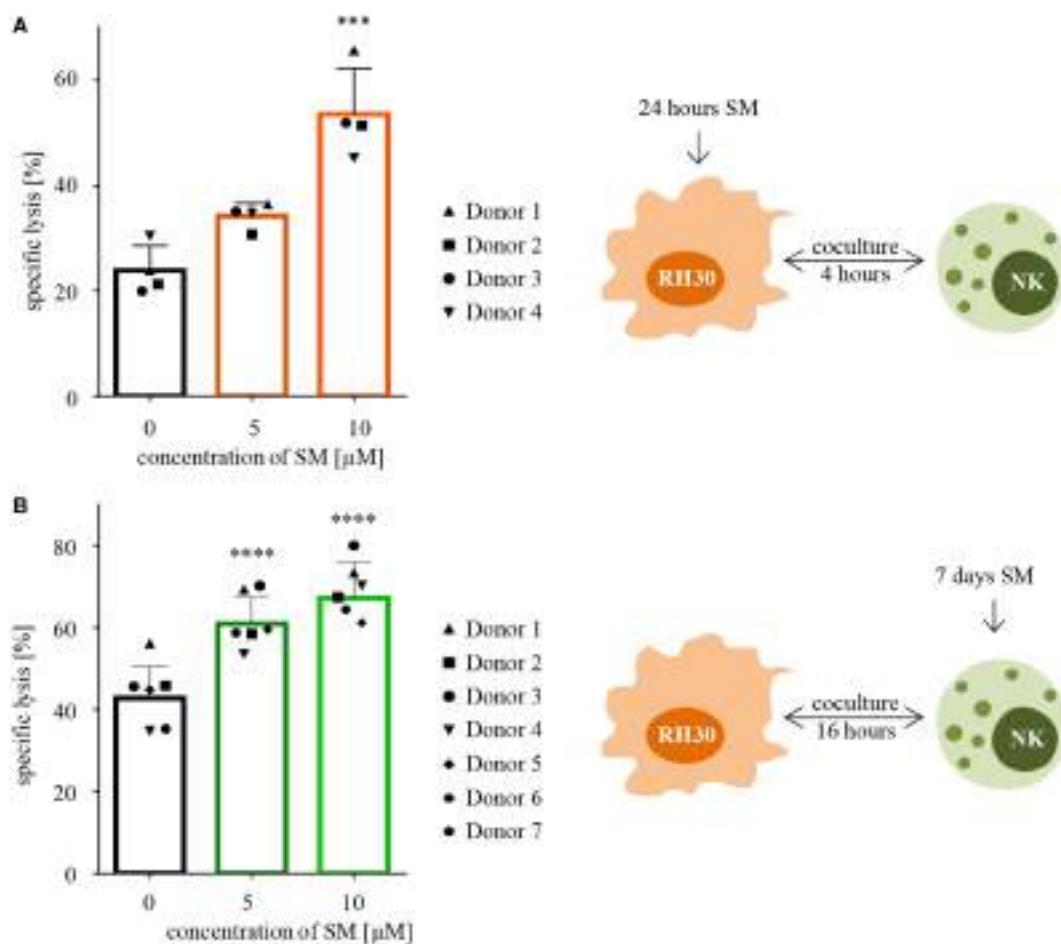


FIGURE 2 | Pretreatment with SM effects both RH30 and NK cells. **(A)** SM sensitize RH30 cells toward NK cell mediated killing. RH30 cells were pretreated with 0, 5, or 10 μM SM for 24 h prior to being used as targets for IL-2-stimulated NK cells on day six of culture (as depicted in the scheme on the right-hand side). The cytotoxicity assay was repeated with NK cells of four different donors ($n = 4$). E:T ratio = 10:1, coculture time 4 h. **(B)** SM increase the cytotoxic potential of NK cells. NK cells were cultured with different doses of SM (0, 5, and 10 μM) in addition to IL-2 for 7 days (as depicted in the scheme on the right-hand side). On day seven, cytotoxicity assays were performed using untreated RH30 cells as targets. The experiment was repeated with NK cells from seven different donors ($n = 7$). E:T ratio = 10:1, coculture time 16 h. Statistical analysis through repeated measures one-way ANOVA + Dunnett's multiple comparison, *** $p < 0.001$, **** $p < 0.0001$, each referred to 0 μM .

while Enbrel prevented SM-induced cell death in MDA-MB231 cells that were used as a positive control, since they have been reported to die in a TNF α -dependent manner upon treatment with SM (38) (Figure S6A in Supplementary Data). Also, the amount of secreted cytokines during the described cytotoxicity assays was not altered by pretreatment of RMS with SM (Figure S3 in Supplementary Data).

Further, we recently identified TRAIL receptor ligand signaling as another critical mediator of SM-induced cell death (38). We therefore asked whether TRAIL is required for the SM-mediated sensitization of RH30 cells to NK cell-mediated killing. To address this question, we used a TRAIL-blocking antibody to neutralize TRAIL on NK cells prior to coculture (Figure S6B in Supplementary Data). The presence of the TRAIL-blocking antibody significantly reduced the SM-conferred sensitization of RH30 cells to NK cell-mediated killing (Figure 3B). While neither surface expression of TRAIL-R1 or TRAIL-R2

on RH30 cells, nor TRAIL expression on NK cells was altered by treatment with SM (Figure S4 in Supplementary Data), SM significantly increased mRNA levels of the NF- κ B target genes TRAIL-R1, TRAIL-R2 and TRAIL in RH30 cells (Figure 4). SM-stimulated upregulation of TRAIL-R1, TRAIL-R2, and TRAIL mRNA expression was confirmed in RD cells (Figure S5 in Supplementary Data).

Furthermore, SM have been reported to promote activation of caspases by neutralizing XIAP proteins (34), wherefore we tested whether caspases are required for the SM-mediated sensitization of RH30 cells. To this extent, we added the pan-caspase inhibitor zVAD.fmk to the medium during cytotoxicity assays after functionality of this caspase-blocking inhibitor on RH30 cells has been proven in standardized cell death assays (Figure S6C in Supplementary Data). Importantly, the presence of zVAD.fmk significantly rescued RH30 cells from NK cell-induced killing (Figure 3C).

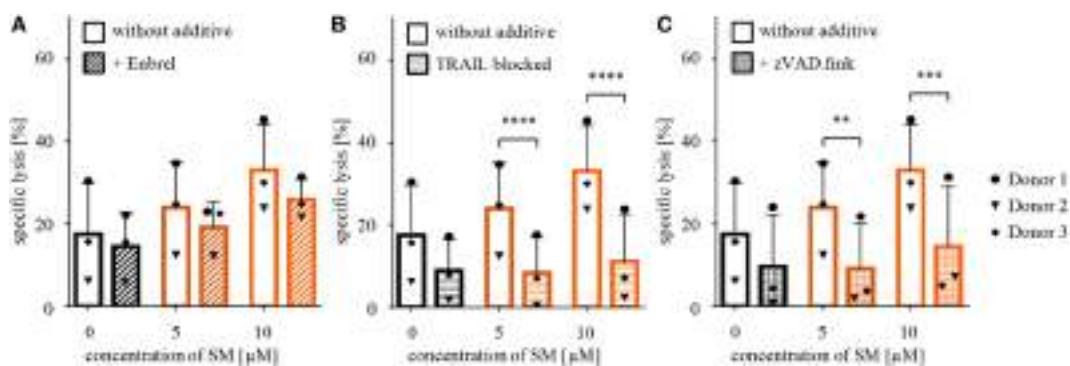


FIGURE 3 | Defining underlying mechanisms behind the SM-induced sensitization of RH30 cells. Cytotoxicity assays with pretreated RH30 cells as targets for IL-2-stimulated natural killer (NK) cells as shown in **Figure 2A** were performed with the additional presence of **(A)** Enbrel, a TNF α blocker (concentration 250 μ g/ml) or **(C)** zVAD.fmk (concentration 40 μ M) during coculture. In panel **(B)**, TRAIL on NK cells was blocked through incubation with an antibody (concentration 50 μ g/ml) prior to coculture. Each experiment was repeated with NK cells from three different donors ($n = 3$), E:T ratio = 10:1, coculture 4 h. Statistical analysis with repeated measures one-way ANOVA with Turkey's multiple comparison, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

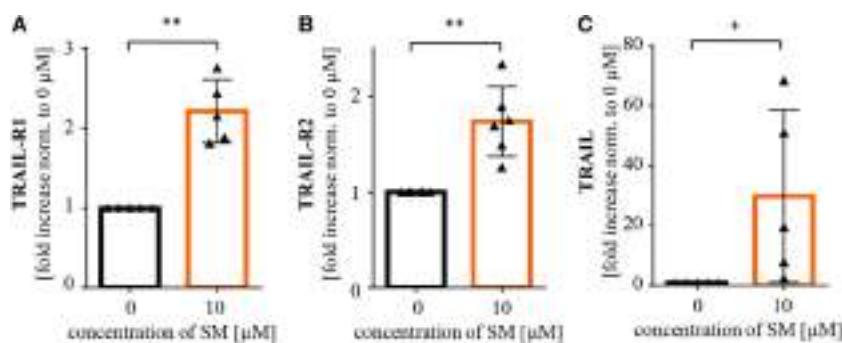


FIGURE 4 | SM induces upregulation of NF- κ B target genes in RH30 cells. Tumor cells pretreated with 10 μ M SM for 24 h as well as untreated cells were analyzed for mRNA levels of the NF- κ B target genes **(A)** TRAIL-R1, **(B)** TRAIL-R2, and **(C)** TRAIL through real time (rt)-PCR. The experiment was repeated with RH30 cells from four to six different cell passages, depicted is the fold increase normalized to 0 μ M. Statistical analysis through paired *t*-test, ** $p < 0.01$, + $p < 0.1$.

TNF α Signaling Contributes to SM-Induced Increase in the Cytotoxic Potential of NK Cells against RH30 Cells

To elucidate which mechanisms are responsible for the SM-induced increase in the cytotoxic potential of NK cells, we first screened a broad range of NK cell surface molecules for changes upon treatment with SM: activating NK cell receptors (NKP30, NKP44, NKP46, NKG2D, DNAM-1, and CD16) (Figure S7A in Supplementary Data), chemokine receptors (CCR7, CX3CR1, and CXCR4) (Figure S7B in Supplementary Data), inhibitory NK cell receptors (NKG2A and KIR2D) (Figure S7C in Supplementary Data), and activation markers (CD25, CD107a, CD69, CD62L) (Figure S7D in Supplementary Data). Generally speaking, we observed a donor-dependent influence of SM on the expression of NK cell surface proteins. Only two of the tested molecules showed a consistent, donor-independent change: CX3CR1, a chemokine receptor, was upregulated, while NKP46 was downregulated upon treatment with SM.

In further search of underlying mechanisms, we investigated the role of TNF α during cytotoxicity assays with SM-pretreated NK cells against RH30 cells by adding Enbrel to the medium during coculture. In fact, the SM-induced increase in NK cell-mediated killing was reduced by the presence of Enbrel (**Figure 5A**). Although this protection by Enbrel was partial, it was consistently found in each of the four tested donors (**Figure 5A**). Correspondingly, we detected a significant increase in secreted TNF α in supernatants of SM-pretreated compared to untreated NK cells at the end of the coculture period of the cytotoxicity assays. Besides TNF α , the levels of IFN- γ were significantly increased in these supernatants (**Figure 5B**), while IFN- α and FasL levels remained unchanged (data not shown). Interestingly, we could only observe this increase in TNF α and IFN- γ levels when the untreated and SM-pretreated NK cells were challenged with their target cells and not upon SM-induction alone (Figure S8 in Supplementary Data).

There are probably further mechanisms contributing to the increased level of activation of NK cells. In line with our findings

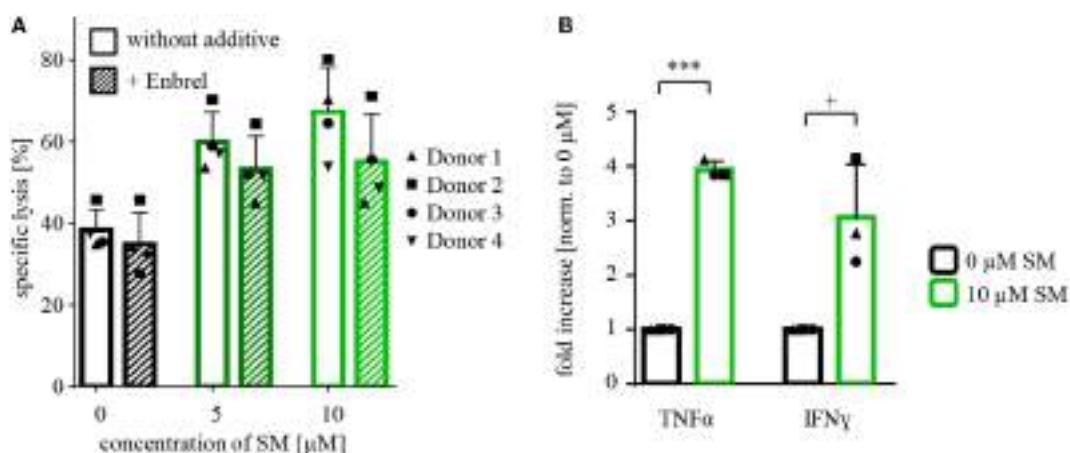


FIGURE 5 | Defining mechanisms behind the SM-mediated stimulation of NK cells. (A) Cytotoxicity assays with pretreated NK cells (SM in addition to IL-2 for 7 days) against untreated RH30 cells (as shown in Figure 2B) were repeated under the presence of Enbrel, a TNF α blocker (concentration 250 $\mu\text{g/ml}$). The experiment was performed with NK cells of four different donors ($n = 4$), E:T ratio = 10:1, coculture time 16 h. Statistical analysis through repeated measures one-way ANOVA were not significant. (B) At the end of coculture of the experiments described under (A), supernatants were taken away and analyzed using cytometric bead array. The graph shows the fold increase in the amount of TNF α and IFN- γ normalized to 0 μM . The experiment was repeated from three different cytotoxicity assays ($n = 3$). Statistical analysis through paired t -test, $^+p < 0.1$, $^{***}p < 0.001$.

in tumor cells, we detected an increase in RelB and IkB α mRNA levels, also indicating an activation of NF- κ B signaling in SM-treated NK cells. Also, the NF- κ B target gene TRAIL was found to be upregulated, while mRNA levels of TNF α were lower than in the untreated control (Figure 6).

DISCUSSION

Since immune effector cells including NK cells mainly function by inducing apoptosis in their targets, the efficacy of immunotherapy critically depends on intact apoptosis signaling pathways within the targeted cancer cells. Here, we report that SM, which antagonize IAP proteins, can prime RMS cells toward NK cell-mediated cytotoxicity (as shown for RD and RH30 cells), and increase the cytotoxic potential of NK cells toward RH30 cells. Of note, the simultaneous targeting of tumor and immune cells with one single drug was restricted to the more aggressive alveolar RH30 cell line.

Importantly, we found that TRAIL signaling contributes to SM-induced sensitization of RMS cells toward NK cell-mediated cytotoxicity, as the addition of a neutralizing TRAIL antibody on NK cells prior to coculture with tumor cells significantly reduced tumor lysis. While we confirmed the overall expression of TRAIL on IL-2-stimulated NK cells, as well as the upregulation of the NF- κ B target gene TRAIL in RMS cells upon treatment with SM, we did not detect any changes in either TRAIL-R1 or TRAIL-R2 expression on RMS cells, nor TRAIL expression on NK cells upon exposure to SM. Overall, our findings are consistent with our previous reports showing that SM or Smac peptides can prime cancer cells toward TRAIL *in vitro* and *in vivo* (39, 40). In addition, we recently identified TRAIL receptor ligand signaling as one critical mediator of SM-induced cell death (38). Also, cooperative TRAIL production has been shown to mediate

SM/IFN α -induced cell death in TNF α -resistant solid cancer cells (41). By comparison, TRAIL signaling turned out to be dispensable for SM/glucocorticoid-induced cell death in leukemia cells (42) or in SM/temozolomide-triggered cell death in glioblastoma cells (43). This indicates that the TRAIL system contributes to SM-induced cell death in a context-dependent manner.

Interestingly, we found a differential role of TNF α in SM-imposed sensitization of RMS cells to NK cell killing, depending on whether RMS or NK cells were pretreated with SM. TNF α contributes, at least to some extent, to the enhanced cytotoxicity when NK cells were pretreated with SM, since the addition of TNF α -blocking Enbrel to the medium during the killing assay significantly, although partially, decreased the NK cell-mediated killing of RH30 cells. In addition, SM-pretreated NK cells produce significantly higher amounts of TNF α and IFN- γ than their untreated counterpart, when cocultured with their tumor target cells. However, beside the possible relevance of TNF α , there are likely additional mechanisms contributing to the SM-induced activation of NK cells, for example, activation of NF- κ B signaling through SM.

On the contrary, TNF α was found to be dispensable for the enhanced cytotoxicity of NK cells when RMS cells were pretreated with SM, since the addition of Enbrel to cytotoxicity assays failed to rescue RMS cells from NK cell-mediated killing. This is underlined by the fact that the supernatants of pretreated RMS cells did not contain more TNF α than the untreated cells. These findings are consistent with previous studies on a context-dependent impact of TNF α as a mediator of SM-induced cytotoxicity. On the one hand, there are several studies showing that an autocrine/paracrine TNF α loop plays a critical role in SM-induced cell death (34, 44–48). On the other hand, blockage of TNF α signaling has also been reported to fail in providing protection against SM in other settings (38, 42, 43). We previously demonstrated that

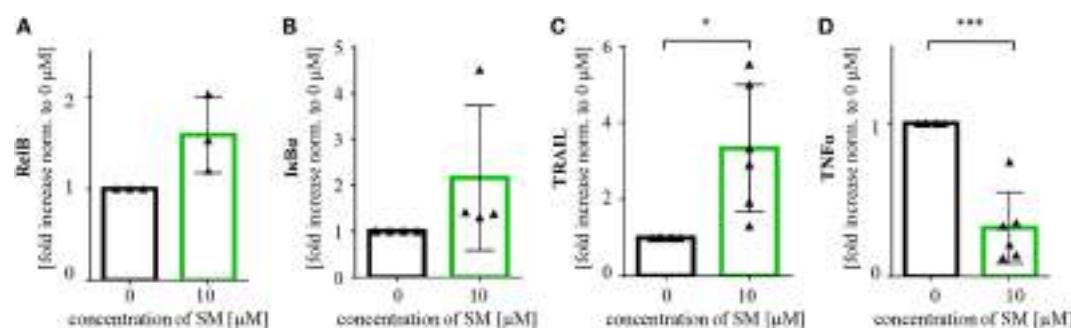


FIGURE 6 | SM induces upregulation of NF- κ B target genes in natural killer (NK) cells. NK cells cultured for 7 days with IL-2 alone and in addition with SM were analyzed for mRNA levels of the NF- κ B target genes **(A)** RelB, **(B)** I κ B α , **(C)** TRAIL, and **(D)** TNF α through rt-PCR. The experiment was repeated with NK cells from three to six different donors, depicted is the fold increase normalized to 0 μ M. Statistical analysis through paired *t*-test, * p < 0.05, *** p < 0.001.

cell type-dependent sensitivity to TNF α can determine whether a cell line depends on TNF α signaling to mediate BV6-induced cell death (41). In TNF α -resistant types of cancer, we showed that TRAIL as another death receptor ligand can mediate SM-induced cell death instead of TNF α (41). Also, differential upregulation of TNF α upon SM might explain TNF α dependency in some but not other instances.

While the monovalent SM LCL161 has previously been reported to upregulate ligands for the activating NK cell receptor NKG2D such as MICA and MICB (49), we did not detect changes in NK cell receptor ligands on RMS cells upon treatment with the SM BV6, which might be due to different tumor types or different SM.

Moreover, caspase-dependent as well as caspase-independent effector pathways may be involved in the SM-conferred increased sensitivity of RH30 cells toward NK cell killing. Our finding that the presence of zVAD.fmk, a pancaspase inhibitor, rescues tumor cells from the increase in killing indicates an apoptosis-dependent cell death in line with previous reports that zVAD.fmk rescues tumor cells from increased cytotoxicity of NK or cytokine-induced killer cells (50, 51).

It is also interesting to note that a 24-h pretreatment with BV6 was necessary to adequately sensitize RMS cells to NK cell cytotoxicity, while a pretreatment of only three hours turned out to be insufficient. By comparison, we previously reported that a 4-h pretreatment with BV6 primed RMS cells for CIK cell-mediated killing (50). One possible explanation for the different requirement of preincubation time is a difference in the cytotoxicity of NK versus CIK cells. Alternatively, these findings may indicate that neither the depletion of cIAPs, nor the direct release of caspases from XIAP proteins, which has been reported to occur within minutes or hours upon exposure to BV6 (34), are responsible for the BV6-conferred sensitization of RMS cells to NK cell-mediated killing. Rather, induction and subsequent expression of proteins or cytokines, for example, as the result of SM-stimulated engagement of alternative NF- κ B signaling upon cIAP1/2 depletion, might be necessary which requires some time. Consistently, we found upregulation of several NF- κ B target genes, including proapoptotic genes such as TRAIL-R1, TRAIL-R2, and TRAIL, in SM-treated RMS cells.

On the NK cell side, the expression of killer immunoglobulin like receptors (KIRs) was slightly decreased as detected by an antibody recognizing the common extracellular Ig-like domains of the KIR2D receptor family—a family whose greater part belongs to the inhibitory receptors. Therefore, the decrease observed might minimize the inhibitory signals within the NK cell and lower the threshold for NK cell activation. This in addition to the increase of CX3CR1 might contribute to the activating effect, even though it is difficult to envisage one of these molecules operating as a key player. In contrast, NKp46, another activating receptor, shows a clear tendency to be downregulated upon exposure to SM, possibly lowering the state of activation in NK cells. All in all, as the increase of the cytotoxic potential through stimulation with SM observed during the cytotoxicity assays was clearly donor-independent, we conclude that the activating effect of SM on NK cell function is not absolutely attributable to changes in the expression of surface molecules.

Consistent with our findings demonstrating that SM at non-toxic concentrations enhance NK cell cytotoxicity, there is increasing evidence showing that SM can potentiate cancer immunotherapy not only by promoting apoptosis of cancer cells but also by modulating immune cell functions without inducing cell death in the majority of immune cells. For example, SM have been shown to augment human and mouse T-cell responses to physiologically relevant stimuli via activation of alternative NF- κ B signaling (52). In addition, SM were described to increase *in vitro* expansion of antigen-specific naive and memory T cells to enhance T-lymphocyte function (53), to trigger phenotypic maturation of monocyte-derived dendritic cells (DCs) (53), and to stimulate maturation of immature DCs (54).

Taken together, SM represent an interesting strategy in optimizing NK cell therapy for the treatment of RMS by sensitizing the tumor cells to NK cell-mediated cell death on the one hand, and by directly activating NK cells on the other (Figure 7).

AUTHOR CONTRIBUTIONS

KF, ST, CB, SR, and RS performed experiments and analyzed data. KF, ST, SR, AS, PB, and TK discussed data. SF and EU designed

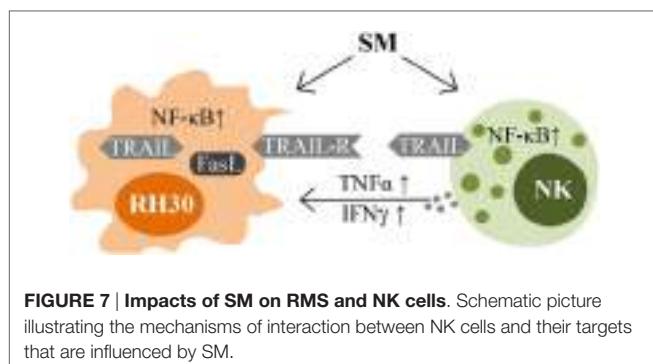


FIGURE 7 | Impacts of SM on RMS and NK cells. Schematic picture illustrating the mechanisms of interaction between NK cells and their targets that are influenced by SM.

the project. KF, SF, and EU wrote the manuscript with support of all other co-authors. All the authors agreed to be accountable for the content of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Data for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00202/full#supplementary-material>.

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Interferon Gamma Induces Changes in Natural Killer (NK) Cell Ligand Expression and Alters NK Cell-Mediated Lysis of Pediatric Cancer Cell Lines

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Natural killer (NK) cells have therapeutic potential for cancer due to their capacity for targeting tumor cells without prior sensitization. Our laboratory has developed an NK cell expansion protocol that generates large quantities of NK cells for therapeutic infusion that secrete 20 times the amount of interferon gamma (IFN γ) than resting NK cells. IFN γ can upregulate major histocompatibility complex (MHC)-class I, an inhibitory ligand for NK cells, but can also upregulate intercellular adhesion molecule 1 (ICAM-1) which promotes NK:target cell interaction for an efficient lysis. Due to the opposing effects reported for IFN γ on tumor sensitivity to NK cells, we evaluated a panel 22 tumor cell lines from the pediatric preclinical testing program corresponding to different tumor types. We determined the impact of IFN γ on their expression of NK cell activating and inhibitory ligands, death receptors, and adhesion molecules using mass cytometry. We also evaluated the effect of IFN γ on their sensitivity to NK cell-mediated lysis. Our results show upregulation of PD-L1, ICAM-1, MHC-class I, HLA-DR, CD95/FasR, and CD270/HVEM after IFN γ treatment, this upregulation is variable across different tumor types. We also observed a variable impact of IFN γ in NK cell-mediated lysis. For six of the cancer cell lines IFN γ resulted in increased resistance to NK cells, while for three of them it resulted in increased sensitivity. Modeling of the data suggests that the effect of IFN γ on NK cell-mediated tumor lysis is mostly dependent on changes in MHC-class I and ICAM-1 expression. For three of the cell lines with increased resistance, we observed higher upregulation of MHC-class I than ICAM-1. For the cell lines with increased sensitivity after IFN γ treatment, we observed upregulation of ICAM-1 exceeding MHC-class I upregulation. ICAM-1 upregulation resulted in increased conjugate formation between the NK cells and tumor cells, which can contribute to the increased sensitivity observed. However, the effects of MHC-class I and ICAM-1 are not readily predictable. Due to the high IFN γ secretion of NK cell infusion products, a better understanding of the NK ligands on tumor cells and how they are affected by IFN γ is essential to optimize NK cell immunotherapy.

Keywords: interferon gamma, natural killer cells, pediatric cancer, mass cytometry, natural killer ligands, immunotherapy, intercellular adhesion molecule 1, major histocompatibility complex

INTRODUCTION

Current chemotherapeutic approaches for pediatric cancer are associated with high morbidity and late effects in survivors. By the age of 50, many survivors experience cardiovascular, renal, and hepatic complications, in addition to the risk of subsequent malignancies (1). Therefore, there is a need to develop new therapies capable of eradicating disease with reduced late effects. Immunotherapy has emerged as a promising new approach for cancer treatment. Natural killer (NK) cells, an important component of our first-line innate immunity, have activity against tumor cells without prior sensitization and are increasingly recognized for their important role in preventing and eradicating cancer. Because NK cells have the ability to selectively target tumor cells without affecting healthy cells, they are an attractive approach for pediatric cancer therapy.

Natural killer cells are typically defined as CD56+/CD3- lymphocytes. Their activity is regulated by a series of inhibitory and activating receptors that recognize ligands on target cells. The balance between activating and inhibitory signals will determine whether an NK cell is activated. The three main receptor families present on NK cells include natural cytotoxicity receptors (NCRs), C-type lectin (CD94/NKG2), and killer cell immunoglobulin-like receptors (KIRs) (2). NCRs (NKp30, NKp44, NKp46) are activating receptors, as are CD244/2B4, CD226/DNAM-1, and CD314/NKG2D. KIRs and C-type lectin receptors may be activating or inhibitory. Among the most important NK cell, inhibitory receptors are the KIRs and NKG2A because they recognize major histocompatibility complex (MHC) on target cells as evidence of self, leading to inhibition of NK cell activity (3). Many tumor cells downregulate MHC to escape T cell immunity, and upregulate activating ligands, making them susceptible targets for NK cell attack (4–7). However, in tumor cells where both inhibitory and activating ligands are present, the balance of these signals determines whether the NK cell is activated.

We developed an expansion protocol that allows production of large quantities of NK cells for adoptive immunotherapy (8). We have observed that, as compared to primary NK cells or IL-15-expanded NK cells, IL-21-expanded NK cells secrete 20-fold or 100-fold more interferon gamma (IFN γ , median 2,493 vs. 24 or 111 pg/mL, respectively), in response to target recognition (8). IFN γ has been reported to upregulate the inhibitory MHC-class I in target cells, making them more resistant to NK cell-mediated lysis (9, 10). However, IFN γ has also been reported to promote NK:target cell interaction through upregulation of intercellular adhesion molecule 1 (ICAM-1), promoting increased target cell death (11, 12). These findings suggest that IFN γ can have opposing effects on tumor cell sensitivity to NK cell-mediated lysis. To optimize the use of expanded NK cells as an immunotherapy it is imperative that we better understand how IFN γ affects NK cell-mediated lysis of the tumor cells.

Opposing effects reported for IFN γ may be due to a focus on specific tumor types, therefore this study aimed to evaluate the effect of IFN γ on a broad selection of 22 tumor cell lines from the pediatric preclinical testing program (PPTP) *in vitro* panel. This panel was designed to evaluate new therapies against childhood

leukemias and solid tumors and has already been used for *in vitro* testing of over 50 pediatric cancer therapies (13). Using these cancer cells corresponding to six different types of pediatric malignancies, we evaluated the effects of IFN γ treatment in tumor cell sensitivity to NK cell-mediated lysis. Also we evaluated the effects of IFN γ treatment on tumor expression of NK cell ligands, including activating and inhibitory ligands, death receptors, and adhesion molecules.

MATERIALS AND METHODS

Isolation and Expansion of Human NK Cells

Buffy coats from four anonymized donors were obtained from Gulf Coast Regional Blood Center (Houston, TX, USA). Exemption and waiver of consent for the research use of buffy coat fractions obtained from anonymized donors at Gulf Coast Regional Blood Center (Houston, TX, USA) was granted by the Institutional Review Board of the University of Texas MD Anderson Cancer Center under protocol PA13-0978. NK cells were isolated using the RosetteSep Human NK cell enrichment cocktail (Stem Cell Technologies) and expanded as described previously using K562 Clone9.mbIL21 as feeder cells for 21 days (8). Expanded NK cells were cryopreserved, and subsequently thawed and recovered for 1–2 days prior to their use. During recovery NK cells were cultured in NK cell media consisting of RPMI 1640 (Corning) supplemented with 50 IU/mL recombinant human IL-2 (Proleukin, Novartis Vaccines and Diagnostics, Inc.), 20% Fetal Bovine Serum (Thermofisher), L-glutamine (Gibco), and penicillin/streptomycin (Corning).

Tumor Cells

TC-71, NALM-6, and Ramos-RA1 were obtained as kind gifts from colleagues (Drs. Eugenie S. Kleinerman, L. J. N. Cooper, and J. Chandra, respectively). Karpas-299 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). RS4;11, MOLT-4, and CCRF-CEM were obtained from the America Type Culture Collection (ATCC). The remaining cell lines were obtained from the Children's Oncology Group (COG) Cell Line and Xenograft Repository. Brain tumor cell lines BT-12, SJ-GBM2, CHLA-266, Ewing sarcoma (EWS) cell lines CHLA-9, CHLA-10, CHLA-258, TC-71, neuroblastoma (NB) cell lines NB1643, NB-EBC1, CHLA-90, CHLA-136, rhabdomyosarcoma (RMS) cell line RD, and leukemia cell line COG-LL-317 were cultured in IMDM (Lonza) supplemented with 20% FBS (Thermofisher), 4 mM L-glutamine (Gibco), 1× ITS (Lonza), and penicillin/streptomycin (Corning). Lymphoma cell lines Karpas-299, Ramos-RA1, leukemia cell lines NALM-6, RS4;11, MOLT-4, CCRF-CEM, Kasumi-1, and RMS cell lines Rh41, Rh30, were cultured in RPMI 1640 (Corning) supplemented with 10% Fetal Bovine Serum (Thermofisher), L-glutamine (Gibco), and penicillin/streptomycin (Corning). Cultures were periodically tested to confirm absence of mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza). Identity was confirmed by STR DNA fingerprinting either using the AmpFLSTR Identifiler

kit (Applied Biosystems) or the Power Plex 16HS Kit (Promega) according to manufacturer instructions. The STR profiles were compared to known fingerprints as published by ATCC or the COG cell STR Genotype Database (<http://strdb.cogcell.org>). STR profiles were last performed on March 2016 (SJ-GBM2, NB1643, MOLT-4), October 2015 (RD, Rh41, Rh30, BT-12, CHLA-10, NB-EBc1, NALM-6, and Ramos-RA1), or September 2012 (CHLA-266, CHLA-9, CHLA-258, TC-71, CHLA-90, CHLA-136, RS4;11, COG-LL-317, CCRF-CEM, Kasumi-1, and Karpas-299). Banks of STR validated, mycoplasma-free cell lines were cryopreserved. Cell lines were kept in culture no longer than eight passages or 4 weeks prior to use.

IFN γ Treatment of Tumor Cells

Cell lines that grow in suspension were seeded at 0.5×10^6 cells/mL and treated with 50 ng/mL of IFN γ (Peprotech) for 48 h. Adherent cells were cultured to a 60–70% confluence and treated with 50 ng/mL of IFN γ (Peprotech) for 48 h. Untreated tumor cells were seeded in parallel. After treatment cells were washed in IFN γ free media, and adherent cells were detached with non-enzymatic cell dissociation buffer (Gibco) to avoid degradation of cell surface proteins. Treated and untreated cells were evaluated for surface expression of NK cell ligands by mass cytometry and sensitivity to NK cell-mediated lysis by calcein release assay.

Cytotoxicity

The fluorescence based calcein release assay was used to assess cytotoxicity, as previously described (8, 14). Adherent cells were detached with non-enzymatic cell dissociation buffer (Gibco) and cells were filtered by using a 70 μ m cell strainer (Corning) to obtain a single-cell suspension. Target cells were labeled with 5 μ g/mL of calcein-AM (Sigma-Aldrich) for 1 h at 37°C. NK cells were cocultured with target cells at different effector to target (E:T) ratios (10:1, 5:1, 2.5:1, 1.25:1, 0.6:1, and 0.3:1) for 4 h at 37°C. Supernatant fluorescence was determined at 485 nm^{Exc}/530 nm^{Emm} using the SpectraMax Plus³⁸⁴ spectrophotometer.

Mass Cytometry

Antibodies for mass cytometry were labeled with heavy metals using Maxpar-X8 labeling reagent kits (DVS Sciences) according to manufacturer's instructions and titrated for determination of optimal concentration. The antibodies and their respective heavy metal labeling can be found in Table S1 in Supplementary Material. Since NK cell receptors may have multiple ligands (e.g., NKG2D binds to MICA, MICB, and ULBP1-5), or unknown ligands, chimeric receptor:IgG-Fc fusion proteins were tagged with heavy metals and used for identification of ligands on tumor cells. Then, 1.5×10^6 cells were stained for viability with 2.5 μ M cell ID cisplatin (Fluidigm, 201064) in serum free RPMI for 1 min and washed twice with complete media. Subsequently, surface staining was performed as previously described (15). Staining media were prepared by adding 5% FBS and 0.1% sodium-azide to PBS. During the intracellular staining step of tumor cells, two

different isotopes of cisplatin Pt-194 (Fluidigm, 201194) and Pt-198 (Fluidigm, 201198) were used to barcode untreated and IFN γ -treated samples, respectively, allowing samples to be combined in a single tube, minimizing acquisition time, and variability between runs. Data were acquired on a CyTOF instrument (DVS Sciences). Files containing only live single cells were exported using FlowJo V10 Software and uploaded into Cytobank for further analysis (Figure S1 in Supplementary Material) (16).

Conjugation Assay

The determination of effector conjugation to target cells was performed as described by Burshtyn et al., with some minor modifications (17). Briefly, NK cells and tumor cells were stained with green dye PKH67-GL (Sigma, MINI67) and red dye PKH26-GL (Sigma, MINI26), respectively, in 5 μ M dye at 5×10^6 cells/mL for 5 min at room temperature. Dye staining was stopped by adding two volumes of FBS and two volumes of complete media. Cells were washed twice with complete media and let rest for at least 1 h at 37°C. Then, 10^5 NK cells were combined with 2×10^5 tumor cells in 200 μ L, centrifuged at 20 g for 1 min (to initiate contact), and incubated at 37°C for 30 min. Cells were resuspended by gentle vortexing, fixed with 200 μ L of 4% formaldehyde, and analyzed by flow cytometry. For antibody blocking experiments, NK cells were pre-incubated with 5 μ L of Fc blocker (Biolegend, 422302) for 10 min to avoid antibody-dependent cell cytotoxicity. ICAM-1 was blocked on tumor cells by adding 10 μ g/mL of anti-CD54/ICAM-1 clone HCD54 (Biolegend, 322703) for 20 min at room temperature.

Statistical Analysis

Statistics were performed in GraphPad Prism Software. For determination of Δ in % Lysis after IFN γ treatment (Figure 1), we used data from six different E:T ratios. For each cell line, we calculated the average difference in lysis after IFN γ treatment (%lysis IFN γ treated – %lysis untreated) using four NK cell donors. Significance was determined by using the *t*-test with a hypothetical value of 0 for comparison. Heatmaps were generated using Cytobank (16). Conjugation data significance was determined by using the *t*-test, statistical significance was determined by a $p < 0.05$.

RESULTS

IFN γ Has a Variable Impact on Tumor Cell Sensitivity to NK Cell-Mediated Lysis

We determined whether tumor IFN γ treatment affected NK cell-mediated lysis for 22 pediatric cancer cell lines from the PPTP *in vitro* panel. Tumor cell lines evaluated were derived from brain tumors, EWS, NB, leukemia, lymphoma, and RMS. The Rh18 cell line was excluded because of repeated problems maintaining the cell line in culture. We observed that 6 of the 22 cell lines evaluated show a significant decrease in NK cell-mediated lysis after IFN γ treatment (Figure 1). The group of cell lines with decreased lysis after IFN γ treatment includes leukemia cell lines Molt-4 ($p = 0.0030$) and Kasumi-1 ($p = 0.0231$), EWS cell lines CHLA-9

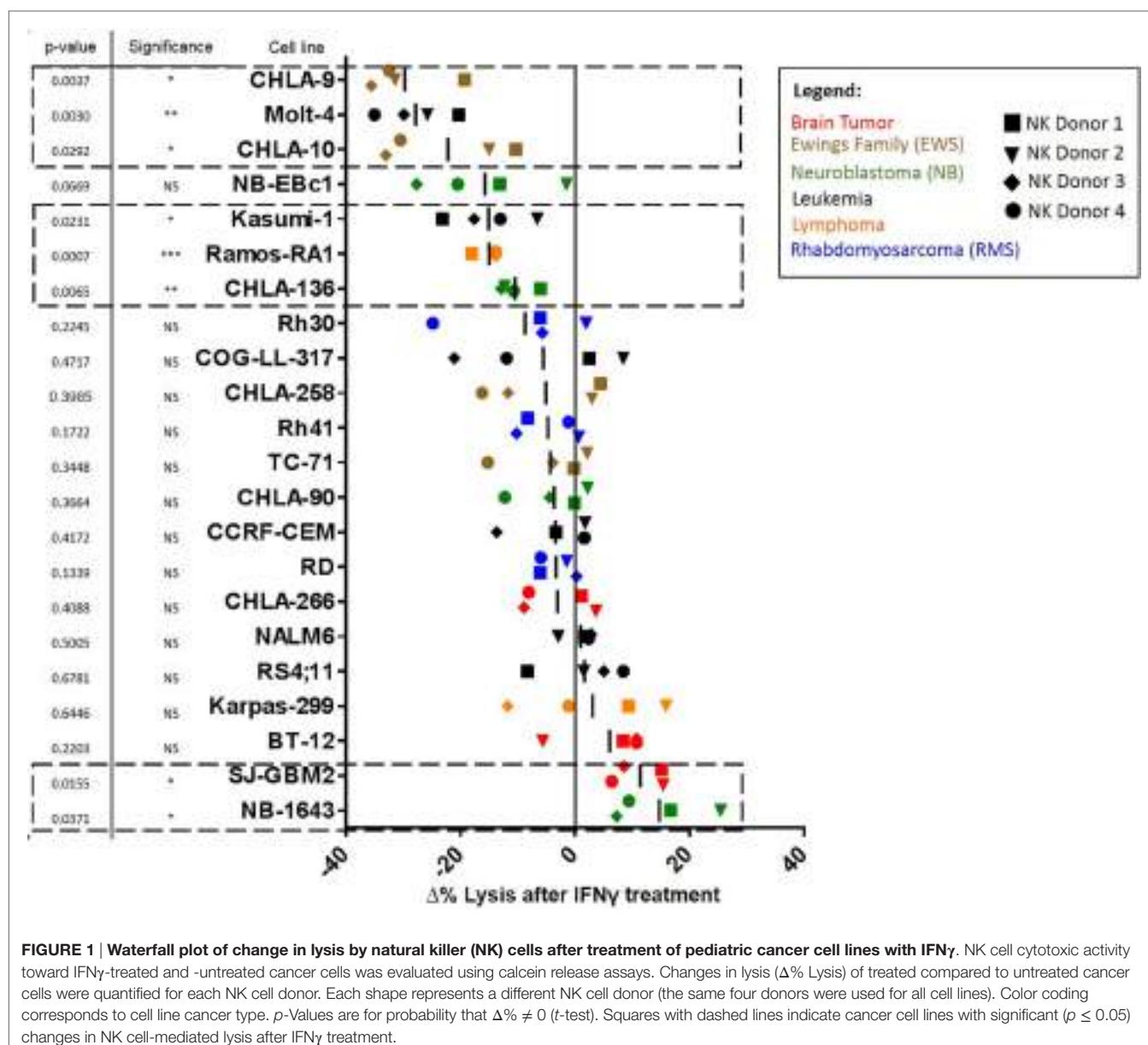
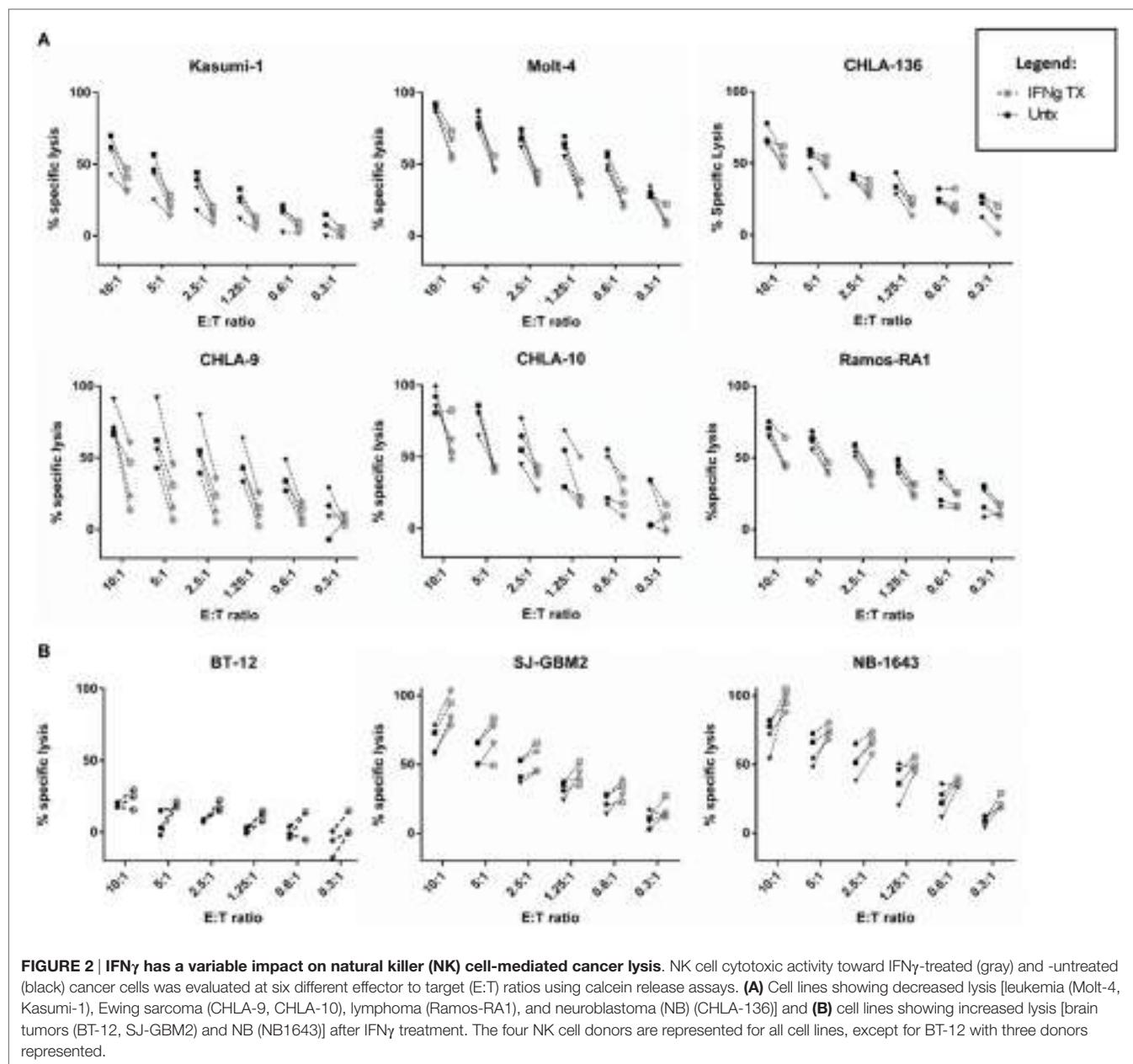


FIGURE 1 | Waterfall plot of change in lysis by natural killer (NK) cells after treatment of pediatric cancer cell lines with IFN γ . NK cell cytotoxic activity toward IFN γ -treated and -untreated cancer cells was evaluated using calcein release assays. Changes in lysis ($\Delta\%$ Lysis) of treated compared to untreated cancer cells were quantified for each NK cell donor. Each shape represents a different NK cell donor (the same four donors were used for all cell lines). Color coding corresponds to cell line cancer type. p -Values are for probability that $\Delta\% \neq 0$ (t -test). Squares with dashed lines indicate cancer cell lines with significant ($p \leq 0.05$) changes in NK cell-mediated lysis after IFN γ treatment.

($p = 0.0037$) and CHLA-10 ($p = 0.0292$), lymphoma cell line Ramos-RA1 ($p = 0.0007$), and NB cell line CHLA-136 ($p = 0.0065$). The NB cell line NB-EBC1 appears to have a decreased lysis after treatment, however, it was not statistically significant. When decreased lysis after treatment was observed, it was consistent for all four donors tested and across E:T ratios (Figure 2A).

By contrast, two of the cell lines showed an increase in NK cell-mediated lysis after IFN γ treatment. These cell lines include the glioblastoma cell line SJ-GBM2 ($p = 0.0155$) and the NB cell line NB1643 ($p = 0.0371$) (Figure 1). In addition, although the brain tumor cell line BT-12 shows a non-significant increase in sensitivity, we observed that for three of the four NK cell donors there was an increased sensitivity after IFN γ treatment, this increase is significant if these three donors are

evaluated ($p = 0.0061$). Increased lysis after IFN γ treatment was a consistent finding with most donors mostly at high NK cell doses (Figure 2B). The remaining cell lines evaluated showed no differences in NK cell-mediated lysis after IFN γ treatment, and this group includes cell lines corresponding to all the tumor types evaluated (Figure 1). After stratifying our data by tumor type, we can observe that the only tumor type for which IFN γ had no effect on lysis was RMS. In EWS, leukemia, and lymphoma IFN γ had no effect or resulted in decreased lysis, whereas IFN γ had no effect or resulted in increased lysis for brain tumors. Interestingly, for NB cell lines, the effect of IFN γ on NK cell-mediated lysis was variable. Lysis of some NB cell lines was unaffected by IFN γ treatment, some became more resistant, and some more sensitive.

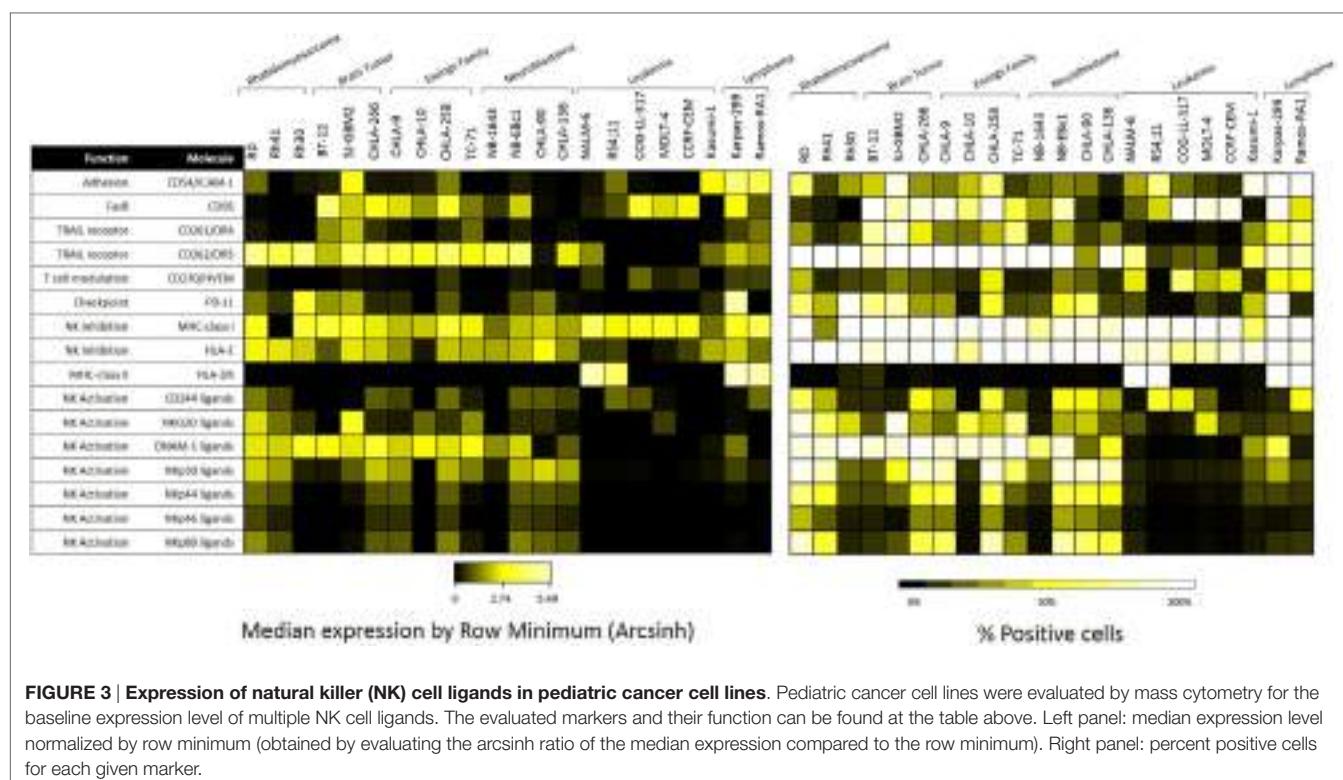


IFN γ Alters Surface Expression of NK Cell Ligands in Pediatric Cancer Cell Lines

The 22 pediatric cancer cell lines obtained from the PPTP *in vitro* panel were evaluated by mass cytometry for the expression of 16 NK cell ligands. Baseline expression of NK cell ligands in terms of median expression and percentage positive cells is provided in Figure 3. The inhibitory ligands MHC-class I and HLA-E are homogeneously expressed (>70%) for all PPTP cell lines with the exception of the RMS cell line Rh41 where MHC-class I was expressed in only 35% of the cells (Figure 3, right panel). Interestingly, we observe that solid tumor cell lines (RMS, brain tumor, EWS, and NB) have higher median expression levels of TRAIL receptor CD262/DR5, and ligands for the activating

receptors NKG2D, DNAM-1, and NCRs, when compared to leukemia cell lines (Figure 3, left panel).

Baseline expression levels of NK cell ligands were compared to the levels after IFN γ treatment and changes were quantified. The percentage change in mean mass intensity (MMI) after IFN γ treatment was determined for each of the 22 cell lines (Figure 4A). CD274/PD-L1, CD54/ICAM-1, HLA-DR, MHC-class I, CD95/FasR, and CD270/HVEM were the most affected by IFN γ (Figure 4A), with at least three cell lines showing fivefold increase or more in MMI. Data were also evaluated in terms of median expression. IFN γ induced changes in median expression were quantified, and a heatmap corresponding to the fold increase in median expression (obtained from the arcsinh ratio



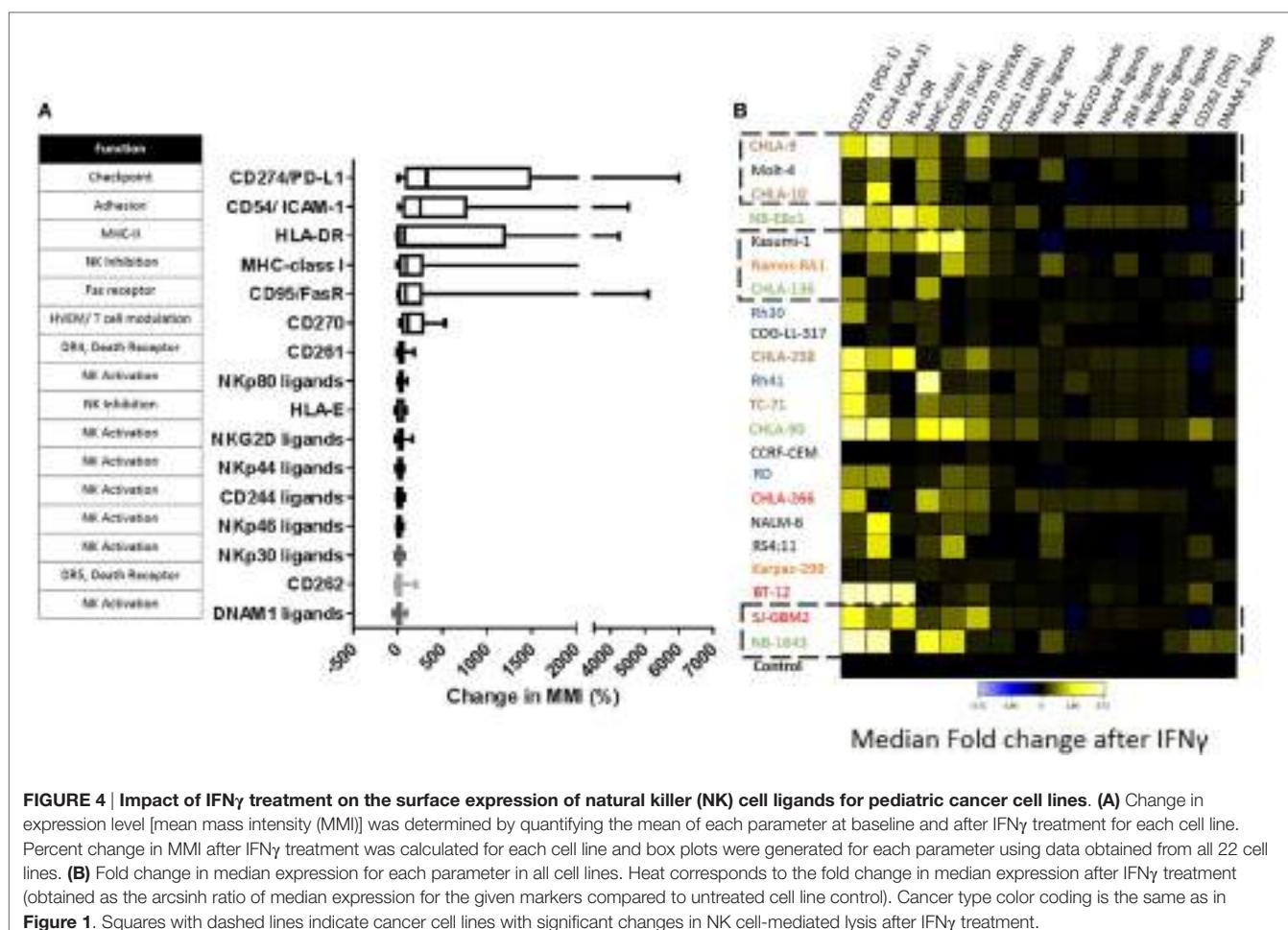
of median) was generated (**Figure 4B**). The changes in median expression were similar to changes in mean expression. CD274/PD-L1, CD54/ICAM-1, HLA-DR, MHC-class I, CD95/Fas, and CD270/HVEM were the markers mostly upregulated by IFN γ (**Figure 4B**). When stratified by tumor type (**Figure 5**; Figure S2 in Supplementary Material), we observe that PD-L1 was most upregulated by IFN γ in solid tumor cell lines (RMS, brain, EWS, and NB), but was relatively unaffected on leukemia and lymphoma cell lines. ICAM-1 upregulation was higher for brain tumors, EWS, NB and leukemia, and lower for RMS and lymphomas. HLA-DR was upregulated by IFN γ on some brain tumor, EWS, and NB cell lines. MHC-class I upregulation was variable even within the same tumor type. However, IFN γ -induced MHC-class I upregulation was consistently observed in all NB cell lines. Finally, CD270/HVEM upregulation was induced by IFN γ in some brain tumor, EWS, and NB cell lines. Our data show that lymphoma cell lines were the least affected by IFN γ in terms of NK cell ligand expression (**Figure 5**; Figure S2 in Supplementary Material).

IFN γ -Induced Upregulation of MHC-Class I and ICAM-1 Correlates with Changes NK Cell-Mediated Lysis

Next, we wanted to determine whether there was a correlation between changes in ligand expression after IFN γ treatment and changes in NK cell-mediated tumor lysis. We first assessed our expanded NK cells for expression of the receptors corresponding to the most upregulated ligands—PD-L1, ICAM-1, and MHC-class I. Expression of PD-1, the receptor for PD-L1, was observed

on only 7% of the expanded NK cells (**Figure 6**). This low percentage of PD-1+ NK cells in our expanded product suggests that PD-L1 upregulation is unlikely to play a role in the IFN γ -induced changes in lysis we observed.

By contrast, LFA-1, the integrin that binds ICAM-1, was expressed in 99.6% of our expanded NK cells (**Figure 6**), and inhibitory KIR receptors were also highly expressed in our expanded NK cells. KIR2DL2/3 expression was 83.9%, KIR2DL1/2DS5 was 97.18%, and KIR3DL1 was 89.15% (**Figure 6**). Also we observed expression of the inhibitory receptor NKG2A in 97% of expanded NK cells. Knowing that the majority of our expanded NK cells express LFA-1, KIR, and NKG2A receptors, we focused on changes in ICAM-1 and MHC-class I expression (**Figure 7**). The ratio of change in MHC-class I over change in ICAM-1 after IFN γ treatment was evaluated and plotted for cell lines with altered sensitivity (**Figure 7C**). We observed that all the cell lines that became more resistant after IFN γ treatment had an increase in MHC-class I expression (**Figures 2A** and **7A**). However, MHC-class I upregulation was also observed in some of the cell lines with increased sensitivity after IFN γ treatment (**Figures 2B** and **7B**). Interestingly, MHC-class I/ICAM-1 change ratio was <1 and, indicating that upregulation of ICAM-1 exceeded MHC-class I upregulation, for all the cell lines with increased sensitivity after IFN γ treatment (**Figures 7B,C**). The opposite pattern, with an MHC-class I/ICAM-1 change ratio >1 and MHC-class I upregulation exceeding ICAM-1 upregulation, was observed in three of the six cell lines where IFN γ induced resistance (Kasumi-1, MOLT-4, and CHLA-136) (**Figures 7A,C**). However, ICAM-1 upregulation exceeded MHC-class I upregulation (ratio <1) in



the EWS cell lines CHLA-9 and CHLA-10 and the lymphoma cell line Ramos-RA1, for which IFN γ treatment resulted in decreased NK cell-mediated lysis.

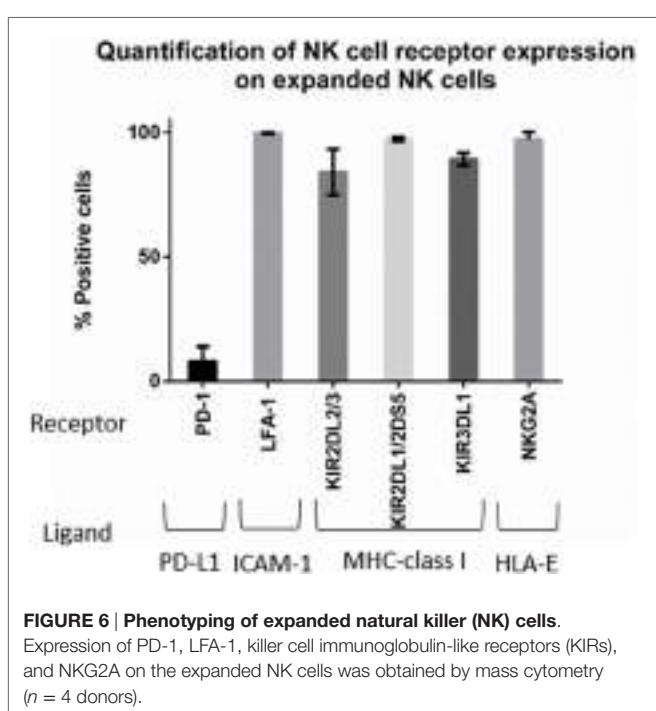
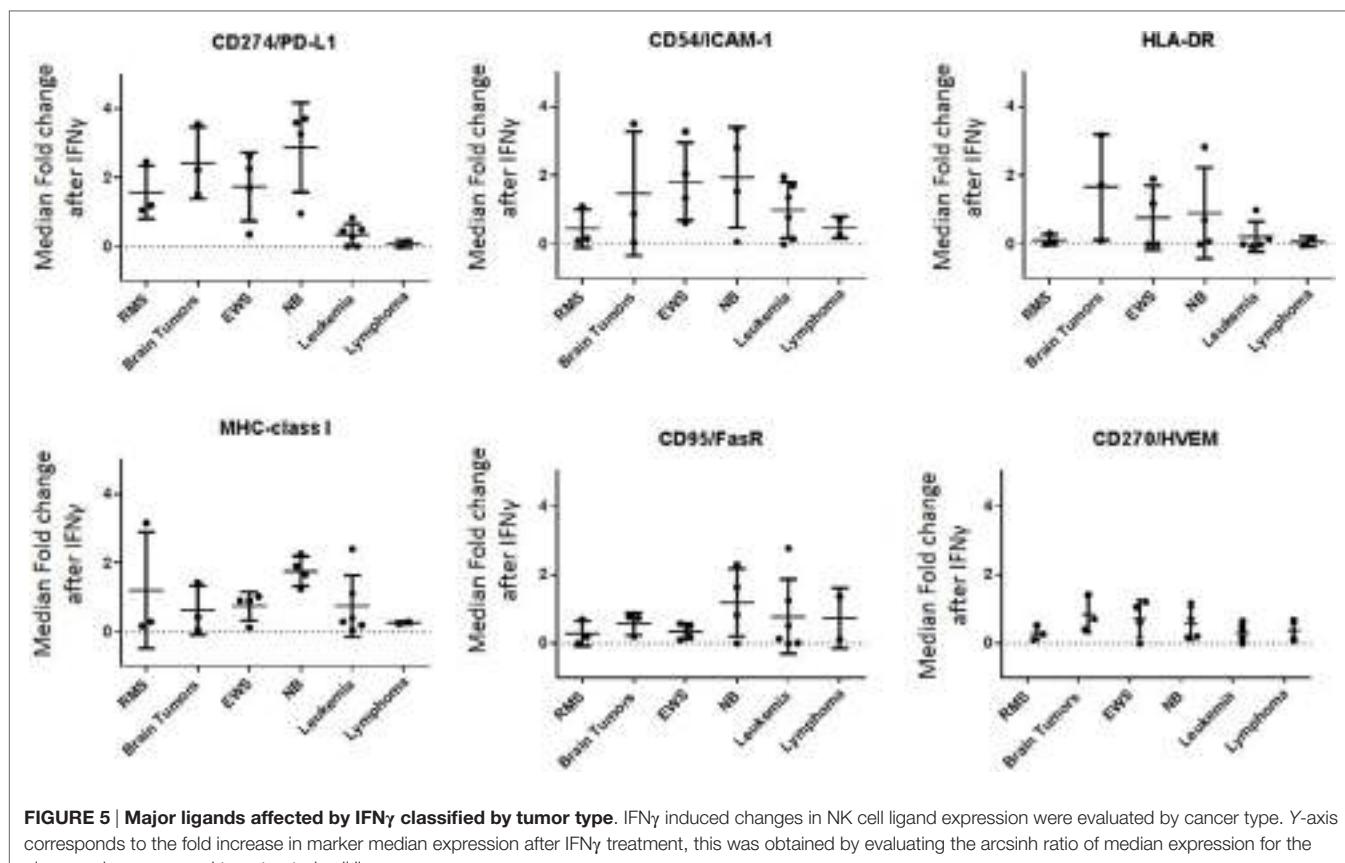
IFN γ -Induced ICAM-1 Upregulation Increases Conjugate Formation for Cell Lines with Increased Sensitivity

Since IFN γ is known to enhance MHC-class I expression, resulting in resistance of target cells to NK cell-mediated lysis (9, 10), we investigated possible mechanisms for the enhanced sensitivity observed after treatment for the cell lines SJ-GBM2, NB1643, and BT-12. These showed increased sensitivity to NK cell-mediated lysis after IFN γ treatment despite presence of high levels of MHC-class I. Since upregulation of the adhesion molecule ICAM-1 exceeded MHC-class I upregulation for these tumors, we determined whether IFN γ -mediated ICAM-1 upregulation was capable of overcoming MHC-class I inhibition through increased conjugate formation between the NK cells and the target cells (Figure 8A). For comparison, we also evaluated IFN γ -mediated changes in conjugate formation between NK cells and Ramos-RA1, a cell line having high ICAM-1 expression but for which IFN γ resulted in more resistance. We observed that treatment of BT-12, SJ-GBM2, and NB1643 with IFN γ resulted in

increased conjugate formation (Figure 8B) and this was statistically significant for BT-12 ($p = 0.026$) and SJ-GBM2 ($p = 0.011$). By contrast, IFN γ treatment did not increase conjugate formation for Ramos-RA1. Next, to confirm whether the increased conjugate formation was specifically mediated by ICAM-1 upregulation, we used monoclonal antibodies to block ICAM-1 on IFN γ -treated cells (BT-12, SJ-GBM2, and NB1643). Blocking of ICAM-1 resulted in a significant decrease in conjugate formation for BT-12 ($p = 0.042$), SJ-GBM2 ($p = 0.008$), and NB1643 ($p = 0.041$) (compared to isotype control, Figure 8C).

DISCUSSION

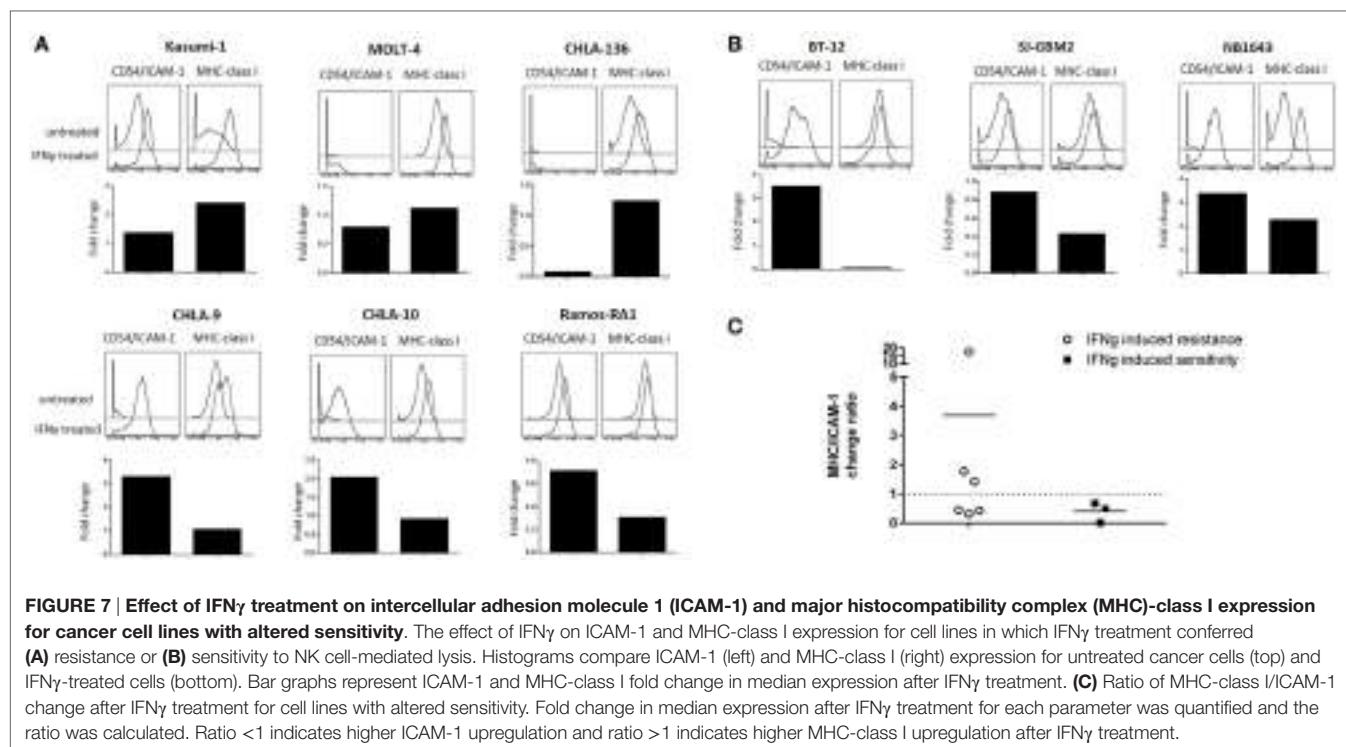
Natural killer cells are an attractive approach for cancer immunotherapy. Our laboratory has developed an *ex vivo* NK cell expansion platform that allows us to generate large quantities of NK cells for patient infusion. We have shown that our expanded NK cells, currently used in several clinical trials for myeloid malignancies and posterior fossa tumors (NCT01787474, NCT01904136, NCT01823198, NCT02271711), secrete large amounts of IFN γ compared to primary NK cells (8). Similarly, memory-like NK cells used for adoptive transfer to AML patients exhibited enhanced IFN γ production when compared to control NK cells



(18). Due to the high levels of IFN γ secreted by NK cell infusion products, we sought to determine the effects of IFN γ on NK cell interactions with the tumor cells.

Through this study we evaluated a broad selection of pediatric tumor cells, representing at least six different types of malignancies, for the effect of IFN γ on their sensitivity to NK cell-mediated lysis using expanded NK cells from four independent donors. This enabled a broad study across tumor types demonstrating opposing effects of IFN γ , a finding that may not have been evident in other studies that focus on a single tumor type or modulation of a single surface ligand. Of the 22 cell lines evaluated, six showed a significant decrease in NK cell-mediated lysis after IFN γ treatment, including leukemia, EWS, lymphoma, and NB cells. By contrast, treatment with IFN γ resulted in enhanced sensitivity to NK cells for three cell lines, BT-12, SJ-GBM2, and NB1643 (two brain tumors and a NB, respectively). For the remaining cell lines, IFN γ treatment did not significantly affect NK cell-mediated lysis, though some showed trends that may be significant with more donor replicates. The effect of IFN γ treatment was variable within the same tumor type, with the exception of RMS cell lines for which IFN γ treatment had no effect. These results suggest that the effect of IFN γ on NK cell-mediated lysis of tumor cells is variable and cell line dependent. Our findings warrant more focused investigation and validation for specific tumor types using primary tumor samples or patient-derived xenografts where feasible.

To better understand this variability, we used mass cytometry to evaluate the effect of IFN γ on expression of NK cell ligands by cancer cells. First, we observed broad heterogeneity in baseline expression levels of ligands between and within tumor types (Figure 3B). Solid tumor cell lines had higher median expression of TRAIL receptor



CD262/DR5 and ligands for the activating receptors NKG2D, DNAM-1, and NCRs, compared to leukemia cell lines (Figure 3A). This suggests NK cells may be a promising therapy for solid tumors. We also observed that none of these ligands were downregulated by IFN γ ; however, CD274/PD-L1, CD54/ICAM-1, HLA-DR, MHC-class I, CD95/FasR, and CD270/HVEM were upregulated in a variety of tumor types. Other than CD270/HVEM, these findings are consistent with previously published studies (11, 12, 19–24). HVEM is involved in T cell regulation (25), but no studies have yet reported regulation of this ligand by IFN γ and its role in NK cell biology has not been well described.

Although IFN γ -mediated upregulation of MHC-class I, PD-L1, and ICAM-1 has been previously described, this study uncovers the variability of IFN γ responses across different pediatric tumor types. In terms of PD-L1, our results show upregulation by IFN γ for most pediatric solid cancers (RMS, brain tumors, EWS, and NB), but no effect on pediatric leukemia and lymphoma cells. On the other hand, the adhesion molecule ICAM-1 was upregulated by IFN γ on brain tumors, EWS, NB, and leukemia, but not on RMS and lymphoma cells. Although IFN γ -induced ICAM-1 upregulation has been previously described in NB and leukemia cells (11, 12), to our knowledge no studies have shown its upregulation on EWS.

According to our data, the ligands most upregulated by IFN γ were PD-L1, ICAM-1, and MHC-class I; therefore, we determined whether changes in their expression correlated to changes in tumor lysis after IFN γ treatment. PD-1/PD-L1 is associated with immune cell suppression; however, we observed that <7% of expanded NK cells express PD-1. In addition, changes in expression of PD-L1 did not correlate with changes in cytotoxicity, suggesting that PD-L1 did not play a role in our model.

By contrast, changes in ICAM-1 and MHC-class I cooperate in affecting NK cell responsiveness. For the cell lines in which IFN γ increased sensitivity to NK cells, we observed ICAM-1 upregulation exceeding MHC-class I upregulation (MHC-class I/ICAM-1 < 1). This suggests that the LFA-1/ICAM-1 interaction augments NK cell-mediated lysis even in the presence of high levels of inhibitory MHC-class I molecules. MHC-class I molecules mediate NK cell inhibition through binding with KIRs (HLA-A, HLA-B, HLA-C) and NKG2A (HLA-E). The anti-MHC-class I antibody clone used in this study, W6/32, recognizes both classical (HLA-A, HLA-B, HLA-C) and non-classical (HLA-E) HLA (26). We observed that for three of the six cell lines with increased resistance after IFN γ treatment, MHC-class I upregulation exceeded ICAM-1 upregulation (MHC-class I/ICAM-1 > 1). This suggests that the NK cell balance was shifted toward inhibition due to the increased expression of MHC-class I, which binds NK cell inhibitory receptors. However, in the other three cell lines with increased resistance after IFN γ treatment we observed that ICAM-1 upregulation exceeded MHC-class I upregulation (MHC-class I/ICAM-1 < 1) for which this model would have predicted increased sensitivity after IFN γ treatment. Our results suggest that the differential effects on MHC-class I and ICAM-1 expression can explain some but not all of the effects of IFN γ on sensitivity to NK cell lysis.

We investigated possible mechanisms for the enhanced sensitivity observed after IFN γ treatment for the cell lines SJ-GBM2, NB1643, and also BT-12. After IFN γ treatment these cell lines showed increased sensitivity to NK cell-mediated lysis, even in the presence of high levels of MHC-class I (Figures 2B and 7B). Since IFN γ treatment caused ICAM-1 upregulation exceeding MHC-class I upregulation in these cell lines (MHC-class I/

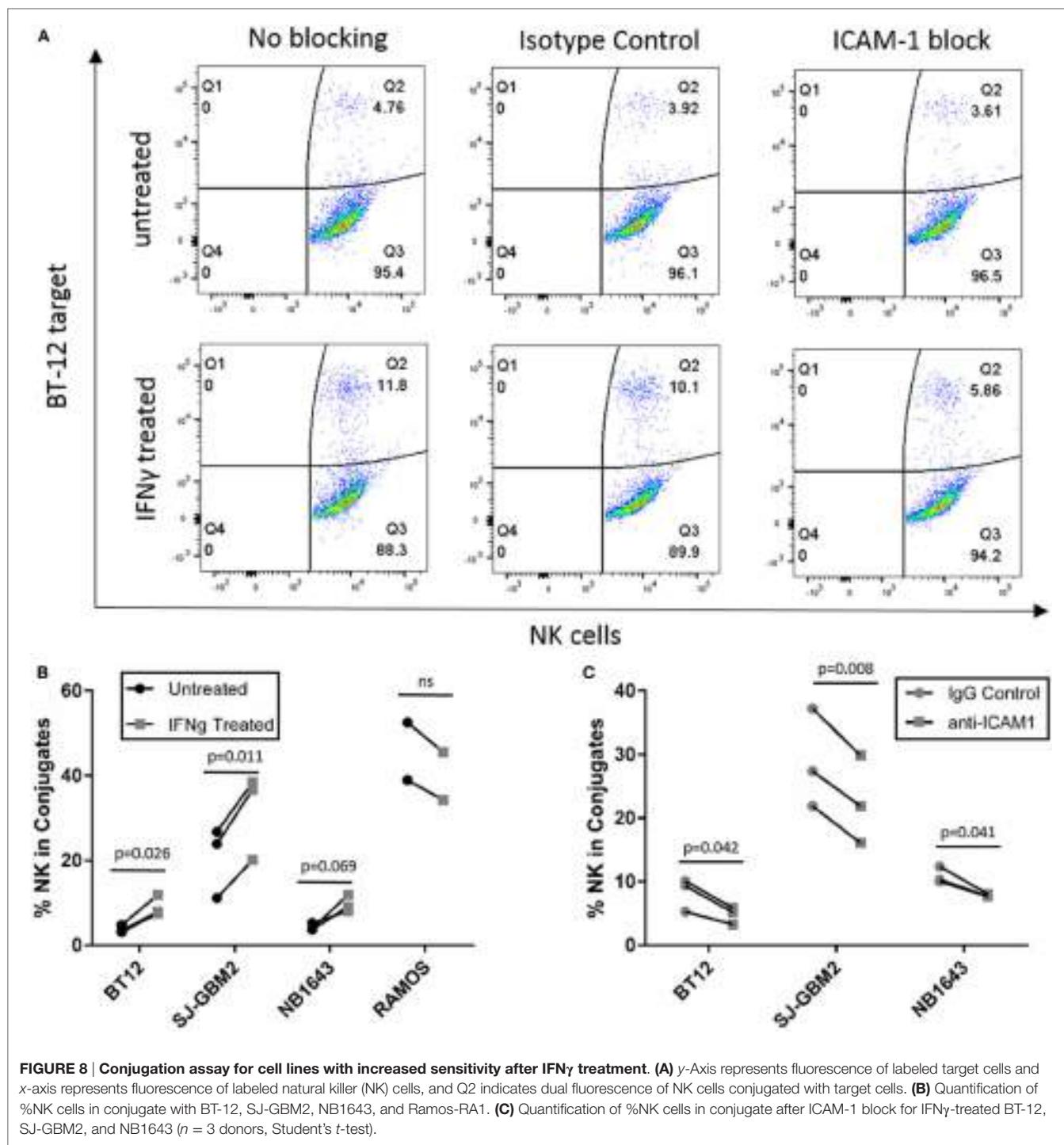


FIGURE 8 | Conjugation assay for cell lines with increased sensitivity after IFN γ treatment. **(A)** y-Axis represents fluorescence of labeled target cells and x-axis represents fluorescence of labeled natural killer (NK) cells, and Q2 indicates dual fluorescence of NK cells conjugated with target cells. **(B)** Quantification of %NK cells in conjugate with BT-12, SJ-GBM2, NB1643, and Ramos-RA1. **(C)** Quantification of %NK cells in conjugate after ICAM-1 block for IFN γ -treated BT-12, SJ-GBM2, and NB1643 ($n = 3$ donors, Student's *t*-test).

ICAM-1 <1), we evaluated conjugate formation after IFN γ treatment. Our results show an increase in the formation of conjugates between the NK cells and the target cells SJ-GBM2, BT-12, and NB1643 after IFN γ treatment, and conjugate formation was decreased for IFN γ -treated cells after blocking ICAM-1 on the target cells. Ramos-RA1 did not show increased conjugate formation or killing in response to IFN γ , possibly because it has such high baseline ICAM-1 expression and conjugate formation.

This suggests that the mechanism of increased sensitivity from IFN γ treatment is, at least in part, mediated by increased ICAM-1 upregulation leading to enhanced effector-target conjugation.

Our study uncovers the complexity behind cancer cell responses to IFN γ . Published literature has shown increased resistance to NK cell-mediated lysis due to MHC-class I upregulation in some cancers (9, 10), but increased sensitivity to NK cell-mediated lysis due to ICAM-1 upregulation in others (11, 12). These studies

reported IFN γ effects on MHC-class I or ICAM-1 expression individually, and the contradictory results stem from analysis of a small number of different targets or focus on particular tumor types. This is, to our knowledge, the first study to evaluate the effect of IFN γ on a broad range of NK cell ligands and across nearly two dozen cell lines representing six broad tumor types. We observed variable effects of IFN γ on tumor sensitivity to NK cells that are cell line dependent and attributable to individual MHC-class I and ICAM-1 responses, suggesting that IFN γ effects on cancer cell sensitivity to NK cells should not be broadly generalized, although further experiments with cell lines and patient samples may determine whether specific tumor types have generalizable IFN γ responses. Our data suggest that increased IFN γ secretion from expanded NK cells can mediate ICAM-1 upregulation and enhanced NK cell conjugate formation in brain tumors and NB, enhancing NK cell activity in adoptive immunotherapy. Thus, a better understanding of the effects of NK cell-mediated inflammation on the tumor microenvironment is essential to optimize cellular immunotherapy of cancer.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AA-L, VS, EK, and DL. Performed the experiments and acquired data: AA-L, VS, and ZV. Analyzed the data: AA-L, EK, and DL. Wrote the paper: AA-L

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00391/full#supplementary-material>.

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Conflict of Interest Statement: DL declares a potential conflict of interest in licensing of intellectual property to Intrexon Corporation and Ziopharm Oncology, and equity/leadership in Cyto-Sen Therapeutics. The other authors declare no conflict of interest.

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The Rise of Allogeneic Natural Killer Cells As a Platform for Cancer Immunotherapy: Recent Innovations and Future Developments

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Natural killer (NK) cells are critical immune effector cells in the fight against cancer. As NK cells in cancer patients are highly dysfunctional and reduced in number, adoptive transfer of large numbers of cytolytic NK cells and their potential to induce relevant antitumor responses are widely explored in cancer immunotherapy. Early studies from autologous NK cells have failed to demonstrate significant clinical benefit. In this review, the clinical benefits of adoptively transferred allogeneic NK cells in a transplant and non-transplant setting are compared and discussed in the context of relevant NK cell platforms that are being developed and optimized by various biotech industries with a special focus on augmenting NK cell functions.

Keywords: hematopoietic stem cell transplantation, autologous natural killer cells, allogeneic natural killer cells, adoptive natural killer cell therapy, natural killer cell biotech companies, natural killer cell combinatorial studies

NATURAL KILLER (NK) CELLS IN ONCOLOGY

So far T cells have been the mainstay of cancer immunotherapy; however, it is generally recognized that NK cells also play an essential role in antitumor immunity. Certainly in the prevention of metastases through the elimination of circulating cancer stem cells with a high metastatic potential, NK cells are recognized as main immune effector cells (1). Moreover, as solid tumors have a propensity to particularly down-regulate MHC-I, NK cells provide a failsafe mechanism in these circumstances where cytotoxic T cells, which depend on MHC-I for tumor recognition and elimination, are debilitated. NK cells have recently been more intensely explored as a viable therapeutic platform next to T cell-based approaches. This review aims to summarize the latest developments in the clinical translation of adoptive transfer of NK cells in the oncology field.

NK CELLS AND THEIR ACTIVATING AND INHIBITORY RECEPTORS

Human NK cells are generally categorized by their level of CD56 and CD16 expression into two subsets: CD56^{bright}CD16^{dim} and CD56^{dim}CD16^{bright} NK cells. Most NK cells in the peripheral blood and spleen are CD56^{dim}CD16^{bright} and are cytotoxic against a variety of tumor cells, whereas CD56^{bright}CD16^{dim} NK cells are immune regulatory in function and constitute the majority in secondary lymphoid tissues, producing abundant cytokines but exerting weak cytotoxicity compared to CD56^{dim}CD16^{bright}.

NK cells (2). The ability of NK cells to discriminate between a cancer cell and a healthy cell is regulated by a balance between its activating and inhibitory receptors. NK-activating receptors such as DNAM-1 and NKG2D; natural cytotoxicity receptors (NCRs) such as NKp30, NKp44, NKp46, CD94/NKG2C, CD94/NKG2E, and CD16a; and activating killer cell-immunoglobulin like receptors (KIRs) contribute to NK cell activation, triggering the release of cytotoxic granules and proinflammatory cytokines such as interferon gamma (IFN γ) from NK cells to lyse cancer cells (3). The NK cell-activating receptor NKG2D (CD314) recognizes MHC class-I-chain related proteins A and B (MICA and MICB) and ULBPs (1–6), while DNAM-1 binds to CD112 (Nectin-2) and CD155 (poliovirus receptor) (5) on stressed, infected, and cancer cells. The ligands for NCRs are widely expressed on cells infected by viruses or by intracellular bacteria and on tumor cells, but their exact modes of action are yet to be characterized to define their role in NK cytotoxicity (6). In addition to this, the heterodimers of the NKG2 family; CD94/NKG2C and CD94/NKG2E recognize the non-classical MHC class I molecule HLA-E and associate with DAP-12 molecule to trigger an NK activation signal (7, 8). Another very important activation mechanism of NK cells is through the interaction of CD16a (Fc γ RIIIa, a low affinity Fc receptor) with the Fc portion of IgG $_1$ antibodies, forming an immunological synapse to engage antibody opsonized targets for NK cell-mediated antibody-dependent cell mediated cytotoxicity (ADCC) (9). Besides engaging activating receptors, NK cells also induce target cell death using tumor necrosis factor α (TNF- α), Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) (10). The most prominent NK cell inhibitory receptors include inhibitory KIRs that recognize MHC class I (HLA-ABC) molecules, which are universally expressed on healthy tissues. Similarly, CD94/NKG2A, an inhibitory receptor from the NKG2 family, binds to HLA-E and induces NK cell tolerance through the activation of an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) (8). Hence, knowing that NK cell functions are determined by an array of receptors, which can either potentiate an activating or inhibitory signal, depending on different ligand interactions with tumor cells, it is critical to shift the balance in a therapeutic setting toward an activating NK phenotype to expedite enhanced NK tumor killing mechanisms.

NK CELL DYSFUNCTIONALITY IN CANCER

Natural killer cells can control circulating tumor cells and prevent formation of tumor metastases (11). However, tumors employ different strategies to evade killing by NK cells. Upregulation of inhibitory ligands such as MHC class I molecules (HLA-ABC, HLA-G and HLA-E) has been associated with a stronger inhibitory signal to NK cells (12–15). Furthermore, increased expression of the inhibitory NKG2A receptor reported in renal cell carcinoma resulted in decreased functionality of tumor infiltrating NK cells (16). On the other hand, downregulation of NK-activating ligands for NKG2D such as MICA and MICB and increased shedding of tumor-derived soluble MIC also impair NKG2D-mediated NK cell tumor recognition (17). Another important necessity for

optimal NK cell function is the ability to home and migrate to tumor sites. Several studies have correlated increased homing of NK cells to tumor tissues with improved treatment outcomes in solid tumors (18–22). However, the immunosuppressive tumor stroma comprising regulatory T cells (T-reg) (23), myeloid-derived suppressor cells (MDSCs) (24), M2 macrophages (25), and immature dendritic cells severely restricts NK cell functionality and their entry into solid tumors. In chronic diseases, such as those associated with human immunodeficiency virus and cytomegalovirus infections, mainly exhausted NK cells with decreased cytokine production and reduced cytolytic activity are observed (26, 27). In a study with breast cancer patients, the NK cell expression levels of activating receptors (NKG2D, DNAM, CD16, and NKp30) were decreased, whereas inhibitory receptor (NKG2A) expression levels were increased and this apparent dysfunctionality of NK cells was found to directly affect NK cell cytotoxicity (28). Similarly, the effector subset of NK cells (CD56 $^{\text{dim}}$ CD16 $^{+}$) from head and neck and breast cancer patients, when tested *in vitro*, was highly prone to apoptosis, thus pointing to low NK cell activity in these patients (29). Impaired NK cell functionality may result from tumor-imposed suppressive mechanisms and presents a major hurdle for NK cell-targeted immunotherapies. Therefore, approaches to restore or replace impaired NK cell cytotoxicity may prove essential for an effective host defense against cancers.

NK CELLS IN THE CLINIC

Novel NK cell-based immunotherapeutic strategies are being developed to overcome the functional limitations of the use of cancer patients' autologous NK cells. To increase the number of functional NK cells even in case of a high tumor load, adoptive transfer of autologous NK cells served as a very feasible approach, as this ruled out the need for immunosuppression, HLA-matching, and prevented the risk of graft versus host disease (GvHD). These advantages sparked the initiation of large-scale expansion protocols and clinical trials using autologous NK cells as a treatment modality for cancer. Though adoptive transfer of autologous NK cells resulted in an increased number of circulating NK cells in peripheral blood, it failed to produce significant therapeutic effects in hematological malignancies, metastatic melanoma, and renal cell carcinoma patients due to the inhibition by self-HLA molecules (30–32). Moreover, the expansion efficiency and functional status of autologous NK cells were still limited when compared to allogeneic NK cells, as autologous cells were often obtained from heavily pretreated patients (33). In addition to this, it was difficult to track infused autologous NK cells in patients and to study their antitumor effects from peripheral blood analyses due to the inability to differentiate *ex vivo* manipulated and transferred autologous NK cells from the non-manipulated circulating NK cells. These limitations motivated researchers shifting their focus to allogeneic NK cells to treat cancer.

In patients with leukemia undergoing allogeneic hematopoietic stem cell transplantation (HSCT), NK cells, being the first lymphoid subset to appear after allogeneic HSCT (34), play a crucial role in controlling host defense against infections and residual cancer cells before T cells are reconstituted (35). These

donor T cells are prime mediators of GvHD (36), and the life-threatening complications that arise due to GvHD have completely overshadowed the beneficial effects of alloreactive NK and T cells, fueling efforts to use T cell depleted grafts (37). Further, this led to the development of NK cell-based therapies coupled with T cell depleted HSCs to enhance the graft versus tumor effect (GvT) without causing GvHD. Unlike autologous NK cells, allogeneic NK cells are not restricted by the patient's tumor's HLA expression, which is an added advantage to mount an improved anti-tumor effect (38, 39). Current translational efforts that are explored as anticancer therapies include adoptive transfer of *ex vivo* activated and/or expanded allogeneic NK cells, either alone or in combination with HSCT.

SOURCES OF ALLOGENEIC NK CELLS USED IN THE CLINIC

Commonly used allogeneic NK cells are apheresis products collected from haploidentical and unrelated donor PBMC (40). Another source is umbilical cord blood (UCB), where NK cells are generated from CD34+ progenitor cells that undergo expansion and differentiation using cytokines and growth factors and thereby mature into cytolytic NK cells (41). Apart from PBMC and UCB, NK cells have also been obtained from the clonal cell line NK-92, derived from immortalized lymphoma NK cells (42, 43).

ALLOGENEIC NK CELL THERAPY IN A TRANSPLANT SETTING

Autologous or allogeneic HSCT serves as a curative regimen by reconstituting the immune system in hematological malignancies. At an earlier stage post HSCT, NK and T cells developing from the graft are immature and less in number with reduced functionality. Under those circumstances, the infusion of purified allogeneic NK cells was explored as a viable option to target minimal residual disease (MRD), prevent graft failure, and relapse. Grafts for allogeneic HSCT and allogeneic NK cell treatments were obtained from HLA matched/mismatched and related/unrelated donors (38, 39). Earlier clinical trials performed by Passweg et al. (44), Koehl et al. (45), Shi et al. (46), Yoon et al. (47), Rizzieri et al. (48), and Brehm et al. (49) have shown that NK cells can be safely administered prior to or post HSCT in patients with different types of hematological diseases. Immune suppression is a prerequisite prior to most of the allogeneic HSCT and NK-cell infusions. A non-myeloablative conditioning regimen usually consisting of cyclophosphamide (Cy) and fludarabine (Flu) was found to facilitate NK cell persistence and expansion *in vivo* (50). High doses of Cy/Flu caused pancytopenia and resulted in high plasma IL-15 levels, which also correlated with the detection of adoptively transferred NK cells up to 14 days after infusion, thus suggesting that excess IL-15 was probably utilized by the NK cells to proliferate and persist longer *in vivo* (51). A summary of clinical trials with allogeneic NK-cell infusions in a HSCT setting with published data is summarized in **Table 1**, and selected clinical trials from recent years are reviewed below.

In 2013, Stern et al. treated acute myeloid leukemia (AML), Acute Lymphocytic Leukemia, Hodgkin's lymphoma (HL), and sarcoma patients with allogeneic NK cells (CD3 depleted and CD56 selected) after a haploidentical HSCT, using the same donor as NK cell source. An overall survival (OS) of 25% was achieved during a median follow-up of 5.8 years. And 4/16 patients developed acute GvHD (aGvHD) due to high T-cell impurities present in two NK cell products and two from stem cell grafts, both containing $\geq 0.5 \times 10^5$ cells/kg T cells (52). Although this prospective Phase II study reported the safety and feasibility of NK-cell infusion following allo-HSCT, it failed to yield results in support of anti-leukemia effects, raising questions as to whether the NK cell dosage of $0.3\text{--}3.8 \times 10^7$ cells/kg used was sufficient to induce a clinical effect. In the same year, Klingemann et al. published data highlighting the safety and alloreactivity of HLA-mismatched (CD3 depleted) NK cells, transfused after autologous HSCT in multiple myeloma (MM), non-Hodgkin's lymphoma (NHL), and HL patients. In this study, 13 patients were enrolled; 6/13 relapsed and 7/13 were in remission during a follow-up between 144 and 1,158 days following autologous stem cell transplantation. The allogeneic NK cells were well tolerated without GvHD. In addition, this study also demonstrated that NK cells generated and processed at distant centers can be shipped and transfused without significantly affecting the viability and cytotoxicity of the NK cell product (53).

Choi et al. (54) summarized their observations from a study, in which allogeneic *ex vivo* expanded and activated NK cells derived from the same donor were administered 14 and 21 days post HLA haploidentical HSCT to patients with hematological malignancies ($n = 41$). The data set from this study was compared with a group of 31 patients, who underwent only HLA haploidentical HSCT. A significantly higher progression-free survival (PFS) was seen in the HSCT + NK group compared to HSCT only group (74% versus 46%). In addition to this, the occurrence of chronic GvHD (cGvHD) (15% versus 10%) and transplant-related mortality (27% versus 19%) was reduced in the HSCT + NK group compared to the HSCT only group (54). In another study by Killig et al. (55), AML patients were treated with haploidentical HSCT followed by NK-cell infusions (CD3 depleted and CD56 selected) from the same donor, on days +1 and +2 post HSCT. aGvHD was highly prevalent in 20/24 patients in this study and histological analysis of skin revealed that GvHD was associated with infiltration by perforin⁺CD8⁺ T cells. Allogeneic NK cells contributed to an increased OS in the HSCT + NK group compared to the HSCT only group (37% versus 14%) over a median follow-up of 2 years (55).

Subsequently, Shah et al. published data from a Phase I study treating patients with Ewing sarcoma, rhabdomyosarcoma, and desmoplastic small round cell tumors ($n = 9$), using donor-derived IL-15/4-1BBL-activated allogeneic NK cells (CD3 depleted and CD56 selected) following allogeneic HSCT from the same donor. aGvHD was highly prevalent in the patient group that received stem cells from matched unrelated donors and was directly linked with a faster T cell recovery and higher T cell chimerism from reconstituted HSCT grafts. About 4/9 patients were alive in this study with a median follow-up of 23.1 months (56). Lee et al. reported results from a Phase I study

TABLE 1 | Summary of allogeneic NK cell clinical trials in a transplantation setting.

Study	Malignancy	Clinical trial design	Culture method^a	Infused dose NK cells	Final product characteristics	Outcome
Phase I (NCT01729091) Shah et al. (59)	MM (<i>n</i> = 12)	Conditioning with Mel on day 7 and Lnd from days 8 to 2 prior to UCB-NK-cell infusion (day 5), followed by autologous-HSCT on day 0	<i>Ex vivo</i> expanded MNCs from unrelated UCB donors. Culture duration: 14 days with irradiated K562 clone 9.mbil-21 aAPCs and IL-2 ^a CD3 depleted (on day 7)	Four escalating doses: 5 × 10 ⁶ , 1 × 10 ⁷ , 5 × 10 ⁷ , and 1 × 10 ⁸ cells/kg	Mean purity: 98.9% CD56+/CD3– cells	Well tolerated. No GvHD. 4/12 progressed or relapsed (median of 21 months follow-up)
Phase I (NCT01795378) Choi et al. (58)	AML (<i>n</i> = 45) and ALL (<i>n</i> = 6)	Hapo-HSCT followed by DNKI from the same donor on days 6, 9, 13, and 20 post HSCT	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: 2–3 weeks with IL-15 and IL-21	Four escalating doses: median DNKIs are 5 × 10 ⁷ , 5 × 10 ⁷ , 1 × 10 ⁸ , and 2 × 10 ⁸ cells/kg	Median viability: 80%. Purity: 48–98% CD56+ CD122+ cells. 0–22% CD3+ CD56+ cells. 0–10.4% CD3+ CD56– cells	Toxicity observed in 73% of patients, 9/45 aGvHD. 29/51 CR (9.3–34.7 months follow-up), 35/51 PD
Phase I (NCT00402558) Phase II (NCT01390402) Lee et al. (57)	AML (<i>n</i> = 8), MDS (<i>n</i> = 6), and CML (<i>n</i> = 7)	Conditioning with Flu/Bu prior to haplo-allo NK-cell infusion, followed by IL-2 therapy (5x, daily); conditioning with Thy/Tac prior to HLA-matched related unrelated allo-HSCT	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: o/n with IL-2. ^a CD3 depleted and CD56 selected (in three infusions)	Four escalating doses: 1 × 10 ⁶ , 5 × 10 ⁶ , 3 × 10 ⁷ , and 3 × 10 ⁷ cells/kg in Phase I study. Four escalating doses of 5 × 10 ⁶ cells/kg in Phase II study	Median purity: 0.02% CD3+ cells. 11.41% CD14+ cells. 21.84% CD19+ cells. 14.1% CD56+ CD3– cells	Well tolerated, no GvHD. 5/21 CR, 5/21 died of transplantation related issues and 11/21 died of relapse
Phase I (NCT01287104) Shah et al. (56)	EWS (<i>n</i> = 5), DSRCT (<i>n</i> = 3), RMS (<i>n</i> = 1)	HLA matched haplo- or unrelated allo-HSCT followed by aNK-DLI from the same donor on day 7 and 35 post HSCT	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: 9–11 days with KT64.4-BBL artificial antigen presenting cells. ^a CD3 depleted and CD56 selected	Repeated doses (2x doses 1, 2, and 3): 1 × 10 ⁵ cells/kg (dose 1), 1 × 10 ⁶ cells/kg (dose 2), and 1 × 10 ⁷ cells/kg (dose 3)	Median purity: CD3+ cells 1.4 × 10 ⁴ cells/kg. CD56+ cells ≥90%. Viability: ≥70%	5/9 aGvHD. 2/9 SD, 7/9 CR. 4/9 are still alive (12.5–27.4 months after treatment)
Phase I/II (NCT01220544) Killig et al. (55)	AML (<i>n</i> = 24)	Hapo-HSCT followed by NK-cell infusion from same donor and OKT3 treatment from days –5 to +3	PBNK cells from haploidentical donors. ^a CD3 depleted and CD56 selected	Single dose: 1.61–32.2 × 10 ⁶ CD56+/CD3– cells/kg	Purity: CD56+ CD3– cells 99.97%. CD3+ cells 0.95–7.4 × 10 ⁴ cells/kg	Toxicity correlated with haplo-HSCT. Deaths: 2/24 GvHD, 6/24 infections and 7/24 died of relapse. 9/24 CR (0.1–8.6-year follow-up)
Phase I/II (NCT00823524) Choi et al. (54)	AML (<i>n</i> = 32), ALL (<i>n</i> = 7), MDS (<i>n</i> = 1), DLBCL (<i>n</i> = 1)	HLA haplo-HSCT followed by DNKI from the same donor, 14 days and 21 days after HSCT	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donor. Culture duration: 13–20 days with IL-15, IL-21, and hydrocortisone	Escalating doses (2x): 0.2 × 10 ⁸ cells/kg (3 pts), 0.5 × 10 ⁸ cells/kg (3 pts), 1.0 × 10 ⁸ cells/kg (8 pts), and ≥1.0 × 10 ⁸ cells/kg (27 pts)	Viability: 71–85%. Median purity: CD56+ CD122+ cells >90%. CD3+ CD56+ cells <3%. Fold expansion: 0.8–70 (after 13–20 days of culture)	Well tolerated. 9/41 aGvHD, 10/41 cGVHD. In total, 11 patients died of TRM. In AML (21/29) (4/8) ALL/lymphoma are in CR
Phase I (IND # 12971) Klingemann et al. (53)	NHL (<i>n</i> = 6), MM (<i>n</i> = 5), and HL (<i>n</i> = 2)	MHC-mismatched haploidentical NK-MC infusion, 49–191 days post auto-HSCT	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: o/n with IL-2	4 Escalating doses: 1 × 10 ⁵ , 1 × 10 ⁶ , 1 × 10 ⁷ , and 2 × 10 ⁷ MC/kg	Median purity: 26% CD56+ CD3– cells. 0.15% CD3+ cells. Median viability: 95% post wash	Well tolerated. No GvHD. 6/13 relapsed and 7/13 in remission
Phase II (NCT01386619) Stern et al. (52)	AML (<i>n</i> = 8), ALL (<i>n</i> = 5), HL (<i>n</i> = 2) sarcoma (<i>n</i> = 1)	Hapo-HSCT followed by NK-DLI from the same donor, +day 3, +day 40, and +day 100 post HSCT	PBNK cells from haploidentical donors. ^a CD3 depleted and CD56 selected	Repeated doses (2–3): 0.3–3.8 × 10 ⁷ cells/kg	Median purity: CD3+ cells 0.03 × 10 ⁵ cells/kg. Median viability: 84%	Safe and feasible. 4/16 aGvHD. Median follow-up of 5.8 years 4/16 are alive. 3/16 died from graft failure

(Continued)

TABLE 1 | Continued

Study	Malignancy	Clinical trial design	Culture method^a	Infused dose NK cells	Final product characteristics	Outcome
Phase I/II (NCT01386619) Brehm et al. (49)	AML (n = 6), ALL (n = 5), NB (n = 5), RMS (n = 1) HL (n = 1)	Haplo-HSCT followed by IL-2 stimulated NK-cell infusion (cryo) or unstimulated NK-cell infusion (fresh) from the same donor, +da 3, +day 40, and +day 100 post HSCT	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: 9–14 days with (group II) or without (group I) IL-2 (fresh or cryo). ^a CD3 depleted and CD56 selected	Repeated doses (1–3 doses): Group I: 3.2–38.3 × 10 ⁶ cells/kg; Group II: 6.0–45.1 × 10 ⁶ cells/kg	Purity: CD56+ CD3– cells 84.4–98.6%. CD3+ cells group I: 0.4–53.4 × 10 ³ cells/kg. CD3+ cells group II: 7.7–98.3 × 10 ³ cells/kg. Viability: freshly NK-cell unstimulated median 93%. Cryo NK-cell IL-2 stim 30–70%	Well tolerated without GvHD >grade II. Group I: 5/9 died (126–498 days post SCT), 3/9 CR (742–2,218 days). Group II: 5/9 died (27–373), 2/9 CR, and 2/9 in remission
Phase I (NCT00586690) Rizzieri et al. (48)	Lymphoma (n = 30)	3–6/6 HLA-matched haploidentical NK-cell infusion, ~8 weeks post haplo-HSCT from the same donor	PBNK cells from haploidentical donors. ^a Only CD56 selected	Repeated dose (1–3): median dose in 3–5/6 HLA match: 9.21 × 10 ⁶ CD3+/CD56– cells/kg, median dose 6/6 HLA match: 10.6 × 10 ⁶ CD3+/CD56– cells/kg	6/6 HLA-matched: Purity: 87–100% CD56+ cells. 0.53 ± 1.1 × 10 ⁶ cells/kg CD3+ CD56–, 3–5/6 HLA-matched: Purity: 86–100% CD56+ cells. 0.27 ± 0.78 × 10 ⁶ cells/kg CD3+ CD56–	Safe. Low toxicity. 6/6 HLA-matched: 6/14 aGvHD (1 severe) and median OS 12 months. 3–5/6 HLA-matched: 8/16 aGvHD and median OS 27 months
Phase I (NCT00569283) Yoon et al. (47)	AML (n = 12) MDS (n = 2)	HLA-mismatched HSCT followed by allo NK-cell infusion from the same donor	<i>Ex vivo</i> expanded, differentiated and activated CD34+ progenitor cells (PB-derived) from haploidentical donors. Culture duration: 21 days with FLT3, IL-7 and hydrocortisone followed by 21 days with IL-15, IL-21 and hydrocortisone	Single dose: 0.33–24.5 × 10 ⁶ cells/kg	Mean purity: CD56+ CD122+ cells 64%. CD3+ cells 1.0%. Mean viability: 88%	1/14 aGvHD and 4/14 cGvHD. 9/14 died (between 1.7 and 15.5 months), 4/14 CR (between 16.2 and 21.6 months) 1/14 PD (25.9 months)
(BB-IND-11347) Shi et al. (46)	MM (n = 10)	Conditioning with Flu/Dex/Mel followed by haplo-KIR-ligand-mismatched NK-cell infusion on day 0 and day +2; IL-2 therapy daily (11×) starting on day +1 after NK-cell infusion; auto-HSCT on day +14	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: o/n with IL-2 (pts 1–5) and brief incubation with IL-2 and anti-CD3 beads (pts 5–10)	Combined dose (day 0 and day +2): 2.7–92 × 10 ⁶ cells/kg	Purity: median CD3+ cells 5.5 × 10 ⁴ cells/kg. Viability: 95%	Safe and no GvHD. 5/10 CR, 1/10 PR, 1/10 MR, 1/10 SD, and 2/10 PD. 4/10 are alive at 1.4, 1.5, 2.3, and 3 years post NK-cell therapy
Pilot study Koehl et al. (45)	AML (n = 1) ALL (n = 2)	Haplo-HSCT followed by KIR-mismatched NK-cell infusion on day +1 or +2 post HSCT and additional infusions every 4–6 weeks; IL-2 therapy +2 days post HSCT, every second day for 2–4 weeks	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: >12 days with IL-2. ^a CD3 depleted and CD56 selected (in three infusions)	Repeated doses (1–3): 8.9–29.5 × 10 ⁶ cells/kg (first infusion), 3.3 and 11.1 × 10 ⁶ cells/kg (second infusion), 33.8 × 10 ⁶ cells/kg (third infusion)	Purity: CD56+ CD3– cells 95%. Median CD3+ cells 0.04%, 45–1,100 × 10 ³ cells. Viability: 95%. Fold expansion: median 5 (after 14 days of culture)	Well tolerated, no GvHD. 1/3 CR (152 days), 2/3 died (80 days and 45 days after NK-cell infusion)
Passweg et al. (44)	AML (n = 4), CML (n = 1)	Haplo-HSCT followed by NK-DLI from the same donor 3–12 months post HSCT	PBNK cells from haploidentical donors. ^a CD3 depleted and CD56 selected	Single dose: 0.21–1.41 × 10 ⁷ cells/kg	Median purity: CD56+ CD3– cells 97.3%. T-cell 0.22 × 10 ⁵ cells/kg	Well tolerated and feasible. 4/5 continuous remission (8–18 months), 1/5 PD

CNS, central nervous system; MPDs, myeloproliferative disorders; LPD, lymphoproliferative disorder; MM, multiple myeloma; MDS, myelodysplastic syndromes; MDN, myelodysplastic neoplasms; MPN, myeloproliferative neoplasms; AML, acute myeloid leukemia; LBLL, lymphoblastic leukemia-lymphoma; ALL, acute lymphoblastic leukemia; NB, neuroblastoma; RMS, rhabdomyosarcoma; CML, chronic myelogenous leukemia; NHL, non-Hodgkin's lymphoma; MCL, mantle cell lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; ALCL, anaplastic large cell lymphoma; HL, Hodgkin's lymphoma; RCC, renal cell cancer; SCLC, small cell lung cancer; CLL, chronic lymphocytic leukemia; HCC, hepatocellular carcinoma; PNET, primitive neuroectodermal tumor; ACC, adrenal cortical carcinoma; EWS, Ewing sarcoma; DSRCT, desmoplastic small round cell tumor; Cy, cyclophosphamide; Flu, fludarabine; Bor, bortezomib; Dex, dexamethasone; Clo, clofarabine; Eto, etoposide; Cis, cisplatin; Pac, paclitaxel; Doc, docetaxel; Vin, vinorelbine; Gem, gemcitabine; Car, carboplatin; Pem, pemetrexed; TBI, total body irradiation; Tac, tacrolimus; Pred, prednisolone; mPred, methylprednisolone; Thy, thymoglobulin; Vin, vincristine; Adr, adriamycin; Predn, prednisone; Mel, melphalan; OKT3, muromonab-CD3; HSPC, human stem and progenitor cells; aGvHD, acute graft versus host disease; DNKI, donor NK-cell infusion; Stim, stimulated; Unstim, unstimulated; DFS, disease-free survival; CR, complete remission; PR, partial response; MR, minimal response; SD, stable disease; PD, progressive disease; aNK-DLI, donor-derived IL-15/4-1BBL activated NK-cell infusion; TRM, transplant-related mortality; MC, mononuclear cell; TLS, tumor lysis syndrome; PLS, passenger lymphocyte syndrome; NE, not evaluable.
^aCulture method displays CD3 depleted PBMC's, otherwise deviated selection method is mentioned.

in patients with AML, myelodysplastic syndromes (MDS), and chronic myelogenous leukemia (CML), in which alloreactive haploidentical NK cells (CD3 depleted and CD56 selected) were administered along with IL-2 injections, followed by thymoglobulin conditioning and allogeneic HSCT. Thymoglobulin was administered to prevent NK cells from hampering engraftment of allogeneic HSCT. Out of 20 evaluable patients, 16 had GvHD (10/16 aGvHD and 6/16 cGvHD) after transplantation. In this study, GvHD was not directly associated with donor T cell or NK cell contents. From this study, also it was concluded that the lack of anti-leukemic effect was mainly due to the low dose of infused NK cells and it was further suggested that thymoglobulin conditioning could also have potentially affected NK cells survival *in vivo* (57).

Later, Choi et al. presented results from a modified treatment protocol of four consecutive infusions of *ex vivo* activated and expanded haploidentical NK cells after HLA-matched HSCT and compared the outcomes to their previous study, in which they administered two infusions. In the subsequent study, additional donor NK-cell infusions were given on days 6 and 9 (i.e., at days 6, 9, 13, and 20). Out of 51 patients with ALL ($n = 6$) and AML ($n = 45$), 24/51 (47%) had four NK infusions. Out of 45 evaluable patients, the 3-year OS rate was 9% in AML and 21% for ALL and 9/45 had aGvHD. Early administration of NK cells after HSCT caused significant toxicities with no improvements in anti-leukemic effects, compared to the previous study. In this study group, a higher CR rate correlated with higher expression levels of NK activating receptors NKG2D and NCRs (NKP44, NKP46, and NKP30) on donor NK cells. In addition, NKP30 expression was significantly higher than that of NKG2D and other NCRs, thus suggesting a role for NKP30 as a predictive biomarker for anti-leukemic effects of NK cells (58).

In 2017, Shah et al. published data from a Phase I study, treating MM patients with UCB-derived NK cells (day 5) along with autologous-HSCT (day 0), following high-dose chemotherapy and low-dose lenalidomide. Mononuclear cells (MNCs) isolated from UCB units (CD3 depleted) were cultured with K562-based artificial antigen presenting cells (aAPCs) expressing membrane bound IL-21. No treatment related toxicities or GVHD was reported in this study. During a median follow-up of 21 months in 12 patients, 4/12 patients had progressive disease (PD) or relapsed. Stable expression of NKG2D and increased expression of CD16 and NKP30 of UCB-NK cells were observed in six patients. This study further reiterates the safety of NK-cell infusions in high doses; however, due to combinatorial set up with HSCT and lenalidomide, it is difficult to interpret the clinical efficacy of UCB-NK cells alone from this study (59).

Taken together, it is evident from these studies, as well as from many others, that GvHD, which is mainly caused by T cells from transplanted grafts, is a major concern in the field of allogeneic HSCT. Under these circumstances, it is difficult to reliably study the safety of allogeneic NK-cell infusions. The timing of NK-cell infusion, NK cell dosage and NK cell promoting conditioning regimens are critical factors that need to be more extensively studied to assess the safety and efficacy of allogeneic NK-cell infusions.

ADOPTIVE NK CELL THERAPY IN A NON-TRANSPLANT SETTING

To gain a better understanding of the safety and efficacy of allogeneic NK cell transfer, investigators started to study NK cells in a non-transplant setting. Landmark clinical trials were performed by Miller et al. (50), Iliopoulou et al. (60), Rubnitz et al. (61), Bachanova et al. (62) Curti et al. (63), and Geller et al. (33) predominantly in hematological malignancies, but also in various solid tumors. These studies demonstrated the safety and in part the efficacy of allogeneic NK-cell infusions in the absence of GvHD. A summary of allogeneic NK cell clinical trials in a non-transplant setting with published results is presented in **Table 2**.

Here, we focus on the latest reports from clinical trials using allogeneic NK cells in a non-transplant setting. Bachanova et al. developed a recombinant cytotoxic protein, i.e., an IL-2/diphtheria toxin fusion protein (IL2DT), which functions by selectively depleting the IL-2 receptor CD25 expressing cells, including regulatory T cells (T-reg). In total, 57 AML patients were treated with KIR and HLA-mismatched haploidentical NK cells and 15 of them in cohort 3 received IL2DT, 1 or 2 days prior to NK-cell infusion, to deplete T-reg. In addition to IL2DT treatment, three different processing methods were used, i.e., a CD3-depleted cohort (cohort 1, $n = 32$), a cohort using CD3 depletion followed by CD56 selection (cohort 2, $n = 10$), and a cohort using CD3 and CD19 depletion (cohort 3, $n = 15$). Higher NK cell doses were obtained from cohort 3, and it could possibly be the reason for the observed longer disease-free survival (DFS) (33% versus 5% at 6 months) in cohort 3 compared to cohorts 1 and 2. In this study, endogenous IL-15 serum levels correlated with reduced T-reg levels in patients treated with IL2DT (64).

Szmania et al. investigated the effect of infusing cryopreserved and freshly prepared NK cells (CD3 depleted), either allogeneic or autologous, given after bortezomib with or without lymphodepletion in high risk relapsed MM patients. NK cells were cocultured with K562-mb15-41BBL cells for 8–9 days. Initially, 6 out of 8 NK products were cultured with low dose IL-2 (10 U/ml), however, increasing the dosage of IL-2 to 500 U/ml resulted in enhanced expression of NKP30 and NK cytolytic activity without affecting the T cell content of the NK cell product and, therefore the last 2 patients in this study were treated with high dose IL-2 cultured NK cells. The highest post-transfer number of circulating NK cells was observed in the high dose IL-2 group. Patients treated with fresh NK cells showed a median 21-fold increase in peripheral NK cell rates by day 7, while no *in vivo* expansion of NK cells was seen in patients treated with cryopreserved NK cells. Overall, the NK-cell infusions were well tolerated and no GvHD was observed. From 7 evaluable patients, 6 had (PD), and 1 had a partial response (PR) for up to 6 months post infusion (65).

In the same year, Kottaridis et al. presented data from a clinical trial in AML, for the first time using tumor-primed NK cells from related haploidentical donors. During a 6-month follow-up period ($n = 7$), three patients in CR remained in CR, one patient in PR achieved CR, two patients relapsed, and one patient died. Median OS was 468 days post NK-cell infusion (66).

Rubnitz and his team reported on the safety and feasibility of haploidentical NK cell therapy in children with relapsed or

TABLE 2 | Summary of allogeneic NK cell clinical trials in a non-transplantation setting.

Study	Malignancy	Clinical Trial design	Culture method ^a	Infused dose NK cells	Final product characteristics	Outcome
Phase I (EudraCT number: 2010-018988-41) Dolstra et al. (73)	AML (<i>n</i> = 10)	Conditioning with Cy/Flu followed by KIR-mismatched UCB-NK-cell infusion	<i>Ex vivo</i> expanded, differentiated and activated UCB-NK cells from unrelated donors. Culture duration: 42 days with GM-CSF, G-CSF, IL-6, SCF, Flt3L, TPO, IL-7, IL-2, and IL-15. ^a CD34+ selected HSPC's	Escalating doses: 3 × 10 ⁶ cells/kg (cohort 1), 10 × 10 ⁶ cells/kg (cohort 2), and 30 × 10 ⁶ cells/kg (cohort 3)	Mean purity: 74 ± 13% CD34+ cell product. 75 ± 12% generated CD56+ CD3– NK cells. 0.03 ± 0.04% CD3+ cells. 0.16 ± 0.21% CD19+ cells. Mean viability: 94%	Well tolerated, no GvHD nor toxicity. 4/10 DFS for 55, 47, 17, and 12 months after infusion
Phase I (NCT01898793) Romee et al. (72)	AML (<i>n</i> = 13)	Conditioning with Cy/Flu followed by cytokine-induced memory-like NK-cell infusion and subsequent IL-2 therapy (every other day, 6x)	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: 12–16 h with IL-15, IL-12, and IL-18. ^a CD3 depleted and CD56 selected	Repeated dose: level 1: 0.5 × 10 ⁶ NK cells/kg level 2: 1 × 10 ⁶ NK cells/kg level 3: 10 × 10 ⁶ NK cells/kg	Purity: >90% CD56+ CD3– cells	Well tolerated, no GvHD. 4/13 NE, 4/13 TF-PD, 3/13 CR, 1/13 Cri, and 1/13 MLFS
Phase I (NCT00799799) Curti et al. (71)	AML (<i>n</i> = 16)	Conditioning with Cy/Flu followed by KIR ligand-mismatched NK-cell infusion; IL-2 therapy (3x weekly for 2 weeks)	PBNK cells from haploidentical donors. ^a CD3 depleted and CD56 selected	Single dose: 1.29–5.53 × 10 ⁶ cells/kg	Median purity: infused CD3+ cells: 0.65 × 10 ⁵ cells/kg. Mean viability: 95%	Feasible study, moderate toxicity. 9/16 DFS, 7/16 in relapse (3–51 months), 1/16 died of bacterial pneumonia
Phase II (NCT00526292) Shaffer et al. (70)	AML (<i>n</i> = 6) and MDS (<i>n</i> = 2)	Conditioning with Cy/Flu followed by HLA-mismatched NK-cell infusion; IL-2 therapy (6x) starting 1 day before and after NK-cell infusion	PBNK cells from haploidentical donors. ^a CD3 depleted and CD56 selected	Single dose: 4.3–22.4 × 10 ⁶ cells/kg	Purity: ≥90% CD3– CD56+ cells. CD3+ cells <0.1%. Viability: 82–100%	No GvHD. 3/8 PR, 5/8 no response. Median survival is 12.9 months
Phase I (NCT01212341) Yang et al. (69)	Lymphoma (<i>n</i> = 2) and solid tumor (<i>n</i> = 18)	KIR ligand-mismatched NK-cell infusion	<i>Ex vivo</i> expanded and activated PBNK cells from unrelated donors. Culture duration: 14 days with irradiated auto-PBMCs, OKT3 and IL-2	Single dose: 1 × 10 ⁶ cells/kg (cohort 1) 1 × 10 ⁷ cells/kg (cohort 2) Repeated dose: 1 × 10 ⁶ cells/kg (cohort 3) 3 × 10 ⁶ cells/kg (cohort 4) 1 × 10 ⁷ cells/kg (cohort 5), and 3 × 10 ⁷ cells/kg (cohort 6)	Purity: CD16 +/CD56+ cells: 98.13 ± 1.98%; CD3+ cells: 0.41 ± 0.43%; CD14+ cells: 0.40 ± 0.37%; CD19+ cells: 0.15 ± 0.25%. Fold expansion: 757.5 ± 232.2. Viability: 92.9 ± 2.1%	No GvHD nor severe toxicities. 8/20 SD, 9/20 PD, 3/20 NE. Median PFS in SD patients: 4 months (2–18 months)
Phase I (NKAML: NCT00697671) Pilot study (NKHEM: NCT00187096) Rubnitz et al. (67)	Relapsed leukemia post HSCT (<i>n</i> = 15) Refractory/relapsed leukemia (no prior HSCT) (<i>n</i> = 14)	Conditioning with Clo/Eto/Cy followed by KIR-matched or -mismatched NK-cell infusion; IL-2 therapy (6x) starting 1 day before and after NK-cell infusion	<i>Ex vivo</i> expanded PBNK cells from haploidentical donors. Culture duration: >12 h. ^a CD3 depleted and CD56 selected	Single dose: 3.5–103 × 10 ⁶ cells/kg	Median purity: 98.4% CD56+ cells. 0% CD3+ CD56– T cells. 0.31% CD19+ B-cells	Well tolerated, no GvHD. 6/29 PR, 14/29 CR, 8/29 no response, and 1/29 NE. 4/29 are alive and DFS
Phase I (EudraCT number: 2005-006087-62) Kottaridis et al. (66)	AML (<i>n</i> = 7)	Conditioning with Flu and TBI followed by haploidentical tumor primed NK-cell infusion	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: o/n with CTV-1 lysate and cryopreserved for infusion. ^a Only CD56 selected	Single dose: 1 × 10 ⁶ cells/kg	Purity: CD56+ cells 97.17% of which 80% CD56+ CD3– cells	Serious adverse reactions, no GvHD. 3/7 in CR remained in remission, 1/7 in PR achieved CR, 2/7 relapsed and 1/7 died (6 months follow-up). Median OS: 141–910 days

(Continued)

TABLE 2 | Continued

Study	Malignancy	Clinical Trial design	Culture method ^a	Infused dose NK cells	Final product characteristics	Outcome
Phase I (BB-IND-14560) Szmania et al. (65)	MM (<i>n</i> = 8)	Conditioning with Bor (+/−Cy/Flu/Dex) followed by fresh haplo-(<i>n</i> = 6) or cryopreserved auto (<i>n</i> = 2) NK cells	<i>Ex vivo</i> expanded and activated PBNK cells from haploididentical (fresh) and autologous (cryopreserved) donors. Culture: 8–9 days with K562-mb15-41BBL stimulator cells and IL-2	Single dose: 2×10^7 – 1×10^8 cells/kg	Median purity: 78% CD3– CD56+ cells. CD3+/CD56– 0.1%. Viability cryopreserved: 94%. Viability fresh: 93%. Recovery cryopreserved: 16%. Recovery fresh: 119%	Feasible and safe. 1/8 PR, 6/8 PD, 1/8 NE, and 3/8 died between days 11 and 98 after NK-cell infusion
Phase II (NCT00274846) Bachanova et al. (64)	AML (<i>n</i> = 57)	Conditioning with Cy/Flu; IL2DT in cohort 3 followed by haploididentical NK-cell infusion 1 day later; IL-2 therapy (14x, daily)	<i>Ex vivo</i> expanded and activated PBNK cells from haploididentical donors. Culture duration: o/n with IL-2. ^a CD3 depleted (cohort 1) or CD3 depleted/CD56 selected (cohort 2) or CD3/CD19 depleted (cohort 3)	Single dose: $0.96 \pm 0.3 \times 10^7$ cells/kg (cohort 1) $0.34 \pm 0.05 \times 10^7$ cells/kg (cohort 2) $2.6 \pm 1.5 \times 10^7$ cells/kg (cohort 3)	Purity: NK cells $39 \pm 9\%$, T cells: 0.7% (cohort 1) NK cells $75 \pm 6\%$, T cells: 1.3% (cohort 2) NK cells $54 \pm 16\%$, T cells: 0.3% (cohort 3)	Well tolerated, no GvHD and mild toxicities. 9/42 in remission (1.8–15 months) (cohorts 1 and 2, <i>n</i> = 42). 8/15 in remission (1–32 months) (cohort 3, <i>n</i> = 15). DFS: 5% (cohorts 1 and 2) and 33% in cohort 3
Tonn et al. (43)	Solid tumors/ sarcoma (<i>n</i> = 12) Leukemia/ lymphoma (<i>n</i> = 2)	Pretreatment with mPred following NK-92 cell infusion	<i>Ex vivo</i> expanded and activated allogeneic NK-92 cells. Culture duration: 100–300 h with IL-2. ^a No selection	Repeated doses (2 \times 48 h apart): 1×10^9 (cohort 1), 3×10^9 (cohort 2) and 1×10^6 (cohort 3) cells/m ² and additional dose level of 10^{10} cells/m ² in some patients	Viability: >80%. Fold expansion: 32	Infusion of 10^{10} NK-92 cells/m ² were well tolerated. 12/15 PD, 2/15 MR, 1/15 SD for 2 years, OS: 13–801 days
Pilot study (NCT00799799) Curti et al. (63)	AML (<i>n</i> = 13)	Conditioning with Cy/Flu followed by KIR ligand-mismatched NK-cell infusion; IL-2 therapy (3x weekly for 2 weeks)	PBNK cells from haploididentical donors. ^a CD3 depleted and CD56 selected	Single dose: $1.11\text{--}5 \times 10^6$ CD3– CD56+ cells/kg	Mean viability: 95%. Median purity: 93.5% NK cells. Maximum T-cell dose 10^5 cells/kg	Feasible and safe, no GvHD. 5/13 active disease: 1/5 CR (6 months), 4/5 died of PD. 3/6 treated in CR are DFS (34, 32, and 18 months), 2/13 in MR in CR (4 and 9 months)
Phase II (BB-IND 8847) Geller et al. (33)	Refractory metastatic breast cancer (<i>n</i> = 14) Ovarian cancer (<i>n</i> = 6)	Conditioning with Cy/Flu with or without TBI followed by allogeneic NK-cell infusion; IL-2 therapy (3x weekly for 2 weeks)	<i>Ex vivo</i> expanded and activated PBNK cells from haploididentical donors. Culture duration: o/n with IL-2	Single dose: 8.33×10^6 – 3.94×10^7 cells/kg	Viability: >70%. Median T cells: 0.11% CD3+ cells	TLS and PLS and limited infusion or IL-2 related toxicities. 1/20 died due to grade 5 toxicity. 4/20 PR, 12/20 SD, and 3/20 PD (between 31 and 109 days)
Pilot study Bachanova et al. (62)	B-cell NHL (<i>n</i> = 6)	Conditioning with Cy/Flu and mAb (rituximab, 4x) before and after haplo NK-cell infusion followed by IL-2 therapy (6x, every other day)	<i>Ex vivo</i> expanded and activated PBNK cells from haploididentical donors. Culture duration: 8–16 h with IL-2	Single dose: $21 \pm 19 \times 10^6$ NK cells/kg	Purity: $43 \pm 11\%$ NK cells. 0.16 \pm 0.12% T cells	Feasible and safe. 2/6 CR, 2/6 relapsed at 6 months, 2/6 died
Pilot study NKAML Rubnitz et al. (61)	AML (<i>n</i> = 10)	Conditioning with Cy/Flu followed by KIR-mismatched NK-cell infusion; IL-2 therapy (6x) starting 1 day before and after NK-cell infusion	PBNK cells from haploididentical donors. ^a CD3 depleted and CD56 selected	Single dose: $5\text{--}81 \times 10^6$ cells/kg	Median purity: B-cells 0.097×10^6 cells/kg. T cells 1×10^3 cells/kg	Feasible and safe. 10/10 in remission (569–1,162 days)

(Continued)

TABLE 2 | Continued

Study	Malignancy	Clinical Trial design	Culture method ^a	Infused dose NK cells	Final product characteristics	Outcome
Phase I (EudraCT number: 2005-005125-58) Iliopoulos et al. (60)	Non-SCLC (n = 16)	Haploidentical NK-cell infusion after chemotherapy	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: 21–23 days with IL-15 followed by 1 h with IL-15 and hydrocortisone. ^a Only CD56 selected	Repeated doses (2–4): 0.2–29 × 10 ⁶ cells/kg per dose	Median purity: (T cells) CD3+ CD56+ CD28– 0.12 × 10 ⁶ cells/kg. CD56+ CD3 cells 97.9% (after 20 days culture). Fold expansion: 23	Safe, no GvHD. 2/16 PR, 6/16 SD, 7/16 PD, 1/16 not treated. 1-year OS 56% (9/16), 2-year OS 19% (4/16)
Phase I Arai et al. (42)	Metastatic RCC (n = 11) or Malignant Melanoma (n = 1)	NK-92-cell infusion	<i>Ex vivo</i> expanded and activated allogeneic NK 92 cells. Culture duration: 15–17 days with or without IL-2. ^a No selection	Repeated doses (3x in cohort): 1 × 10 ⁸ (cohort 1), 3 × 10 ⁸ (cohort 2), 1 × 10 ⁹ (cohort 3), and 3 × 10 ⁹ (cohort 4) cells/m ²	Fold expansion: 200 over 15–17 days. Viability: ≥80%	Safe and feasible, mild toxicities (1 grade 4, hypoglycemia). 10/12 PD (died between day 101 and 1,059), 1/12 alive (1,450 days) and 1/12 died of bronchopneumonia (day 832)
Phase I (BB-IND 8847) Miller et al. (50)	Metastatic Melanoma (n = 10), Metastatic RCC (n = 13), Refractory HL (n = 1), and AML (n = 19)	Conditioning with low Cy/ mPred or Flu or high-Cy/Flu followed by NK-cell infusion; IL-2 therapy (14x, daily)	<i>Ex vivo</i> expanded and activated PBNK cells from haploidentical donors. Culture duration: o/n with IL-2	Escalating doses: low cy/ mPred: 1 × 10 ⁵ , 1 × 10 ⁶ , 1 × 10 ⁷ , or 2 × 10 ⁷ cells/kg (at least three per cohort). Flu or high-Cy/Flu: 2 × 10 ⁷ cells/kg	Viability: >70%. Purity: NK cells 40 ± 2%. T cells 1.75 ± 0.3 × 10 ⁵ cells/kg is 0.9 ± 0.1%. Monocytes 25 ± 1.6% and B-cells 19 ± 2%	Feasible and tolerated without toxicities. Low-Cy/mPred: 2/17 with MRCC SD for 20 and 21 months. 4/17 with MM SD for 4–9 months (n = 17) High-Cy/Flu: 5/19 AML pts in CR (n = 19)

CNS, central nervous system; MPDs, myeloproliferative disorders; LPD, lymphoproliferative disorder; MM, multiple myeloma; MDS, myelodysplastic syndromes; MDN, myelodysplastic neoplasms; MPN, myeloproliferative neoplasms; AML, acute myeloid leukemia; LBLL, lymphoblastic leukemia-lymphoma; ALL, acute lymphoblastic leukemia; NB, neuroblastoma; RMS, rhabdomyosarcoma; CML, chronic myelogenous leukemia; NHL, non-Hodgkin's lymphoma; MCL, mantle cell lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; ALCL, anaplastic large cell lymphoma; HL, Hodgkin's lymphoma; RCC, renal cell cancer; SCLC, small cell lung cancer; CLL, chronic lymphocytic leukemia; HCC, hepatocellular carcinoma; PNET, primitive neuroectodermal tumor; ACC, adrenal cortical carcinoma; EWS, Ewing sarcoma; DSRCT, desmoplastic small round cell tumor; Cy, cyclophosphamide; Flu, fludarabine; Bor, bortezomib; Dex, dexamethasone; Clo, clofarabine; Eto, etoposide; Cis, cisplatin; Pac, paclitaxel; Doc, docetaxel; Vin, vinorelbine; Gem, gemcitabine; Car, carboplatin; Pem, pemtrexed; TBI, total body irradiation; Tac, tacrolimus; Pred, prednisolone; mPred, methylprednisolone; Thy, thymoglobulin; Vin, vincristine; Adr, adriamycin; Predn, prednisone; Mel, melphalan; OKT3, muromonab-CD3; HSPC, human stem and progenitor cells; aGvHD, acute graft versus host disease; DNKI, donor NK-cell infusion; Stim, stimulated; Unstim, unstimulated; DFS, disease-free survival, CR, complete remission; PR, partial response; MR, minimal response; SD, stable disease; PD, progressive disease; aNK-DLI, donor-derived IL-15/4-1BBL activated NK-cell infusion; TRM, transplant-related mortality; MC, mononuclear cell; TLS, tumor lysis syndrome; PLS, passenger lymphocyte syndrome; NE, not evaluable.

^aCulture method displays CD3 depleted PBMC's, otherwise deviated selection method is mentioned in product characteristics.

refractory leukemia. NK cells were administered along with IL-2 injections in 29 patients, out of which 14 had not undergone HSCT (cohort I) and the other 15 have relapsed after HSCT (cohort II). In total, 90% of the NK cell donors were KIR mismatched and when the outcomes from both cohorts were combined, 14/29 were in CR, 6/29 showed PR, and 8/29 patients showed no response to the treatment (67).

The first clinical trial to adoptively transfer allogeneic NK cells derived from peripheral blood of unrelated donors without immune suppression was performed by Yang et al. In this study, allogeneic IL-2-activated NK cells (MG4101) were expanded at Green Cross Lab Cell (68) and administered to patients with advanced lymphoma and recurrent solid tumors. Following NK cell-adoptive transfer, an increase in NKG2D expression levels on CD8⁺ T cells, a reduction in the number of T-reg, and MDSCs followed by a decrease in serum levels of transforming growth factor-beta were noted. An enhanced PFS was noted in KIR-mismatched NK cell recipients. In addition, a KIR B haplotype was associated with a higher incidence of stable disease (SD). This study demonstrated that KIR-ligand mismatched donor NK cells can be safely administered without any sign of GvHD and with a GvT effect. Though an antitumor effect of the adoptively transferred NK cells could be observed, their persistence *in vivo* was shorter (between 1 and 4 days) in comparison to other clinical trials. This stresses the potential need for an effective NK cell-promoting conditioning regimen, to increase the life span and migration of NK cells in patients (69).

Shaffer et al. published results from a Phase II study in patients with relapsed or progressive AML ($n = 6$) or MDS ($n = 2$), treated with allogeneic NK-cell infusions and supported by IL-2 injections *in vivo*. NK cell donor chimerism was not detected post infusion, and no signs of GvHD were reported from this study. About 3/8 patients achieved PR of which 1/6 patients with AML and 1/2 patients with MDS achieved a CR after treatment but relapsed within 2 months. Of note, both these patients survived for 20.2 months post NK infusion, while the remaining 5 patients without response had a median survival of 5.4 months (70).

Around the same time, Curti et al. published results from a Phase I trial using KIR ligand-mismatched haploidentical NK cells to treat AML patients in CR ($n = 16$). About 7/16 patients relapsed, while 9/16 remained disease free at a median follow-up of 22.5 months. Overall, 69% (11/16) responded to therapy, with no signs of GvHD. Prolonged DFS was higher in patients with an absolute increase in the number of circulating alloreactive NK cells in this study (71).

Romee et al. investigated the antitumor effects of cytokine induced memory-like NK cells, which were adoptively transferred in relapsed or refractory AML patients after overnight activation with IL-12, IL-15, and IL-18. They were defined as memory-like NK cells based on their enhanced responsiveness upon restimulation with cytokines. NK-cell infusions were safe and well tolerated and no GvHD was reported in this study. Out of nine evaluable patients, four had CR and five showed disease responses (72).

Most recently, a first clinical trial using UCB CD34+ progenitor cell-derived NK cells was published by Dolstra et al. Allogeneic NK cells were generated from UCB CD34+ cells using an *ex vivo*

expansion and differentiation method developed by Glycostem Therapeutics (41). In this study, 10 AML patients in morphologic CR, who were ineligible for HSCT transplantation, received partially HLA-matched (5/10 KIR ligand-ligand mismatched and 7/10 KIR receptor-ligand mismatched) UCB-NK cells (oNKord®). Following Cy/Flu conditioning, lymphocytopenia was induced and found to correlate with elevated IL-15 levels, which peaked at day 6 after NK-cell infusion. Out of 10 treated patients, 5 were alive and 4 had a DFS of 60, 52, 22, and 16 months after infusion. About 2/4 patients with very poor prognosis (i.e., with detectable MRD in bone marrow before NK infusion) became MRD negative for 6 months post NK infusion, indicating a potential GvT effect. UCB-NK-cell infusions were safe and well tolerated without signs of GvHD. Interestingly, UCB-NK cells expressing low levels of KIRs and CD16a at the end of the *ex vivo* culture, underwent further maturation post-transfer *in vivo*, resulting in the upregulation of KIRs and CD16a, but continued to preserve the activated phenotype denoted by high expression of NKp30, NKp44, NKp46, NKG2D, and DNAM (73).

In addition to NK cells from PBNK and UCB-NK, two clinical studies reported on the use of the NK-92 cell line. A Phase I trial conducted by Arai et al. investigated the safety and feasibility of allogeneic NK-92 cells in advanced renal cancer and melanoma. A total of 12 patients were evaluated and 6/12 had PD, 4/12 had SD, while 1/12 had minor response and 1/12 had mixed response, 4 weeks post infusion (42). Similarly, in another study with NK-92 conducted by Tonn et al., 15 patients were included with advanced solid ($n = 13$) and hematological malignancies ($n = 2$). About 1/7 tested patients produced antibodies against the HLA antigens expressed by NK-92 cells, 1/15 showed SD, 1/15 a mixed response, and the rest of the patient group had disease progression, being treated with a maximum tolerated dose of 10^{10} cells/ m^2 . NK-92 cells had a very short persistence (48 h) *in vivo* (43). As NK-92 cells are derived from cancer (lymphoma) cells and require irradiation before infusion, which could hamper their ability to proliferate and home *in vivo*, potentially limiting their efficacy.

Overall, analyzing the data from adoptive allogeneic NK cell therapy trials in a non-transplant setting, we conclude that such treatments are very safe and well tolerated and efficacious in hematological malignancies, especially in AML, but as yet relatively ineffective in solid tumors. Trials using allogeneic NK cells alone yielded valuable information on the *in vivo* persistence, donor chimerism, and antitumor potential in different indications. Furthermore, unlike combined approaches with HSCT, the absence of life-threatening GvHD and major treatment-related toxicities makes this method advantageous and provides an opportunity to further enhance the cytotoxic effects of allogeneic NK cells.

APPROACHES TO AUGMENT NK CELL FUNCTIONS: A VIEW ON BIOTECH INDUSTRIES

As reviewed above, various clinical trials have been published, mainly initiated by academia, proposing allogeneic NK cells as an

effective therapeutic option. As a result of these studies, interest in NK cell-based immunotherapy strategies has been engendered in an increasing number of biotech companies. Clinical trials conducted in academia are often restricted to Phase I or II, as progression of experimental therapies to Phase III clinical trials and further on to commercialization and marketing requires a level of funding that surpasses the capacity of academic institutions. The financing of market enabling studies is coming mainly from industry. Although NK cells can be effective in some types of cancer as a monotherapy, considering their heterogeneity, complex networking, and the inherent adaptability of several tumors to evade killing by immune cells, one believe is that it is necessary to improve on the efficacy of currently available NK cell products. In this respect, it is worthwhile to consider combinatorial approaches of different treatment strategies involving NK cell functions. A summary of biotech companies involved in NK cell research is listed in **Table 3** and **Figure 1**. Here, we review for a selected group of NK cell companies, which develop NK cell-specific treatments, the underlying scientific principles and findings of their product pipelines, revealing highly innovative concepts that herald future clinical applications.

Fc OPTIMIZED MONOCLONAL ANTIBODIES (mAbs)

The potential of NK cells to mediate ADCC with therapeutic mAbs has been well described over the years (74). However, concerns have been voiced based on results from certain clinical trials, showing that polymorphisms in NK CD16 (V158V, V158F, and F158F) could influence the efficacy of mAb treatment and ADCC (75). To address this issue and limit the variations between different CD16 sequences, Fc glyco-engineered (defucosylated) mAbs with enhanced binding affinities to NK CD16a were developed. The Fc optimized anti-CCR4 mAb mogamulizumab (76) (Kyowa Hakko Kirin) has entered Phase III clinical testing in patients with adult T cell leukemia, emerging as the lead NK cell ADCC product to reach the market soon. Fc-optimized anti-CD20 mAbs Obinutuzumab (Genentech) (77) and Ocaratuzumab (Mentrik Biotech, LLC) (78) are currently tested in patients with chronic lymphocytic leukemia and follicular lymphoma. Similarly, the Fc-optimized anti-EGFR mAb imgatuzumab (Roche Glycart) is tested in Phase I/II clinical trials for head and neck cancer and in KRAS mutant colorectal cancer (79, 80). Although Fc-engineered mAbs address NK-mAb-binding issues, reports of serious side effects, like from the imgatuzumab study (81), have made the scientists rethink this strategy and call for the careful study of the advantages and disadvantages of this approach.

BISPECIFIC ANTIBODIES

In the last decade, several bispecific and trispecific Ab platforms, simultaneously targeting immune cells and tumor cells, have been developed in the field of cancer immunotherapy (82). To date, the majority of bispecific Abs that has been developed targets T cells, while only a limited number of bispecific approaches targets NK cells (83). Affimed is a clinical stage pharmaceutical company

developing bifunctional antibodies that recruit immune cells such as T and NK cells to tumor sites. These bispecifics (TandAbs) are tetravalent in nature, thus offering four binding sites, two aimed at tumor antigens and two aimed at immune cells. Currently, Affimed's AFM13 that targets CD30 on cancer cells and CD16a on NK cells is in clinical Phase II testing in patients with HL. In Phase I studies AFM13 was found to be safe and well tolerated and resulted in an overall response rate of 23%. Furthermore, AFM13 treatment resulted in an increase in NK cell activation and a decrease in soluble CD30 levels in peripheral blood (NCT01221571) (84). Further, two other bispecific CD16a-based tumor targeting antibodies are in preclinical phase development, i.e., AFM22 and AFM24 that bind to EGFRvIII expressed by several solid tumors, including glioblastoma (GBM), and wild-type EGFR, respectively. Another promising NK cell-focused bispecific platform is developed by AvidBiotics to target tumors that evade NK killing via downregulation or shedding of the NKG2D ligand MICA, which is a major limiting step in NK-mediated tumor targeting. To overcome this, AvidBiotics designed MicAbody proteins that bind to the NK cell NKG2D receptor with high affinity. Further, this MicAbody was engineered with an additional binding site to target tumor antigens of interest, thus enabling recruitment of NK cells to tumors (85).

NK CELL CHECKPOINT INHIBITORS

Another strategy to increase NK cell functionality is the disruption or blocking of NK inhibitory signals. Innate Pharma is a clinical stage pharmaceutical company focused on developing NK cell checkpoint inhibitors. Lirilumab (IPH2102/BMS 986015) is a fully humanized IgG₄ anti-KIR mAb against the inhibitory KIRs KIR2DL1, L2, and L3, which are expressed predominantly on NK cells and on some T cells. Lirilumab induced significant anti-tumor activity of NK cells against HLA-C-expressing tumor cells, contributing to increased survival in lirilumab-treated mice (86). Similar to KIRs, the NK cell inhibitory receptor NKG2A binds to its ligand HLA-E on tumor cells resulting in an inhibition of NK cell function. HLA-E is overexpressed in colon, cervical, and ovarian cancers, thus serving as an escape mechanism for NK killing in these tumors (87, 88). The anti-NKG2A mAb monalizumab was developed to block the interaction between NKG2A and HLA-E and is currently under clinical investigation. IPH4102, which targets KIR3DL2, is under Phase I clinical investigation in cutaneous T cell lymphoma (CTCL). Clinical trials testing lirilumab, monalizumab, and IPH4102 are listed in **Table 3**.

GENETIC MODIFICATION OF NK CELLS

In addition to successful expansion, differentiation, and demonstrable anti-tumor effects of NK cells, NK cell tumor targeting can be made more specific by employing chimeric antigen receptors (CARs) as demonstrated for T cell adoptive transfer strategies (89). CARs are recombinant Ab-based molecules that upon expression in immune effector cells bind antigens of interest on target cells, resulting in immune activation and enhanced immune effector cell survival through specific

TABLE 3 | List of biotech NK cellular therapies and NK cell function enhancing compounds.

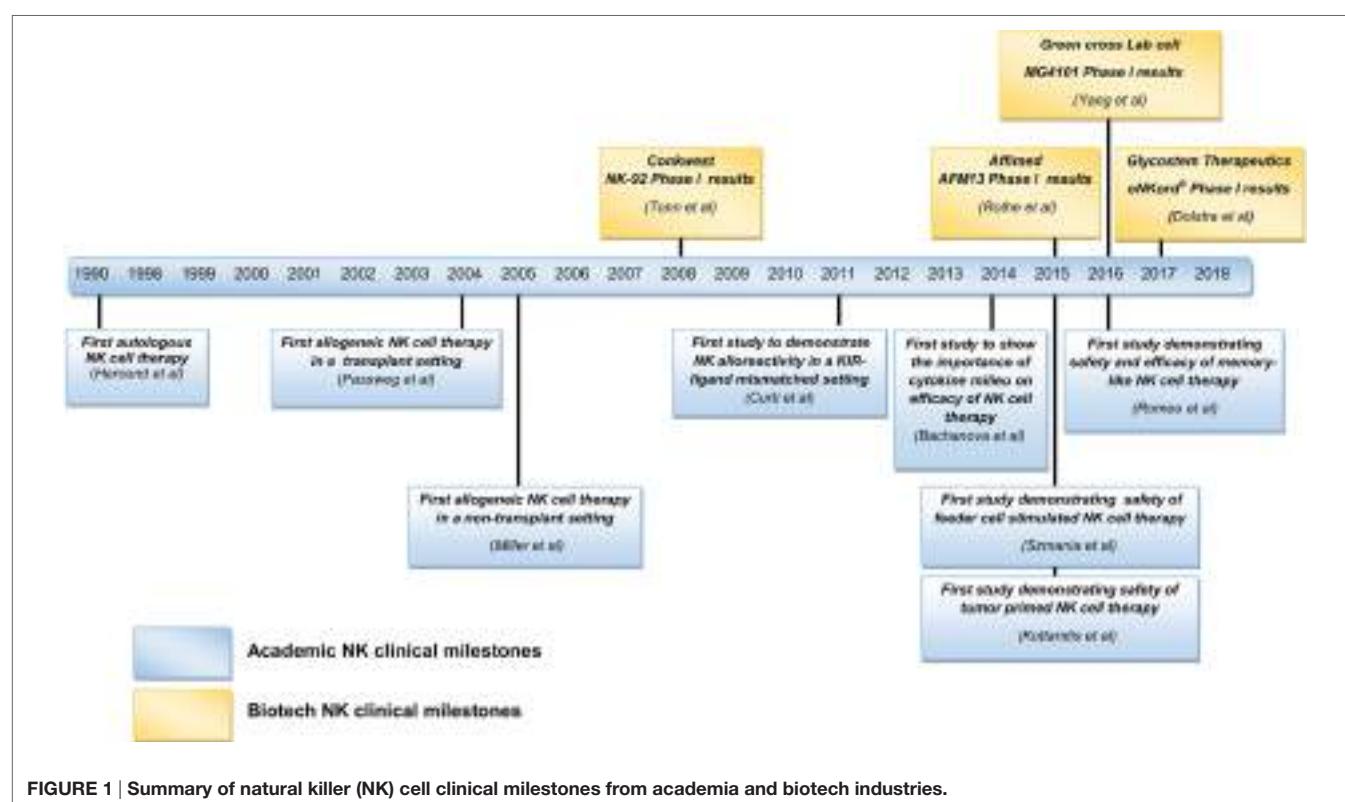
Company	NK cell product	Product characteristics	Disease target	Product stage
Fortress Biotech Inc.	CNDO-109	Tumor primed NK cells	AML	Phase I/II
Multimmune GmbH	ENKASTIM-ev	A synthetic peptide which mimics Hsp70 and activates NK cells ex vivo	Metastatic colon and non-small cell lung cancer	Phase II
Glycostem Therapeutics	oNKord®	NK cells derived from umbilical cord blood (UCB) progenitor cells	AML and solid tumors	Phase I (AML)
NantKwest Inc.	Activated NK-92 cells (aNK cell)	IL-2-dependent tumor cell-derived NK cell line	Solid tumors and hematological malignancies	Phase I
	High affinity NK cells (haNK)	aNK cells genetically modified to express CD16 for ADCC with therapeutic mAbs	Ideally in combination with IgG ₁ therapeutic mAbs in solid tumors (e.g., cetuximab) and hematological malignancies (e.g., rituximab)	Preclinical
	Target-activated NK cells (taNK)	aNK cells genetically modified to express CARs	NK-92 CARs are developed targeting tumor antigens in neuroblastoma, melanoma, breast cancer, MM and leukemias	Preclinical
Green Cross Lab Cell	MG4101	<i>Ex vivo</i> expanded NK cells derived from CD3 depleted unrelated donors	Solid tumors and lymphoma (NCT01212341)	Phase I
Gamida Cell	NAM-NK cells	Nicotinamide-based PBNK cell culture system	Solid tumors and hematological malignancies	Preclinical
Celgene Cellular Therapeutics	NK cells	NK cells derived from UCB and placenta.	Solid tumors and hematological malignancies	Preclinical
Fate Therapeutics Inc.	iNK cells	NK cells derived from induced pluripotent stem cells	Solid tumors and hematological malignancies	Preclinical
Sorrento Therapeutics Inc	CARs to enhance tumor homing of NK-92 cells	NK-92 cells CAR targeting programming death ligand-1 and NK-92 CAR targeting receptor tyrosine kinase like orphan receptor to increase NK-92 tumor homing	Solid tumors and hematological malignancies	Preclinical
Nkarta Therapeutics	NKG2D CARs	NKG2D CARs developed with NK-92 and PBNK to enhance the functions of NKG2D receptor in NK cells	Osteosarcoma and hepatocellular carcinoma	Preclinical
Ziopharm Oncology Inc.	HLA gene editing	Zinc finger nuclease technology to delete HLA-A sequences from allogeneic NK cells, allowing them to evade recipient T cell killing	Solid tumors and hematological malignancies	Preclinical
Company	NK cell enhancing products	Product characteristics	Disease target	Product stage
Kyowa Hakko Kirin	Mogamulizumab	Fc optimized anti CCR4 CD20 mAb	CTCL	Phase III
Genentech	Obinutuzumab	Fc optimized anti CD20 mAb	CLL	Phase II
Mentrik Biotech, LLC	Ocaratuzumab	Fc optimized anti CD20 mAb	CLL	Phase II
Roche Glycart	Imgatuzumab	Fc optimized anti EGFR mAb	Head and neck and KRAS mutant colorectal cancer	Phase I/II
Affimed N.V.	AFM13	Bispecific antibody binding to CD16a on NK cells and CD30 on tumor cells	Hodgkin's lymphoma and lymphomas	Phase II
	AFM22	Bispecific antibody binding to CD16a on NK cells and EGFR vIII on tumor cells	Head and neck and solid tumors	Preclinical
	AFM24	Bispecific antibody binding to CD16a on NK cells and wild type EGFR on tumor cells	EGFR-expressing solid tumors	Preclinical
Innate Pharma S. A.	Lirilumab	mAb to block NK cell inhibitory signaling from KIRs (KIR2DL1-3)	As monotherapy (Phase II, NCT02399917), with nivolumab (Phase I, NCT01592370), with ipillimumab (Phase I, NCT01750580), 5-azacytidine (Phase I, NCT02399917), with nivolumab + 5-azacytidine (Phase II, NCT02599649), with elotuzumab (NCT02252263) and with rituximab (Phase I, NCT02481297)	Phase I/II

(Continued)

TABLE 3 | Continued

Company	NK cell product	Product characteristics	Disease target	Product stage
Innate Pharma S. A.	Monalizumab	mAb to block NK cell inhibitory receptor NKG2A	As monotherapy (Phase I/II, NCT02459301, NCT02331875) with cetuximab (NCT02643550), with ibrutinib (NCT02557516) and with durvalumab (NCT02671435)	Phase I/II
	IPH4102	mAb to block NK cell inhibitory receptor KIR3DL2	As monotherapy in CTCL (NCT02593045)	Phase I
	IPH4301	mAb to target NKG2D ligands MICA/MICB and it also mediates ADCC with NK cells	Solid tumors and hematological malignancies	Preclinical
Altor Biosciences corporation	ALT-803	IL-15 super agonist reported to stably express IL-15. Increases NK cell proliferation <i>in vivo</i> , also enhances expansion of migratory NK subsets	Advanced solid tumors (NCT01946789), MM (NCT02099539), HIV patients (NCT02191098), with nivolumab in NSCLC (NCT02523469), with rituximab (NCT02384954) in B cell Non-Hodgkin Lymphoma (NHL) (NCT02384954), with (BCG) in Non-Muscle Invasive Bladder Cancer (NCT02138734), with chemotherapy drugs gemcitabine and Nab-paclitaxel in advanced pancreatic cancer (NCT02559674)	Phase I/II
NOXXON Pharma	NOX-A12	Functions as chemokine receptor CXCL12 inhibitor, enables the release of CXCL12 from the surface of tumor stromal cells, thus facilitating migration of tumor cells toward NK cells	Solid tumors and MM	Preclinical
AvidBiotics	MicAbody proteins	Dual role: binds to NKG2D receptor in NK cells and to target antigens of interest simultaneously	Solid tumors and hematological malignancies	Preclinical

KIRs, killer cell immunoglobulin-like receptors; ADCC, antibody-dependent cell mediated cytotoxicity; aNK, activated NK cells; hNK, high affinity NK cells; taNK, target activated NK cells; mAbs, monoclonal antibodies; NAM, nicotinamide; CARs, chimeric antigen receptors; EpCAM, epithelial cell adhesion molecule; AML, acute myeloid leukemia; KIR2DL1-3, killer cell immunoglobulin like receptor two domains long cytoplasmic tail 1–3; KIR3DL2, killer cell immunoglobulin like receptor three domains long cytoplasmic tail 2; MICA/MICB, MHC class-I-chain related protein A and B; HIV, human immunodeficiency virus; NSCLC, non-small-cell lung cancer; BCG, Bacillus Calmette Guerin; MM, multiple myeloma; CLL, chronic lymphocytic leukemia; CTCL, cutaneous T cell lymphoma; EGFR, epidermal growth factor receptor.

**FIGURE 1 | Summary of natural killer (NK) cell clinical milestones from academia and biotech industries.**

intracellular signaling motifs fused to the antigen binding domain [usual a single-chain Fv fragment (scFv)]. PBNK-CARs against breast cancer (HER-2), NB (CD244), and CD19 + B-cell precursor cell ALL (CD19) (90) have demonstrated efficacy in preclinical studies, while two clinical trials are ongoing using modified haplo-identical PBNK cells with anti-CD19 CARs in B cell malignancies (NCT00995137 and NCT01974479) (89). NantKwest, is actively involved in enhancing the functions of its lead product, parental NK-92 cells (activated NK cells, aNK), through gene modifications employing CARs to make them target specific. NK-92 CARs (taNK) are developed against tumor markers in NB (GD2), melanoma (GPA7) (91), breast cancer (EpCAM, HER-2, EGFR) (92, 93), MM [CS1 (94), CD138 (95)], and leukemias (CD19, CD20) (96) and have shown efficacy in preclinical studies. In an alternative approach, NK-92 cells have also been modified to express CD16a (high affinity NK cells, haNK) to promote ADCC (97). NantKwest has also partnered with Sorrento Therapeutics to develop NK-92 CARs targeting programmed death-ligand1 (PD-L1) (98) and receptor tyrosine kinase-like orphan receptor 1 (ROR-1) (99).

Besides specific targeting of tumor antigens and strategies to promote ADCC, Nkarta therapeutics developed NKG2D CARs (NKG2D-CD3 ζ -DAP10) using NK-92 cells and PBNK cells, which exhibited enhanced cytotoxicity against osteosarcoma and hepatocellular carcinoma when compared to activated and expanded PBNK cells (100, 101). mRNA-based genetic engineering has been used to enhance migration of NK cells to tumors.

Apart from gene modification, gene editing is also widely used to overexpress or knock out genes of interest to augment NK cell function. Expression of HLA-A on allogeneic NK cells leads to rejection of allogeneic NK cells by the recipient's T and NK cells. Cooper and colleagues from Ziopharm Oncology used zing finger nuclease (ZFN) technology to remove HLA-A sequences from allogeneic NK cells, thus enabling these immune effector cells to escape rejection from recipient T cells. However, in that case, there is yet a high probability of being attacked by endogenous NK cells targeting HLA-A negative allogeneic cells. This was further addressed by retaining HLA-B and HLA-C genes in donor NK cells (102–104). To increase NK cell persistence *in vivo*, scientists at oNKo-innate identified a group of proteins called suppressor of cytokine signaling (CIS, SOCS 1–7), which negatively regulate CIS pathways. SOCS1 and SOCS3 bind to JAK1, JAK2, and TYK2 molecules and inhibit JAK activity. Similarly, CIS protein binds to JAK1 and suppresses IL-15 signaling in NK cells. It became evident from *in vivo* studies in mice with *Cish*^{-/-} knockout NK cells that loss of CIS led to prolonged IL-15 signaling, resulting in an increased proliferation, survival, and functionality of NK cells (105).

NK CELLS FROM iPSCs

In recent years, NK cells generated from induced pluripotent stem cells (iPSC-NK) and human embryonic stem cells (hESC-NK) have been gaining more interest as an NK cell therapeutic product. Fate Therapeutics developed a platform technology to generate NK cells from iPSC. hESC/iPSC were made into aggregates by centrifugation to form so-called embryoid bodies (spin

EBs) (106), giving rise to hematopoietic progenitor cells expressing CD34 and CD45, which were then differentiated into mature NK cells using a specific cytokine cocktail. iPSC/hESC-derived NK cells were shown to express common NK cell markers, such as KIRs, CD16, NKp44, NKp46, NKG2D, and TRAIL, and were cytotoxic against several hematological and solid tumor cells *in vitro* (107, 108). In the next stage, iPSC/hESC-derived NK cells were successfully expanded using IL-2 and K562-based aAPCs with membrane-bound IL-21 to generate sufficiently high numbers for clinical applications (109).

NK CELLS FROM HUMAN UCB CELLS

Stem cell progenitors from cord blood offer a unique platform to be expanded and differentiated into cytotoxic NK cells. The low immunogenicity of cord blood cells strongly reduces the risk of relapse and GvHD after transplantation (110). Considering the advantages of using cord blood, Glycostem Therapeutics, a clinical stage biotech company, which in the last decade has developed a flexible platform technology to expand and differentiate NK cells from CD34+ cells (111), upgraded this into a large scale GMP UCB-NK platform for clinical implementation (oNKord[®]) (41). UCB-NK cells were infused at up to 30×10^6 cells/kg/bodyweight in elderly AML patients, resulting in excellent safety and initial efficacy in a Phase I trial. Infused oNKord[®] cells showed active migration to the marrow and further matured in the absence of any exogenous cytokine injections. This confirms previous findings from a preclinical model, showing migration to the bone marrow and upregulation of KIRs and CD16a *in vivo* as well as antileukemic activity (112). oNKord[®] is well characterized and was found to have a similar functionality and gene expression profile as PBNK cells (113). Furthermore, oNKord[®] is highly cytotoxic against solid tumor targets such as cervical cancer cells, in which killing was independent of HLA expression levels, tumor histology and HPV types (114), or colorectal cancer cells, in which killing was independent of tumor EGFR levels, and RAS and RAF mutations (115), thus paving the way for oNKord[®] as immunotherapy for advanced solid tumors.

CYTOKINES TO ENHANCE NK CELL FUNCTIONS

To improve the antitumor activity of autologous NK cells, systemic administration of clinical grade recombinant IL-2 (rIL-2) and single chain IL-15 (scIL-15) has been used in high doses and this has resulted in severe grade 3/4 toxicities (116–118). Since then, their safety and efficacy have been tested in low doses following NK cell-adoptive transfer in cancer patients (50, 63, 119). However, IL-2 resulted in expansion and mobilization of inhibitory T-reg, severely limiting NK cell cytotoxicity (120). This shifted the focus toward the use of IL-15 for clinical trials involving NK cells. Currently more potent and advanced heterodimeric IL-15, which has a longer shelf life than scIL-15, is being tested in several studies (121). IL-15 is known to be more effective in membrane-bound form (i.e., bound to its receptor),

engaging target immune cells in a cell contact dependent manner. Campana and his team (from Nkarta Therapeutics) addressed this by stably transducing the membrane bound IL-15 (mbIL-15) gene into proliferating PBNK cells, which were stimulated with K562-mb15-41BBL. mbIL-15 resulted in increased survival, proliferation, and enhanced cytotoxic functions of NK cells (122). Further, Cyto-Sen Therapeutics compared mbIL-15 to K562-based aAPCs with mbIL-21. From their findings, it was evident that mbIL-21 NK cells have a significantly higher expansion and proliferation ability compared to mbIL-15 NK cells (123). Cyto-Sen also developed plasma membrane particles (PM21) engineered from K562-mb21-41BBL cells and found that these PM21 particles stimulated efficient NK cell expansion in AML patient's PBMC samples (124).

Compared to IL-2, the use of IL-15 minimizes capillary leak syndromes and has less side effects overall, thus providing a strong rationale to use IL-15 instead of IL-2. However, the use of mammalian recombinant IL-15 in the clinic has been limited due to its short half-life and decreased functional activity *in vivo*. Altor BioSciences corporation came up with a unique design to overcome these limitations. It developed an IL-15 super agonist known as ALT-803. It consists of a human IL-15 mutant N72D variant, which is stably complexed with a soluble human IL-15R α

sushi-Fc dimer protein. Enhanced biological activity of ALT-803 was reported in several preclinical studies showing durable anti-tumor activity in various solid and hematological malignancies (125–128). Furthermore, ALT-803 facilitated expansion of effector and migratory NK cell subsets and significantly decreased the metastatic activity of tumor cells in a murine colon cancer pulmonary metastasis model (129). ALT-803 stimulated primary human NK cells to exhibit increased degranulation, IFN γ production, and ADCC when exposed to B cell lymphoma cell lines coated with IgG $_1$ therapeutic anti-CD20 mAbs (130). Several clinical trials are currently ongoing with ALT-803 as monotherapy in patients with advanced solid tumors, hematological malignancies, and AIDS as summarized in **Table 3**.

PRIMING NK CELLS TO ENHANCE TUMOR KILLING

Mark Lowdell and his team proposed that for a NK cell to be able to kill tumor cells, it requires a priming and triggering signal. NK cells failing to kill tumor cells, though they are exposed to the triggering signal, remain inactive due to the absence of a priming ligand. To address this, Fortress Biotech (previously known

Strategies to augment NK cell functions

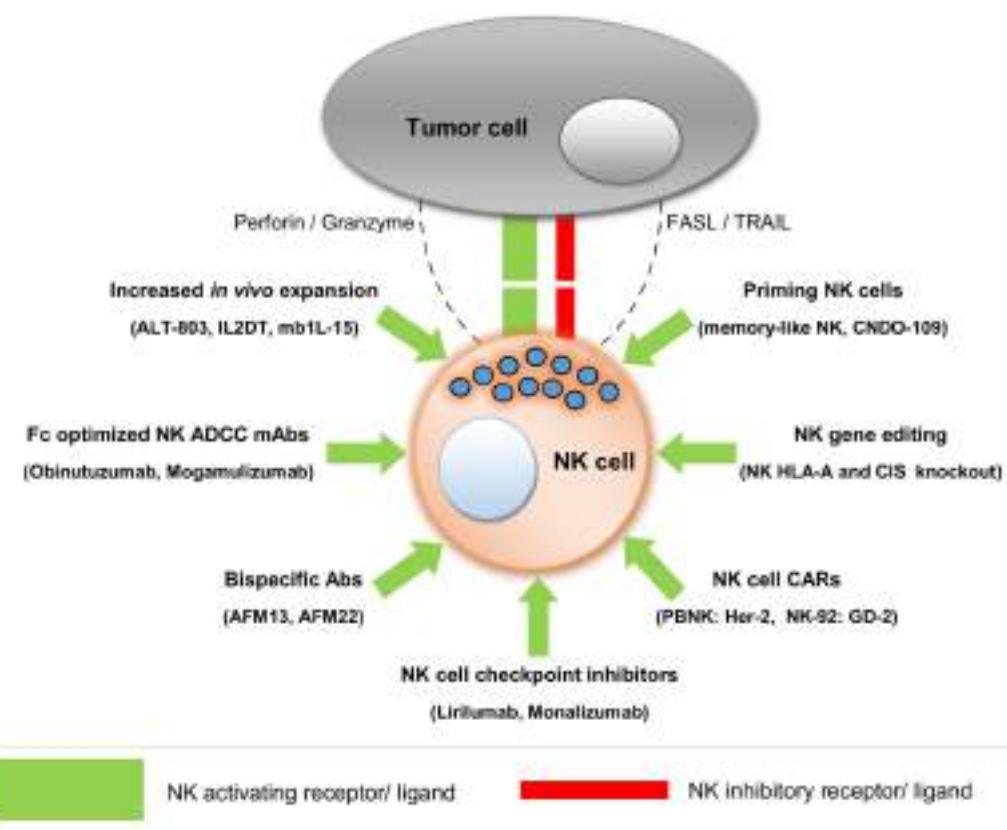


FIGURE 2 | Strategies to augment natural killer (NK) cell functions.

as Coronado Biosciences) developed a technology to increase NK cell tumor killing using cell lysates from the leukemia cell line CTV-1, known as CNDO-109, to prime NK cells. A Phase I/II clinical trial of activated PBNK cells from haploidentical donors coincubated with CNDO-109, infused at doses of up to 3×10^6 kg/recipient/body weight was tolerable without any adverse reactions. Out of seven evaluable patients, four remained disease relapse free for more than 1 year (131).

Another NK cell-activating product is ENKASTIM-ev, developed by Multimmune GmbH, which mimics the functions of heat shock protein 70 (Hsp70). ENKASTIM-ev resulted in NK specific activation and actively targeted Hsp70 expressing tumors. Safety of Hsp70-activated autologous NK cells has been documented in a Phase I study in patients with metastatic colorectal and non-small cell lung cancer (132).

ENHANCING NK CELL HOMING FUNCTIONS

Gamida-cell developed a feeder cell-free NK cell culture and expansion system containing nicotinamide (NAM) to generate NK cells from PBMC apheresis products. Nicotinamide, a derivative of vitamin B3, serves as a potent inhibitor of NAD dependent enzymes. Results from *in vivo* studies in mice showed that PBNK cells expanded with NAM in feeder free cultures exhibited increased homing potential toward lymphoid organs, with a significant increase in the expression of CD62L (L-selectin) compared to cultures without NAM (133).

TUMOR DISRUPTIVE TECHNOLOGY AIDING NK TUMOR RECOGNITION

NOXXON Pharma target chemokine receptor CXCL12, with the aim of increasing the sensitivity of tumor cells to drugs and immune cells. Their product NOX-A12 functions as a CXCL12 inhibitor and enabled the release of CXCL12 from the surface of tumor stromal cells and blocked its interaction with cell surface receptors CXCR4 and CXCR7. This mechanism facilitated the mobilization of CXCR4-expressing tumor cells from their tissue niches to areas, where they become easily accessible by NK cells or T cells (134, 135). Using tumor spheroids, increased mobilization of T and NK cells toward tumor cells in the tumor microenvironment was demonstrated. NOX-A12 also enhanced NK killing of obinutuzumab-coated Raji cells *in vitro*, mediated by ADCC (136).

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CONCLUSION

From this literature review, we conclude that adoptive transfer of allogeneic NK cells in a non-transplant setting is safe and shows early signs of clinical efficacy against hematological and certain solid tumors. Current data are mostly based on Phase I clinical trials, and hence it is still too early to get an overall picture of NK cell alloreactivity in different kinds of cancer. Most of the clinical studies conducted so far have used primary NK cells but with limited efficacy, pointing to the need to improve the functionality of these NK cells after their transfer to patients. The growing opportunities to augment NK cell functions have attracted several biotech companies to invest in NK cell research, spearheading NK therapy development with different innovative approaches. This review also stresses the need for combining adoptive transfer of allogeneic NK cells with NK function-augmenting products to achieve a maximum anti-tumor effect. As NK cells are safe to infuse, the use of CAR-NK cells may be instrumental in providing a much safer but still very effective platform, to bring CAR-based therapies to broader clinical applications. It may also facilitate effective tumor targeting of NK cells. oNKord® and iPSC-derived NK cells could serve as alternative allogeneic platforms to develop CAR-NK products, besides NK cell lines. In a solid tumor setting, NK cells are challenged by several factors that affect their homing and penetration into the tumor tissues. Moreover, they should achieve and maintain an activated effector state, even in the face of immune suppressive conditions, that are prevalent in patients with cancer. To overcome these bottlenecks in NK therapy of solid tumors, a plethora of creative solutions are being pursued by numerous research labs as well as by biotech companies in clinical or close to clinical phase. Strategies to enhance NK cell functions from leading NK cell products are summarized in Figure 2. With all these exciting developments, NK cells are set to make a considerable impact on the future treatment of patients with hematological as well as with solid tumors.

AUTHOR CONTRIBUTIONS

JV, NK, HVV, TG, and JS wrote the paper. HVV, TG, HV, and JS reviewed the paper.

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Umbilical Cord Blood Natural Killer Cells, Their Characteristics, and Potential Clinical Applications

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Natural killer (NK) cells are lymphocytes of the innate immune system able to kill different targets such as cancer cells and virally infected cells without prior activation making them attractive candidates for cancer immunotherapy. Umbilical cord blood (UCB) has become a source of hematopoietic stem cells for transplantation but as we gain a better understanding of the characteristics of each immune cell that UCB contains, we will also be able to develop new cell therapies for cancer. In this review, we present what is currently known of the phenotype and functions of UCB NK cells and how these cells could be used in the future for cancer immunotherapy.

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INTRODUCTION

Natural killer (NK) cells are lymphocytes of the innate immune system that exhibit cytotoxicity toward cancer cells and virus-infected cells and have the capacity to produce cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) in response to stimuli. NK cells are defined as CD56⁺CD3⁻ cells and can be divided into two main subsets according to their expression of CD56 and CD16. CD56^{dim}CD16⁺ NK cells (CD56^{dim} NK cells) are cytotoxic NK cells capable to mediate direct killing of target cells via exocytosis of granules containing granzyme B and perforin, activation of cell death pathways such as TRAIL or FAS/FAS-L or via antibody-dependent cellular cytotoxicity. CD56^{bright}CD16^{-/low} NK cells (CD56^{bright} NK cells) are the main cytokine-producing NK cells (1). In peripheral blood (PB), up to 90% of NK cells are CD56^{dim} NK cells while most NK cells are CD56^{bright} NK cells in lymph nodes.

Natural killer cell functions are regulated by signals delivered through activating and inhibitory receptors. As opposite to T cells, NK cells are “ready to go” and can eliminate target cells without prior stimulation. However, stimulation of NK cells by cytokines leads to NK cell activation and enhanced functions, in particular enhanced cytolytic activity and proliferation. NK cells have long been considered potential candidates for cancer immunotherapy and their versatility makes them attractive cells to explore. Phase I clinical trials showed autologous NK cell therapies to be feasible and safe without adverse effects in patients with breast cancer or non-Hodgkin’s lymphoma; however, these therapies had no or little impact on relapse rates (2). The potential impact of NK cell alloreactivity in hematopoietic stem cell transplantation (HSCT) was suggested by Valiante and Parham (3). The first evidence that allogeneic NK cells could exert strong anti-leukemic activity and impact on the outcome of haploidentical transplantation stems from the study of Ruggeri et al. (4) who reported NK cell alloreactivity against leukemic cells while reducing the risk of graft-versus-host disease (GvHD) in the context of human leukocyte antigen (HLA) mismatch settings. Other trials

have showed that allogeneic NK cells alone can target different types of cancers such as acute myeloid leukemia (AML), melanoma, renal cell carcinoma, Hodgkin lymphoma (5), breast and ovarian cancer (6), or refractory lymphoma (7). The same group has shown the importance of NK cell expansion *in vivo*, which can be accomplished by infusion of interleukin (IL)-2. However, regulatory T cells were also found to compete for this cytokine and beneficial effects on NK cell expansion were observed when regulatory T cells could be depleted (8). Interestingly, other studies also have indicated that NK cell therapy could also be of interest to treat glioma (9) or neuroblastoma (10).

Umbilical cord blood (UCB) has become an established source of hematopoietic stem cells (11) for transplantation. Advantages for the use of UCB include low risk of viral transmission from donor to recipient, rapid availability of UCB units serving as an immediate “off-the-shelf” product, less stringent requirements for HLA matching, and lower risk of GvHD. However, UCB contains between 10- and 100-fold fewer nucleated cells than other sources of HSC, limiting how many cells of interest can be retrieved from one UCB unit. Interestingly, NK cells are the first lymphocytes to recover after HSCT including after umbilical cord blood transplantation (UCBT) (12). In addition, NK cells are key effectors of the graft-versus-leukemia (GvL) effect. Especially after UCBT, as T cell immune reconstitution is delayed and there is no increased incidence of relapse, it is likely that NK cells are actually the main effectors of the GvL effect in the first year post-UCBT. However, UCB also contains different types of immune cells including NK cells and as we learn more about their specific characteristics, we will identify the conditions which might benefit of an UCB NK cell therapy. This review focuses on providing an overview of the characteristics of UCB NK cells compared to NK cells from PB and explain how they could be used as a cell therapy to cancer.

CHARACTERISTICS OF UCB NK CELLS

Natural killer cells constitute up to 10% of lymphocytes in PB and up to 30% in UCB (13, 14), and both CD56^{dimm} NK cells and CD56^{bright} NK cells can be found in PB and UCB with some groups reporting similar proportions of both subsets or higher frequency of CD56^{bright} NK cells in UCB (14–16). Regarding the phenotype and functions of UCB NK cells, some groups have identified differences when compared to PB NK cells while others found them to be similar to PB NK cells (17) (Figure 1).

Advantages of UCB-Derived NK Cells

Aside from the higher percentage of NK cells present in UCB, the ability to cryopreserve UCB together with the ease of collecting UCB units offers a unique clinical advantage of making UCB an off-the-shelf source for NK cell immunotherapy. Moreover, a more rapid recovery of NK cells was reported after UCBT than PB HSCT (18, 19). This faster recovery could be explained by the fact that UCB contains different NK cell progenitor populations that have the capacity to differentiate into NK cells and are typically absent in PB (20–22). Further, PB and UCB NK cells produced similar amounts of IFN- γ and TNF- α in response to different stimuli (14, 23) and could proliferate in response to cytokines such as IL-2 or IL-15 (14, 16, 24) despite UCB NK cells exhibiting lower expression of the IL-2 receptor subunits and lower phosphorylation of STAT5 (25). Additionally, UCB NK cells have also been described to have a higher expression of the bone marrow homing receptor, CXCR4, compared to PB NK cells indicating that UCB NK cells may contain a greater potential to home to the bone marrow (14). Finally, IL-15 activated UCB NK cells have been reported to impact positively on UCB HSC engraftment by enhancing their migration and clonogenic capacity, and their engraftment in humanized animal model (26).

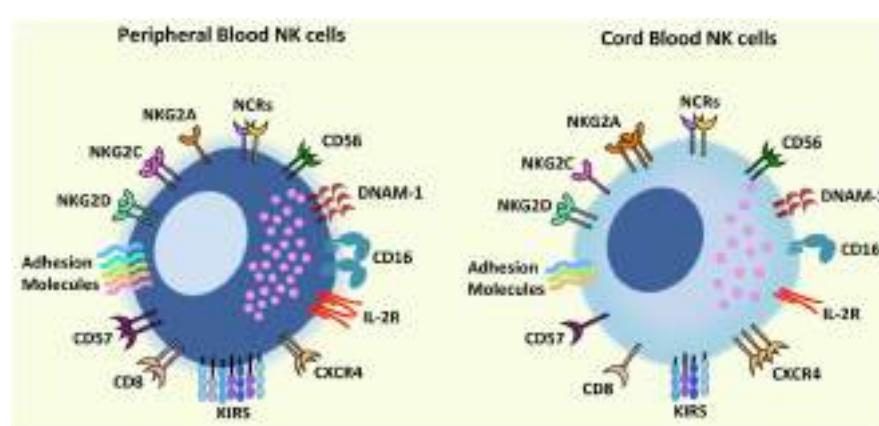


FIGURE 1 | Comparison of phenotypic characteristics between umbilical cord blood (UCB) natural killer (NK) cells and peripheral blood (PB) NK cells. In comparison to PB NK cells, UCB NK cells exhibit similar levels of CD56, NCRs (Nkp46 and Nkp30), and NKG2D but a lower expression of CD16, adhesion molecules (e.g., CD2, CD11a, CD18, CD62L), KIRs, DNAM-1, NKG2C, IL-2R, and CD57 and CD8 (receptors associated with terminal NK cell maturation) together with a higher expression of inhibitory receptor NKG2A indicating that UCB NK cells possess an immature phenotype and reduced cytotoxicity compared to PB NK cells. Further UCB NK cells have a higher expression of the bone marrow homing receptor, CXCR4, compared to PB NK cells proposing that cord blood NK cells may contain a greater potential to home to the bone marrow. Abbreviations: KIRs, killer-cell immunoglobulin-like receptors; NCRs, natural cytotoxicity receptors.

Drawbacks of UCB-Derived NK Cells

The use of cord blood (CB) as a source of NK cells for immunotherapy, however, is also limited as a result of the low numbers and immaturity of CB NK cells. Although, UCB NK cells have been reported to be fully mature and functional (16, 27), some groups found them to have an immature phenotype (14, 28), exhibiting normal levels of degranulation but lower cytotoxicity against K562 cells as compared to PB NK cells (14, 23). This lower activity could be explained by the fact that UCB NK cells have decreased expression of certain adhesion molecules on their surface such as CD2, CD11a, CD18, and CD62L (15, 16), decreased expression of CD16 (15), decreased expression of perforin and granzyme B (14, 23), and lower killer-cell immunoglobulin-like receptors (KIRs) expression together with a higher expression of inhibitory molecules such as NKG2A when compared to PB NK cells indicating an immature phenotype (14, 23). However, activation with cytokines such as IL-2 or IL-15 or the combination of IL-15 with IL-2 or IL-18 was able to restore or enhance their cytotoxicity to the levels observed for PB NK cells (14, 16, 23, 25, 29). Moreover, although the frequencies of NK cells present in UCB is greater than PB (14), low numbers of UCB NK cells are obtained as a result of the limited volume of an UCB unit, which is a major obstacle in obtaining sufficient numbers of NK cells for clinical application. However, different strategies to increase NK cell doses have been developed.

EXPANSION OF UCB NK CELLS

A number of studies have recently explored different platforms to expand UCB NK cells. Increased NK cell numbers can be achieved either by large-scale expansion techniques using artificial antigen-presenting cell (aAPC) or cytokines including IL-2, IL-15, and/or FLT-3 ligand. One such strategy employed to expand purified UCB-derived NK cells on a large scale has been reported using good manufacturing practice (GMP)-grade K562-based aAPCs expressing membrane-bound IL-21 (30). Shah and colleagues have shown that following 14 days of culture in a gas permeable culture system, a 2,389-mean fold expansion of NK cells derived from frozen UCB was achieved. The expanded NK cells presented >95% purity of CD56⁺CD3⁻ NK cells and displayed efficient killing capacity against multiple myeloma *in vitro* and *in vivo*, highlighting the use of aAPCs as an attractive approach to generate large numbers of functionally competent UCB NK cells. A further strategy to evaluate the potential use of expanded NK cells was reported by using aAPCs in the form of genetically modified K562 cells expressing membrane-bound IL-15 and 41BBL (31). The aAPCs were cultured with CB mononuclear cells for 7 days, which led to the generation of expanded UCB NK cells that displayed increased expression of NK cell activating receptors, increased perforin and granzyme expression, and increased cytotoxicity against B-cell non-Hodgkin lymphoma *in vitro* and *in vivo*. The study merits the use of expanded NK cells for adoptive cellular therapy specifically to target relapse or refractory disease after UCBT. Finally, the use of irradiated Epstein–Barr virus-transformed lymphoblastoid cell lines and IL-2 was also recently reported to generate large numbers of CD56⁺ NK cells derived frozen UCB (32). The

generated NK cells exhibited higher levels of cytotoxicity against K562 leukemic cells than expanded PB-derived NK cells (32). The unique advantage of this platform is that only 1 ml of the UCB unit is selectively used to generate expanded NK cells for adoptive therapy and the remaining UCB from the same unit can be cryopreserved and used for future transplantation. It would be interesting to assess whether the use of the same UCB for early NK cell adoptive therapy and transplantation can help to prevent relapse and augment GvL post-UCBT.

DIFFERENTIATION OF NK CELLS FROM UCB CD34⁺ CELLS

Natural killer cells can be directly isolated from PB and UCB but an alternative to these cell sources is the differentiation of NK cells from HSC as a way to generate high numbers of cells (33). NK cells can be differentiated from CD34⁺ cells from the bone marrow, from embryonic stem cells, mobilized PB, or UCB CD34⁺ cells. The expansion of NK cells derived from both fresh and frozen UCB CD34⁺ cells using a cocktail of cytokines in a culture system has also been described as an efficient system to generate large numbers of NK cells. We and others have reported the characteristics of NK cells produced *in vitro* from UCB CD34⁺ cells (34–36). These cells are mostly similar to PB NK cells with the exception that they express low levels of inhibitory receptors. However, NK cells produced in such a way have been shown to be functional, able to kill leukemic cell lines and patient cells *in vitro* and *in vivo* and produce cytokines in response to diverse stimuli (34, 36–38). Interestingly, NK cells produced *in vitro* have been shown to expand to high numbers while preserving their phenotype and functions after cryopreservation (39). Thus, frozen UCB CD34⁺ cells were found to be the best source of NK cells when compared to fresh UCB-derived CD34⁺ cells and frozen PB CD34⁺ cells and could therefore be a readily available off-the-shelf product for NK cell immunotherapy.

NK Cells Alloreactivity in UCBT Setting

Umbilical cord blood NK cells express both inhibitory and activating receptors, which are highly important in mediating self-tolerance or NK cell activity (40). Inhibitory receptors are part of the immunoglobulin superfamily including the KIRs, the immunoglobulin-like transcripts, and C-type lectin receptors CD94/NKG2A. Inhibitory receptors recognize the classical MHC class I molecules on target cells and inhibit NK cell lysis (41). Most KIRs are inhibitory receptors but a limited number of KIRs also function as activating receptors; however, the function and ligands of the later are less well understood. Since KIR genes are not on the same chromosome as HLA, these genes are inherited independently. This allows for donor and recipient HLA-matched UCBT and mismatching between KIRs and their ligands, maintaining the appropriate matching required for HSCT but providing NK cell alloreactivity, which triggers NK cell activation leading to tumor cell lysis (42). This phenomenon of NK cell alloreactivity was proposed as beneficial in reducing relapse after HSCT; however, variable results have been reported

from different studies (4, 43–47). In UCBT setting, only few studies have evaluated the outcome of UCBT using mismatched KIR and its ligands (48–51) with only some of them reporting beneficial results (52, 53). KIR haplotype has also been shown to influence the outcome of HSCT. In this context, the higher the number of activating KIR a donor has the higher NK cell alloreactivity might be. Some studies have reported the beneficial effect of the donor B haplotype that contains more activating gene than a A haplotype on HSCT outcome in particular showing a lower incidence of relapse for patients with AML or lower GvHD incidence depending on the study considered (47, 54–56). Whether KIR haplotype can also influence UCBT outcome needs to be investigated.

Finally, NK cell licensing (57), arming/disarming (58), or education (59) is another factor to be considered. NK cells can express one or more inhibitory receptors recognizing HLA molecules. The process by which NK cells become functional and tolerant to self-HLA can be referred to as NK cell licensing and is defined by the fact that to be functional NK cells must express inhibitory receptors recognizing self-HLA. This concept has been well studied in mice and there are now also evidence in humans (59, 60). However, it has been reported that unlicensed NK cells are able to mount an immune response against cytomegalovirus in mice (61) and can kill neuroblastoma cells in humans (10). Therefore, moving forward it will be essential to gain a better understanding of the impact of NK cell licensing on their functions especially in the context of HSCT including UCBT.

CURRENT CLINICAL STUDIES INVOLVING UCB NK CELLS

Natural killer cells can be isolated from UCB based on CD56 purification methods. One step isolation method can be used in UCB as opposite to PB where two steps are needed in order to eliminate NKT cells. This is not necessary when considering UCB as it contains a very low percentage of that cell subset. In addition, UCB has the advantage of being readily available as UCB is cryopreserved and can be obtained from accredited UCB banks.

Therefore, a NK cell product derived from UCB has the potential to be off-the-shelf. Another advantage of UCB is that HLA is less stringent, although it is not clear what level of matching will be necessary to develop a third party NK cell product from UCB. However, because of the limited volume of blood collected from the umbilical cord there are only a limited number of NK cells that can be isolated from UCB. In addition, as they are immature and have lower functionality as compared to PB NK cells; taking UCB NK cells to the clinics will require a prior activation/expansion step. Several clinical trials are currently ongoing to evaluate the safety and feasibility of UCB NK cells as an “off the shelf product” in transplant and non-transplant settings (Table 1). GMP grade expansion methods for UCB NK cells are currently available as previously described. Notably, only a handful of clinical trials are currently ongoing and recruiting patients using the latest method to expand UCB NK cells to reach the cell dose required. Two clinical phase I studies aim to use expanded UCB NK cells for the treatment of patients with chronic lymphocytic leukemia (NCT01619761, NCT02280525), while another aims to evaluate NK cell therapy in the context of autologous HSCT for patients with myeloma (NCT01729091).

Only a few groups have focused on developing cell therapy approaches based on the differentiation of NK cells from HSC *in vitro*. However, NK cells produced *in vitro* have been shown to be safe and their use feasible when considered in the context of allogeneic HSCT (62). In addition, another trial, oNKord®, is currently ongoing testing the use of NK cells produced *in vitro* from UCB CD34⁺ cells in patients with AML (EudraCT number 2010-018988-41).

CONCLUDING REMARKS

Immunotherapy is a promising treatment for different types of cancer allowing the possibility of personalized medicine for each cancer patient. UCB provides distinct advantages and is an increasingly attractive source for HSCT and cellular therapy. Despite low NK cell numbers within a single UCB unit and their immature phenotype, strategies to expand UCB NK cells using aAPCs or cytokines and feeder cells are

TABLE 1 | UCB NK cells currently in the clinic.

Clinical trial identifier	Diseases	Trial phase	Type of transplant	Conditioning	Method of expansion	Sponsor
NCT01619761	ALL, AML, CLL, CML, HL, MDS, MM, NHL, SLL	I	Double umbilical cord blood transplantation	Fludarabin, melphalan, lenalidomide ± rituximab	<i>Ex vivo</i> expansion of NK cells from 20% UCB unit fraction	MD Anderson Cancer Center
NCT02280525	CLL, ALL, AML, CML, NHL, HL	I	Non-HSCT	Fludarabin, cyclophosphamide, lenalidomide, and rituximab	<i>Ex vivo</i> expansion of NK cells from thawed from UCB unit	MD Anderson Cancer Center
NCT01729091	MM	I/II	Autologous	Melphalan, lenalidomide	<i>Ex vivo</i> expansion of NK cells from thawed from UCB unit	MD Anderson Cancer Center
EudraCT number 2010-018988-41	AML	I	Non-HSCT	Fludarabin, cyclophosphamide	NK cells generated <i>in vitro</i> from UCB progenitor cells	Radboud Medical Centre, Nijmegen, Netherlands

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CB, cord blood; CLL, chronic lymphoblastic leukemia; CML, chronic myeloid leukemia; HL, Hodgkin lymphoma; HSCT, hematopoietic stem cell transplantation; MDS, myelodysplastic syndromes; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NK, natural killer; SLL, small lymphocytic lymphoma; UCB, umbilical cord blood.

paving the way for NK cell adoptive immunotherapy. NK cells have shown great potential in eliminating different types of cancer cells *in vitro* and in animal models. A few clinical trials are currently underway to evaluate the safety and feasibility of using UCB NK cells as an “off the shelf” product for the prevention of relapse. The results from these studies will help in understanding how to maximize the beneficial potential of UCB NK cells for the treatment of hematological malignancies and solid tumors.

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AUTHOR CONTRIBUTIONS

All the authors contributed to writing and reviewing the manuscript.

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Redirected Primary Human Chimeric Antigen Receptor Natural Killer Cells As an “Off-the-Shelf Immunotherapy” for Improvement in Cancer Treatment

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Primary human natural killer (NK) cells recognize and subsequently eliminate virus infected cells, tumor cells, or other aberrant cells. However, cancer cells are able to develop tumor immune escape mechanisms to undermine this immune control. To overcome this obstacle, NK cells can be genetically modified to express chimeric antigen receptors (CARs) in order to improve specific recognition of cancer surface markers (e.g., CD19, CD20, and ErbB2). After target recognition, intracellular CAR domain signaling (CD3ζ, CD28, 4-1BB, and 2B4) leads to activation of PI3K or DNAX proteins (DAP10, DAP12) and finally to enhanced cytotoxicity, proliferation, and/or interferon γ release. This mini-review summarizes both the first preclinical trials with CAR-engineered primary human NK cells and the translational implications for “off-the-shelf immunotherapy” in cancer treatment. Signal transduction in NK cells as well as optimization of CAR signaling will be described, becoming more and more a focal point of interest in addition to redirected T cells. Finally, strategies to overcome off-target effects will be discussed in order to improve future clinical trials and to avoid attacking healthy tissues.

Keywords: natural killer cells, chimeric antigen receptor, chimeric antigen receptor-associated signaling domain, intracellular chimeric antigen receptor-dependent signaling, cancer immunotherapy

INTRODUCTION

Natural killer (NK) cells are peripheral blood lymphocytes that mediate immune surveillance in regard to virus infected and malignant cells (1–3). For early disease detection and killing NK cells rely on several mechanisms such as inflammatory cytokine secretion [e.g., interferon gamma (IFNγ), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10)], receptor ligand binding (e.g., tumor necrosis factor-related apoptosis inducing ligand, Fas ligand) (4), or release of cytoplasmic granule toxins (e.g., perforin, granzyme A, granzyme B, and granzylsin) (5, 6) as a result of antibody-dependent cellular cytotoxicity (ADCC) (7).

Recognition of aberrant and stressed cells occurs by means of activating cell surface receptors including natural killer group 2 member D (NKG2D) (CD314), NKp30 (CD337), NKp46 (CD335), and NKp44 (CD336) (8), receptor complex CD94/NKG2C (9), or FCγRIII (CD16) for ADCC (10–12). The counterpart of these activating complexes comprises various inhibitory receptors that

usually bind to a variety of different major histocompatibility complex I (MHC I) molecules. Examples for these receptors are several receptors of the killer cell Ig-like receptors (KIRs) family (CD158), NKG2A that pairs with CD94 to a heterodimer (binding the non-classical MHC molecule HLA-E), leukocyte immunoglobulin-like receptor (LILR), natural killer cell receptor protein 1 (CD161), sialic acid-binding immunoglobulin-like lectin-7 (CD328), leukocyte-associated Ig-like receptor 1 (LAIR-1; CD305), killer cell lectin-like receptor G1, carcinoembryonic antigen-related cell adhesion molecule (CD66a), paired immunoglobulin-like receptor α , and CD300a. Each NK cell expresses individually a composition of inhibitory and activating receptors (9). In the resting state, NK cells are in balance receiving signals from activating and inhibitory ligands and no signaling pathway dominates. After adaption to self-MHC I environment, NK cells respond to ligands for activating receptors resulting in killing of malignant cells. Presence of self-MHC I demonstrates inhibitory response. Contrarily, lack of constitutive self-MHC I enhances elimination of aberrant cells (13). At least, NK cell activation by ligand receptor interaction sum up signals received from inhibitory and activation receptors, which cumulates to release perforin and granzymes (cytotoxicity) as well as cytokine production (e.g., IFN γ and TNF- α) mediated by adaptor proteins (DNAX activation proteins DAP10 and DAP12, CD3 ζ). These peptides contain immunoreceptor tyrosine-based activation motifs (ITAMs) that become phosphorylated by Src kinase family members and result in at least cytotoxicity and cytokine production.

However, tumors can develop tumor immune escape mechanisms to protect themselves from NK cell attack, e.g., by matrix metalloproteinase-dependent proteolytic cleavage of MHC class I polypeptide-related sequence A and B (MICA and MICB) (14). These soluble immunosuppressive molecules decrease NK cell cytotoxicity by reduction of NKG2D expression that leads to attenuated recognition of target cells. Strategies have been developed to overcome this inhibition using cell modifications such as vector transduction (15) or antibodies bound to the NK cell surface. These bi- and trispecific killer engagers recognize, e.g., CD33 *in vivo* on myelodysplastic syndrome target cells, and induce cell lysis (16). Also, a promising approach is the use of chimeric antigen receptors (CARs) to improve NK cell cytotoxicity. CARs consist of an external recognition domain [single-chain variable fragment (scFv)] combined with a transmembrane domain followed by one or more signaling domains. It has been shown that CARs using CD3 ζ and CD28 domains and/or additional 4-1BB (CD137) or 2B4 domains demonstrate an enhanced killing activity (see Table 1).

Most published preclinical and clinical studies with CAR-modified immune cells comprise T cells. On the NK cell side, publications are mainly restricted to NK cell lines as reviewed in Ref. (31, 32). Less is known about CAR-engineered primary human NK cells as alternative effector cells since the advantages of NK cells are the limited lifespan of several weeks or months (2, 33) and the absent formation of memory cells that persist in patients as observed in CAR T cells. That means multiple dose of CAR NK cells might be safely administered to patients. The present review will discuss the use of primary NK cells isolated from peripheral blood for CAR engineering.

SIGNAL TRANSDUCTION IN NK CELLS

There is a competitive equilibrium between different opposing pathways (13, 34) that culminate at least in activation or inhibition of NK cells depending on the cell surface complexes that are formed by non-covalent associations between distinct transmembrane ligand-binding and signaling adaptor proteins. The Src (sarcoma) family kinases seem to be essential in these interactions because the enzymes are involved in receptor clustering in these microdomains that may facilitate receptor phosphorylation (35, 36).

Starting with NK-cell-target-cell interactions on the surface, this leads to induction of signaling pathways and at least to release of cytotoxic granules (e.g., perforin, granzyme A/B, and granulysin) and/or secretion of cytokines (e.g., IFN γ and TNF- α).

Activation Receptors

Natural cytotoxicity receptors (NCRs) as NKp30 and NKp46 can couple to CD3 ζ that contains several ITAMs (37). NKp44 instead associates with the ITAM-bearing adaptor DAP12. In the next step, tyrosine residues of the ITAM sequences are phosphorylated by protein tyrosine kinases of the Src family. This leads to recruitment of protein tyrosine kinases of the Syk family (e.g., Syk or ZAP70; spleen-associated tyrosine kinase or zeta-chain-associated protein kinase 70) and transmembrane adaptor molecules (e.g., linker for activation of T cells and non-T cell activation linker) that provide multiple docking sites for Syk family kinases. These associations of different signaling partners initialize activation and phosphorylation of multiple partners of signaling pathways such as PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) or members of Vav family resulting in release of lytic granules and leading to cytotoxicity (9). The activation of a single NCR seems to start an activation cascade in which different NCRs cross talk to each another for amplifying activating signals (e.g., cross talk between NKp30, NKp44, and NKp46) (38).

Natural killer group 2 member D (CD314) is non-covalent associated with transmembrane adaptor protein DAP10. This pathway is independent of Syk family tyrosine protein kinases (39) and involved PI3K in its signaling cascade. After ligand binding (MICA, MICB, or divers UL16-binding proteins), phosphorylation of a tyrosine-based DAP10 motif by Src family kinases creates binding sites for p85 subunit of PI3K or for the adaptor protein complex Grb2-Vav1 (growth factor receptor-bound protein 2-vav guanine nucleotide exchange factor 1). The result is exocytosis of lytic granules (e.g., perforin, granzyme A/B, and CD107a) in response to PLC (phospholipase C)- γ 2-induction (40–43).

For NK cell activation, Vav proteins are essential. Depending of the NCR and of the DNAX proteins, different Vav proteins are involved, e.g., Vav1 is part of the signaling with NKG2D/DAP10 (39–41), whereas Vav2 and Vav3 take part of the DAP12 signaling cascade (44). Vav proteins are involved in a GTPase-dependent reorganization of the cytoskeleton to mediate the directed release of the granules (9).

The NK cell-activating receptor CD226 (DNAX accessory molecule 1) lacks any ITAM. Instead, intracellular signaling starts with phosphorylation of a serine and a tyrosine residue by

TABLE 1 | Preclinical and clinical investigations of CAR-modified primary human natural killer cells.

Antigen	Signaling domain	Target cells	Efficacy	Reference or ClinicalTrials.gov identifier
Preclinical studies with cell lines as targets	CD19	4-1BB/CD3 ζ	Acute lymphatic leukemia cell lines	+++ (15)
	HER-2	CD28/CD3 ζ	Ovarian cancer cell line and breast cancer cell line	+ (17)
	Disialoganglioside 2 (GD2)	2B4/CD3 ζ	Neuroblastoma cell line	+++ (18)
	CD19	2B4/CD3 ζ	ALL cell lines	+++ (18)
	CD19	4-1BB/CD3 ζ	B-ALL cell line	+++ (19)
	CD19	4-1BB/CD3 ζ	B-ALL cell lines and B cell lymphoma cell lines	++ to +++ (20)
	Natural killer group 2 member D ligands	DAP10/CD3 ζ	ALL cell lines and several solid tumor cell lines	+ to +++ (21)
	HER-2	CD28/CD3 ζ	HER-2-expressing cell lines	n.a. (22)
	CD19	4-1BB/CD3 ζ	B-ALL cell lines	+ (23)
	CS1	CD28/CD3 ζ	Myeloma cell lines	Data not shown (24)
	CD20	4-1BB/CD3 ζ	CD20 $^+$ B-cell non-Hodgkin lymphoma cell lines	++ to +++ (25)
	Epidermal growth factor receptor (EGFR)	CD28/CD3 ζ	Glioblastoma cell lines	+ (26)
	Prostate stem cell antigen (PSCA)	DAP12	several PSCA $^+$ tumor cells	(+) to +++ (27)
	CD19	CD28/4-1BB/CD3 ζ	CD19 $^+$ leukemia cell line	+ to +++ (28)
Preclinical studies with patient malignant cells as targets	EGFR	CD28/CD3 ζ	Breast cancer cell lines	+ (29)
	GD2	CD28/4-1BB/CD3 ζ	Ewing sarcoma cell lines	+ to ++ (30)
	CD19	4-1BB/CD3 ζ	Acute lymphatic leukemia	+++ (15)
	CD19	2B4/CD3 ζ	Acute lymphatic leukemia	+++ (18)
	CD19	4-1BB/CD3 ζ	B-CLL cells	+++ (19)
Clinical trials	CD19	4-1BB/CD3 ζ	B-ALL cells	+ (23)
	CD19	CD28/CD3 ζ	Glioblastoma stem cells	(+) (26)
	CD19	4-1BB/CD3 ζ	B-lineage acute lymphoblastic leukemia	n.a. NCT 00995137
	CD19	4-1BB/CD3 ζ	B-lineage acute lymphoblastic leukemia	n.a. NCT 01974479
	CD19	CD28/CD3 ζ	B-lymphoid malignancies	n.a. NCT 03056339

(+), cytotoxicity <25%; +, cytotoxicity 25–49%, ++, cytotoxicity 50–75%, +++, cytotoxicity >75%.

protein kinase C. This step is critical for association of CD226 to lymphocyte function-associated antigen 1 at the cell surface and facilitates simultaneously cytoplasmic signaling involving Src kinase, Vav1, and PLC- γ 2 leading to NK cell activation (45).

The receptor 2B4 has been characterized as costimulatory for activation receptors (e.g., for CD226). A complex formed of CD226 and 2B4 triggers NK cell degranulation, activates PLC- γ 2, and increases Ca $^{2+}$ intracellular flux (46). On one hand, 2B4 is sufficient to induce IFN γ release alone (47), on the other hand, 2B4 demonstrated enhanced cytokine secretion after cross linking with NKG2D (48).

Inhibitory Receptors

After binding of MHC class I molecules to inhibitory receptors, the inhibitory signaling cascade starts with phosphorylation of one or more ITIM sequences (immunoreceptor tyrosine-based inhibitory motif). Detailed mechanism of phosphorylation is unknown but tyrosine kinases that are involved in activation pathways have been expected. After tyrosine phosphorylation, the phosphatases Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP1) and SHP2 bind to ITIMs (49) and subsequently recruit additional molecules such as inhibitory C-terminal Src kinase Crk (for LILR and LAIR-1) or β -arrestin 2 (for KIRs). ITIM-bound SHP starts to dephosphorylize specifically Vav1 or other pivotal proteins to inhibit clustering of receptors and cytoskeleton rearrangements (50).

Killer cell Ig-like receptor and CD94/NKG2A initiate an alternative signaling pathway that also results in inhibition. The binding of the tyrosine kinase c-Abl and the subsequent phosphorylation of adaptor protein Crk (CT10 regulator of kinase) by c-Abl cause dissociation of Crk from protein complexes that are involved in NK cell activation (51, 52). Inhibition of NK cells is achieved and lysis of target cells decreased.

A second MHC class I independent pathway is composed of the inhibitory receptor T cell immunoglobulin and ITIM domain (TIGIT) and the putative weak-activating receptor CD96. For inhibitory signaling of TIGIT, the intracellular motifs immunoglobulin tail tyrosine and/or ITIM are phosphorylated after ligand binding following recruitment of SHP1 and Grb2 that result in blocking the pathways of PI3K and mitogen-activated protein kinase. CD96 contains a cytoplasmic ITIM as well as a YXXM motif that is a putative binding sites for the p85 subunit of PI3K that may lead to NK cell activation. Both receptors, TIGIT and CD96, were found to counterbalance the costimulatory receptor CD226 and limit NK cell-mediated cytotoxicity and IFN γ release (46, 53).

OPTIMIZATION OF CAR SIGNALING IN NK CELLS

Chimeric antigen receptors contain an extracellular region of a scFv that was fused to transmembrane domain and cytosolic

signaling components. The antibody-derived scFv domain is involved in antigen recognition and immune synapse formation, whereas the endodomains are responsible for cell activation. Because CAR constructs are premised on a modular system, it is feasible to combine any scFv with any signaling or cosignaling domain. First-generation CARs that included only signaling motifs derived from CD3 (ζ or γ chain) (13, 54) were fully capable to activate murine CTL hybridoma cells (55), although no additional intracellular signaling region was added. But some tumors were able to inactivate CAR-engineered cells and leading them to anergy (54). To prevent this effect and to improve CAR functionality, subsequent CAR designs incorporated additional costimulatory domains (CD28, 4-1BB, OX40, and 2B4) and evolved to CARs of the second- (addition of one costimulatory domain) or third-generation (addition of more than one costimulatory domain) (Figure 1A).

The motif CD28 is most common in these CAR constructs but is not naturally expressed in human NK cells (56). In T cells, the mode of action of this costimulatory molecule starts with phosphorylation of its intracellular tyrosine residues by PI3K following recruitment of Grb2 and results in activation of protein kinase B (PKB/Akt) and in IL-2 production (57). The advantage for NK cells is still in discussion although for T cells CD28 demonstrates high effectiveness (58).

4-1BB is a surface protein discovered on activated T cells (59) that is often used in CAR constructs for NK cells (28). For domain 4-1BB (CD137), costimulation could be clearly detected in T cells (60), but there are conflicting data for NK cells. Navabi et al. demonstrated neither improved NK cell cytotoxicity nor enhanced IFN γ production (61) after NK cell stimulation by 4-1BB ligands in contrast to augmented NK cell-killing capacity as reported in Ref. (62, 63).

The transmembrane adaptor polypeptide DAP10 is originally associated with NKG2D. Comparing the CAR constructs anti-CD19-DAP10 and anti-CD19-CD3 ζ , both CARs evoke NK cell cytotoxicity but anti-CD19-CD3 ζ exhibited higher antitumor activity than anti-CD19-DAP10 molecules (15). The combination of both signaling domains DAP10 and CD3 ζ resulted in secretion of several cytokines (e.g., IFN γ and TNF- α) as well as in a vast release of cytotoxic granules that both increased NK cell cytotoxicity (21).

DAP12 is involved in signal transduction of activated NK cells and is associated with activating receptors such as NKG2C or NKp44. Transmission of intracellular signaling occurs *via* a single ITAM compared to CD3 ζ containing three ITAMs (9). Therefore, DAP12 provides an alternative signaling pathway resulting in antitumor activity of NK cells. First investigations assessed DAP12-based CARs in NK cell line YTS (64) as well as in primary human NK cells (27). Combinations of scFv against prostate stem cell antigen (PSCA) with DAP12 exhibit an improved cytotoxicity and increased IFN γ release in primary NK cells compared to CAR NK cells expressing the first-generation CD3 ζ -based construct anti-PSCA-CD3 ζ (53). This concept without CD3 ζ -signaling domain may promise new opportunities to redirect NK cells to resistant target cells.

2B4 (CD244) is a member of the signaling lymphocytic activation molecule family and contains four immunoreceptor

tyrosine-based switch motifs (ITSMs) of which the first and second is associated with activation of stimulatory pathways in NK cells (65). Altvater et al. (18) investigated the signaling component 2B4 combined with CD3 ζ in primary human NK cells and compared this CAR construct with CAR molecules incorporated either 2B4 or CD3 ζ signaling element. As a result, induction of cytokine secretion failed when 2B4 is the sole signaling compound in CAR molecules.

Instead, combination of the domains 2B4 and CD3 ζ demonstrated enhanced cytokine secretion (IFN γ and TNF- α) and release of cytolytic granules. In addition, comparable results were observed for a 4-1BB-CD3 ζ CAR construct demonstrating equality of 2B4 and 4-1BB signaling domains in combination with CD3 ζ .

Similar to 4-1BB, OX40 (CD134) is a TNF receptor on the surface of lymphatic cells (e.g., T cells, NK cells, and NK-like T cells) (66). This costimulatory molecule is involved in recruitment of TNF receptor-associated factor adaptor proteins and leads to cell survival and cytokine release (67, 68). OX40 is often part of third-generation CARs in T cells that show improved signaling capacities based on putative upregulation of PI3K pathway and lead to enhanced cytokine production and cytotoxicity (69), but was not integrated yet in CAR constructs neither for NK cell lines nor for primary NK cells. Because of its costimulatory potential, OX40 may present a promising candidate for improved endogenous CAR signaling in NK cells.

PRECLINICAL INVESTIGATIONS WITH PRIMARY HUMAN CAR NK CELLS

To date, several preclinical studies have been investigated primary human CAR-modified NK cells directed against various antigens (Table 1). However, compared to CAR T cells that already entered clinical studies, there is only a small number of clinical investigations using CAR NK cells (Table 1).

Most preclinical data describe primary human CAR NK cells directed against CD19 and few against CD20, human epidermal growth factor receptor 2, disialoganglioside 2, epidermal growth factor receptor, and PSCA (references see Table 1).

Mostly, second-generation CARs use CD3 ζ in combination with 4-1BB, DAP10, or 2B4, respectively, and result in strong efficacy based on upregulation of the PI3K/AKT pathway. By contrast, CD3 ζ constructs with CD28 led to less cytotoxicity. High efficacy could also be revealed by third-generation CARs (CD28/4-1BB/CD3 ζ) (28, 30) and a DAP12-based first-generation CAR (27). There is a long-standing discussion that costimulatory domain combines best to CD3 ζ . For CAR T cells, investigations suggest that constructs containing 4-1BB may be superior (70), but this has not been yet evaluated for CAR NK cells. In addition, so far safety aspects have not been addressed extensively in CAR NK cells and are under discussion.

Although feasibility and efficacy could be shown for all mentioned constructs in Table 1, safety aspects have to be clarified in detail in an ongoing discussion.

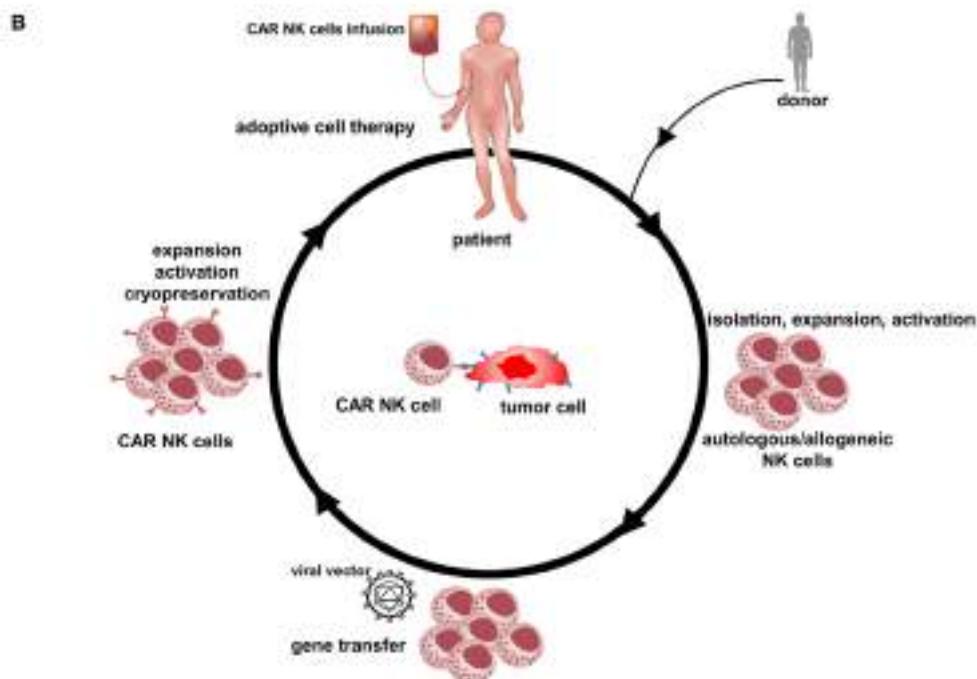
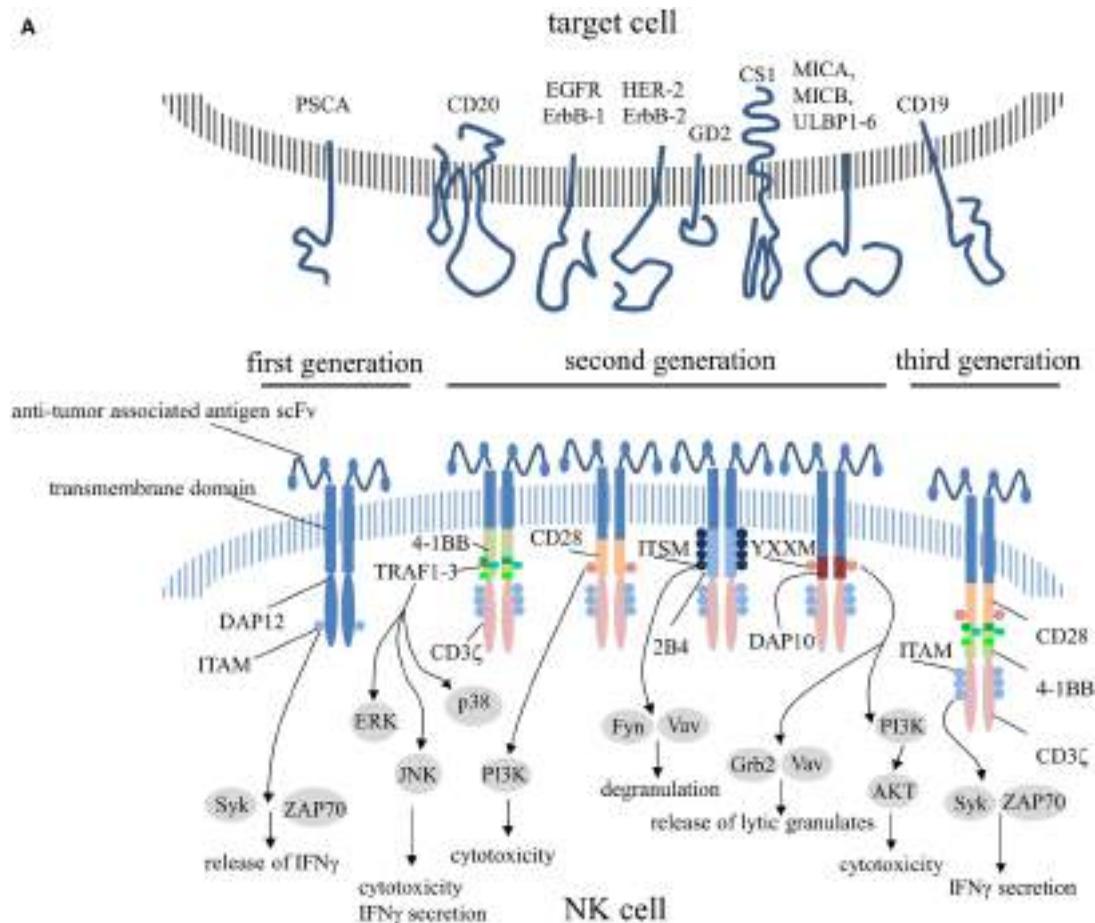


FIGURE 1 | Continued

FIGURE 1 | Continued

(A) Schematic structures of various chimeric antigen receptors applied in engineered primary human NK cells including its intracellular signaling domains. **(B)** CAR NK cell therapy. Autologous NK cells or donor NK cells (allogeneic) are isolated, expanded, and activated by cytokines. After modification of NK cells to express CAR, NK cells are expanded, activated, and administered to the patient or frozen for long-term preservation. PSCA, prostate stem cell antigen; EGFR, epidermal growth factor receptor; HER-2, human epidermal growth factor receptor 2; GD2, disialoganglioside 2; CS1, CD2 subset 1; MICA/B, MHC class I polypeptide-related sequence A/B; ULBP1-6, UL16-binding proteins 1–6; DAP, DNAX-activation protein; ITAM, immunoreceptor tyrosine-based activation motif; Syk, spleen-associated tyrosine kinase; ZAP70, zeta-chain-associated protein kinase 70; TRAF, tumor necrosis factor receptor-associated factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; I3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; ITSM, immunoreceptor tyrosine-based switch motif; Fyn, Src family tyrosine kinase; Vav, vav guanine nucleotide exchange factor; YXXM, phosphorylation motif; Grb2, growth factor receptor-bound protein 2; AKT, protein kinase B.

First clinical studies followed the success of CAR T cell trials redirecting NK cells against CD19. These antiCD19-4-1BB-CD3 ζ CAR NK cells were administered to patients with B-ALL (NCT 00995137; NCT 01974479) but results have not been published to date. The first study comprises expansion of donor-derived NK cells cocultured with irradiated and gene-modified K562 cells that expressed surface bound IL-15 and 4-1BB 1. The second trial expands IL-2-activated haploidentical NK cells before administering to pediatric and adult patients. Recently, a third study (NCT 03056339) started for patients suffering from relapsed and/or refractory B-cell lymphoma or leukemia. Genetically engineered NK cells derive from umbilical cord blood (CB) and express antiCD19-CD28-CD3 ζ CAR, the iCasp9 safety switch as well as IL-15.

OFF-THE-SHELF (OTS) IMPLICATIONS FOR CANCER TREATMENT

Antigen specificity of CAR NK cells is independent of the recipient's human leukocyte antigen (HLA) type. This feature is the prerequisite for targeting the same antigen on several tumor types even if recipients demonstrate a high variability of HLA. There may be no need any more to customize individual therapies for each patient. Implementation of a cell bank with cryopreserved immune cells that are allogeneic and genetically modified may solve availability and reduce cost of treatment. Developing OTS therapies means that portions of immune cells will be manufactured (and modified) in advance, stored in cryopreservation, and infused on demand as required by attending physicians (Figure 1B). It has been shown in several studies that administration of haploidentical NK cells to patients with relapsed acute myelogenous leukemia cause good clinical effects without graft versus host disease (GvHD) as reviewed in Ref. (71). For this reason, it seems to be a successful strategy to set a strong focus on CAR NK cell-based immunotherapies (see Table 1), although Shah et al. recently observed GvHD after infusion of *ex vivo* expanded activated allogeneic NK cells (72).

The ideal source for CAR NK cells as OTS products is still in discussion. The cell line NK92 has been described as an option that can be easily transduced and irradiated before administration (73, 74). On the other hand, umbilical CB is well known to be a good source for primary NK cells (75, 76). But limitations as immature phenotype or restriction of NK cell amount should be kept in mind (77, 78), which might be circumvent by refined

protocols for primary NK cell *ex vivo* expansion and activation (79) especially in regard to GMP compliance.

A subset of NK cells has been described in mouse and human that demonstrated long-lived capacity for several months (80). These "memory-like" NK cells respond to antigens in second confrontation and show enhanced effector function and expansion. They even may prevent leukemia relapse by a robust cytokine production (81, 82) and may therefore be beneficial in general in long-term antitumor responses. For safety reason, a CAR suicide system should be integrated in CAR NK cells to limit circulation of CAR effector cells in patients (83) and to restrict putative toxic side effects as demonstrated for CAR T cells (84, 85).

STRATEGIES TO OVERCOME OFF-TARGET TOXICITIES

The choice of tumor antigens that can be recognized by CARs depends on the unique and selective character of the antigen for target cancer cells. These regular antigens mainly show increased expression on tumor tissues but are also detectable on normal tissues, often in a minute amount. For this reason, on-target toxicities may appear in clinical studies that have been described for CAR T cells (86–88). On the other hand, off-target toxicity attacks tissues and organs that do not express the antigen but CAR constructs can bind unspecifically. For primary human CAR NK cells, toxicity reports have not been published yet but recognition of specific tumor targets are the base for safe and effective CAR constructs.

To increase selectivity of CAR molecules and reduce putative off-target effects, different strategies have been developed, e.g., combination of two extracellular domains in a tandem structure (89, 90) or of two independent constructs to form bispecific CAR molecules (91). A second concept describes CAR constructs that triggers the release of pro-inflammatory IL-12. The composition of CAR resulted in expression of IL-12 after antigen binding to the extracellular CAR domain (92). Recently, Wu et al. developed a split CAR construct that needs a dimerizing small molecule to form a functional unit. This new strategy promises control of timing, location, and dosage of CAR activity and thereby a possible mitigation of toxicities (93). A similar concept demonstrates the use of an inducible molecular switch off (94). When exposed to a dimerizing drug, the fusion protein iCasp9 is activated and triggers apoptosis in all gene-modified cells. In general, all strategies have been shown for engineered CAR T cells, except the last one that has also been evaluated in the murine model using modified

NK cells (95) and even for primary CB-derived NK cells expressing antiCD19 CAR molecules [unpublished data mentioned in Ref. (96)].

CONCLUSION

In the next years, the possibility of unlimited access to cryopreserved NK cells from CB or third party donors may revolutionize therapy options for cancer patients. Although discussions about best source of NK cells and the question of long-living NK cells have not been finished yet, generation of redirected NK cells against new targets is in rapid progress. Demonstrated results using CAR technologies are auspiciously and may improve cancer therapy also by implemented novel safety strategies. Furthermore, combined immunotherapies

using checkpoint blockade monoclonal antibodies to overcome inhibitory signals (e.g., anti-KIR or anti-TIGIT) may enhance CAR NK cell activity.

AUTHOR CONTRIBUTIONS

OO performed the review of the literature and wrote the manuscript. SK and UK edited the manuscript.

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Chimeric Antigen Receptor-Engineered NK-92 Cells: An Off-the-Shelf Cellular Therapeutic for Targeted Elimination of Cancer Cells and Induction of Protective Antitumor Immunity

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Significant progress has been made in recent years toward realizing the potential of natural killer (NK) cells for cancer immunotherapy. NK cells can respond rapidly to transformed and stressed cells and have the intrinsic potential to extravasate and reach their targets in almost all body tissues. In addition to donor-derived primary NK cells, also the established NK cell line NK-92 is being developed for adoptive immunotherapy, and general safety of infusion of irradiated NK-92 cells has been established in phase I clinical trials with clinical responses observed in some of the cancer patients treated. To enhance their therapeutic utility, NK-92 cells have been modified to express chimeric antigen receptors (CARs) composed of a tumor-specific single chain fragment variable antibody fragment fused via hinge and transmembrane regions to intracellular signaling moieties such as CD3ζ or composite signaling domains containing a costimulatory protein together with CD3ζ. CAR-mediated activation of NK cells then bypasses inhibitory signals and overcomes NK resistance of tumor cells. In contrast to primary NK cells, CAR-engineered NK-92 cell lines suitable for clinical development can be established from molecularly and functionally well-characterized single cell clones following good manufacturing practice-compliant procedures. In preclinical *in vitro* and *in vivo* models, potent antitumor activity of NK-92 variants targeted to differentiation antigens expressed by hematologic malignancies, and overexpressed or mutated self-antigens associated with solid tumors has been found, encouraging further development of CAR-engineered NK-92 cells. Importantly, in syngeneic mouse tumor models, induction of endogenous antitumor immunity after treatment with CAR-expressing NK-92 cells has been demonstrated, resulting in cures and long-lasting immunological memory protecting against

tumor rechallenge at distant sites. Here, we summarize the current status and future prospects of CAR-engineered NK-92 cells as off-the-shelf cellular therapeutics, with special emphasis on ErbB2 (HER2)-specific NK-92 cells that are approaching clinical application.

Keywords: natural killer cells, NK-92, chimeric antigen receptor, adoptive cancer immunotherapy, leukemia, lymphoma, solid tumors

INTRODUCTION

Natural killer (NK) cells are specialized effectors of the innate immune system and central players in the defense against viral infections and cancer. Natural cytotoxicity of NK cells can be triggered rapidly upon appropriate stimulation and is regulated by a complex balance of signals from germ-line encoded activating and inhibitory cell surface receptors (1, 2). The antitumoral activity of NK cells has been well documented in mouse models (3, 4). In humans, a correlation between low peripheral blood NK-cell activity and an increased cancer risk was demonstrated (5), and numbers and phenotype of tumor-infiltrating NK cells likely influence the course of the disease (6–8). Mechanisms involved in tumor immune evasion can be diverse and include upregulation of the non-classical MHC molecules HLA-E and HLA-G that trigger inhibitory NK-cell receptors (9), selective loss of ligands for activating NK-cell receptors (10, 11), as well as shedding of soluble forms of MHC class I polypeptide-related sequence A/B (MICA/B) and B7-H6 (12–14). Furthermore, the tumor microenvironment plays a crucial role in preventing infiltration by NK and other immune cells and interfering with the activity of NK cells already present in the tumor (15, 16). Hypoxia as well as immunosuppressive factors such as transforming growth factor (TGF)- β , indoleamine 2,3-deoxygenase (IDO), prostaglandin E2, nitric oxide (NO), and reactive oxygen species (ROS), which are produced by regulatory immune cells like regulatory T (T_{reg}) cells and myeloid-derived suppressor cells, by stromal cells like cancer-associated fibroblasts, and by tumor cells themselves can inhibit expression of activating NK-cell receptors, disrupt the interactions between NK and other immune cells, and avert the contact of NK cells with tumor cells (17).

To bypass deficiencies in endogenous NK-cell activity, current NK-cell therapies are typically based on adoptive transfer of *ex vivo*-expanded allogeneic NK cells derived from a suitable donor (18–20). While displaying graft-versus-leukemia (GvL) or graft-versus-tumor (GvT) activity, such donor-derived NK cells do not carry a high risk of inducing graft-versus-host-disease (GvHD) frequently associated with donor lymphocyte infusion (DLI) of allogeneic T cells (20). In addition, antibodies that block inhibitory NK-cell receptors such as killer cell immunoglobulin-like receptors (KIRs) and NKG2A/CD94, or link activating NK-cell receptors to tumor cell surface antigens are being investigated as activity enhancers for endogenous or adoptively transferred NK cells (21, 22). Sparked by the clinical success of chimeric antigen receptor (CAR)-engineered T cells in the treatment of B-cell malignancies, genetic modification of NK cells with CAR constructs is receiving increasing attention. CAR engagement in

NK cells can override inhibitory signals deployed by tumor cells and directly trigger the effector cells' intrinsic cytolytic effector functions as well as the release of pro-inflammatory cytokines (23, 24). Nevertheless, despite the close similarity of NK cells to T cells with respect to their cytotoxic mechanisms, the development of CAR-engineered NK cells for adoptive cancer immunotherapy is still in its early stages, owing mainly to the complexity of isolating, activating, expanding, and manufacturing large numbers of peripheral blood-derived NK cells, the lower efficiency of gene transfer when compared to T cells, and the limited *in vivo* proliferation and persistence in recipients. While efforts are being made to overcome these hurdles by improving *ex vivo* expansion of NK cells to allow multiple infusions (25), results from clinical trials with CAR NK cells are not yet available.

Continuously expanding NK cell lines provide an unlimited source of effector cells to investigate and improve concepts for genetic engineering of NK cells (23, 26–29) but also hold potential for development as standardized off-the-shelf therapeutics for adoptive cancer immunotherapy. Different human NK cell lines have been established, including NK-92, HANK-1, KHYG-1, NK-YS, NKG, YT, YTS, NKL, and NK3.3 (30). Among them, NK-92 cells (also termed “aNK” for activated NK) have been investigated most thoroughly and already been applied in a clinical setting (31, 32). NK-92 express many activating NK-cell receptors such as NKP30, NKP46, and NKG2D but lack most of the inhibitory KIRs, except for low levels of KIR2DL4 (33, 34). Other inhibitory receptors expressed by NK-92 are Ig-like transcript 2 (ILT-2) and NKG2A/CD94. This unique profile renders NK-92 cells highly cytotoxic against a broad spectrum of malignant cells of hematologic origin and other cancers (32). General safety of infusion of irradiated NK-92 cells has been established in phase I clinical trials in patients with advanced cancers (35, 36), and results from other phase I and phase II studies may soon become available (NCT00990717, NCT00900809, NCT02465957; <https://clinicaltrials.gov>).

As outlined in the following sections, the robust *ex vivo* expansion of NK-92 cells to high cell numbers, their exquisite safety profile, as well as the ease of genetic modification make this cell line an ideal platform for the development of CAR-engineered variants. Here, we provide an overview of the diverse approaches that have been taken to date to target NK-92 cells to various hematological malignancies and solid tumors, summarize pre-clinical *in vitro* and *in vivo* studies with special emphasis on ErbB2 (HER2)-specific CAR NK-92 cells (NK-92/5.28.z) that are ready to enter clinical trials, and discuss general advantages and challenges associated with the use of CAR NK-92 cells as an off-the-shelf cellular therapeutic.

ADVANCES FROM THE CAR T CELL FIELD ENABLING THE GENERATION OF TUMOR-SPECIFIC NK CELLS

Since introduction of the basic CAR design with a single chain fragment variable (scFv) antibody for target recognition fused to CD3 ζ or Fc ϵ RI γ chains for signaling (first-generation CARs) by Eshhar and colleagues (37), many groups have contributed to further improve and develop this concept, facilitating the clinical success of CAR T cell therapy seen today (38, 39). The most significant CAR modification was thereby the inclusion of costimulatory protein domains derived from CD27, CD28, CD134 (OX40), CD137 (4-1BB), CD244 (2B4) or CD278 (ICOS) (second-generation CARs), or their combinations (third-generation CARs) in addition to CD3 ζ to improve T-cell activation, proliferation, and persistence (40). Other advances enhancing CAR functionality and providing additional benefits with respect to stimulating innate immunity, improving safety, or alleviating tumor immune escape have been reviewed extensively by Fesnak et al. (41). They include for instance interleukin (IL)-12-armed T cells redirected for universal cytokine-mediated killing (TRUCKs) (42), universal CARs activated by modular antibody-based targeting molecules (43), and dual-targeting tandem CARs (TanCARs) (44, 45). In patients with lymphomas and leukemias of B-cell origin, remarkable efficacy was demonstrated and durable responses were achieved with both, T-cell products harboring CD19-specific second-generation CD28- or CD137-containing CARs. While in experimental models CD28-CD3 ζ CARs led to stronger T-cell activation, CD137-CD3 ζ CARs prolonged *in vivo* T-cell persistence and reduced exhaustion (46, 47).

Already early on, it was postulated for first-generation CARs that they would be functional in NK cells (37), which was formally demonstrated for a CAR-like CD4-CD3 ζ fusion receptor in human NK3.3 cells (26). In the first report proposing CAR-engineered NK-92 cells as a continuously expanding off-the-shelf cell therapeutic, we also applied a first-generation CAR consisting of an ErbB2-specific scFv antibody fused to CD3 ζ through a CD8 α hinge region, which resulted in high and specific cytotoxicity of the genetically modified cells toward ErbB2-expressing breast cancer cells and other targets of solid tumor origins (23). Similar first-generation CAR designs were successfully used in subsequent studies with NK-92 cells targeting the B-cell differentiation antigens CD19 and CD20 (48–53), CD138 for recognition of multiple myeloma (54), and various surface antigens expressed by solid tumors including the disialoganglioside GD2, epithelial cell adhesion molecule (EpCAM), and a peptide epitope of the melanoma antigen gp100 in complex with HLA-A2 (55–59) (Table 1). In studies with CD19- and GD2-targeted primary human NK cells, inclusion of costimulatory CD137 or CD244 domains in the CAR in addition to CD3 ζ enhanced both specific cytotoxicity and production of interferon (IFN)- γ and granulocyte-macrophage colony stimulating factor (GM-CSF) when compared to first-generation CARs (24, 60). This clearly demonstrates that at least primary NK cells benefit from CAR-induced costimulatory signals. In preclinical studies, also an ErbB2-specific CD28-CD3 ζ CAR and a CD20-specific CD137-CD3 ζ CAR were shown to be functional in donor-derived human

NK cells, but no comparison with respective CD3 ζ -only CARs was performed (61, 62). Clinical trials with CAR-engineered primary NK cells for the treatment of B-cell acute lymphoblastic leukemia (B-ALL) employ CD19-specific CD137-CD3 ζ receptors (NCT00995137, NCT01974479; <https://clinicaltrials.gov>), but results from these trials are not yet available.

INFLUENCE OF THE CAR DESIGN ON FUNCTIONALITY OF RETARGETED NK-92 CELLS

In the presence of IL-2, NK-92 cells persistently exhibit a phenotype similar to activated NK cells (33). Hence, CAR-engineered NK-92 variants may be less dependent on costimulation than T cells and primary NK cells (80). Nevertheless, second-generation CARs employing a composite CD28-CD3 ζ signaling domain have been shown to be functional in NK-92 cells targeting EpCAM and ErbB2 on breast cancer cells (28, 67, 68), epidermal growth factor receptor (EGFR) on glioblastoma cells and breast cancer brain metastases (70–72), EGFRvIII, a glioblastoma-specific mutant form of EGFR arising from an in-frame deletion of exons 2–7 of the receptor (71, 72), CD19 on B-cell malignancies (74), CS1 on multiple myeloma cells (77), and CD33 on acute myeloid leukemia cells (81). Likewise, second-generation CARs harboring CD137-CD3 ζ domains and targeting ErbB2 (67), CD19 (74), or peptide epitopes of Epstein–Barr virus (EBV) latent protein EBNA3C, and Wilms tumor protein in complex with HLA-A2 (78, 79) have been used successfully with NK-92 cells as well as third generation CD28-CD137-CD3 ζ CARs that recognize CD3 or CD5 for elimination of malignant T cells (75, 76) (Table 1).

Only two reports compared the functionality of NK-92 cells harboring CD3 ζ -based first-generation or CD28-CD3 ζ - and CD137-CD3 ζ -based second-generation CARs directly (67, 74), using a general CAR design as depicted in Figure 1A. NK-92 cells express high levels of CD3 ζ and moderate levels of CD28 and CD137 (23, 34, 67) (Figure 1B), suggesting that the CARs could readily link to respective endogenous signaling pathways. Indeed, while differences were relatively small, ErbB2-targeted NK-92 cells expressing CD28-CD3 ζ and CD137-CD3 ζ CARs displayed more pronounced cytotoxicity in short-term assays when compared to a corresponding CD3 ζ -only CAR (67). Conversely, CD19-targeted NK-92 cells harboring a CD137-CD3 ζ CAR were much less effective in cell killing than cells expressing a CD3 ζ -only or a CD28-CD3 ζ CAR containing the same cell targeting domain (74). With respect to cytokine production, highest amounts of IFN- γ were found in cultures of CD19-specific NK-92 expressing a CD28-CD3 ζ CAR, while less pronounced levels were secreted upon CAR activation by cells harboring a CD3 ζ -only CAR, and only marginally enhanced levels by cells carrying the CD137-CD3 ζ CAR.

The first-generation CARs included in these studies for comparison utilized the endogenous transmembrane domain of CD3 ζ . This allowed formation of both, disulfide-linked CAR homodimers, and heterodimers of the CAR with endogenous CD3 ζ of NK-92 cells (67, 74). Such preformed receptor complexes may get activated more rapidly and by lower target antigen densities than the CARs with CD28-CD3 ζ and CD137-CD3 ζ

TABLE 1 | Preclinical studies with CAR NK-92 cells.

Target	Antibody	Hinge	TM	Signaling	Gene transfer	Cancer type	In vivo model	Treatment	Reference
ErbB2 (HER2)	FRP5	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	Breast ca. Ovarian ca. SCC	CD-1 nude	Local co-injection	Uhrek et al. (23)
ErbB2 (HER2)	FRP5	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	Breast ca.	BALB/c nude	Systemic	Daldrup-Link et al. (63), Meier et al. (64)
ErbB2 (HER2)	FRP5	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	Brain metastasis	Athymic nude rats	Systemic (with FUS)	Alkins et al. (65), Alkins et al. (66)
ErbB2 (HER2)	4D5-8	hlgG2	hFcεRⅠγ	hFcεRⅠγ	Retrovirus	Breast ca.	NSG	Systemic	Clemenceau et al. (58)
ErbB2 (HER2)	FRP5	hCD8α	hCD3ζ hCD28 hCD137	hCD3ζ hCD28-CD3ζ hCD137-CD3ζ	Lentivirus	Breast ca. Ovarian ca. Melanoma RCC	NSG	Systemic	Schönfeld et al. (67)
ErbB2 (HER2)	n.s.	hCD8α	hCD28	hCD28-CD3ζ	Electroporation	Breast ca.	BALB/c nude	Systemic	Liu et al. (68)
ErbB2 (HER2)	FRP5	hCD8α	hCD28	hCD28-CD3ζ	Lentivirus	GBM	NSG C57BL/6	Local	Zhang et al. (69)
Epidermal growth factor receptor (EGFR)	528	n.s.	n.s.	hCD28-CD3ζ	Lentivirus	Brain metastasis	NSG	Local (combined with HSV-1)	Chen et al. (70)
EGFR	R-1	hCD8α	hCD28	hCD28-CD3ζ	Lentivirus	GBM	NSG	Local	Genßler et al. (71)
EGFR/EGFRvIII	528	n.s.	hCD28	hCD28-CD3ζ	Lentivirus	GBM	NSG	Local	Han et al. (72)
EGFR/EGFRvIII	225	hCD8α	hCD28	hCD28-CD3ζ	Lentivirus	GBM	NSG	Local	Genßler et al. (71)
EGFRvIII	MR1-1	hCD8α	hCD28	hCD28-CD3ζ	Lentivirus	GBM	NSG	Local	Genßler et al. (71)
GD2	ch14.18	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	NB Breast ca. Melanoma	NSG	Local	Esser et al. (56), Seidel et al. (59)
Epithelial cell adhesion molecule (EpCAM)	MOC31	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	Prostate ca.	Athymic nude rats	Systemic	Tavri et al. (55), Meier et al. (73)
EpCAM	MOC31	hCD8α	hCD28	hCD28-CD3ζ	Lentivirus	Breast ca.	–	–	Sahm et al. (28)
CD19	FMC63	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	B-ALL	–	–	Romanski et al. (48), Romanski et al. (53)
CD19	FMC63	mCD8α	mCD3ζ	mCD3ζ	mRNA transfection	B-ALL CLL	–	–	Boissel et al. (50)
CD19	FMC63	mCD8α	mCD3ζ	mCD3ζ	mRNA transfection Lentivirus	B-ALL CLL Burkitt's lymphoma	–	–	Boissel et al. (51)
CD19	FMC63	mCD8α	mCD3ζ	mCD3ζ	Lentivirus	B-ALL CLL	NOD/SCID NSG	Local Systemic	Boissel et al. (52)
CD19	FMC63	hCD8α	hCD3ζ hCD28 hCD137	hCD3ζ hCD28-CD3ζ hCD137-CD3ζ	Lentivirus	B-ALL Burkitt's lymphoma	NSG	Systemic	Oelsner et al. (74)
CD20	Leu-16	mCD8α	mCD3ζ	mCD3ζ	Retrovirus	B-ALL CLL Burkitt's lymphoma	NSG	Local co-injection	Müller et al. (49)
CD20	Leu-16	mCD8α	mCD3ζ	mCD3ζ	mRNA transfection Lentivirus	B-ALL CLL Burkitt's lymphoma	–	–	Boissel et al. (51)
CD20	Leu-16	mCD8α	mCD3ζ	mCD3ζ	Lentivirus	B-ALL CLL	NOD/SCID NSG	Local Systemic	Boissel et al. (52)

(Continued)

TABLE 1 | Continued

Target	Antibody	Hinge	TM	Signaling	Gene transfer	Cancer type	In vivo model	Treatment	Reference
CD3	n.s.	hCD8 α	hCD8 α	hCD28-CD137-CD3 ζ	Lentivirus	PTCL T-ALL	NSG	Systemic	Chen et al. (75)
CD5	n.s.	hCD8 α	hCD8 α	hCD28-CD137-CD3 ζ	Lentivirus	PTCL T-ALL Sézary syndrome	NSG	Systemic	Chen et al. (76)
CD138	4B3	hCD8 α	hCD3 ζ	hCD3 ζ	Lentivirus	MM	NOD/SCID	Systemic	Jiang et al. (54)
CS1	Luc90	n.s.	n.s.	hCD28-CD3 ζ	Lentivirus	MM	NSG	Systemic	Chu et al. (77)
EBNA3C peptide	EBNA Clone 315	hCD8 α	hCD8 α	hCD137-CD3 ζ	Retrovirus	BLCL	–	–	Tassev et al. (78)
gp100 ₂₀₉₋₂₁₇ peptide	GPA7	n.s.	HLA-A2	hCD3 ζ	Electroporation	Melanoma	NOD/SCID	Systemic	Zhang et al. (57)
WT1 ₁₂₆ peptide	Q2L	hCD8 α	hCD8 α	hCD137-CD3 ζ	Retrovirus	B-ALL AMoL NB	–	–	Zhao et al. (79)

TM, transmembrane domain; n.s., not specified; m, murine; h, human; SCC, squamous cell carcinoma; RCC, renal cell carcinoma; GBM, glioblastoma; NB, neuroblastoma; B-ALL, B-cell acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; PTCL, peripheral T-cell lymphoma; T-ALL, T-cell acute lymphoblastic leukemia; MM, multiple myeloma; BLCL, Epstein-Barr virus (EBV)-transformed lymphoblastoid B cell line; AMoL, acute monocytic leukemia; FUS, MRI-guided focused ultrasound.

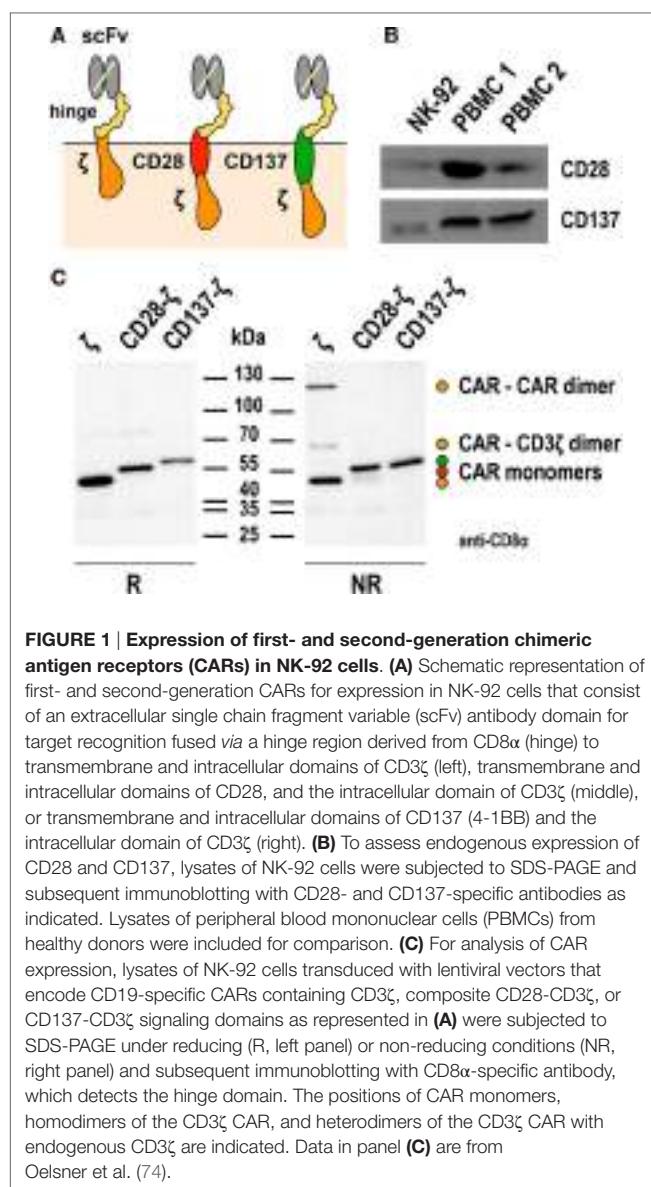
domains, which contained the transmembrane domains of CD28 and CD137 and did not form covalent dimers as assessed by SDS-PAGE and immunoblot analysis (74) (**Figure 1C**). Of note, while this was not the case for the CD19-specific CD137-CD3 ζ CAR tested in NK-92 cells, a different CD19-specific CD137-CD3 ζ CAR that contained the transmembrane domain of CD8 α and formed covalent CAR homodimers showed enhanced activity in comparison to a respective CD3 ζ -only CAR in primary NK cells (24). Also sterical effects such as distance of the target epitope to the cell surface and CAR accessibility can play a role in determining the activation threshold of individual CARs (82, 83). This may explain why in NK-92 cells otherwise identical ErbB2- and CD19-targeted CD137-CD3 ζ CARs in one case led to higher and in the other case to lower-specific cell killing when compared to the respective CD3 ζ -only CAR (67, 74). Hence, while the data available so far suggest that inclusion of a costimulatory protein domain in the CAR can be beneficial at least for particular functions of NK-92 cells, continuing research efforts are needed to clarify whether cytotoxicity, cytokine production, and resistance to immunosuppressive mechanisms can be improved with a single, generalized CAR design. Possibly, the most optimal CAR composition has to be determined experimentally in each case, taking into consideration CAR-binding affinity, location of the binding epitope within the target antigen, length of hinge region, and nature of the transmembrane domain (84).

CONTINUOUS EXPANSION OF CAR NK-92 CELLS

Isolation and *ex vivo* expansion of peripheral blood-derived NK cells for therapeutic applications can be demanding, time-consuming, and costly (85). Since KIR-mismatched allogeneic NK cells are superior to autologous cells, a suitable donor needs to be identified to allow for efficient GvL or GvT activity (18–20).

Moreover, owing to the intricate heterogeneity of human NK cells with respect to cytotoxic and regulatory activity, NK-cell licensing, unlicensing, and memory, selecting the most appropriate NK subpopulations for cancer therapy is difficult (86, 87). Sufficient numbers of NK cells are critical for a better clinical outcome, which is complicated by the limited *ex vivo* expansion potential of NK cells that remains a challenge despite the development of genetically engineered feeder cells supporting NK-cell growth and improved protocols for cytokine stimulation (25, 88, 89). These issues are also relevant for the development of CAR-engineered primary NK cells, which may explain the slow progress in this field with respect to CAR T cells.

Chimeric antigen receptor-engineered NK-92 could offer a valid and cost-effective alternative to primary CAR NK or T cells, in particular, in cases, where a suitable donor is not available or the sophisticated infrastructure needed for cell isolation, expansion, and genetic modification is missing. Methodology for continuous good manufacturing practice (GMP)-compliant expansion from an established master cell bank has been validated in the framework of early phase clinical trials with unmodified NK-92 cells and can easily be adapted for large-scale production in centralized facilities (32, 90). This advantage may readily be extended to CAR-engineered NK-92 variants. In contrast to CAR approaches based on autologous or donor-derived primary cells, genetic modification of NK-92 cells is thereby not performed in a patient-individual setting under tight time constraints. Instead, a molecularly and functionally well-characterized cell product can be established for a particular target specificity independent from the time point of therapeutic application. The resulting cells are stable with respect to CAR expression and functionality during extended expansion, as recently demonstrated for ErbB2-specific NK-92/5.28.z cells (also termed “HER2.taNK” for HER2-specific target-activated NK), a single-cell clone derived under GMP-compliant conditions that is intended for clinical use (67).



As NK cells, NK-92 as well as their CAR-expressing derivatives are dependent on exogenous IL-2 for growth and maintenance of their activated phenotype (23, 33). To ease cell expansion, different groups have engineered NK-92 by retroviral transduction or particle-mediated non-viral gene transfer to ectopically produce IL-2, leading to IL-2 secretion and growth of the cells in the absence of IL-2 supplementation (91, 92). Similarly, IL-15 and stem cell factor (SCF) have been ectopically expressed in NK-92 using plasmid DNA transfection (93, 94). While the resulting cells proliferated in medium with lower IL-2 concentrations than parental NK-92, in contrast to the IL-2-engineered variants, they were not completely independent from exogenous cytokines. In humans, high concentrations of IL-2 are associated with severe toxicity. Furthermore, in contrast to IL-15, IL-2 preferentially enhances the activity of T_{reg} cells, which is not desired in the context of cancer immunotherapy (95). Hence, a modified version

of IL-2 was developed for expression in NK-92, which carries a C-terminal KDEL endoplasmic reticulum retention signal. This still allowed activation of IL-2 receptor complexes in the secretory pathway of the producer cells but limited release of IL-2 and availability to bystander cells (96, 97). A similar effect was achieved by expression of unmodified IL-15 in NK-92 using a lentiviral vector, which supported growth in the absence of exogenous IL-2 but also restricted cytokine activity to the producer cells. When IL-15 was coexpressed with an EpCAM-specific CAR from a bicistronic lentiviral vector, transduced cells could be enriched merely by IL-2 withdrawal, with the selected CAR NK-92 cells displaying high and specific cytotoxicity in the absence of exogenous cytokines (28).

CAR-ENGINEERED NK-92 CELLS EXHIBIT ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)-LIKE ACTIVITY AND SERIAL KILLING

Antibody-dependent cell-mediated cytotoxicity of NK cells is triggered by Fc γ RIIIa (CD16), which associates with CD3 ζ and Fc ϵ RI γ that are linked to overlapping as well as distinct intracellular signaling pathways (98, 99). NK-92, which is phenotypically CD16-negative, readily mediates ADCC in the presence of a suitable IgG antibody when engineered to express Fc γ RIIIa (27, 97, 100). This has sparked efforts to clinically develop genetically modified NK-92 cells that harbor the high affinity V158 variant of CD16 (termed haNK) in combination with antibodies of IgG1 isotype (32, 97). Initial safety assessment of such cells in cancer patients is expected to begin soon (NCT03027128; <https://clinicaltrials.gov>). Interestingly, side by side comparison of NK-92 cells carrying a CD20-specific first-generation CAR with a CD3 ζ domain showed more pronounced killing of otherwise NK-resistant primary CLL cells than CD16-engineered NK-92 applied together with rituximab (52). Similarly, NK-92 cells harboring an EBV EBNA3C-specific CAR lysed peptide-pulsed B-cell lymphoblastic cells more efficiently than CD16-engineered NK-92 in the presence of an anti-EBNA3C-Fc fusion protein (78) and NK-92 cells expressing a trastuzumab-based ErbB2-specific CAR with an Fc ϵ RI γ signaling domain displayed more enhanced cytotoxicity against breast carcinoma cells than NK-92 harboring a CD16-Fc ϵ RI γ hybrid receptor in combination with trastuzumab antibody (58).

Successful triggering of ADCC through CD16 requires its non-covalent interaction with the Fc portion of an antibody that is simultaneously bound to its antigen on the surface of a neighboring target cell, as well as association with intracellular CD3 ζ and Fc ϵ RI γ . Direct linkage of extracellular target recognition and intracellular signaling functions in one molecule as implemented in a CAR can bypass such complex stoichiometry and intermolecular interactions, likely accelerating kinetics of NK-cell activation. CAR signal strength is further enhanced by integrating CD3 ζ , which in monomeric form contributes three immunoreceptor tyrosine-based activation motifs (ITAMs) that are crucial for downstream signaling, while an Fc ϵ RI γ monomer only provides one ITAM sequence (101). Accordingly, specific

target-cell recognition by CAR NK-92 results in immediate and effective ADCC-like activity, characterized by orientation of cytotoxic granules toward the immunological synapse, release of high levels of perforin and granzyme B, and rapid induction of target-cell apoptosis as demonstrated for various tumor-associated antigens (23, 49, 67, 71, 74). Live cell imaging and cytotoxicity experiments at effector to target ratios below 1:1 showed that one CAR-engineered NK-92 cell can thereby kill multiple targets within a few hours (49, 67, 74). This includes tumor cells exhibiting only moderately enhanced expression of the chosen target antigen, as demonstrated for established and tumor-initiating primary glioblastoma cells exposed to ErbB2-specific NK-92/5.28.z cells (69). NK-92/5.28.z cells also killed trastuzumab-sensitive and trastuzumab-resistant ErbB2-positive breast carcinoma cells to a similar extent (**Figure 2**), attesting to the different mode of action of the retargeted NK cells and suggesting their application in a disease setting with existing resistance to other targeted therapies.

IN VIVO ANTITUMOR ACTIVITY OF CAR NK-92 CELLS

Initial studies performed with ErbB2-, CD20-, and GD2-targeted CAR NK-92 cells showed that these cells retain specific cytotoxicity in simplified *in vivo* models in immunocompromised nude and NOD-SCID IL2R γ^{null} (NSG) mice, where effector cells were either subcutaneously coinjected together with tumor cells, or established subcutaneous tumors treated by peritumoral NK-cell injection. This resulted in delayed tumor onset and extended survival when compared to animals receiving parental NK-92 cells (23, 49, 59). Similar intratumoral treatment may be an option for cancer indications such as glioblastoma and brain metastasis, where disease is locally restricted. This has been investigated with NK-92 cells-expressing second-generation CARs targeting

ErbB2, EGFR, or mutant EGFRvIII, which are expressed by a large proportion of human glioblastomas. In orthotopic xenograft models in NSG mice, repeated stereotactic injection of ErbB2-specific NK-92/5.28.z cells into the tumor area effectively inhibited tumor progression and resulted in a marked extension of survival, while parental NK-92 cells were ineffective (69). Similar effects were seen upon local application of NK-92 cells equipped with CARs that recognize EGFR, mutant EGFRvIII, or both antigens against orthotopic EGFR- and/or EGFRvIII-positive glioblastoma xenografts or breast cancer brain metastases growing in NSG mice (70–72). In contrast to EGFR- or EGFRvIII-targeted monospecific NK-92 variants, dual targeting of EGFR and EGFRvIII with a cetuximab-based CAR recognizing a common epitope of the receptors, thereby circumvented immune escape in mixed tumors that similar to the clinical situation, consisted of EGFR-positive and EGFR/EGFRvIII-double positive glioblastoma cells (71).

For broad applicability in metastatic and disseminated disease, CAR effector cells must cross tissue barriers and reach distant tumor sites to be effective. Magnetic resonance imaging, bioluminescence imaging, and positron emission tomography experiments as well as direct analysis of tumor infiltration revealed rapid and specific accumulation of intravenously injected NK-92 carrying first- and second-generation ErbB2-specific or EpCAM-specific CARs in orthotopic breast and subcutaneous prostate carcinoma xenografts in rodents (55, 63, 64, 67, 73), while parental NK-92 cells showed no tumor homing and were mainly localized to spleen and liver (55). Focused ultrasound has been demonstrated to allow systemically applied CAR NK-92 cells to cross the blood–brain barrier and reach breast cancer brain metastases in a xenograft model in immunocompromised rats (65, 66). Specific antitumor activity of intravenously applied CAR NK-92 cells has also been found in a model of locally growing breast carcinoma (68), in an experimental renal cell carcinoma metastasis model (67), and models of disseminated leukemia, lymphoma, and

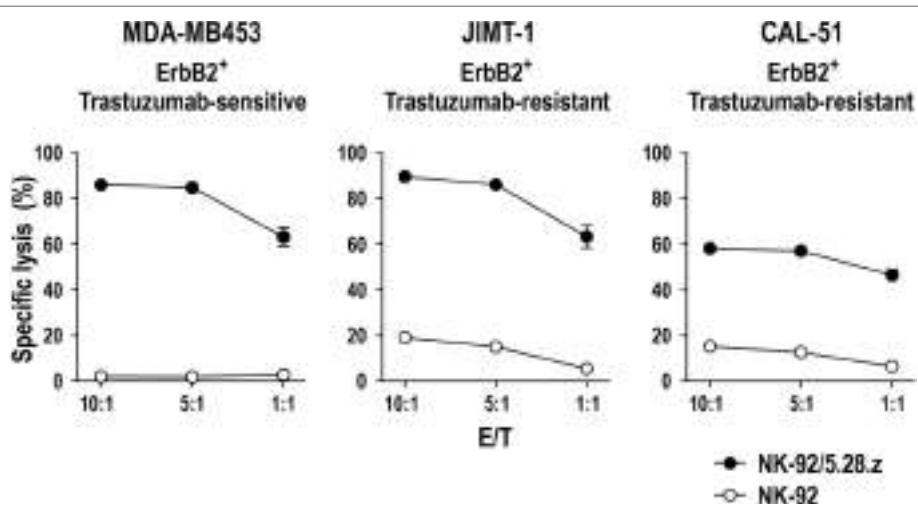


FIGURE 2 | Activity of NK-92/5.28.z against ErbB2-expressing breast carcinoma cells. Cytotoxicity of CAR-engineered ErbB2-specific NK-92/5.28.z cells (filled circles) against ErbB2-overexpressing and trastuzumab-sensitive MDA-MB453 (left), or ErbB2-overexpressing and trastuzumab-resistant JIMT-1 (middle) and CAL-51 (right) breast carcinoma cells was investigated in flow cytometry-based cytotoxicity assays after coincubation of NK cells and tumor cells at different effector to target ratios (E/T) for 2 h. Parental NK-92 cells were included for comparison (open circles). Mean values \pm SEM are shown; $n = 3$.

multiple myeloma (52, 74–77), underscoring the potential of CAR-engineered NK-92 cells for the treatment of a large variety of different cancers.

NK CELLS: A BRIDGE BETWEEN INNATE AND ADAPTIVE ANTITUMOR IMMUNITY

Natural killer cells do not only play a critical role in antitumor immunity by directly eliminating malignant cells, but also by regulating tumor-specific adaptive immune responses through cross talk with other immune cells. In particular, the interaction between NK cells and dendritic cells (DCs) is important in this context (Figure 3). On the one hand, DCs enhance the direct antitumor activity of NK cells (102). On the other hand, NK cells regulate DC maturation, thereby determining the effectiveness of subsequent DC-mediated T-cell activation (103, 104). Once activated by target cells or soluble factors, NK cells secrete high amounts of IFN- γ and tumor necrosis factor (TNF)- α , which synergistically contribute to the maturation of immature DCs (iDCs). This leads to enhanced expression of costimulatory molecules such as CD80, CD83, and CD86 by the DCs and

favors Th1 polarization during subsequent DC-mediated T-cell activation (105–107). Mature DCs (mDCs) release IL-12, IL-15, and IL-18, which in turn enhance IFN- γ expression by NK cells and NK-cell cytotoxicity against virus-infected and tumor cells (103, 108). Likewise, cytotoxicity of NK cells can be boosted by type I interferons such as IFN- α secreted by plasmacytoid DCs (pDCs) (109).

Dendritic cell maturation and reciprocal NK-cell activation are also strongly dependent on the engagement of activating receptors like NKp30, NKG2D, and NKp46 on NK cells (107, 110–113). Concurrent with inducing DC maturation, NK cells control the quality of the mDC population by killing iDCs (DC editing), which can otherwise induce immune tolerance through T-cell depletion or T_{reg} expansion. Discrimination and lysis of iDCs by NK cells is mainly regulated by activating signals through NKp30 and inhibitory signals through KIRs and the NKG2A/CD94 complex (107, 114). Accordingly, inhibition of NKp30 signaling or reduced NKp30 expression results in impaired NK-mediated killing of iDCs (110, 115, 116). Both mDCs and iDCs express NKp30 ligands. However, higher amounts of HLA class I and HLA-E molecules expressed by mDCs protect them from lysis

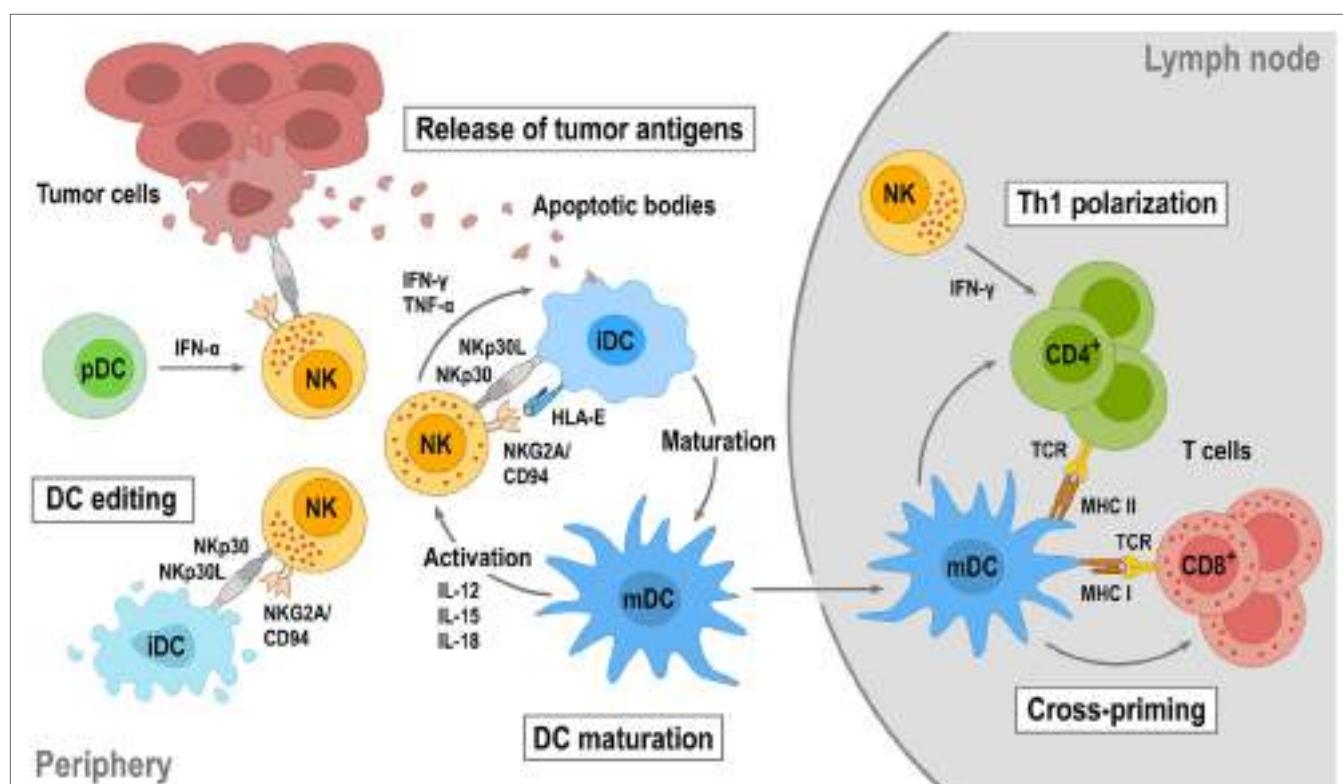


FIGURE 3 | Reciprocal natural killer (NK)–dendritic cell (DC) cross talk. Upon activation by target tumor cells or cytokines, NK cells produce IFN- γ and tumor necrosis factor (TNF)- α that can promote DC maturation. DC maturation is also strongly dependent on the engagement of activating receptors on NK cells such as NKp30 and NKG2D. Mature DCs (mDCs) will in turn produce interleukin (IL)-12, IL-15, and IL-18, which enhance cytotoxicity and IFN- γ secretion of NK cells. NK cells can also distinguish immature (iDC) and mDCs through activating NKp30 and inhibitory killer cell immunoglobulin-like receptors and NKG2A/CD94 and eliminate immature DCs (iDCs), thereby maintaining the quality of the mDC population (DC editing). NK-cell cytotoxicity can be further augmented by IFN- α secreted by plasmacytoid DCs (pDCs). NK-induced tumor cell lysis provides antigens, which can be taken up by DCs for antigen presentation. Once matured, antigen-loaded mDCs will migrate into tumor-draining lymph nodes, cross-present tumor antigens to naïve T cells, and induce their differentiation toward tumor-specific CD8 $^{+}$ cytotoxic T cells and CD4 $^{+}$ T helper 1 (Th1) cells.

by NK cells, whereas lower HLA class I and HLA-E expression makes iDCs vulnerable (114, 117). Importantly, by direct lysis of malignant cells, NK cells also provide tumor antigens for uptake and processing by DCs, which upon maturation and migration into a tumor-draining lymph node can cross-present such antigens to T cells, thereby inducing Th1 polarization of CD4⁺ T cells and differentiation of CD8⁺ T cells into tumor-specific cytotoxic T-lymphocytes (CTLs). NK cells can also migrate into tumor-draining lymph nodes and provide an early source of IFN- γ for Th1 polarization (118).

CAR-ENGINEERED NK-92 CELLS OVERCOME IMMUNOSUPPRESSIVE MECHANISMS AND ENHANCE ADAPTIVE ANTITUMOR IMMUNITY

As discussed above, efficient NK-cell activation is a prerequisite for productive NK-DC interaction. However, in cancer patients, NK-cell abnormalities are frequently found, including reduced NK-cell numbers, impaired cytotoxicity, and inefficient tumor infiltration (119). Especially in solid tumors, NK-cell activity is negatively affected by immunosuppressive factors in the tumor microenvironment (17, 120). High levels of TGF- β , IDO, and PGE2, as well as hypoxic conditions strongly inhibit the ability of NK cells to upregulate cytokine production and expression of activating cell surface receptors, while decreasing expression of ligands for activating NK-cell receptors by tumor cells. Under these conditions, tumor cells can also upregulate the non-classical MHC class I molecule HLA-G, a ligand for the NK-cell inhibitory receptors KIR2DL4 and ILT-2 (17, 121). Hence, therapeutic approaches that restore diminished NK-cell function may not only enhance direct NK-mediated tumor cell lysis but also improve clinical outcome by reinforcing DC activity and induction of adaptive antitumor immune responses.

Killing of cancer cells by CAR-engineered NK-92 is largely independent from the activation of endogenously expressed activating NK receptors and the presence of their ligands on target cells but mainly mediated by CAR-activation through binding to a cognate tumor-associated surface antigen (23, 49, 56, 67, 69, 71, 74). As recently demonstrated for ErbB2-specific NK-92/5.28.z carrying a CD28-CD3 ζ CAR, such cells retain efficient CAR-mediated cell killing even under hypoxic conditions and in the presence of TGF- β concentrations exceeding the elevated TGF- β levels found in the plasma of cancer patients (69, 122). Furthermore, target tumor cells ectopically overexpressing human HLA-G were unable to block specific cell killing by CAR-engineered NK-92 (Zhang et al., unpublished data), although NK-92 cells express the immunoregulatory receptors KIR2DL4 and ILT-2, which are activated by HLA-G (34, 123). These findings show that activated CAR NK-92 cells can maintain their cytotoxic potential in an immunosuppressive environment similar to the one found within a solid tumor. In addition, NK-92 readily express activating NK receptors such as NKP30 and NKG2D while most of the inhibitory KIRs are absent (34), which may make CAR NK-92 cells particularly effective in aiding DC maturation and editing, and

enhancing DC-mediated cross-priming of tumor-specific T cells and induction of adaptive antitumor immunity.

We recently investigated this possibility in an immunocompetent mouse model for glioblastoma and could indeed demonstrate the induction of endogenous antitumor immunity following therapy with CAR-engineered NK-92 cells (69). In this model, the majority of mice carrying syngeneic intracranial GL261/ErbB2 glioblastomas were cured upon repeated intratumoral injection of ErbB2-specific NK-92/5.28.z cells, while unmodified parental NK-92 cells were unable to inhibit tumor progression (Figure 4A). Human NK-92 and CAR NK-92 cells do not permanently engraft in immunodeficient mice and are quickly rejected by immunocompetent animals (67, 69, 124, 125). Nevertheless, without any further treatment, all mice that were cured from their initial tumors also rejected a rechallenge with GL261/ErbB2 cells injected into the other brain hemisphere 4 months after initial therapy, mediated by an endogenous memory immune response induced in the animals by initial treatment with NK-92/5.28.z (69). Sera from these mice contained IgG antibodies reactive with both GL261/ErbB2 and ErbB2-negative, but otherwise isogenic GL261 cells (Figure 4B), indicating that the induced protective antitumor immune response was broadly directed against the glioblastoma cells and not limited to the CAR target antigen. Accordingly, mice that were cured of GL261/ErbB2 tumors also rejected a subsequent challenge with GL261 cells (Zhang et al., unpublished data). When animals that had rejected the initial tumor and the first rechallenge with GL261/ErbB2 were injected once again with GL261/ErbB2 cells but this time after depletion of CD4⁺ and CD8⁺ T cells, tumors formed in a large proportion of the mice. This demonstrates that protective immunity induced by initial treatment with NK-92/5.28.z cells was also dependent on T-cell memory (Figure 4C). Similarly, in a later study by Boissel et al., intratumoral injection with NK-92 cells expressing a CAR specific for murine CD19 induced protective antitumor immunity in a syngeneic A20 lymphoma model in immunocompetent mice (126).

These data suggest that the release of tumor antigens through the cytotoxic activity of CAR NK-92 cells, most likely augmented by the demonstrated CAR-induced production of high levels of pro-inflammatory cytokines (67, 69, 71, 74), can not only induce a humoral immune response directed against the tumor but also enhance cross-presentation of tumor antigens by murine DCs for the activation of tumor-specific CTLs. In a clinical setting, this may be further enhanced by IFN- γ , which is released at high levels by activated CAR NK-92. In murine models, the effects of human IFN- γ are limited due to the species-specificity of IFN- γ /IFNGR1 interactions (127, 128). Of note, apoptotic tumor cells have been shown to be superior to cell lysates or tumor cell RNA in inducing a tumor-specific T-cell response (129, 130). Hence, tumor-cell apoptosis induced by the release of cytotoxic granules from activated CAR NK-92 cells provides tumor antigens in a most effective form for uptake, processing, and presentation by DCs. Whether this vaccine or adjuvant effect of CAR NK-92 cells is a particular consequence of direct intratumoral administration as performed in our study and the study by Boissel et al. (69, 126) is subject of ongoing investigations.

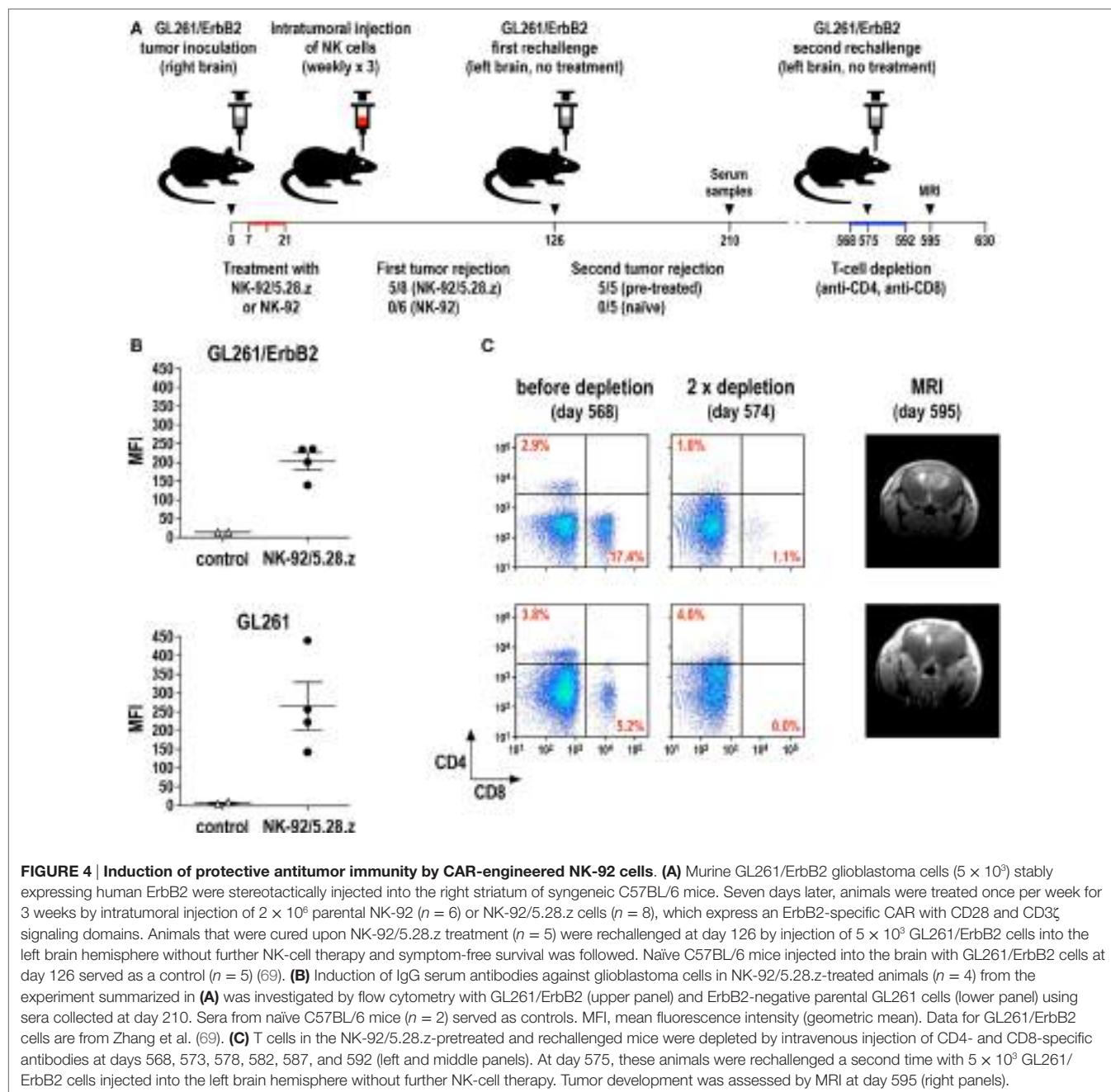


FIGURE 4 | Induction of protective antitumor immunity by CAR-engineered NK-92 cells. **(A)** Murine GL261/ErbB2 glioblastoma cells (5×10^3) stably expressing human ErbB2 were stereotactically injected into the right striatum of syngeneic C57BL/6 mice. Seven days later, animals were treated once per week for 3 weeks by intratumoral injection of 2×10^6 parental NK-92 ($n = 6$) or NK-92/5.28.z cells ($n = 8$), which express an ErbB2-specific CAR with CD28 and CD3 ζ signaling domains. Animals that were cured upon NK-92/5.28.z treatment ($n = 5$) were rechallenged at day 126 by injection of 5×10^3 GL261/ErbB2 cells into the left brain hemisphere without further NK-cell therapy and symptom-free survival was followed. Naive C57BL/6 mice injected into the brain with GL261/ErbB2 cells at day 126 served as a control ($n = 5$) (69). **(B)** Induction of IgG serum antibodies against glioblastoma cells in NK-92/5.28.z-treated animals ($n = 4$) from the experiment summarized in **(A)** was investigated by flow cytometry with GL261/ErbB2 (upper panel) and ErbB2-negative parental GL261 cells (lower panel) using sera collected at day 210. Sera from naïve C57BL/6 mice ($n = 2$) served as controls. MFI, mean fluorescence intensity (geometric mean). Data for GL261/ErbB2 cells are from Zhang et al. (69). **(C)** T cells in the NK-92/5.28.z-pretreated and rechallenged mice were depleted by intravenous injection of CD4- and CD8-specific antibodies at days 568, 573, 578, 582, 587, and 592 (left and middle panels). At day 575, these animals were rechallenged a second time with 5×10^3 GL261/ErbB2 cells injected into the left brain hemisphere without further NK-cell therapy. Tumor development was assessed by MRI at day 595 (right panels).

GENERAL SAFETY ASPECTS OF CAR NK-92 CELLS

In addition to the well-defined chimeric receptor, polyclonal CAR T cells carry endogenous, MHC-restricted T-cell receptors (TCRs) of unknown specificity. In an autologous setting, it can be expected that due to thymic selection, only very few autoreactive T cells are present in the periphery. Nevertheless, in donor-derived CAR T cells, CAR-induced activation and expansion may accidentally result in increased TCR-mediated reactivity with the recipient's tissues, leading to severe GvHD (131). Unlike T cells, NK cells do not carry genetically rearranged clonogenic receptors. Ligands for germline-encoded activating

NK-cell receptors are typically upregulated only by stressed cells after virus infection or malignant transformation, which is the basis of the NK cells' intrinsic antitumor activity (2). Hence, the activating receptors NKp30, NKp46, and NKG2D expressed by NK-92 can be expected to contribute to the antitumor activity of CAR-engineered variants rather than causing adverse effects (34). The natural cytotoxicity of NK-92 is largely retained by CAR-expressing variants as demonstrated in different studies using K562 cells as targets (23, 49).

Donor lymphocyte infusion with allogeneic NK cells is mostly performed in the context of hematopoietic stem cell transplantation and generally considered safe, without a high risk of inducing GvHD (20). Nevertheless, depending on donor

selection and *ex vivo* activation, development of acute GvHD after NK DLI has been described, attributed to NK-dependent augmentation of T-cell alloreactivity (132). In two clinical trials with NK-92 cells, only mild infusion-related side effects were noted, while no severe treatment-related toxicities were observed even at a cell dose as high as $1 \times 10^{10}/\text{m}^2$ body surface (31, 35, 36). While HLA-specific antibodies reactive with the allogeneic cells were found in some patients after NK-92 therapy, this was not linked to adverse effects. To prevent potential engraftment of NK-92 that was initially derived from a non-Hodgkin lymphoma patient (33), the cells were irradiated with 10 Gy prior to infusion, prohibiting further proliferation but only resulting in a gradual decline of cytotoxicity over several days (31). Since it is presently unknown whether non-irradiated NK-92 cells have the potential to form secondary lymphoma in a human host, irradiation of the cells before infusion will also be included as a safety measure in a planned phase I clinical trial investigating intratumoral injection of ErbB2-specific NK-92/5.28.z cells in patients with recurrent ErbB2-positive glioblastoma (69, 133). Like parental NK-92, CAR-engineered NK-92 variants transiently retain specific cytotoxicity after γ -irradiation with 10 Gy, with unchanged *in vitro* and *in vivo* antitumor activity (23, 56, 67, 69). This extends to the immunostimulatory activity of CAR NK-92 cells, which was also found for irradiated ErbB2-specific NK-92/5.28.z cells in an immunocompetent glioblastoma mouse model similar to the one described in **Figure 4** (Zhang et al., unpublished data). Hence, in contrast to CAR T cells, which are capable of uncontrolled *in vivo* expansion, the effective dose of CAR NK-92 cells can be tightly managed to establish a therapeutic window, albeit at the price of potentially higher cell numbers and more frequent treatment intervals needed.

A major concern with CAR T-cell therapy is cytokine release syndrome (CRS) frequently observed in clinical trials with CD19-specific effector cells, which can be severe and even cause fatalities. IL-6 production and IL-6 trans-signaling after massive activation of infused CAR T cells were found to play a critical role in CRS (134). CRS can be managed with the IL-6 receptor (IL-6R) blocking antibody tocilizumab or steroid treatment (134–136). While the latter inhibits CAR T-cell expansion and activity, it constitutes an important option for patients who do not respond to the IL-6R antagonist. In contrast to CAR T cells, activated CAR NK-92 cells do not produce measurable amounts of IL-6 and IL-4 as demonstrated for NK-92 variants targeted to EGFR, EGFRvIII, ErbB2, or CD19 (67, 69, 71, 74). Instead, upon CAR activation, these cells secrete high levels of IFN- γ , macrophage inflammatory protein (MIP)-1 α (CCL3), GM-CSF, and moderate levels of TNF- α . This cytokine/chemokine profile appears more favorable and less likely to induce CRS, while supporting the CAR NK-92-induced activation of endogenous antitumor immunity described above.

POTENTIAL ON-TARGET/OFF-TUMOR TOXICITY

Chimeric antigen receptor effector cells specifically targeting mutated tumor antigens and viral antigens not expressed in normal tissues do not carry the risk of inducing on-target/off-tumor toxicity. This safety feature is given for CAR NK-92

cells selective for the tumor-specific EGFR mutant EGFRvIII frequently expressed in glioblastoma (71) and genetically modified NK-92 cells recognizing an epitope of the EBV latent protein EBNA3C in complex with HLA-A2 (78). However, most CARs currently available are directed to non-mutated self-antigens differentially expressed by the cancer cells. Consequently, there is a possibility for on-target/off-tumor activity against antigen-positive healthy tissues, which can result in severe toxicities. B-cell aplasia is typically observed after CD19 CAR T-cell therapy but can easily be managed by infusion of immunoglobulins. This may be different if a tumor-associated target antigen is also present in vital tissues. In a clinical trial conducted at the National Cancer Institute, a fatal adverse event occurred after infusion of autologous T cells modified to express an ErbB2-specific third generation CAR based on trastuzumab (137). Although antigen-independent CAR activation due to the combination of three signaling domains (CD137, CD28, CD3 ζ) cannot be excluded (46), massive T-cell activation and respiratory failure immediately after CAR T-cell infusion may have been triggered at least in part by ErbB2 expressed at low levels on normal lung epithelium.

In addition to CAR affinity, the location of the CAR-binding epitope within the target antigen can play a decisive role in effector cell activation and influence on-target/off-tumor effects (82, 138). In the case of CAR T cells, CARs directed to membrane-distal epitopes were shown to be superior in binding but less potent in mediating activation than CARs directed to membrane-proximal epitopes of the same antigen (82, 83). We recently showed a similar effect for CAR NK-92 cells targeting EGFR, where a second-generation CAR based on antibody cetuximab, which interacts with domain III of EGFR mediated more potent cytotoxicity than an otherwise identical CAR based on antibody R1 that recognizes an epitope within the N-terminal EGFR domain I (71). The trastuzumab-derived ErbB2-specific scFv antibody fragment used by Morgan et al. binds to the juxtamembrane region (domain IV) of the target receptor (137, 139). In contrast, antibody FRP5 used for the generation of ErbB2-specific NK-92/5.28.z cells recognizes a discontinuous epitope within domain I of ErbB2 facing away from the cell surface (44, 140). Consequently, FRP5-based CARs are less likely than trastuzumab-based CARs to get activated by ErbB2 expressed at moderate levels, which is supported by data from a clinical trial in sarcoma patients with ErbB2-specific T cells carrying an FRP5-based second-generation CAR, where no on-target/off-tumor toxicities were observed (141).

Irradiated CAR NK-92 cells do not expand and persist *in vivo*. Hence, they may even be applicable in a clinical setting to target more abundantly expressed self-antigens such as non-mutated EGFR (70–72), since side effects due to reactivity with normal tissues would be expected to be transient. In the future, more sophisticated safety measures like expression of inducible caspase-9 (iCasp9) as a suicide gene may thereby replace γ -irradiation of NK-92 and CAR NK-92 cells, allowing to rapidly eliminate the cells in case of toxicities, but also to extend *in vivo* activity with a reduction in the cell dose and treatment frequency needed. iCasp9 represents a fusion of the human FK506-binding protein FKBP12 harboring an F36V mutation, and truncated human caspase-9 lacking the caspase activation and recruitment domain (142). In the presence of otherwise inert FK506 analogs such as

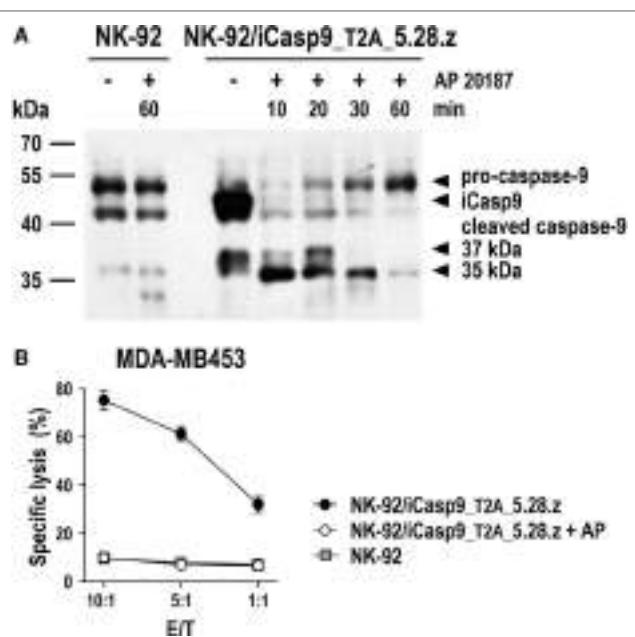


FIGURE 5 | Inducible caspase-9 (iCasp9) as a safety switch for CAR-engineered NK-92 cells. **(A)** NK-92 cells transduced with a lentiviral vector that encodes iCasp9 and ErbB2-specific CAR 5.28.z separated by a *Thosaea asigma* virus self-cleaving peptide (T2A) (NK-92/iCasp9_T2A_5.28.z) were incubated in the presence of 10 nM of the homodimerizer AP20187 for iCasp9 activation. Lysates of cells collected after 10, 20, 30, or 60 min of exposure to AP20187 were subjected to SDS-PAGE and subsequent immunoblotting with a caspase-9-specific antibody. Lysates of NK-92/iCasp9_T2A_5.28.z cells kept without dimerizer and parental NK-92 cells incubated in the absence or presence of AP20187 served as controls. **(B)** Cytotoxicity of NK-92/iCasp9_T2A_5.28.z cells against ErbB2-overexpressing MDA-MB453 breast carcinoma cells was investigated in flow cytometry-based cytotoxicity assays after co-incubation of NK cells and tumor cells at different effector to target ratios (E/T) for 2 h in the absence (filled circles) or presence of AP20187 (open circles). Parental NK-92 cells were included for comparison (gray boxes). Mean values \pm SEM are shown; $n = 2$.

AP1903 or AP20187, iCasp9 dimerizes, inducing caspase activation and apoptotic cell death. NK-92 cells coexpressing iCasp9 and a CAR are viable in the absence of dimerizer and retain high and specific CAR-mediated cytotoxicity. In contrast, addition of AP20187 rapidly induces activation of iCasp9 and cleavage of endogenous caspases, precluding any further cell killing by the CAR-engineered cells (Figure 5).

CONCLUSION AND FUTURE PERSPECTIVES

Over the past 25 years, NK-92 cells have transformed from a readily available model for studies on human NK cell biology to a promising cell therapeutic for applications in adoptive cancer immunotherapy. As outlined above, genetic modification of NK-92 with CARs has emerged as a successful strategy to enhance the cells' intrinsic antitumor activity and provide them with the capacity for selective target recognition. The ability of CAR NK-92 cells to bypass the immunosuppressive effects of TGF- β

and hypoxia in preclinical studies and to enhance or initiate adaptive antitumor immunity is encouraging (69). Nevertheless, the potential impact of immunosuppressive factors like IDO, PGE2, IL-4, NO, and ROS abundant in the tumor microenvironment has not yet been investigated. Concurrent interference with these mechanisms may offer an opportunity to further improve the direct antitumor activity of CAR NK-92 and enhance their immunostimulatory potential. Also, clarifying the relevance of checkpoint regulators such as PD-1 for CAR NK-92 functionality (74) and investigating combination therapies with checkpoint inhibitors and other immunomodulatory regimens appears warranted. Specific cytotoxicity of the NK cells may be enhanced by ectopic expression of components of the cytolytic machinery (143). Chemoattractants like CXCR3 ligands and chemerin can increase accumulation of NK cells at tumor sites (144, 145) and modulation of chemokine receptor expression would likely augment the tumor homing capability of CAR NK-92 cells (146).

Clinical responses seen in individual patients treated with parental NK-92 and ease of improvement by genetic modification with Fc receptors and CARs not only increased efforts of academic researchers to design tailor-made variants for specific disease entities and target antigens but also sparked commercial interest, which is essential to address the challenges associated with standardization of such cell products, large-scale expansion, logistics for distribution, and advanced clinical development (32, 90). Current efforts in the CAR T-cell field are aimed at generating similar universal cell products by eliminating endogenous TCR and MHC with the help of sophisticated gene editing procedures (147–149). This underscores the relevance of truly off-the-shelf CAR cell products like CAR NK-92 for broader applicability of this therapeutic strategy. Early phase clinical trials with CAR NK-92 cells are expected to commence in the near future. Insights from these studies will be essential to judge the therapeutic potential of CAR NK-92 in comparison to *ex vivo* expanded and CAR-engineered primary NK cells and determine the direction of further development.

ETHICS STATEMENT

Animal experiments were approved by the responsible government committee (Regierungspräsidium Darmstadt, Darmstadt, Germany) and were conducted according to the applicable guidelines and regulations.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design of this review article and critically evaluated the cited literature. CZ and SO performed experiments and analyzed data. CZ, PO, AW, AL, and WW drafted the initial version of the manuscript with support from all other co-authors. All authors revised the manuscript and approved the final version of this study. All authors agree to be accountable for the content of this work.

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The reviewer AC declared a shared affiliation, though no other collaboration, with several of the authors CZ, TT and WW to the handling Editor, who ensured that the process nevertheless met the standards of a fair and objective review.

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In Vivo Efficacy of Umbilical Cord Blood Stem Cell-Derived NK Cells in the Treatment of Metastatic Colorectal Cancer

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Therapeutic monoclonal antibodies against the epidermal growth factor receptor (EGFR) act by inhibiting EGFR downstream signaling and by eliciting a natural killer (NK) cell-mediated antitumor response. The IgG₁ mAb cetuximab has been used for treatment of RAS^{wt} metastatic colorectal cancer (mCRC) patients, showing limited efficacy. In the present study, we address the potential of adoptive NK cell therapy to overcome these limitations investigating two allogeneic NK cell products, i.e., allogeneic activated peripheral blood NK cells (A-PBNK) and umbilical cord blood stem cell-derived NK cells (UCB-NK). While cetuximab monotherapy was not effective against EGFR⁻ RAS^{wt}, EGFR⁺ RAS^{mut}, and EGFR⁺ BRAF^{mut} cells, A-PBNK were able to initiate lysis of EGFR⁺ colon cancer cells irrespective of RAS or BRAF status. Cytotoxic effects of A-PBNK (but not UCB-NK) were further potentiated significantly by coating EGFR⁺ colon cancer cells with cetuximab. Of note, a significantly higher cytotoxicity was induced by UCB-NK in EGFR-RAS^{wt} (42 ± 8 versus 67 ± 7%), EGFR⁺ RAS^{mut} (20 ± 2 versus 37 ± 6%), and EGFR⁺ BRAF^{mut} (23 ± 3 versus 43 ± 7%) colon cancer cells compared to A-PBNK and equaled the cytotoxic efficacy of the combination of A-PBNK and cetuximab. The antitumor efficacy of UCB-NK cells against cetuximab-resistant human EGFR⁺ RAS^{mut} colon cancer cells was further confirmed in an *in vivo* preclinical mouse model where UCB-NK showed enhanced antitumor cytotoxicity against colon cancer independent of EGFR and RAS status. As UCB-NK have been proven safe in a recently conducted phase I clinical trial in acute myeloid leukemia, a fast translation into clinical proof of concept for mCRC could be considered.

Keywords: EGFR, RAS mutation, cetuximab, metastatic colorectal cancer, A-PBNK, UCB-NK, allogeneic NK cell immunotherapy

INTRODUCTION

Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths in the world (1). Despite substantial advances in the treatment of metastatic CRC (mCRC) over the last decades that have contributed to better survival rates (2, 3), the disease is still frequently fatal. Monoclonal antibodies (mAbs) targeting the epidermal growth factor receptor (EGFR) pathway, such as panitumumab

and cetuximab, are approved for the treatment of patients with advanced CRC either in combination with chemotherapy or, as monotherapy, in chemorefractory conditions (4). Cetuximab (CET) and panitumumab block the interaction between EGFR and its ligands, thus inhibiting the downstream RAS-signaling cascade and tyrosine kinase activation (5). However, mutations in tumor suppressor genes and proto-oncogenes in EGFR signaling pathways, such as in RAS, BRAF, and PIK3CA, are common in patients with CRC. These mutations represent a poor prognostic marker and render anti-EGFR mAbs ineffective, leaving 42% of the chemorefractory mCRC population without this standard treatment option (6, 7).

Besides the blockade of the EGFR-ligand interaction on tumor cells, therapeutic mAbs can also interact with natural killer (NK) cells triggering antibody-dependent cell-mediated cytotoxicity (ADCC) (8–10), and this can translate into superior antitumor effects (11). Two NK cell subsets can be identified based on the expression of CD16, the low affinity Fc γ RIIIa receptor. The majority of NK cells are CD56^{dim}CD16⁺ and play an active role in NK cell cytotoxicity and are capable of performing ADCC upon IgG₁ engagement via CD16, whereas CD56^{bright}CD16⁻ NK cells are mainly immune regulatory in function, secreting cytokines, and are less cytotoxic than CD56^{dim} cells (12). NK cell functions are tightly regulated by a delicate balance between activating receptors (like the natural cytotoxicity receptors NKp46, NKp30, and NKp44, or C-type lectin-like receptor NKG2D) (13) and major histocompatibility complex (MHC) class I binding inhibitory receptors, including killer cell immunoglobulin-like receptors (KIRs), LIR1/ILT2, and NKG2A/CD94 (14). The importance of NK cells in controlling tumors has been extensively demonstrated since their identification 40 years ago (15–17).

Several studies have shown a dysfunctional phenotype and poor infiltration of NK cells in the CRC tissue from early stages on, together with an immunosuppressive tumor microenvironment (18, 19). Hence, various strategies, e.g., using cytokines or therapeutic ADCC enhancing mAbs, have been explored to increase NK cell numbers and function and to enhance their trafficking to tumor sites (20). Another approach entails the adoptive transfer of *in vitro* manipulated and expanded autologous or allogeneic NK cells. Autologous NK cells so far have failed to demonstrate significant therapeutic benefits in solid tumors (21–23). Therefore, the focus has shifted to the development of allogeneic NK cells as a potential adoptive cell therapy for treatment in solid tumors. Previously, we demonstrated that the combination of allogeneic activated peripheral blood NK cells (A-PBNK) and CET can effectively target RAS mutant (RAS^{mut}) CRC tumors (24).

Here, we compared two feeder cell-free allogeneic NK cell products, i.e., A-PBNK and umbilical cord blood stem-cell derived NK cells (UCB-NK), alone or in combination with cetuximab for antitumor effects against RAS^{mut} CRC.

MATERIALS AND METHODS

Cell Lines

Cell lines A431 (epidermoid carcinoma), COLO320, SW480, and HT-29 (colon carcinoma) were obtained from American Type

Culture Collection and cultured in Dulbecco's modified medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands). Cell cultures were passaged every 5 days and maintained in a 37°C, 95% humidity, 5% CO₂ incubator.

PBNK Isolation and Activation

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of healthy donors (six males, four females, age range = 56–64 years and CRC patients (eight males, two females, age range = 66–74 years) after written informed consent and according to protocols approved by the institutional review board of VU University Medical Center, Amsterdam (NCT01792934). Blood samples were collected at baseline and after the first cycle of first-line palliative chemotherapy consisting of oral capecitabine (1,000 mg/m², bid, days 1–14), i.v. oxaliplatin (130 mg/m², day 1), and i.v. bevacizumab (7.5 mg/kg, day 1, in 4/10 mCRC patients). PBMCs were isolated using Lymphoprep™ (STEMCELL Technologies, Cologne, Germany) density gradient centrifugation. CD56⁺ NK cells were isolated from PBMC using a MACS Human NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. PBNK cells purity and viability were checked using CD3 VioBlue, CD56 APC Vio 770, and CD16 APC (Miltenyi Biotech) and 7-AAD (Sigma Aldrich, Zwijndrecht, The Netherlands). Isolated PBNK cells were activated overnight with 1,000 U/ml IL-2 (Proleukin®; Chiron, München, Germany) and 10 ng/ml IL-15 (CellGenix) for use in cytotoxicity assays. The parameters compared before and after stimulation with cytokines were NK purity (87 ± 5 versus 84 ± 2%), NK CD16⁺, (92 ± 12 versus 88 ± 8%) and NK viability (89 ± 5 versus 84 ± 8%), respectively.

Flow Cytometry

The antibody staining mix for the assessment of NK cell functionality consisted of CD45 VioGreen, CD14 VioBlue, CD19 VioBlue, and SYTOX® Blue, together with CD3 PerCP-Vio 700 and TCRγδ PerCP-Vio700 to exclude dead cells, debris, and non-NK populations from PBMCs. NK cells were identified by the expression of CD45⁺CD3⁻CD56⁺ cells, and further characterized for NK functionality by plotting against CD16 APC, CD25 VioBrightFITC, CD107a PE, and NKp44 PE-Vio770 and for NK cell phenotype by plotting against NKG2A PE-Vio770, NKG2C PE, NKG2D PerCP-Cy5.5, and PanKIR2D FITC. All antibodies were supplied by Miltenyi Biotec except SYTOX® Blue (Thermo Fisher Scientific, Berlin, Germany).

UCB-NK Cultures

Allogeneic NK cells (UCB-NK) were generated from cryopreserved umbilical cord blood (UCB) hematopoietic stem cells as previously described (25). CD34⁺ UCB cells from six UCB-donors were plated (4 × 10⁵/ml) into 12-well tissue culture plates (Corning Incorporated, Corning, New York, NY, USA) in Glycostem Basal Growth Medium (GBGM®) (Clear Cell Technologies, Beernem, Belgium) supplemented with 10% human serum (HS; Sanquin Bloodbank, Amsterdam, The Netherlands), 25 ng/ml of SCF, Flt-3L, TPO, and IL-7 (CellGenix, Freiburg, Germany). In the

expansion phase II, from day 9 to day 14, TPO was replaced with 20 ng/ml IL-15 (CellGenix). During the first 14 days of culture, low molecular weight heparin (Clivarin®; Abbott, Wiesbaden, Germany) in a final concentration of 20 µg/ml and a low-dose cytokine cocktail consisting of 10 pg/ml GM-CSF (Neupogen), 250 pg/ml G-CSF and 50 pg/ml IL-6 (CellGenix) were added to the expansion cultures. Cells were refreshed with new medium twice a week and maintained at 37°C, 5% CO₂. On day 14, the NK cell differentiation process was initiated by addition of NK cell differentiation medium consisting of the same basal medium with 2% HS but with high-dose cytokine cocktail consisting of 20 ng/ml of IL-7, SCF, IL-15 (CellGenix), and 1,000 U/ml IL-2 (Proleukin®; Chiron, München, Germany). Cultures were refreshed every 2–3 days and maintained till day 42. Five UCB-NK cultures were used for cytotoxicity assays and one UCB-NK culture for *in vivo* studies (both with a CD56⁺ cell purity of >95%). UCB-NK CD16 levels in matured UCB-NK cells were monitored using an antibody mix of human CD45VioGreen (1:11), CD56 APC-Vio770 (1:11), and CD16 APC (1:11). Similarly, UCB-NK CD16 expression in BRGS mice was monitored using an antibody mix of BV650 anti-mouse CD45 (clone 30-F11), Alexa Fluor® 700 anti-human CD45 (clone HI30), PE-CF594 anti-human CD56 (clone B159), all from BD, and APC-Vio770 anti-human CD56 (clone REA196) and APC CD16 (clone REA423) both from Miltenyi Biotec.

NK Cell Cytotoxicity Assays

Flow cytometry was used for the readout of cytotoxicity assays. Target cells (COLO320, SW480, and HT-29) were labeled with 5 µM pacific blue succinimidyl ester (PBSE; Molecular Probes Europe, Leiden, The Netherlands) at a concentration of 1 × 10⁷ cells/ml for 10 min at 37°C. The reaction was terminated by adding an equal volume of FCS, followed by incubation at room temperature for 5 min after which stained cells were washed twice and suspended in DMEM + 10% FCS to a final concentration of 5 × 10⁵/ml. Overnight activated PBNK cells and UCB-NK cells were washed with PBS and suspended in GBGM + 2% FCS to a final concentration of 5 × 10⁵/ml. Target cells were cocultured with effector cells at an E:T ratio of 1:1 in a total volume of 250 µl in 96-well flat-bottom plates (5 × 10⁴ targets in 100 µl of DMEM + 10% FCS incubated with 5 × 10⁴ effectors in 100 µl of GBGM + 2% FCS, further supplemented with 25 µl of GBGM + 2% FCS and DMEM + 10% FCS medium). NK cells and target cells alone were plated out in triplicate as negative controls. Target cells were coated with 5 µg/ml cetuximab (Merck, Darmstadt, Germany) for 1 h at 4°C. To measure degranulation of NK cells, anti-CD107a PE (Miltenyi Biotech) was added in 1:20 dilution at the beginning of the assay. After incubation for 4 h at 37°C, cells were harvested and stained with CD56 APC Vio 770 (1:25) and CD16 APC (1:25) (Miltenyi Biotech) and 7-AAD (1:500) (Sigma Aldrich). Degranulation of NK cells was measured by detecting cell surface expression of CD107a.

In Vivo Studies

The EGFR⁺RAS^{mut} SW480 cell line and EGFR⁺⁺⁺RAS^{wt} A431 cell line were stably transduced with Gaussia Luciferase (Gluc) for *in vivo* studies. Lentiviral (LV) supernatant of Cerulean

Fluorescent Protein (CFP)-positive Gluc virus (LV-CFP-Gluc) was kindly provided by Dr. Tom Würdinger (26). SW480 and A431 cells with Gluc expression of 95% were used for mouse studies.

Immunodeficient BRGS mice (BALB/c *Rag2*^{tm1Fwa} *Il2rg*^{tm1Cgn} *Sirpa*^{NOD}) were used in this study. Twenty-four adult mice (male, 8 weeks old) received an intravenous (i.v.) tail vein injection with 0.5 × 10⁶ SW480 Gluc cells at day 0 and were randomized into four groups. Group A only received SW480 cells, group B received SW480 in combination with cetuximab intraperitoneally (i.p., 0.5 mg, days 1, 4, and 7), group C received SW480 in combination with UCB-NK i.v. (1 × 10⁷, days 1, 4, and 7), and group D received SW480 cells in combination with UCB-NK i.v. (1 × 10⁷, days 1, 4, and 7) and cetuximab i.p. (0.5 mg, days 1, 4, and 7). Groups C and D received i.p. 0.5 µg IL-15 + 7.5 µg IL-15Rα every 2–3 days from day 0 till day 14. Further, three adult mice received i.v. tail vein injection of 0.5 × 10⁶ A431 Gluc cells at day 0 and were treated with 0.5 mg cetuximab (i.p., 0.5 mg, days 1, 4, and 7), which were used as a cetuximab efficacy control. Treatment effects were monitored using blood Gluc levels and bioluminescence imaging (BLI). All manipulations of BRGS mice were performed under laminar flow conditions.

Blood Gluc Quantification In Vitro

Secreted Gluc was measured according to a protocol described previously (27). A total of 10 µl of blood was collected by capillarity into EDTA containing Microvette® CB tubes. Blood samples were distributed in 96-well black plates then mixed with 100 µl of 100 mM Gluc substrate native coelenterazine in PBS (P.J.K. GmbH, Kleinblittersdorf, Germany), and 5 min later, light emission was quantified. Blood that was withdrawn before tumor inoculation served to determine a baseline value. Measurements were done twice a week until day 35. Gluc activity was measured using IVIS spectrum luminescence detector (PerkinElmer, Villebon-sur-Yvette, France). Data obtained were quantified using Living Image 4.0 software (PerkinElmer, Villebon-sur-Yvette, France).

BLI In Vivo

Mice were anesthetized using isofluorane gas in an induction chamber at a gas flow of 2.5 pm. Retro-orbital injection of coelenterazine (4 mg/kg body weight) was administered and mice were placed in the anesthesia manifold inside the imaging chamber and imaged within 5 min following substrate injection. Mice were placed into the light chamber and overlay images were collected for a period of 15 min using IVIS spectrum *in vivo* imaging system (PerkinElmer, Villebon-sur-Yvette, France). Images were then analyzed using Living Image 4.0 software (PerkinElmer, Villebon-sur-Yvette, France).

Ethics Statement

Animals were housed in isolators under pathogen-free conditions with humane care and anesthesia was performed using inhalational isoflurane anesthesia to minimize suffering. Experiments were approved by the Institut Pasteur's ethical committee for animal use in research, Comité d'éthique en expérimentation animale (CETEA) #89, protocol reference # 2007–006

and validated by the French Ministry of Education and Research (Reference # 02162.01).

Statistical Analysis

Data were analyzed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Differences between conditions were determined using one-way ANOVA or two-way ANOVA with multiple comparisons between column means, unpaired *t* test and log-rank (Mantel–Cox) test as deemed appropriate. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Highly Dysfunctional NK Cells in CRC Patients

Flow cytometry was used to determine the frequency, phenotype, and functionality of NK cells in PBMC of healthy volunteers (*n* = 10, age range 56–64 years, 6 males/4 females) and patients with metastatic CRC (*n* = 10, age range 66–74 years, 8 males/2 females) before and after the first cycle of first-line palliative chemotherapy consisting of oral capecitabine (1,000 mg/m², bid, days 1–14), i.v. oxaliplatin (130 mg/m², day 1), and i.v. bevacizumab (7.5 mg/kg, day 1, in 4/10 mCRC patients). As illustrated in **Figure 1A**, mCRC patients harbored on average a 20% lower percentage of CD3[−]CD56⁺NK cells in the total CD45⁺ lymphocyte population

as compared to healthy controls (*P* < 0.05). These lower NK rates, which are in line with a previous report in CRC (28), further declined after the first cycle of chemotherapy (*P* < 0.01).

We next evaluated whether this quantitative NK cell defect was also accompanied by functional defects in the NK cell population. For this purpose, the ability of NK cells from healthy volunteers and mCRC patients to induce both natural cytotoxicity and mediate ADCC of the epidermoid carcinoma cell line A431 (MHC-I^{low}, EGFR^{high}, KRAS^{wt}) was assessed. For ADCC, tumor target cells were coated with cetuximab before the addition of NK cells. It was evident that the cytotoxic potential of NK cells from mCRC patients, as reflected by degranulation (i.e., CD107a surface expression), was highly impaired both before chemotherapy and after the first cycle of chemotherapy. Though NK cells of mCRC patients were capable of ADCC, as evidenced by significant increases in degranulation when target cells were coated with cetuximab (*P* < 0.05), levels were still low compared to those observed in healthy volunteers (**Figure 1B**). Of note, although the NK cells of healthy volunteers and mCRC patients expressed similar levels of CD16 (**Figure 1C**), this did not translate into comparable levels of ADCC. Nkp44 expression, known to reflect the activation status of NK cells, was similar between the HD and mCRC groups used in NK cytotoxicity experiments (**Figure 1D**). Furthermore, no significant differences were observed in expression levels of NK activating (NKG2D, NKG2C) and NK inhibiting

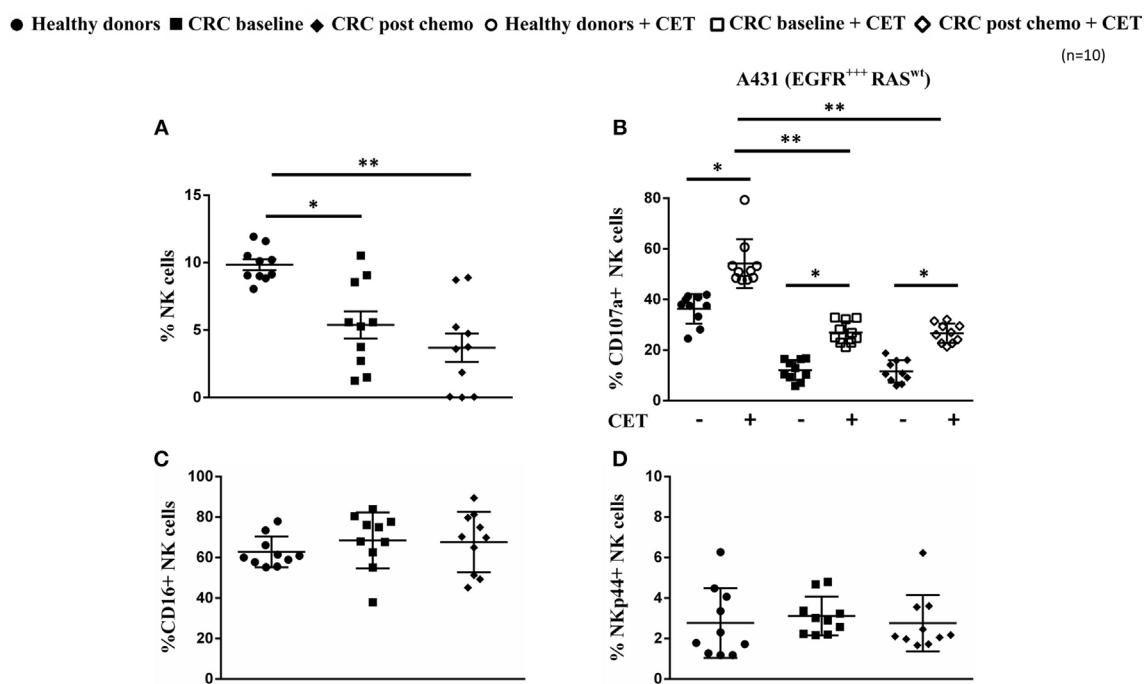


FIGURE 1 | Low prevalence and functionally impaired natural killer (NK) cells in colorectal cancer (CRC) patients. **(A)** Frequency of NK cells within peripheral blood mononuclear cells from healthy controls and from metastatic CRC (mCRC) patients at baseline and after the first cycle of chemotherapy. **(B)** NK cell degranulation in healthy controls and mCRC patients after a 4-h coculture of resting NK cells with A431 cells in the presence (open symbols) or absence (closed symbols) of cetuximab at an E:T ratio of 1:1. **(C)** Expression levels of resting NK cell CD16 and **(D)** Nkp44 in healthy controls and in mCRC patients before and after one cycle of chemotherapy. Data represent mean \pm SEM from 10 mCRC patients and 10 age- and sex-matched healthy controls. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, calculated with one-way ANOVA, multiple comparison between column means.

(NKG2A, KIR2D) receptors between healthy controls and CRC patients (Figure S1 in Supplementary Material).

Enhanced *In Vitro* Cytotoxicity of Colon Cancer Cells Mediated by UCB-NK Cells

In order to explore novel therapies to replace dysfunctional NK cells in patients with advanced CRC, we tested two different sources of allogeneic NK cell products (A-PBNK and UCB-NK) that could eventually be used for adoptive transfer strategies. We next compared the activity of A-PBNK cells (age range 22–37 years) and UCB-NK cells using a flow based NK cell cytotoxicity assay based on detection of 7-AAD accumulation in tumor cells. Three different cell lines of colon cancer origin were compared, i.e., COLO320 (EGFR⁻ RAS^{wt}), SW480 (EGFR⁺ RAS^{mut}), and HT-29 (EGFR⁺ RAS^{wt} BRAF^{mut}). As expected, addition of cetuximab to EGFR⁻ RAS^{wt} COLO320 cells did not result in increased killing. Of interest, lysis was consistently and significantly higher ($P < 0.01$) using UCB-NK compared to A-PBNK. As reported previously, the combination of cetuximab and A-PBNK resulted in increased killing of EGFR⁺RAS^{mut} SW480 and EGFR⁺ BRAF^{mut} HT-29 via ADCC (24). CD16 was expressed by 88 ± 8% ($n = 5$) of A-PBNK after overnight stimulation with cytokines and by 7 ± 2% ($n = 5$) of UCB-NK cells at the end of the 35-day culture period. No added effect of cetuximab was observed when using UCB-NK cells, which is possibly related to their lower *in vitro*

CD16 levels (29). Of note, tumor cell lysis induced by UCB-NK cells was comparable to that observed with the combination of A-PBNK and cetuximab (Figures 2A,B,C). Measurements of NK cell degranulation reflected equivalent trends observed for tumor cell lysis (Figures 2D,E,F). These results show that UCB-NK cells have superior cytotoxic efficacy over A-PBNK cells against cetuximab-resistant colon cancer cells *in vitro*.

UCB-NK Cells Inhibit *In Vivo* Tumor Growth and Increase Survival

To address whether UCB-NK cells exhibit similar antitumor efficacy *in vivo*, we transferred Gluc transduced SW480 cells to immunodeficient mice (BRGS; see Materials and Methods). SW480 cells are EGFR⁺RAS^{mut} and cetuximab monotherapy resistant. Mice were divided into four groups of six mice per group: SW480 only (group A), SW480 + cetuximab (group B), SW480 + UCB-NK (group C), and SW480 + UCB-NK + cetuximab (group D). Gaussia luciferase activity in whole blood was measured every 3 days to monitor the tumor burden (Figure S2 in Supplementary Material). These data confirmed our *in vitro* observations that SW480 cells were resistant to cetuximab-mediated growth inhibition (blue line). Of note, while treatment with UCB-NK cells alone significantly decreased the tumor load (green line), this effect was not increased by combining UCB-NK cells with cetuximab and thereby further confirmed both the

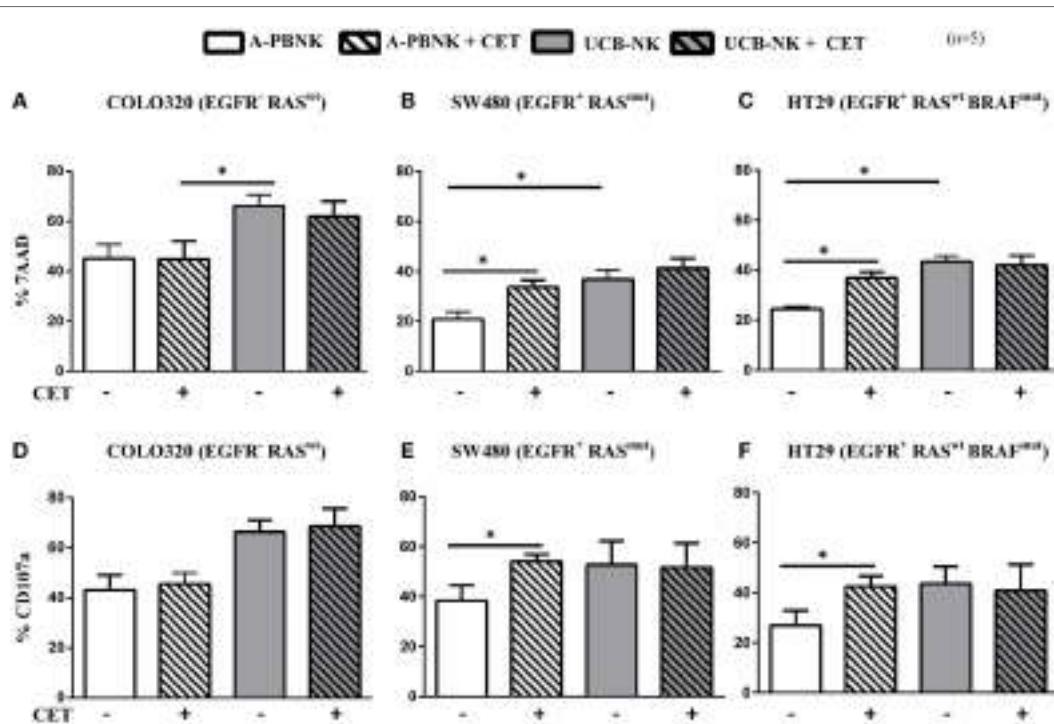


FIGURE 2 | *Ex vivo* cytotoxic efficacy of A-PBNK and UCB-NK cells against colorectal cancer (CRC) cells. CRC cell lines COLO320 (EGFR⁻, RAS^{wt}), SW480 (EGFR⁺, RAS^{mut}), and HT-29 (EGFR⁺, RAS^{wt}, BRAF^{mut}) were subjected to natural killer (NK) killing using two allogeneic NK cell products, i.e., A-PBNK and UCB-NK cells. 7-AAD (A,B,C) and CD107a (D,E,F) were measured after a 4-h coculture of A-PBNK and UCB-NK cells with CRC targets in the presence or absence of cetuximab at an E:T ratio of 1:1. Experiments were carried out in triplicate. Bars represent mean ± SEM, $n = 5$. * $P < 0.05$ and ** $P < 0.01$, calculated with two-way ANOVA, multiple comparison between column means.

inefficacy of cetuximab in treating RAS mutated tumors as well as the inability of cetuximab to induce ADCC of UCB-NK cells *in vivo* (orange line) (**Figure 3**). CD16 expression levels on UCB-NK cells were monitored in two mice upon adoptive transfer and increased from 6.0% before transfer to 14.0% (mouse 1) and 19.1% (mouse 2) at day 5 post UCB-NK cell infusion (data not shown).

While the blood Gluc assay measurements provided evidence of a reduction in the total tumor burden after UCB-NK treatment, we wanted to explore the impact of the therapy on the localization and size of the metastases. For that purpose, BLI was performed at day 35 after tumor inoculation. **Figure 4A** depicts four representative BLI images from each group at day 35 posttumor injection and average radiance from range of interest measurements are shown in **Figure 4B**. It is clear that mice from groups A and B showed a higher and more diffuse tumor load compared to mice treated with UCB-NK alone or in combination with cetuximab. In order to demonstrate the possibility of antitumor efficacy of cetuximab in the BRGS mouse model, we performed a similar tumor challenge using the cetuximab-sensitive A431 cell line, which bears wild-type RAS and overexpresses EGFR. A significant decrease in tumor load was observed when A431 tumors were treated with the same concentration of cetuximab as in the SW480 study (**Figure 4C**), confirming the *in vivo* functionality of cetuximab. We next assessed whether treatment of SW480 bearing mice with UCB-NK cells alone or in combination with cetuximab translated

into a survival advantage (**Figure 5**). Indeed, treatment of mice with UCB-NK cells alone resulted in a significant prolongation in their life span ($P = 0.01$), whereas combinatorial therapy did not add significantly to this. Treatment with cetuximab alone did not translate into a significant survival advantage, consistent with the observed effects on tumor growth.

DISCUSSION

In order to test the cytotoxic potential of NK cells for treating advanced CRC patients, we compared their functional status before and after chemotherapy. We observed that peripheral blood NK cell numbers were reduced in mCRC patients and that residual NK cells were dysfunctional and unable to mount a strong effector response when stimulated with an NK cell sensitive tumor target. Though an increase in NK cell cytotoxicity was observed when tumor target cells were coated with the anti-EGFR mAb cetuximab, reflecting a capacity for ADCC, cytotoxicity was still significantly lower (both before and after chemotherapy) than that observed in healthy controls. These data indicate a decreased functional state of NK cells in patients with mCRC, which is in line with studies in mice where the cytokine production and antitumor activity of adoptively transferred NK cells were highly affected following long-term exposure to tumors (30). Through recognition of MHC class I molecules, KIRs prevent NK cells from targeting healthy cells while allowing them to detect tumor or

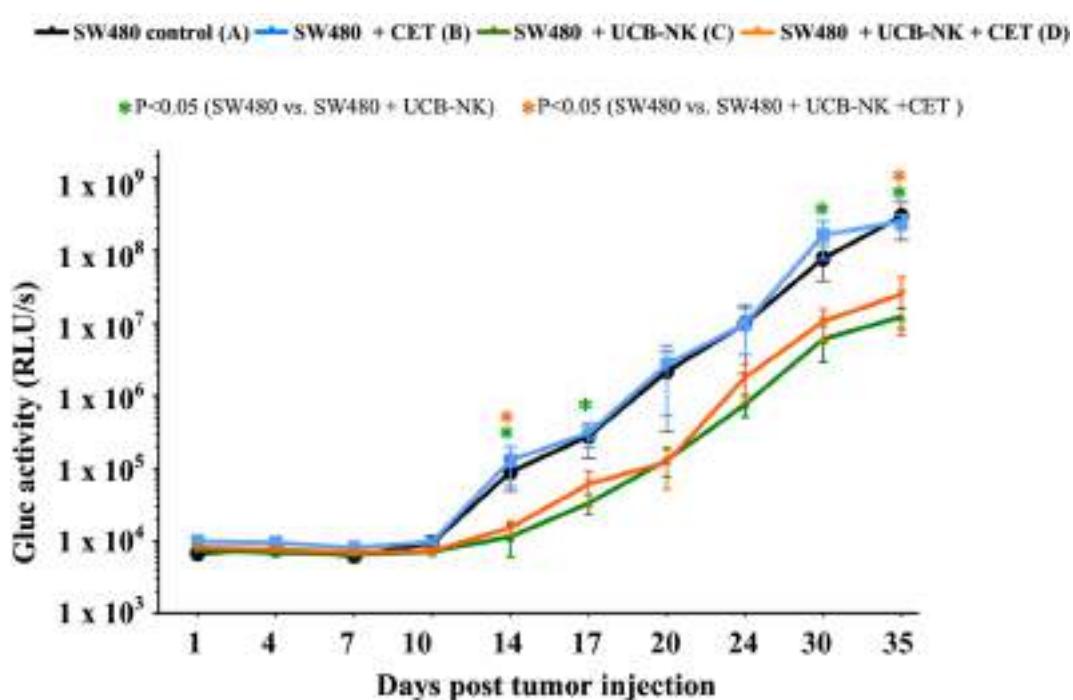


FIGURE 3 | Significant antitumor effects of UCB-NK cells *in vivo*. Real-time monitoring of tumor progression and treatment response was performed measuring Gluc levels from mice blood twice a week. Baseline Gluc values were obtained from all mice a day before tumor injection (day 1), and further monitoring continued until day 35. Blood Gluc levels were compared between control SW480 only (A) group and treatment groups SW480 + cetuximab (B), SW480 + UCB-NK (C), and SW480 + UCB-NK + cetuximab (D) for statistical significance. Data presented is from six mice per group ($n = 6$). Scatter plots represent mean \pm SEM. * $P < 0.05$, calculated with unpaired t test.

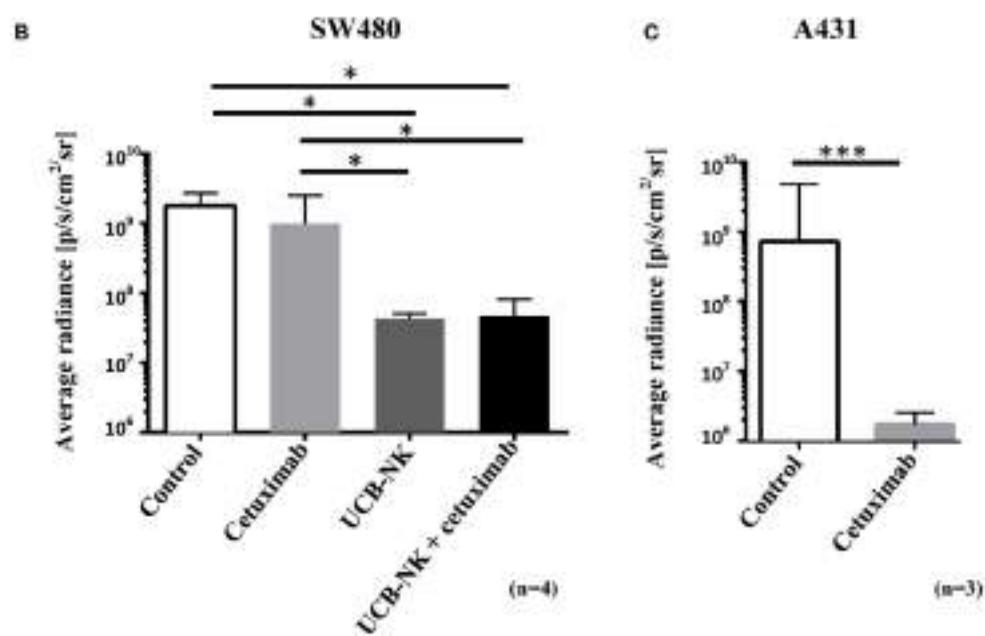
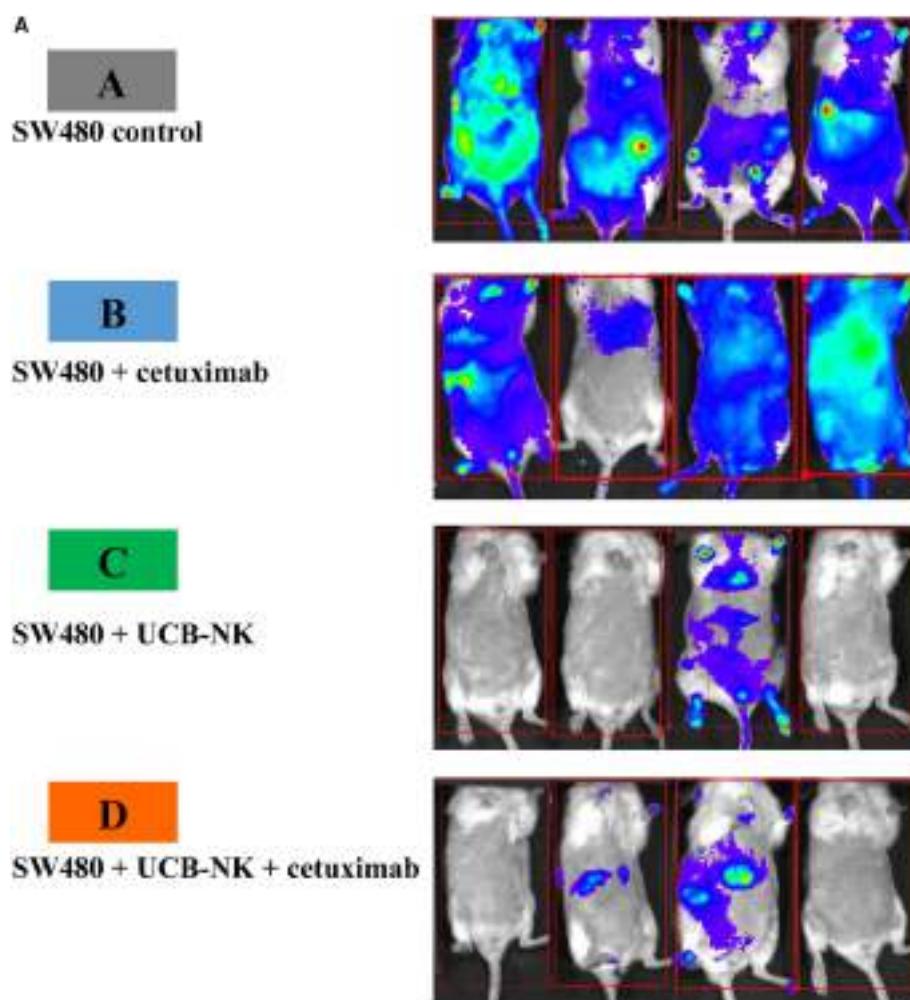


FIGURE 4 | Continued

FIGURE 4 | Continued

Successful tumor elimination by UCB-NK cells as revealed by bioluminescence imaging *in vivo*. (A) Four mice from control and treatment groups were imaged at day 35 for tumor load and distribution. Mice were injected retro-orbitally with Gluc substrate coelenterazine and images were acquired for 5 min. In SW480 control and SW480 + cetuximab groups, tumor growth was extensive and highly disseminated, spreading to most parts of the body. However, in UCB-NK and UCB-NK + cetuximab groups, there was a significantly lower tumor load, which was further verified by calculating the average radiance between groups as shown in panel (B) ($n = 4$ mice per group). (C) Cetuximab functionality against EGFR⁺⁺⁺ RAS^{wt} A431 cells was tested in parallel to SW480 studies in BRGS mice ($n = 3$ mice per group). For panels (B,C), bars represent mean \pm SEM. * $P < 0.05$ for panels (B,C) was calculated with unpaired t test.

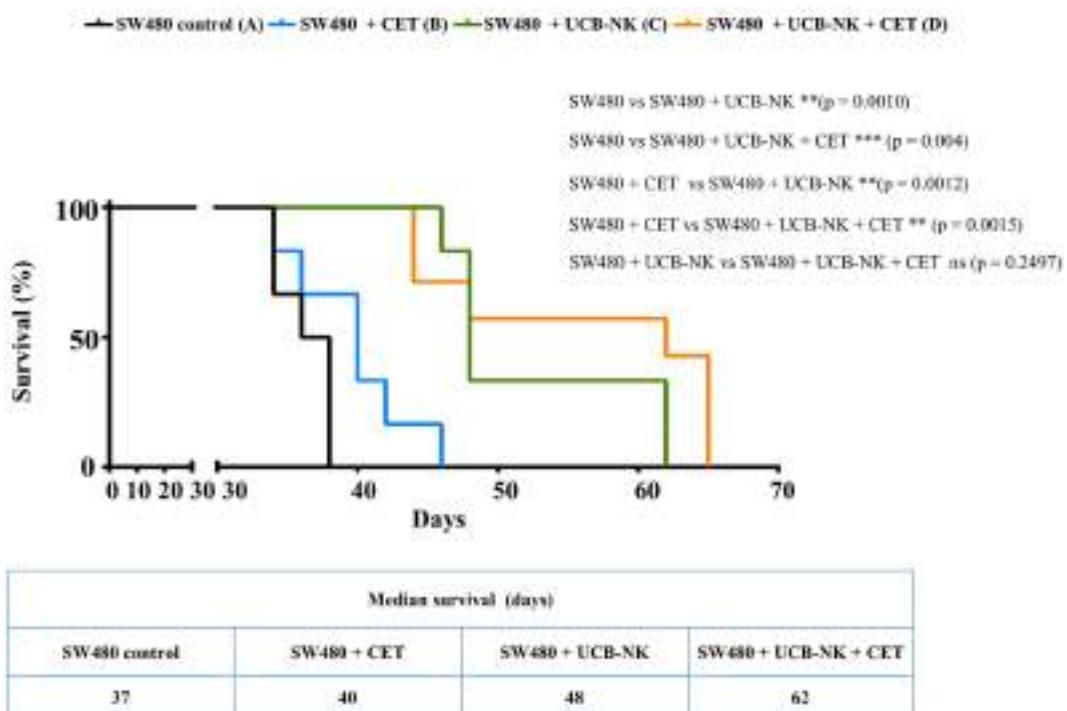


FIGURE 5 | Significant survival benefit in cetuximab-resistant RAS mutant tumor bearing mice treated with UCB-NK cells. Kaplan–Meier survival curves were plotted for the total experimental study period from day 0 until day 65. Survival rates of SW480 (EGFR⁺, RAS^{mut}) tumor-bearing mice ($n = 6$ per group) following treatment with PBS only (black line), cetuximab only (blue line), UCB-NK only (green line), and UCB-NK + cetuximab (orange line) were plotted over time to monitor treatment outcome. Statistical differences between groups were calculated using log-rank (Mantel–Cox) test and indicated in the figure.

infected cells with low or downregulated expression of MHC class I in a process known as “missing self” (31). Severely diminished or aberrant expression of MHC class I has been reported in the majority of colorectal adenocarcinomas (32, 33), which makes them an ideal target for NK cell-mediated killing. Although NK cells are infrequent in colorectal tissues (18), several independent studies investigated the clinical impact of NK cell infiltration on the prognosis of CRC, as well as in other types of carcinoma. These clinical studies, including a recent tissue microarray of 1,414 CRC biopsies, led to the conclusion that NK cell infiltration in tumors correlated with better overall response rates and progression-free survival in CRC patients (34–37), suggesting that therapies aimed at boosting NK cell functions could be beneficial in mCRC and possibly also in other types of cancer.

We evaluated and compared the cytotoxic efficacy of two different sources of feeder cell free allogeneic NK cells, i.e., A-PBNK cells and *in vitro* expanded and differentiated UCB-NK cells. *In*

vitro NK cell cytotoxicity experiments revealed that the cytotoxic activity of UCB-NK cells against CRC cells was significantly higher than that of A-PBNK cells and in addition demonstrated that, while an increase in cytotoxicity through ADCC was not evident with UCB-NK cells, their cytotoxic potential was still comparable to that observed with A-PBNK potentiated by cetuximab-mediated ADCC. It is possible that the stronger cytotoxic effects of UCB-NK cells result from a more intense stimulation with cytokines in comparison to A-PBNK cells. The failure to observe ADCC-enhanced cytotoxicity with UCB-NK cells *in vitro* can be explained by their low expression levels of CD16 (29). As we previously observed *in vivo* upregulation of CD16 on UCB-NK cells upon their transfer to NOD/SCID/IL2Rgnull (NSG) mice (38), we decided to also test the efficacy of cetuximab treatment in combination with UCB-NK cells in an *in vivo* model. Treatment of SW480 RAS^{mut} tumors in BRGS mice with UCB-NK cells resulted in control of disease progression

and translated into a significantly longer survival. As expected, cetuximab monotherapy did not result in a decreased SW480 tumor load or improvement in survival, recapitulating the clinical data from patients bearing RAS^{mut} CRC tumors. Unexpectedly, we failed to demonstrate superior *in vivo* antitumor effects or survival when we combined the transfer of UCB-NK cells with cetuximab infusions. The underlying causes for this latter finding remain obscure but may be related to suboptimal *in vivo* upregulation of CD16 in the used mouse model or CD16 polymorphisms in the employed batch of UCB-NK cells, both of which could have hampered efficient ADCC.

Taken together, UCB-NK cells displayed significant antitumor efficacy, suggesting a potential beneficial role for UCB-NK cells in the treatment of RAS and BRAF mutant CRC. As an important present limitation in treating mCRC patients is related to resistance to anti-EGFR mAbs, adoptive transfer of cytolytic UCB-NK cells could thus constitute a viable treatment option. Our *in vitro* and *in vivo* data demonstrating that adoptive transfer of UCB-NK cells alone was as effective as the combination of A-PBNK and cetuximab raises the possibility that UCB-NK administration could obviate the use of cetuximab in RAS^{wt} mCRC. Furthermore, UCB-NK can also lyse RAS^{mut} CRC cells at levels higher than those observed with A-PBNK. Importantly, allogeneic NK cells have demonstrated their safety in clinical trials in several solid tumors (39, 40), and more specifically, the UCB-NK cell product used in our experiments was found to be safe in a clinical trial in acute myeloid leukemia (AML) patients (Dolstra et al., 2016 manuscript submitted).

Several features make UCB-NK attractive for further clinical development. For example, our GMP-based expansion and differentiation protocol reproducibly resulted in a more than 10,000-fold expansion of cytotoxic UCB-NK cells from single donors. Furthermore, UCB-NK cells can be supplied as an “off the shelf” product, stored in large aliquots facilitating multiple infusions. Also, the low immunogenicity by UCB grafts prevents adverse reactions that are prevalent after repeated PBNK transfusions (41). In this respect, it is relevant to mention that while NK cells in general are often inhibited by recognition of MHC class I molecules on the surface of tumor cells, UCB-NK display relatively low levels of KIRs supporting their ability to effectively lyse MHC class I-expressing tumor cells (29). Finally, the ability of UCB-NK cells to proliferate and home to liver, lungs, spleen, and bone marrow after adoptive transfer has been previously demonstrated in NSG mice (38), though additional studies are required to determine whether UCB-NK cells have a similar migratory pattern upon adoptive transfer in solid tumor patients. Together, these features and observations provide UCB-NK cells with several unique advantages for further development as a universal NK cell platform.

Considering the size and heterogeneity of the tumor mass in advanced stages of CRC and other types of cancer, UCB-NK may not provide a sufficient therapeutic effect as a single agent. However, rational combinations of UCB-NK cells with existing drugs or drugs that are in clinical development can be envisioned to further increase their efficacy. Previous studies have pointed out that the proteasome inhibitor (bortezomib) (42) and the immunomodulatory drug (lenalidomide) (43) sensitize tumor

cells to NK-mediated killing. In addition, UCB-NK cell application together with bispecific or trispecific antibodies that bind to tumor and UCB-NK cell-activating receptors can also increase NK cell tumor specificity (44). Though we did not specifically assess ADCC induced by other mAbs, it is very likely that the failure of UCB-NK to mediate ADCC is a more general phenomenon as this depends on binding to CD16/FcγRIII, which was found to be expressed at only low levels in the UCB-NK cell product. However, recent data from a clinical phase 1 study with the same UCB-NK cell product in patients with AML revealed significant upregulation of CD16 on UCB-NK cells post transfusion suggesting that the UCB-NK cell product may acquire the capacity to mediate ADCC in patients following adoptive transfer (Dolstra et al., manuscript submitted). Further, this phenomenon may also provide a strong rationale for combining UCB-NK cells with bispecific or trispecific killer cell engagers (45). Taken together, these approaches can substantially increase UCB-NK cell responses to advanced solid tumors, including mCRC.

In conclusion, in this study, we have demonstrated the *in vitro* efficacy of UCB-NK cells against multiple CRC cell lines independent of EGFR expression and EGFR downstream signaling mutations, and in addition have demonstrated the *in vivo* antitumor efficacy of adoptively transferred UCB-NK cells against EGFR⁺RAS^{mut} tumors. As the adoptive transfer of UCB-NK cells (oNKord[®]) has been shown to be safe in patients with AML (CCMO no. NL31699 and Dutch trial register no 2818), our data provide a rationale for the clinical exploration of UCB-NK cells in the treatment of mCRC.

ETHICS STATEMENT

Approval for human subjects use: this study was carried out in accordance with the recommendation of the institutional review board of VU University Medical Center, Amsterdam (NCT01792934) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Committee for Scientific research of the VU University Medical Center, Cancer Center Amsterdam. Approval for animal subjects use: this study was carried out in accordance with the recommendations of the ethical committee at the Institut Pasteur (Reference # 2007–006). The protocol was approved by the French Ministry of Education and Research (Reference # 02162.01).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JV, SL-L, JPD, TG, HHV, JS, and HV. Performed the experiments: JV, SL-L, NK, and FB. Analyzed the data: JV and SL-L. Contributed reagents/materials/analysis tools: DH. Wrote the paper: JV and SL-L.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.00087/full#supplementary-material>.

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Conflict of Interest Statement: JV, JS, NK, and FB are employees of Glycostem Therapeutics; DH serves on the scientific advisory boards of Amgen and Pfizer. JPD is a stakeholder and founder of Axenix, SAS (France). The authors declare no conflict of interest.

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Triplebody Mediates Increased Anti-Leukemic Reactivity of IL-2 Activated Donor Natural Killer (NK) Cells and Impairs Viability of Their CD33-Expressing NK Subset

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Natural killer cells (NK) are essential for the elimination of resistant acute myeloid and acute lymphoblastic leukemia (AML and ALL) cells. NK cell-based immunotherapies have already successfully entered for clinical trials, but limitations due to immune escape mechanisms were identified. Therefore, we extended our established NK cell protocol by integration of the previously investigated powerful trispecific immunoligand ULBP2-aCD19-aCD33 [the so-called triplebodies (TBs)] to improve the anti-leukemic specificity of activated NK cells. IL-2-driven expansion led to strongly elevated natural killer group 2 member D (NKG2D) expressions on donor NK cells which promote the binding to ULBP2⁺ TBs. Similarly, CD33 expression on these NK cells could be detected. Dual-specific targeting and elimination were investigated against the B-cell precursor leukemia cell line BV-173 and patient blasts, which were positive for myeloid marker CD33 and B lymphoid marker CD19 exclusively presented on biphenotypic B/myeloid leukemia's. Cytotoxicity assays demonstrated improved killing properties of NK cells pre-coated with TBs compared to untreated controls. Specific NKG2D blocking on those NK cells in response to TBs diminished this killing activity. On the contrary, the observed upregulation of surface CD33 on about 28.0% of the NK cells decreased their viability in response to TBs during cytotoxic interaction of effector and target cells. Similar side effects were also detected against CD33⁺ T- and CD19⁺ B-cells. Very preliminary proof of principle results showed promising effects using NK cells and TBs against primary leukemic cells. In summary, we demonstrated a promising strategy for redirecting primary human NK cells in response to TBs against leukemia, which may lead to a future progress in NK cell-based immunotherapies.

Keywords: natural killer cells, natural killer group 2 member D, triplebodies, ULBP2, CD19, CD33, immunoligands, acute myeloid leukemia

INTRODUCTION

Natural killer (NK) cells are a subset of lymphoid effector cells within the innate immune response and have been shown to be a suitable tool for adoptive immunotherapy because of their ability of anti-tumor surveillance (1–6). In contrast to T cells, NK cells identify and eliminate malignant and virus-infected target cells in a major histocompatibility complex (MHC)-unrestricted way by engaging natural cytotoxicity receptors (NCRs), such as NKP30, NKP44, NKP46, and the activating receptor NKG2D (natural killer group 2 member D) which recognizes a variety of well-defined ligands expressed by transformed cells (7–9).

Major histocompatibility complex class I-related chain A and B (MICA/B) and the UL-16 binding protein family are cancer cell surface ligands that interact with NKG2D on NK cells. These specific bindings between NKG2D and their corresponding ligands (NKG2DL) on cancer cells are responsible for improved cytotoxic properties of NK cells against tumor and leukemia cells (10–12). Based on these receptor-ligand bindings between effector and target cells, an increased secretion of granzyme A (GraA) and B (GraB), granulysin, and perforin induced in NK cells could be demonstrated (13–15).

However, several types of cancer have developed a broad spectrum of immune escape mechanisms that down-modulate the NKG2D-mediated immune surveillance by metalloproteinases-driven proteolytic shedding and release of soluble NKG2DLs (16–20). In addition, elevated DNA-“hyper”-methylations for NKG2DLs could be detected in some malignant cells, mainly in acute myeloid leukemia (AML) cells, resulted in a clearance of NKG2DL surface cell expression, also detected for MICA, ULBP1/2 in AML patients (21–23). Enhanced tumor-shedding and DNA-methylation could contribute to an unhampered proliferation and evasion of immune control in AML patients (21).

Previous reports indicated that human leukocyte antigen (HLA) class I diversities could be responsible for induction of NK cell alloreactions by KIR (killer-cell immunoglobulin-like receptors)-ligand mismatch as shown in acute leukemia patients. The efficacy of this donor NK cell alloreactivity in mismatched hematopoietic transplants resulted in strong graft-versus-leukemia effects, prevented graft rejection and graft versus host disease and protected against AML relapse (24–26). Increased eliminations of AML blasts could be also shown by adoptive transfer of haploididentical NK cells and IL-2 infusions to stimulate *ex vivo* donor NK cell expansion. However, limitations have been observed by lacking of antigen specificity and long-lasting increase of immunosuppressive regulatory T cells that resulted in a reduction of NK cell proliferations and/or cytotoxic properties (27–30).

Some of the current anti-leukemia therapy studies focus on developing antibody constructs that target activated NK cells to specific leukemia antigens to overcome those limitations listed here on the functionality, expansion, and persistence of NK cells. Recent advances were made, including manipulation of receptor-mediated activation, augmentation of antibody-dependent cellular cytotoxicity reactions, gene-modified NK cells engineered by chimeric antigen receptors or, finally,

mono-, bi-, and tri-specific engagers for antigen retargeting on cancer cells (31).

In the past, therapeutic monoclonal antibodies (mAbs) [e.g., rituximab (anti-CD20), cetuximab (anti-EGFR), linterumab (anti-CD33), and alemtuzumab (anti-CD52)] against the corresponding surface antigens on leukemia cells have positively contributed to the treatment but still lead to the development of resistance and an unsatisfactory response rate. Moreover, several high expressed antigens appear on non-transformed cells and, thus, therapeutic antibodies that recognize those target molecules may be scavenged and turned ineffective (32–37). Recently, with the advance in recombinant DNA technology, bispecific (CD16 × CD19 or CD16 × CD33) and trispecific killer engager (CD16 × CD19 × CD22) were developed to redirect NK cell cytotoxicity toward malignant cells, demonstrating significant increase of NK cell cytotoxicity and cytokine release against several CD19 expressing B cell lines. Miller et al. have shown that efficacy with CD16 × CD33 bispecific (BiKE) or IL-15-trispecific killer cell engagers (TriKE) successfully reversed CD33-positive myeloid-derived suppressor cells and stimulated NK cell-induced target cell lysis (38, 39).

Vyas et al. showed clearly that trispecific immunoligands (ULBP2-aCD19-aCD33 and ULBP2-aCD19-aCD19), designated as triplebodies (TBs), successfully retargeted short-time-activated (24 h) NK cells demonstrating increased NK cell-dependent killing activities of several target cells (MEC1, HL60, BV-173, and SEM) by using ULBP2 as a natural ligand to induce high expression levels of NKG2D receptors on activated NK cells. Moreover, activated NK cells in response to control TBs without ULBP2 domains showed a reduced IFN γ release and killing properties compared to full-constructed TBs (ULBP2-aCD19-aCD33) (40).

Based on our review from a clinical phase I/II study using IL-2 activated haploididentical NK cell for adaptive immunotherapy (Clin-Gov-No-NCT01386619) showing not only benefits but also limitations due to tumor immune escape mechanisms (TIEMs), we focused on those TBs in response to NK cells to overcome TIEMs (6, 41, 42). All experiments were performed to investigate specifically the efficacy of the employed ULBP2-aCD19-aCD33 against only CD19/CD33-expressing leukemia cells, which are mainly found in resistant antigen loss variants especially described as mixed lineage leukemia (MLL). In combination with primary donor NK cells, activated up to 14 days, we analyzed the TB-dependent improvement of retargeted recognition and cytotoxicity of those effector cells. In addition, possible side effects due to activated NK cells in the crosslink to these TBs should be evaluated.

MATERIALS AND METHODS

Construction, Expression, and Purification of the Trispecific Immunoligand ULBP2-aCD19-aCD33

The ULBP2-aCD19-aCD33 TBs, kindly provided by Prof. Elke Pogge von Strandmann and Dr. Maulik Vyas, was constructed from immunoligands with high specificity for NKG2D receptors

on NK cells and for CD19 and CD33 on AML cells that were efficiently expressed and secreted by HEK293T cells as previously reported (40).

BV-173 Cell Line

The B cell precursor leukemia cell line BV-173 was purchased from Leibnitz Institute DSMZ (German Collection of Microorganisms and Cell Cultures) and maintained in RPMI-1640 Medium supplemented with 10–20% fetal calf serum (FCS). Cells were split every 3 days under cell culture conditions (37°C, 5% CO₂). For functional assays, the cells were washed once with phosphate-buffered saline (PBS), centrifuged and adjusted to a final concentration of 2.5 × 10⁵/ml in TexMACS (Miltenyi Biotec) containing 5% human serum albumin (HSA).

Toxicity Studies Containing T and B Cells

For toxicity experiments in response to TBs (ULBP2-aCD19-aCD33), T and B cells were isolated from fresh whole blood of healthy donors. The EasySep™ HLA Whole Blood B Cell and CD3 Positive Selection Kit (STEMCELL™ TECHNOLOGIES, Germany) was used to separate CD19⁺/CD20⁺ B or CD3⁺ T cells, respectively, by positive selection according to the manufacturer's recommendations. The isolated cells were expanded in RPMI-1640 medium containing 10% FCS and in presence of a cytokine composition [final concentration: 50 IU/ml (IL-2), 100 IU/ml (IL-4), and 20 IU/ml (IL-10)]. The cells were split every 2–3 days under culture conditions (37°C, 5% CO₂). These cells were washed once with PBS and adjusted to a final concentration of 2.5 × 10⁵/ml in TexMACS (Miltenyi Biotec) supplemented with 5% HSA. For toxicity assays, CD33 on T cells and CD19 surface expression on B cells were characterized by 10-color flow cytometry (FCM) analysis and then co-incubated with IL-2 activated NK cells (E/T ratio: 1:1) pretreated with 1 µg/ml TBs.

Thawed Primary Human AML Cells

Thawed primary AML samples from three different patients [French-American-British classification system: M0 or M5, respectively, kindly provided from Prof. M. Heuser, Hannover Medical School (MHH)] disclosing myeloid CD33 and B lymphoid CD19 surface marker expression were used as examples for antigen loss variants such as MLLs. These primary blasts were washed twice with PBS containing 10% FCS and treated with DNase I to avoid cell clumping. Cells were cultured up to 2 weeks in IMDM supplemented with 10% FCS, Penicillin/Streptavidin, L-Glutamine, and 20 ng/ml each of IL-3, IL-6, SCF, G-CSF, and GM-CSF. As an essential control for differentiation, marker expression, and stability, CD33, CD19 surface levels and cell viability of thawed AML samples were monitored every 2–3 days over a time period about 2 weeks (**Figure 1A**). Afterward, primary leukemic cells were washed with PBS and adjusted to 2.5 × 10⁵/ml in TexMACS (Miltenyi Biotec) containing 5% HSA and used for cytotoxicity assays in different E/T ratios in response to NK cells and TBs. Additional monocultured primary target cell controls were monitored during cytotoxicity assays under normal culture conditions (37°C, 5%

CO₂) and analyzed by FCM to estimate the viability, CD33 and CD19 surface expressions.

Untouched Isolation and Expansion of Primary CD56⁺CD3⁻ NK Cells

Up to 30 ml anticoagulated whole blood from different healthy donors was used to separate “non-touched” primary human NK cells without density gradient centrifugation using MACSxpress® NK Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's recommendations. Based on the expansion protocol from the previous clinical phase I/II NK cell study (42), we improved the protocol and expanded these freshly isolated NK cells (purity: 97.8 ± 1.4%) in NK MACS® basal medium (Miltenyi Biotec, Germany) containing 5% AB serum (human) and 1,000 IU/ml IL-2 up to 14 days (d) as described previously (43).

Cytotoxicity Assay

To assess the NK cell-mediated killing activity in the presence and absence of TBs (ULBP2-aCD19-aCD33), we optimized a no-wash, single platform cytotoxic assay based on FCM (Navios, Beckman Coulter, Germany). This functional assay is based on the recovery of the viable effector and target cells after cytotoxic interaction within a predefined period of time (4 h). Initially, the surface expression of relevant antigens on cultured effector and target cells were determined by FCM as an essential control prior to each approach of this cytotoxicity assay. These phenotypic determinations included specific markers, such as CD45, CD56, CD16, CD33, and NKG2D (CD314) for NK cells and CD9, CD19, CD33, and HLA-DR for target leukemia cells. Further on, freshly isolated and cultured (0–14 days of expansion) NK cells were pre-incubated with various TB doses (TBs: 0.1–30 µg/ml). To determine TB-mediated cytotoxic effects against CD33⁺/CD19⁺ leukemia target cells, pre-coated or non-treated (control) NK cells were co-incubated in different ratios (E:T ratio: 1:1, 5:1) with the leukemic cell line BV-173 and/or primary leukemia blasts. To prevent insufficient stirring of incubated samples or cell sedimentations during cytotoxic cell contacts the co-cultured suspensions were shaken in an CO₂-incubator (CO2 cell, 170-400 Plus, RS Biotech, Scotland) for up to 4 h (37°C, 5% CO₂, 250 rpm). Afterward, effector cells were stained with mAbs by using CD45 KO (Krome Orange), CD56 PC-7 (Phycoerythrin-Cyanine-7) and CD16 APC (Allophycocyanin) in order to exclude the effector cells from leukemia cells stained with CD9 FITC (Fluorescein Isothiocyanate), CD34 PE (Phycoerythrin) and HLA-DR PB (Pacific Blue). Toxicity against effector and/or target cells with and without TBs was calculated as the increased loss of viable cells (43–46):

$$\text{Cytotoxicity} = \left(\frac{1 - \text{concentration}_{[\text{co-cultured target cells}/\mu\text{l}]}}{\text{over concentration}_{[\text{target control cells}/\mu\text{l}]}} \right) \times 100\%$$

To prove TB specificities, the primary NK cells were pre-incubated (20 min, 37°C, 5% CO₂, 250 rpm) with 1 µg/ml anti-NKG2D to block the redirected cytotoxicity in response to TBs against leukemia blasts.

CD107a-Degranulation Assay

Concurrent to our cytotoxicity assays, we assessed the NK cell degranulation by monitoring the cell surface expression of the lysosomal protein CD107a via FCM. NK cells were also co-incubated with leukemia cells at the same E:T ratios in response to TBs (ULBP2-aCD19-aCD33). Cells were stained with PE-conjugated anti-CD107a mAbs and incubated for 1 h at 37°C, 5% CO₂. Phorbol 12-myristate 13-acetate and Ionomycin (I) (Cell stimulation cocktail from eBioscience) were used as a positive control whereas NK cells alone served as unstimulated baseline parameter. After stimulation, Monensin (1:1,000; eBiosciences) and GolgiPlug (1:1,000; BD Biosciences) were added to the samples. These batches were incubated for additional 3 h. Subsequently, cells were washed, stained and analyzed by FCM (see chapter: "Cytotoxicity Assay").

Cytokine Analysis

The multi-analyte flow assay kit (LEGENDPLEX™, BioLegend®, USA) was used for detection of soluble cytokines and pro-apoptotic markers, especially IFNγ, TNFα, perforin, GrA and GrB, and granzylisin. Two sets of beads with known size and fluorescence allowed detections of those soluble molecules in supernatants that previously contained co-cultured effector and target cells. All analysis and evaluations were carried out according to manufacturer's recommendations.

Time-Lapse Microscopy

Redirected cell contacts and interactions between effector and target (E/T) or effector and effector (E/E) cells in presence of TBs could be monitored and followed by fluorescence scanning microscope (IX81, Olympus, USA). As a control before starting for those imaging experiments, surface expression levels of IL-2-expanded NK cells and cultured leukemia cells (BV-173) were characterized for CD3 (PB), CD9 (FITC), NKG2D (PE), CD33 (PE), CD56 (PC-7), CD16 (APC), CD19 (ECD), 7-AAD (PC-5.5), HLA-DR (PB), and CD45 (KO). Afterward, NK cells and BV-173 cells were intracellularly stained with cell proliferation dyes (CFSE/eFluor® 450, affymetrix eBioscience, USA). In the following, NK and BV-173 cells were co-incubated (E/T ratios: 5:1) on chamber slides over a time period of 8 h in response to 10 µg/ml TBs under culture conditions (37°C, 5% CO₂). Beside time-lapse movie experiments to follow specific cell migrations and interactions by designed tracking protocols (time-lapse movie: see Figure S1 in Supplementary Material), it was also possible to evaluate all recorded images containing specific E/T- and E/E-cell contacts and cluster formations by quantitative analyses using the Olympus scanR automated image and data analysis (quantitative evaluations/gating strategy: see Figure S2 in Supplementary Material).

Statistical Analyses

Statistical analysis has been performed using GraphPad Prism v6.02 (GraphPad Software, San Diego, CA, USA). Results of different cytotoxic experiments were compared by the paired Student's *t*-test in order to assess the significance of the NK cell-mediated cytotoxicity incubated in absence and presence of TBs (ULBP2-aCD19-aCD33). Statistical evaluations of surface

expression levels are indicated as median with range in the individual text parts. Differences were stated significant for a *p* ≤ 0.05 and *p* ≤ 0.01 (indicated as * and **, respectively). Minor differences were defined as statistically non-significant (n.s.). Unless otherwise declared, results of statistical evaluations from functional assays are indicated as mean ± SD and represent 4–6 independent experiments and measured in duplicates by FCM.

RESULTS

TBs Increase Killing Activities of IL-2-Expanded NK Cells against Leukemia Cells

The capability of ULBP2-aCD19-aCD33 TBs to induce specific NK cell cytotoxicity against human leukemic cells was determined using the CD19- and CD33-double-positive BV-173 cell line with pre-B phenotype. Purified NK cells (97.8 ± 1.4% CD56⁺CD3⁻) were IL-2 activated and expanded for 14 days. The moderate-to-low expression levels of the NCRs on freshly isolated NK cells were markedly increased approximately 5.2-, 4.9-, and 1.4-fold for NKp30, NKp44, and NKp46, respectively (data not shown). Concomitantly, NKG2D revealed higher median expression levels on these expanded NK cells with 86.4% (range: 64.5–99.2%; 10–14 days) and 57.2% (range: 15.1–97.3%; 6–9 days) compared to unstimulated and early cultured NK cells (Figure 1A, right graph). Activated NK cell cytotoxicity rises with increasing duration of expansion time which correlates also with elevated NKG2D levels on these NK cells (Figure 1A). However, the NK cell-mediated cytotoxicity against BV-173 cells could be further enhanced at increased expansion periods by pre-incubation of NK cells with 1 µg/ml TBs (ULBP2-aCD19-aCD33) (Figure 1B). Accordingly, the NK cell killing activity in presence of TBs reached a maximum of cytotoxic average value of 33.2 ± 5.1% (E/T: 1:1) and 55.2 ± 8.8% (E/T: 5:1) with IL-2-cultured NK cells expanded for 10–14 days compared to significant lower cytotoxic levels of untreated NK cells [24.8 ± 9.4% (E/T: 1:1) and 26.8 ± 5.0% (E/T: 5:1)] (Figure 1B).

In opposite to corresponding control TBs containing depleted ligand (ULPB2) or receptors (anti-CD33/anti-CD19), respectively, specific blocking antibodies were used to inhibit TB-induced cytotoxicity. For these NKG2D blocking experiments, different concentrations of TBs were used to show specific competitive inhibition by saturation of the target epitope with defined concentrations of blocking antibodies (anti-NKG2D) adjusted in several pre-experiments by titration of anti-NKG2D. Thus, inhibition of TB-dependent cytotoxicity by specific blocking of the receptor-ligand-(NKG2D-ULPB2)-binding sites could be achieved partially by pre-incubation of IL-2-expanded NK cells (10–14 days) with anti-NKG2D mAbs (1 µg/ml, 20 min) following treatment with 1 and 10 µg/ml TBs. This resulted in a reduction of cytotoxicity against BV-173 of 20.2-fold (1 µg/ml TB) or 25.8-fold (10 µg/ml TB) (Figure 1D). These results of cytotoxicity assays against BV-173 were largely consistent with the data from the degranulation assays. NK cells

in different expansion periods were pre-incubated in absence or presence of 1 µg/ml TBs and co-cultured for 4 h (E/T ratios: 1:1, 5:1) to detect the lysosomal-associated membrane protein-1 (LAMP-1/CD107a) on NK cells as an mobilized cell surface marker following stimulation-induced granule exocytosis.

TBs were able to elevate the degranulating subpopulation of NK cells in response to BV-173 cells at the indicated ratios with a maximum degranulation average of $11.1 \pm 6.3\%$ (E/T: 1:1) and $9.9 \pm 4.6\%$ (E/T: 5:1) on NK cells expanded for 10–14 days (**Figure 2A**). Interestingly, in some cases, non-significant lower

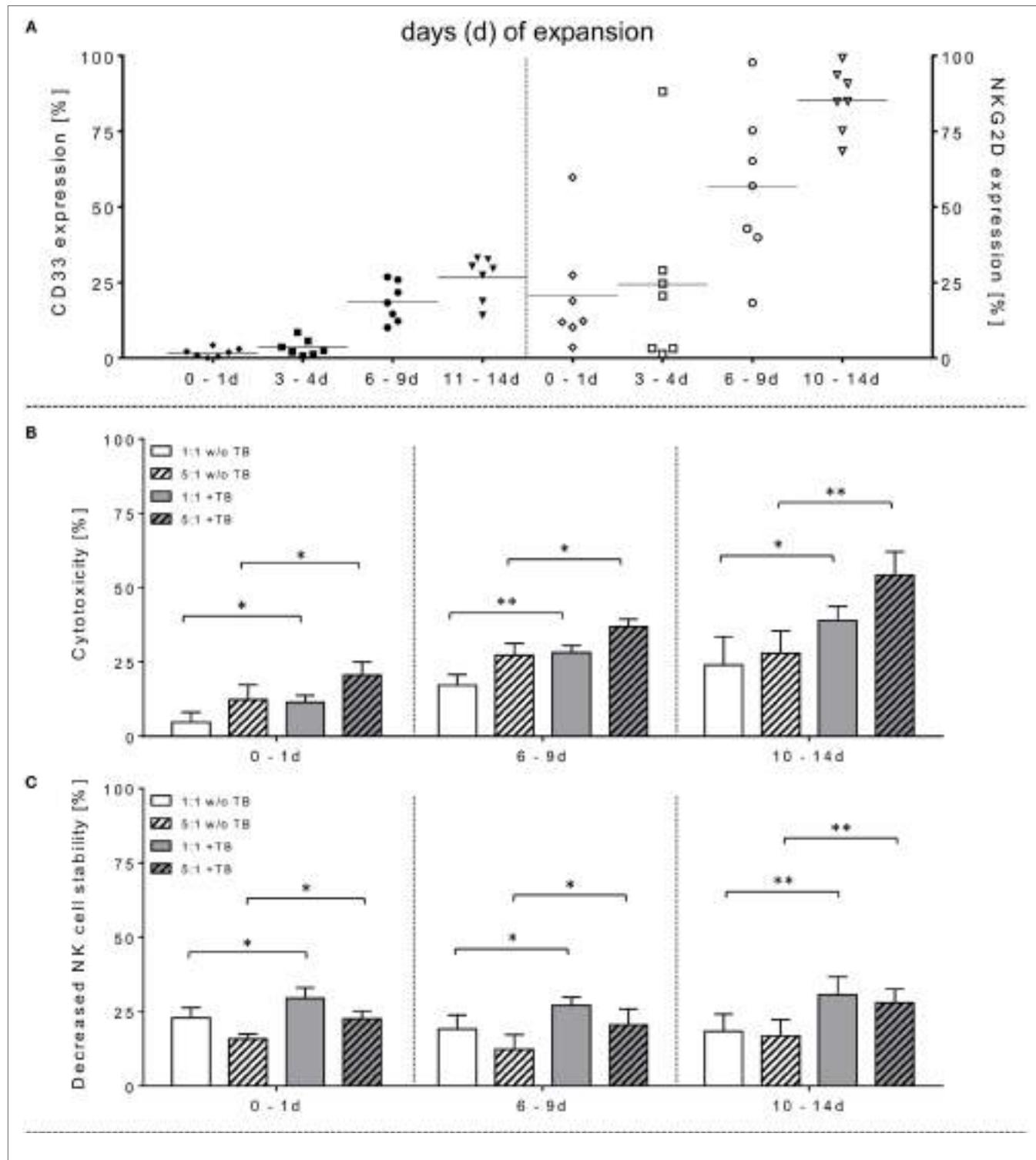
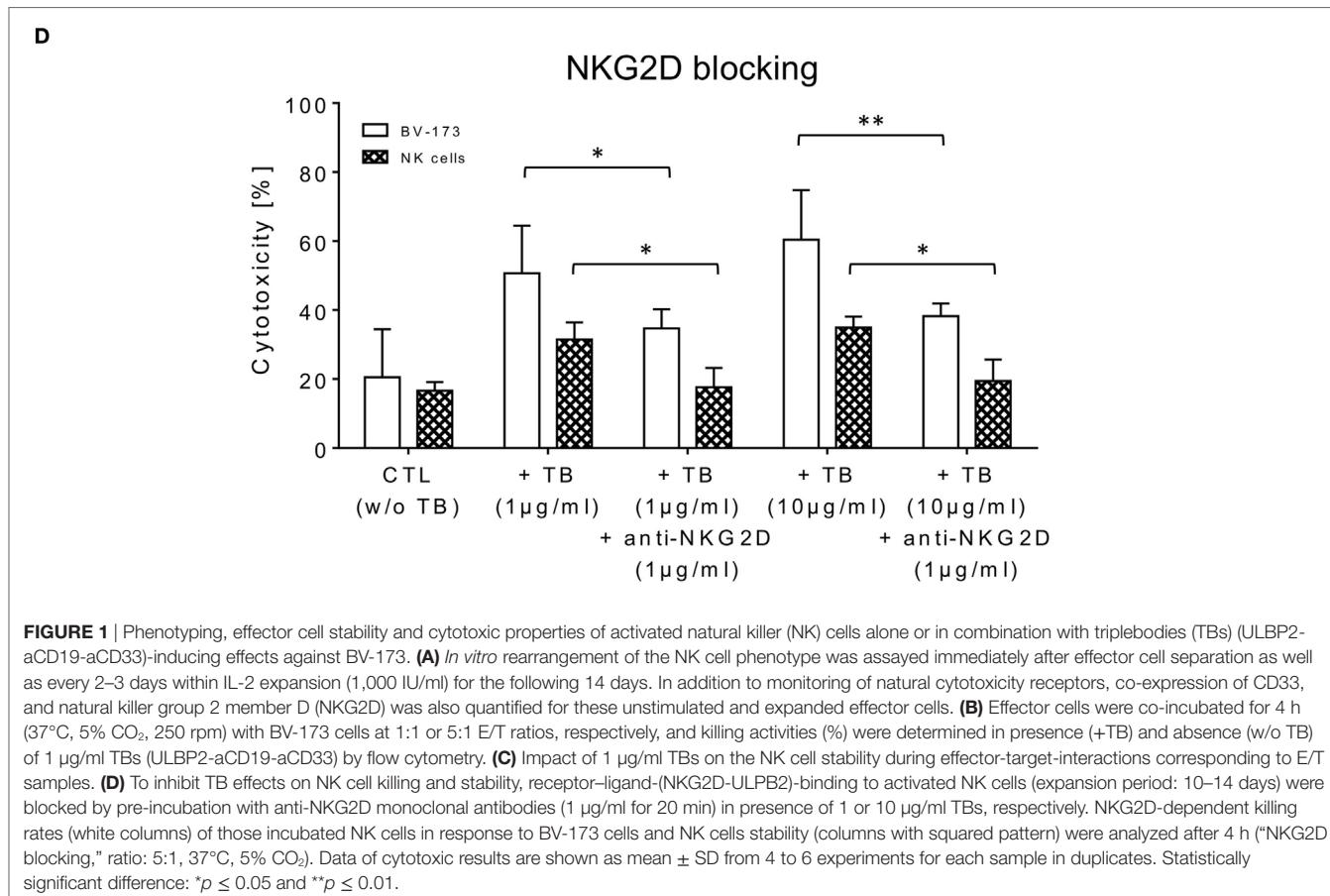


FIGURE 1 | Continued



degranulation of the effector cell at higher (5:1) compared to lower E/T ratios (1:1) in a TB-independent manner was observed (**Figure 2A**). Correspondingly, the analysis of cytokines and apoptotic markers released from TB-incubated NK cells during cytotoxic interaction against BV-173 cells showed increased concentrations for perforin, GrA and GrB, but no significant alterations of TNF α , IFN γ , or granulysin could be detected (**Figure 2C**).

TBs Improve Killing Activity against Native AML Blasts by IL-2-Activated NK Cells

In order to demonstrate that TBs-(ULBP2-aCD19-aCD33)-treated NK cells also promote a cytotoxic effect against primary AML blasts, three different patient samples were thawed and NK cell cytotoxicity was assessed by FCM analyses. In the presence of TBs, the NK cell-mediated cytotoxicity against primary blasts from three AML patients was significantly enhanced compared to NK cell killing activities in absence of TBs. Moreover, the TB-mediated cytotoxic response was more pronounced at higher E/T ratios and TB concentrations. This resulted in improved killing activities of 1.4-fold (1.3-fold) with 1 μg/ml TBs and 1.8-fold (1.6-fold) with 10 μg/ml at E/T ratios of 1:1 or 5:1, respectively (**Figure 3A**). In accordance with our previous degranulation assays in response to BV-173 cells, increased CD107a-positive NK cell subsets could be identified during cytotoxic interaction

at the indicated ratios with a maximum degranulation mean of 9.7 ± 5.2% (E/T: 1:1, 10 μg/ml TBs) and 8.9 ± 4.3% (E/T: 5:1, 10 μg/ml TBs) (**Figure 2B**). Cytokine and apoptotic marker detections in response to AML blasts revealed that pre-incubation of NK cells from expansion period 10–14 days with 1 μg/ml TBs resulted in elevated levels of perforin and GrA and GrB without changes in the amount of TNF α , IFN γ , and granulysin (**Figure 2D**). Corresponding to previous experiments in response to BV-173 cells, TNF α , and IFN γ showed a non-significant tendency to lower cytokine release at higher (5:1) compared to lower E/T ratios (1:1) which was also independent of the impact from TBs (**Figure 2D**).

TBs Decrease NK Cell Viability during Cytotoxic Interactions against Leukemia Cells

Surface expression levels of CD33 were monitored within NK cell expansion over 14 days (**Figure 1A**, left graph). Beside increased NCRs and NKG2D, CD33 levels were also elevated on IL-2-activated NK cells with median expression levels of 27.9% (range: 15.8–33.8%; 10–14 days) and 21.6% (range: 11.0–28.2%; 6–9 days) compared to very low amounts in early expansion stages (**Figure 1A**, left graph). This led to the upcoming question whether the cytotoxic potential of the applied TBs analogous to the shown directed killing effects against AML blasts also has

an unfavorable impact on the expanded NK cells themselves. Therefore, in addition to cytotoxic determinations against AML cells, the stability of the NK cells during cytotoxic interactions in the presence and absence of TBs was investigated in all

cytotoxic experiments. Interestingly, in all E/T ratios pre-incubated with TBs, a marked decrease of these effector cells could be demonstrated both in response to BV-173 cells or AML blasts. Accordingly, TB-induced effector cell decrease reached

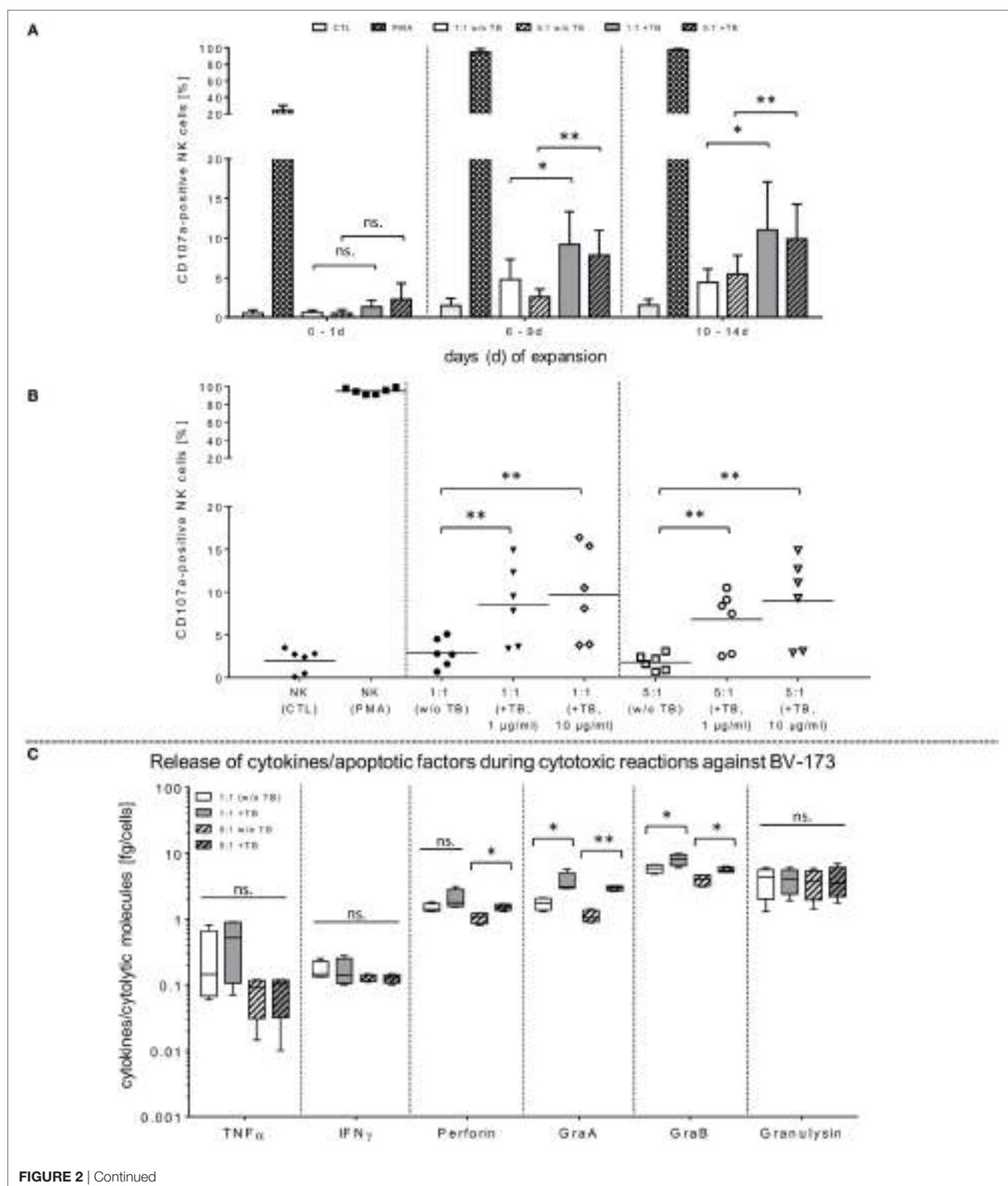


FIGURE 2 | Continued

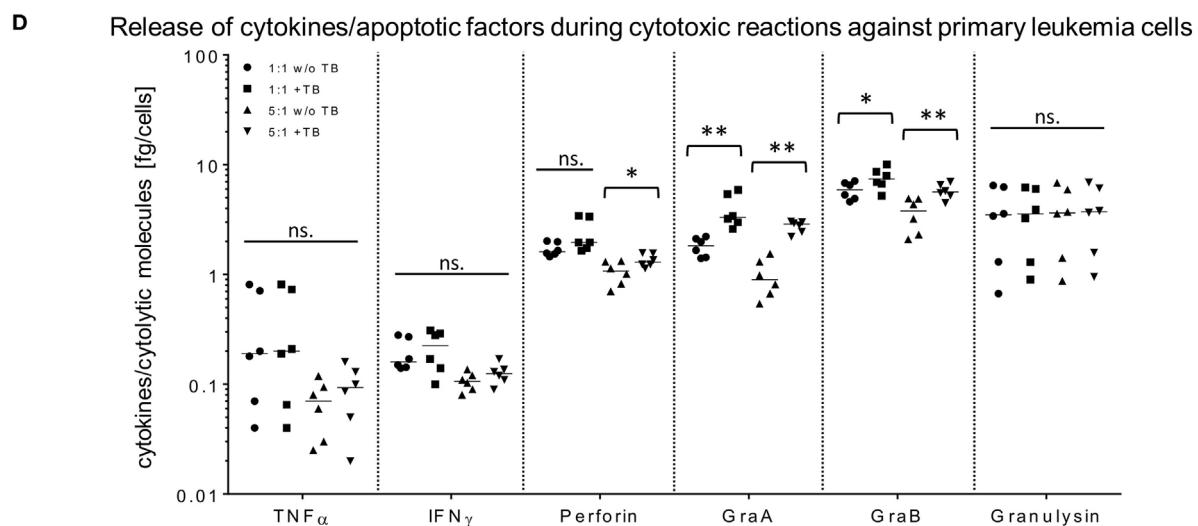


FIGURE 2 | Functional activities of natural killer (NK) cells against BV-173 and primary leukemia cells in response to triplebodies (TBs). **(A,B)** Activated NK cells from latest expansion periods (10–14 days) were co-cultured with BV-173 cell line or with primary blasts from all acute myeloid leukemia patients, respectively. Afterward, the NK cell degranulation were detected by flow cytometry (FCM) analysis using the lysosomal protein CD107a in presence of 1 or 10 µg/ml TBs, respectively, under same experimental conditions as described in **Figure 1**. **(C,D)** Supernatants of different effector–target cell ratios against BV-173 **(C)** or primary patient blasts **(D)**, respectively, were collected after co-incubations over 4 h. Afterward, NK cell-mediated secretion of cytokines and pro-apoptotic markers were quantified by FCM at the indicated ratios in presence or absence of 1 µg/ml TBs. Data show mean ± SD from six experiments measured in duplicates. Statistically significant difference: * $p \leq 0.05$ and ** $p \leq 0.01$.

a maximum average of $31.0 \pm 6.4\%$ (E/T: 1:1; 1 µg/ml TBs) co-cultivated with BV-173 cells (**Figure 1C**) or $24.9 \pm 5.8\%$ (E/T: 1:1, 10 µg/ml TBs) in response to primary blasts (**Figure 3B**), respectively. Interestingly, the NK cell viability seemed to be more reduced in 1:1 than in 5:1 E/T ratios only in presence of 1 µg/ml TBs (**Figures 1C and 3B**). By blocking of the receptor-ligand-(NKG2D-ULPB2)-binding (anti-NKG2D mAbs, 1 µg/ml, 20 min) in presence of TBs and BV-173 cells, this unfavorable effect on the stability of the NK cells could be almost completely abolished (**Figure 1D**).

Dose Escalations of TBs (ULBP2-aCD19-aCD33) in Regard to Effector and Target Cell Stability

In order to estimate in which concentrations those TBs (ULBP2-aCD19-aCD33) affect efficiently CD33⁺/CD19⁺ target cells and the stability of CD33⁺ effector cells, several dose-escalation experiments with highly activated NK (expansion period: 10–14 days) containing elevated CD33 levels in response to BV-173 cells were performed. Dose-escalation experiments of at least 0.1–30 µg/ml resulted in a maximum cytotoxicity against target cells average of $60.2 \pm 2.6\%$ ($52.4 \pm 0.7\%$) from NK cells in response to BV-173 cells starting from a TBs concentration of at least ≥ 1 µg/ml and an E/T ratio of 5:1 or ≥ 7.5 µg/ml and an E/T ratio of 1:1 (**Figure 3C**). Moreover, a maximum toxicity mean response of $41.2 \pm 1.5\%$ ($33.1 \pm 0.8\%$) against co-cultured NK cells could be achieved from TBs concentrations of at least ≥ 7.5 µg/ml and an E/T ratio of 5:1 or ≥ 1 µg/ml at an E/T ratio of 1:1 (**Figure 3D**). Further toxicity experiments with activated NK cells and without any other

target cells in presence of 1 or 10 µg/ml TBs (4 h), respectively, also demonstrated a pronounced decrease in the viability of the effector cells. Accordingly, a maximum reduction of 31.8% (24.0%) for effector cell stability after 4 h could be determined by 10 µg/ml (1 µg/ml) TBs (**Figure 4A**). However, pretreatment of NK cells with anti-NKG2D mAbs (1 µg/ml, 20 min) could partially neutralize the TB-mediated destabilization effects with a maximal blocking efficiency of 62.7% (64.0%) after 4 h in presence of 10 µg/ml (1 µg/ml) TBs (**Figure 4A**). These toxicity data raised the question whether these TBs, in addition to the demonstrated toxicity against activated NK cells, also revealed side effects against other lymphocytes, especially CD33⁺ T cells or CD19⁺ B cells. Therefore, we co-incubated (E/T ratios: 1:1) activated NK cells pretreated with 1 µg/ml TBs in response to T or B cells. This resulted in a moderate decrease of T cells, exhibiting only a weak CD33 surface expression, by approximately 12.1% after 4 h in contrast to higher reductions of 25.1% for B cells containing high CD19 expression levels (**Figure 4B**). However, pre-coating (20 min) of both lymphocyte subsets with the respective AK constructs (anti-CD33 or anti-CD19, respectively; each with 1 µg/ml) allowed blocking of the TB-induced toxicity (1 µg/ml TBs) down to 0.3% for T cells and 10.2% for B cells after 4 h co-incubations (**Figure 4B**).

TBs (ULBP2-aCD19-aCD33) Promote Cell Cluster Formations between NK and Leukemia Cells

Previous toxicity studies showed that our TBs also bind highly activated CD33⁺ NK cells resulted in reduced effector cell stability during the cytotoxic attack on leukemia cells. This led to

the hypothesis that, in addition to increased cytotoxic contacts between NK and leukemia cells, elevated effector-to-effector cell contacts are responsible for higher effector cluster formations in the presence of TBs. This could partially neutralize the improved

effect of TB-mediated NK cell killing activity. Therefore, CD33⁺ NK cells from late expansion periods (10–14 days) and BV-173 cells were intracellular stained with cell proliferation dyes (see Materials and Methods). Before fluorescent microscopy

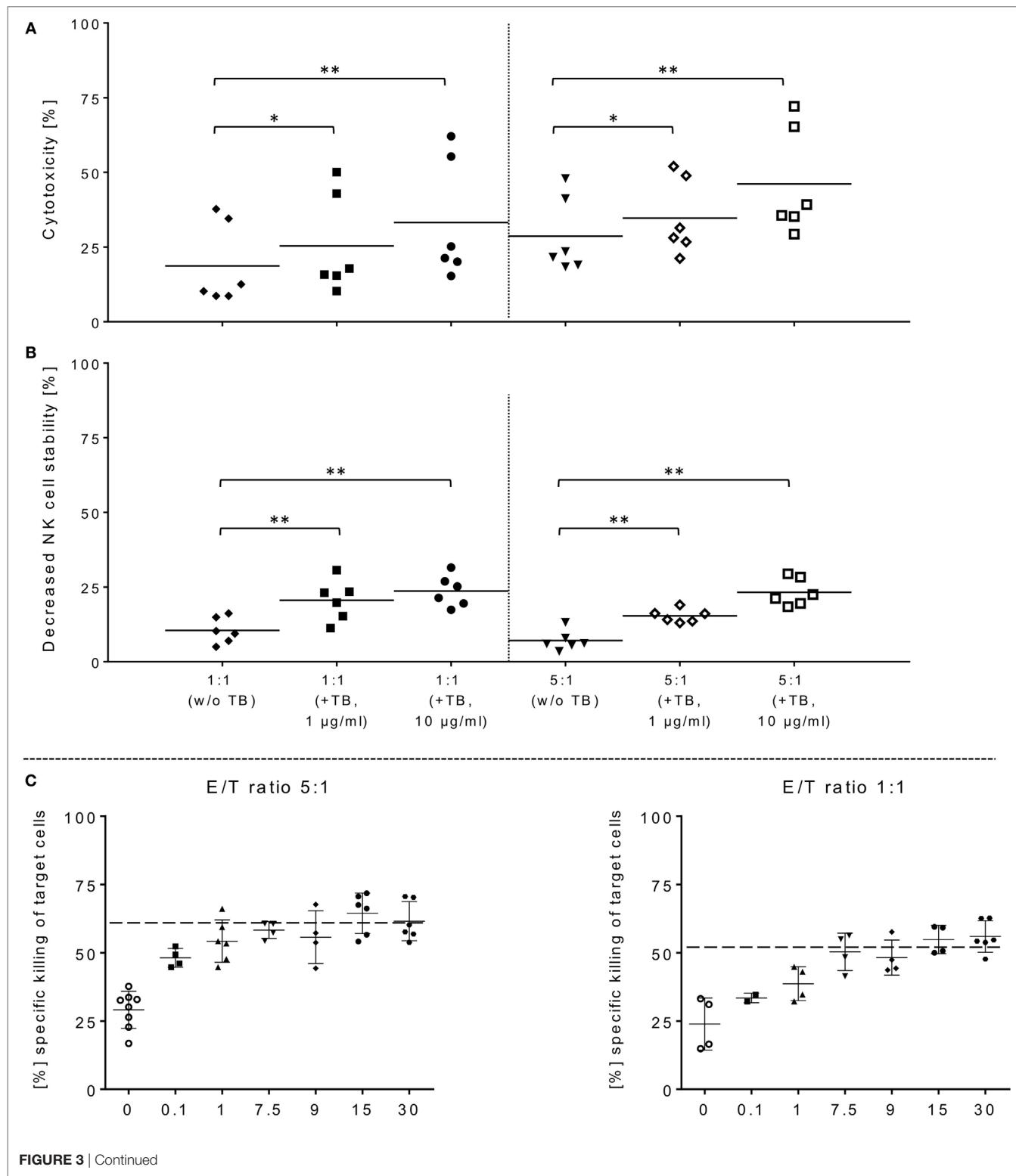


FIGURE 3 | Continued

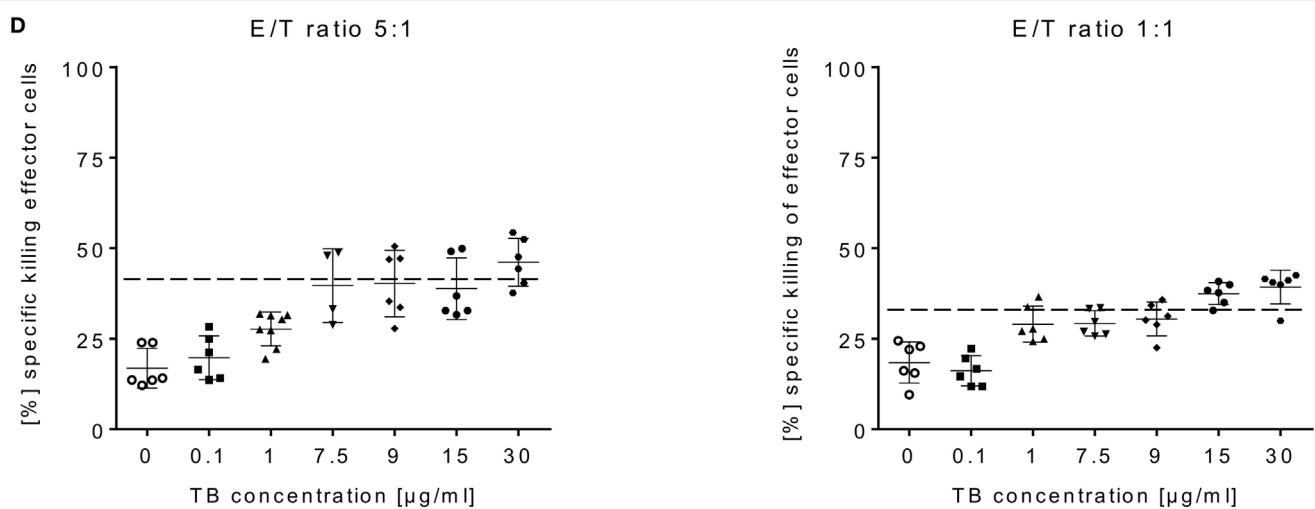


FIGURE 3 | ULBP2-aCD19-aCD33-mediated natural killer (NK) cell killing of primary acute myeloid leukemia (AML) cells. **(A)** Activated NK cells were co-incubated with primary blasts from three different AML patients in presence of 1 or 10 μg/ml triplebodies (TBs), respectively, under same experimental conditions as described in **Figure 1**. **(B)** TBs-induced effects on the stability of NK cells were also determined corresponding to E/T samples (1:1, 5:1). **(C,D)** Estimate the maximum efficiency of ULBP2-aCD19-aCD33 against effector and target cells, different concentrations (0.1–30 μg/ml) of TBs were applied to activated NK cells (time period: 10–14 days) and BV-173 cells at the indicated ratios after 4 h (37°C, 5% CO₂). Data present mean ± SD from six independent experiments measured in duplicates for each patient's sample. Statistically significant difference: **p* ≤ 0.05 and ***p* ≤ 0.01.

experiments were started, activated NK cells were also analyzed for NKG2D or CD33 (PE), respectively, CD56 (PC-7), CD16 (APC), CD3 (PB), and CD45 (KO) surface expression levels. Similarly, target cells were examined for following markers: CD9 (FITC), CD33 (PE), CD19 (ECD), 7-AAD (PC-5.5), HLA-DR (PB), and CD45 (KO). Subsequently, NK and BV-173 cells (E/T ratios: 5:1) were co-cultured over 8 h in presence of TBs (10 μg/ml) monitored by designed tracking protocols. Generated transmission and fluorescent images were quantitatively evaluated by described Olympus scanR acquisition analysis (quantitative evaluations/gating strategy: see Figure S2 in Supplementary Material). It was shown that specific E/T and also E/E cell contacts had increased significantly in presence to TBs (**Figure 5A**) compared to time-limited and unspecific/confused cell contacts in untreated controls (**Figure 5B**), exemplarily shown for two separated tracking runs (time-lapse movie: see Figure S1 in Supplementary Material). Accordingly, subsequent quantitative analyses confirmed the results of time-lapse monitoring by elevated numbers of E/T and/or E/E cell contacts in presence of TBs and resulted in higher cell cluster formations containing up to eight different effector and/or target cells shown in **Tables 1** and **3**. By contrast, only unspecific cell clumping and lower cell clustering containing smaller E/T or E/E cell numbers could be detected in absence of TBs (**Tables 2** and **4**) exemplarily presented for three independent experiments.

DISCUSSION

In our experiments, we could confirm the effectiveness of the (ULBP2-aCD19-aCD33) TBs in the crosslink with activated NK cells showing increased specific killing against a leukemia

cell line (BV-173) and primary AML samples from three different patients compared to single use of NK cells only. Successful targeting was directed against both, the CD19 and CD33 antigen. The transmembrane glycoprotein CD19 (95 kDa) and the early myelopoietic antigen CD33 (approximately 67–75 kDa), seemed to be suitable and prominent surface markers to distinguish myelogenous leukemia cells from lymphoid or erythroid leukemia and were also clinically validated antigens for development of antibody-based immunotherapeutic (bi- or tri-specific) construct's (47–49). However, it should be noted that human-activated NK cells also show a diversity of CD33 surface expression levels within different developmental stages (50–52).

Similar to our cytotoxic assays with the (ULBP2-aCD19-aCD33) TBs in response to leukemia cell lines and primary AML blasts, designed NKG2D-stimulating TBs that contained targeting against CD19 antigens (ULBP2-aCD19-aCD19) only displayed strong affinity to CD19 surface molecules on CLL cells. Vyas et al. (40) showed a significantly higher NK cell-mediated cytolytic activity in response to TBs (ULBP2-aCD19-aCD33 and ULBP2-aCD19-aCD19) against both, target cell lines (MEC1, BV-173, and SEM) and primary CLL blasts. The effects were independent from different E/T ratios (40). In our study as a proof-of-principle-experiment, we were able to inhibit the TB-induced cytotoxic specificity of IL-2-activated NK cells against BV-173 cells by blocking of NKG2D using anti-NKG2D mAbs. However, specific inhibition of these cytotoxic reactions could be achieved only partially and not fully by 1 μg/ml anti-NKG2D. This shows that in addition to the TB-induced killing activity, other cytotoxic mechanisms of activated NK cells are also present, which are NKG2D independent and could not be

blocked in those analyses. In analogous experiments, Vyas et al. (40) achieved a decreasing specificity of TBs by pre-blocking the target antigens CD19 and CD33 on the surface of BV-173 cells. It was also shown that the NK cell-mediated cytotoxicity was strictly NKG2D dependent because control constructs lacking the ULBP2 domain could not induce IFN γ secretion and killing activity of co-incubated NK cells in response to leukemia cells (40). Further functional experiments revealed correlations of TBs-stimulated NK cell degranulations detected

by increased CD107a $^{+}$ effector cell populations. IFN γ secretion were also enhanced in presence of CD33-/CD19-expressing target cells that were inhibited by control constructs lacking the natural ligand ULBP2 for retargeting the NK cells *via* NKG2D receptors (40). By contrast, in our study, the functional assays showed no significant alterations for IFN γ or TNF α in collected supernatant samples after cytotoxic reactions. However, we detected TBs-dependent elevated NK secretion levels of apoptotic markers (perforin, GraA, and GraB) in response to

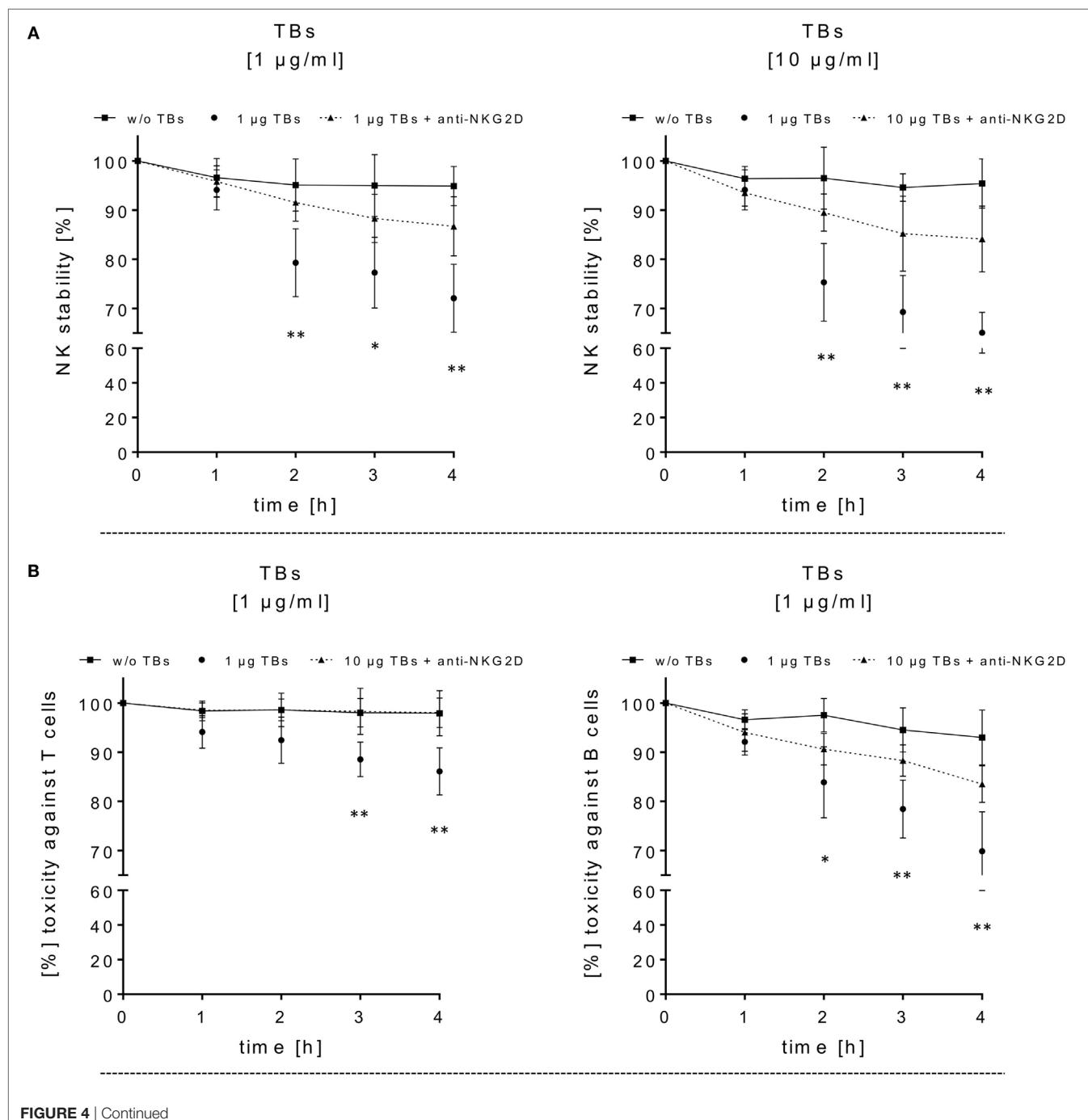
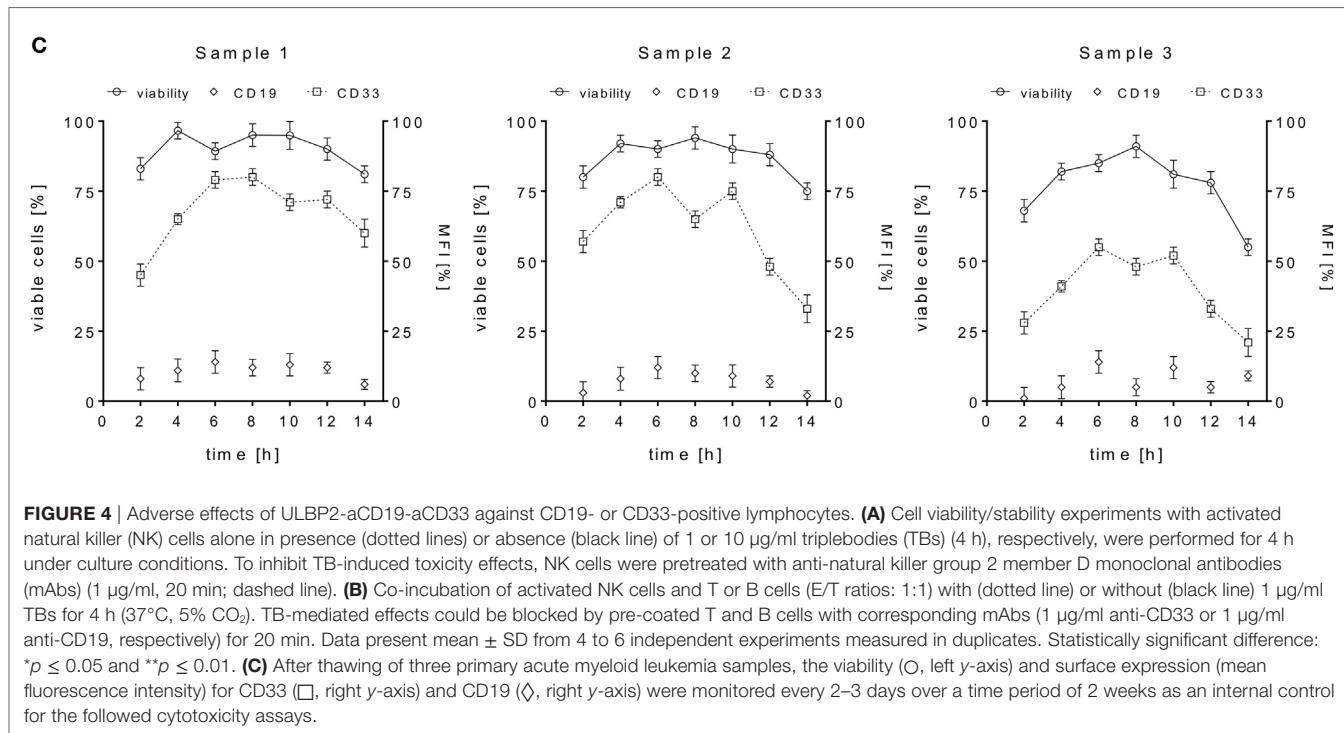


FIGURE 4 | Continued



both BV-173 cells and primary blasts. This correlated with an increased CD107a⁺ NK cell subset, but showed no alterations in the granulysin releases. This could be explained by the fact that probably the maximum time for intracellular productions of IFN γ and TNF α has long been exceeded by the long-term IL-2-driven NK cell expansion over 14 days. In contrast to our NK cell expansion protocol, Vyas et al., cultivated freshly purified NK cells only overnight (37°C, 5% CO₂) in IMDM medium supplemented with 10% heat-inactivated FCS and human IL-2 (200 IU/ml) + IL-15 (10 ng/ml) (40). We concluded that this overnight cultivation in combination with both cytokines could induce an earlier IFN γ and TNF α secretion for these short-activated NK cells. Nevertheless, Vyas and our workgroup were able to show clearly improved cytotoxic properties of activated NK cells that were consistent with an optimized anti-leukemic efficiency. However, we detected a marked decrease in the stability of activated CD33⁺ NK cells during cytotoxic interactions against leukemia blasts confirmed by increased E/E cell contacts and higher effector cell clustering analyzed using fluorescence scanning microscope.

Future immunotherapy approaches containing primary NK cells in combination with examined TB-constructs should ensure that sufficient NK cell numbers and a strongly elevated NKG2D expression are available for an efficient receptor-ligand-(NKG2D-ULPB2)-binding as well as for complete eliminations of remaining leukemia cells, especially shown in high-risk patients. The significance of NKG2D could be also confirmed by several reports dealing with immunosurveillance and development of novel NK-based immunotherapies by using bispecific immunoligands targeting NKG2D receptors (7, 53–55). Concomitant experiments within our previous clinical

phase I/II NK cell study (42) demonstrated that the NKG2D-dependent cytotoxicity against resistant neuroblastoma cells was strongly affected by immunosuppressing NKG2DLs, as one of multiple strategies to escape from immune-mediated eradication. This effect could be blocked by scavenging soluble NKG2DLs with IL-2-activated donor NK cells. As a result, NKG2D-dependent cytotoxic response was restored (41, 56). These results suggest that, in addition to a permanent characterization of NKG2D levels on NK cells, a closed monitoring of such critical immunosuppressive markers in patients' plasma appears to be necessary before TBs are administered.

Since only controversial data concerning myeloid antigen CD33 (SIGLEC-3) expression on NK cells were published so far, this expression was also closely monitored concomitantly to NCRs/NKG2D characterizations. During 14 days of NK cell expansion, we could detect a transient higher CD33 surface expression level on late-expanded NK cells compared to unstimulated and early cultured primary NK cells. In accordance with these results, several subsets of NK cells were also found in human umbilical cord blood (CB) and in diverse distributions at different development stages in the peripheral blood (PB), lymph nodes, and spleen. Because of this distributions NK cell differentiations could occur at different anatomical locations (51). Interestingly, CD56⁺/CD33⁺ NK cell subpopulations identified in human umbilical CB revealed only a low cytotoxic effect against K562 target cells after IL-2-triggered expansion, whereas higher cytolytic effects were observed in response to activated CD56⁺/CD33⁻ NK cell subsets (50, 52).

Correspondingly to our experiments, increased CD33 expression levels were proven only in a subset of IL-2-cultured NK cells compared to ubiquitous elevations of NCRs and

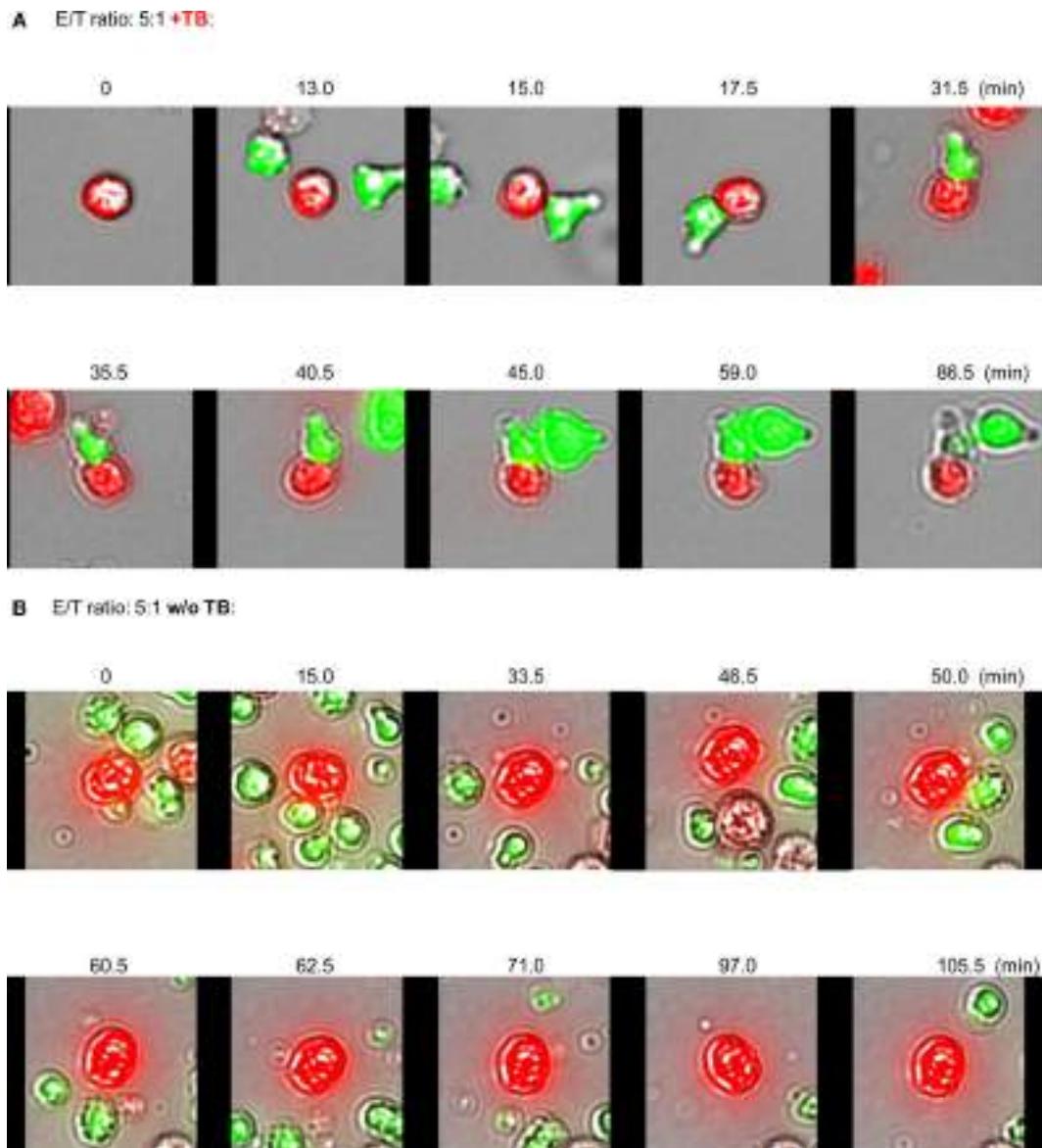


FIGURE 5 | Redirected effector–target cell interactions in response to ULBP2-aCD19-aCD33. Activated natural killer (NK) cells (expansion period: 10–14 days) and BV-173 cells were labeled with CFSE (green NK cells) or eFluor® 450 (red target cells), respectively, and co-cultured on chamber slides at the indicated E/T ratio for 8 h (37°C, 5% CO₂) in presence (**A**) or absence (**B**) of 10 µg/ml triplebodies (TBs) (ULBP2-aCD19-aCD33) exemplarily shown for three different experiments. Specific Effector-to-target (E/T)- and Effector-to-Effector (E/E)-contacts were monitored by scanR analysis allowed the time-limiting tracking of cell migrations [0–86.5 min (**A**) and 0–105.5 min (**B**)] evaluated with a fluorescence scanning microscope (IX81, Olympus, USA), visualized in response to cell morphology and fluorescence.

NKG2D surface expressions detected on all NK cells during 14 days of expansion. The viability of these CD33-expressing effector cells was adversely affected in presence of TBs (ULBP2-aCD19-aCD33) and resulted in a diminished cytotoxic response against leukemic blasts. In addition to the shown toxicity against CD33⁺ NK cell subsets, adverse effects toward the viability of T and B lymphocytes could also be observed, which were explained according to target antigen expressions (CD19 or CD33) on those lymphocytes. Besides the well-studied CD19 surface levels that are expressed during

all development stages of B cells with the exception of differentiated plasma cells (57, 58), human T cells express also a low amount of CD33. Interestingly, both T and NK cells show similarly high surface expressions of activation markers (CD25, CD28, CD38, CD45RO, or CD95) (52). This could explain the observed toxicity of activated NK cells in the presence of TBs (ULBP2-aCD19-aCD33) against T and B lymphocytes. Therefore, a close-meshed patient monitoring and examination of PB-derived immune status from AML patients should be implemented.

TABLE 1 | Quantitative evaluations of E/T cell cluster formations.**Evaluation of E/T cell cluster formations (E/T ratio: 5:1 + TB)**

											
	Viable cells	Total cell clusters	T:T cell clusters	E:T cluster (1x)	E:T cluster (2x)	E:T cluster (3x)	E:T cluster (4x)	E:T cluster (5x)	E:T cluster (6x)	E:T cluster (7x)	E:T cluster (8x)
Gates	R01	R01/R02	R01/R02/ R03	R01/R02/R04	R01/R02/ R05	R01/R02/ R06	R01/R02/ R07	R01/R02/ R08	R01/R02/ R09	R01/R02/ R10	R01/R02/R11
Cell numbers (%)	8,157 100	3,334 40.9	650 8.0	679 8.3	1,003 12.3	715 8.8	132 1.6	95 1.2	41 0.5	16 0.2	3 0.04
32.9% (2,684 E/T cell clusters)											

Activated natural killer and BV-173 cells were co-cultured (E/T ratios: 5:1) on chamber slides over 8 h in presence of 10 µg/ml triplebodies (TBs) (37°C, 5% CO₂). Specific cell interactions between effector and target (E/T) cells were analyzed by fluorescence scanning microscope (IX81, Olympus, USA).

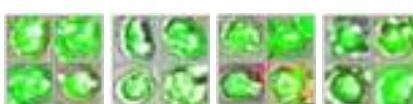
TABLE 2 | Quantitative analyses of E/T cell cluster formations in absence of triplebodies (TBs).**Evaluation of E/T cell cluster formations (E/T ratio: 5:1 w/o TB)**

											
	Viable cells	Total cell clusters	T:T cell Clusters	E:T cluster (1x)	E T cluster (2x)	E:T cluster (3x)	E:T cluster (4x)	E:T cluster (5x)	E:T cluster (6x)	E:T cluster (7x)	E:T cluster (8x)
Gates	R01	R01/R02	R01/R02/ R03	R01/R02/ R04	R01/R02/ R05	R01/R02/R06	R01/R02/ R07	R01/R02/ R08	R01/R02/ R09	R01/R02/ R10	R01/R02/R11
Cell numbers (%)	8,588 100	1,287 15.0	602 7.0	589 6.9	609 7.1	44 0.5	— —	— —	— —	— —	— —
14.5% (1,242 E/T cell clusters)											

Natural killer and BV-173 cells were co-incubated (E/T ratios: 5:1) over 8 h (37°C, 5% CO₂).

Clustering effector and target cells were analyzed by fluorescence scanning microscope.

TABLE 3 | Quantitative analyses of E/E cell cluster formations.**Evaluation of E/E cell cluster formations (E/T ratio: 5:1 + TB)**

						
	Viable cells	Total E:E cell clusters	E:E cluster (1x)	E:E cluster (2x)	E:E cluster (3x)	E:E cluster (4x)
Gates	R01	R01/R02	R01/R02/ R04	R01/R02/ R05	R01/R02/ R06	R01/R02/ R07
Cell numbers (%)	8,157 100	7,537 92.4	6,394 78.4	955 11.7	157 1.9	31 0.4

Activated natural killer in response to BV-173 cells (E/T ratios: 5:1) were co-incubated over 8 h in presence of 10 µg/ml triplebodies (TBs) (37°C, 5% CO₂). Specific effector-to-effector cell contacts were analyzed by fluorescence scanning microscope.

CONCLUSION

Our results indicate that TBs, especially ULBP2-aCD19-aCD33, are able to increase cytolytic properties of activated NK cells. This could be clearly demonstrated against both leukemic cell

TABLE 4 | Quantitative evaluations of E/E cell clusters in absence of triplebodies (TBs).**Evaluation of E/E cell cluster formations (E/T ratio: 5:1 w/o TB)**

						
	Viable cells	Total E:E cell clusters	E:E cluster (1x)	E:E cluster (2x)	E:E cluster (3x)	E:E cluster (4x)
Gates	R01	R01/R02	R01/R02/ R04	R01/R02/ R05	R01/R02/ R06	R01/R02/ R07
Cell numbers (%)	8,588 100	2,661 31.0	2,367 27.6	229 2.7	65 0.76	3 0.03

Natural killer and BV-173 cells were co-cultured (E/T ratios: 5:1) over 8 h (37°C, 5% CO₂). Clustering effector cells were analyzed by fluorescence scanning microscope.

line BV-173 and primary AML blasts, but with some unfavorable toxicity effects against own effector cells and further adverse effects against T and B lymphocytes.

In summary, the experiences of our previous clinical phase I/II NK cell study for adaptive immunotherapy

(Clin-Gov-No-NCT01386619) (6, 41, 42) and our results suggest that highly activated NK cells in combination with TBs, especially ULBP2-aCD19-aCD33, might be an innovative strategy for efficient redirected eliminations of resistant AML cell. Therefore, it is necessary to monitor both CD33 and NKG2D expression levels on *ex vivo* expanded NK cells and leukemic blasts isolated from PB of AML patients to improved therapeutic benefit.

ETHICS STATEMENT

Pre-clinical development of an antibody-based triple body for NK cell-mediated immunotherapy of pediatric acute leukemia was approved and assessed by the Ethics Committee of Hannover Medical School (MHH) (ethical number: 2628-2015).

AUTHOR CONTRIBUTIONS

UK, ES, and SK designed the study, while AS, SK, and UK were mainly responsible for the performance of this study. More in detail, TG, NM, AS, and OO realized the experiments containing trispecific immunoligand ULBP2-aCD19-aCD33 (the so-called triplebodies [TBs]). SK, TG, NM, OO, and AS carried out the quality control analyses, including cell characterizations, effector cell killing activity and degranulation, time-lapse microscopy, and the enumeration of the cytokine secreting

cells. MV and ES were responsible for construction, expression, and purification of the engineered trispecific immunoligand ULBP2-aCD19-aCD33. MH provided primary leukemia samples from different AML patients. SK wrote the manuscript, while UK, LA, and ES contributed to helpful discussions and the careful approval of the final manuscript. LA reworked as a specialist for native English the manuscript in word, sentence and grammar.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2017.01100/full#supplementary-material>.

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Antigen Loss Variants: Catching Hold of Escaping Foes

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Since mid-1990s, the field of cancer immunotherapy has seen steady growth and selected immunotherapies are now a routine and preferred therapeutic option of certain malignancies. Both active and passive cancer immunotherapies exploit the fact that tumor cells express specific antigens on the cell surface, thereby mounting an immune response specifically against malignant cells. It is well established that cancer cells typically lose surface antigens following natural or therapy-induced selective pressure and these antigen-loss variants are often the population that causes therapy-resistant relapse. CD19 and CD20 antigen loss in acute lymphocytic leukemia and chronic lymphocytic leukemia, respectively, and lineage switching in leukemia associated with mixed lineage leukemia (MLL) gene rearrangements are well-documented evidences in this regard. Although increasing number of novel immunotherapies are being developed, majority of these do not address the control of antigen loss variants. Here, we review the occurrence of antigen loss variants in leukemia and discuss the therapeutic strategies to tackle the same. We also present an approach of dual-targeting immunoligand effectively retargeting NK cells against antigen loss variants in MLL-associated leukemia. Novel immunotherapies simultaneously targeting more than one tumor antigen certainly hold promise to completely eradicate tumor and prevent therapy-resistant relapses.

Keywords: antigen loss, leukemia, NK cells, T cells, dual-targeting immunoligand

INTRODUCTION

In what is known as cancer immunoediting, the immune system not only tries to eradicate the evolving tumor but, in doing so, also shapes the immunogenicity of the tumor that may escape the immune control (1). Ultimately, the tumor cells that progress despite the immunosurveillance consist of one or more clones with lower visibility and/or higher resistance to the immune cells (1). For example, tumors often decrease the expression of components required for antigen presentation (MHC) and/or T cell activation (costimulatory molecules) as well as ligands for the NK cell-activating receptors in order to hide from the T and NK cells, respectively (1–4). Alternatively, tumor cells express ligands, which, upon binding to the respective checkpoint receptors such as CTLA-4 and PD-1 on T cells and KIR and CD94/NKG2A on NK cells, suppress their effector functions (5–9). The following sections review the current targeted therapies and the evidences of relapses associated with antigen

loss variants in leukemia. Several therapeutic approaches including a dual-targeting immunoligand to manage this challenging clinical scenario are discussed.

TARGETED IMMUNOTHERAPIES— CURRENT STATUS IN LEUKEMIA

Acute leukemia represents an uncontrolled proliferation of the immature immune precursor cells and are further classified based on the lineage of the affected immune cell. Acute lymphocytic leukemia (ALL) affects the cells from the lymphoid lineage in contrast to the leukemia of myeloid cells, collectively known as acute myeloid leukemia (AML) (10). Both lymphoid and myeloid leukemia exploit the abovementioned and several other immune evasive strategies [reviewed in Ref. (6)]. However, the fact that tumor cells have to evade the immune system in order to be clinically relevant disease also supports the idea that immune system, when properly activated, can fight the cancer.

Over the last three decades, the cancer immunotherapy field has seen much progress and most of its success can be attributed to the targeted therapies against leukemia (11). The most promising immunotherapeutic options for leukemia include targeted approaches such as chimeric antigen receptor (CAR) modified T cells (CAR-T cells) and antibody-based therapies that activate T and NK cells (11). Within the CAR construct, extracellular antibody-derived scFv confers the antigen specificity, while the intracellular signaling domains (from T cell receptor and costimulatory molecule) provide the activation signal to the engineered T cells (12). Various CAR-T cells have entered the clinical studies for leukemia and the most advanced CAR is against CD19, which is being tested for ALL (13, 14). Blinatumomab, a bispecific T cell engager against CD19 and CD3 that recently got the FDA approval for the treatment of ALL, is an antibody-based molecule that also activates T cells, albeit via CD3, against the CD19-bearing target cells (15). NK cells, like T cells, have equally contributed to the clinical success of cancer immunotherapy against leukemia. For example, NK cells serve as an important effector population in chronic lymphocytic leukemia (CLL) patients who mediate antibody-dependent cell-mediated cytotoxicity through FcγRIIIa (CD16a) receptor engagement by the FDA-approved anti-CD20 antibodies (rituximab, obinutuzumab, and ofatumumab), anti-CD52 antibody alemtuzumab, and other promising anti-CD19 antibodies (MEDI-551 and XmAb5574) that are currently in clinical trials (16). In addition to the conventional antibodies, there are numerous novel approaches currently in preclinical development that aim to harness NK cell activity against cancer [reviewed in Ref. (8)].

Although many of the targeted immunotherapies have produced unprecedented responses in leukemia, especially in chemorefractory patients, the complete remissions observed following such therapies are not long-lasting and a large variety of leukemia cases are presented with relapses that are aggressive and difficult to manage. This dismal scenario emphasizes the intratumoral heterogeneity that is driven by the intrinsic factors such as accumulation of genetic and epigenetic mutations during tumor progression and extrinsic factors imposed by therapeutic pressure and tumor microenvironment (17, 18).

OCCURRENCE OF ANTIGEN LOSS VARIANTS IN LEUKEMIA

Around 30% of acute leukemia patients experience a relapse with occasional co-presentation of a phenomenon known as “lineage switch.” Lineage switching occurs when acute leukemia that was initially classified as lymphoid or myeloid subtype according to the standard French-American-British guidelines shows opposite lineage when relapsed (10, 19). This phenomenon is often associated with poor prognosis and therapy resistance regardless of whether it emerged due to the lineage conversion of the original malignant clone or the selective outgrowth of a new leukemic clone (10). Out of the two possibilities, lymphoid to myeloid lineage switch is more frequently observed with more cases reported in children and often associated with the mixed lineage leukemia (MLL) gene rearrangements on chromosome 11q23 (20, 21).

Most cases of lineage switch have been reported in patients who had undergone some sort of targeted therapy. CD19-targeting immunotherapies including a bispecific antibody blinatumomab and CAR-expressing T cells have been very effective in chemorefractory B cell ALL. Anti-CD19/CD3 antibody blinatumomab redirects endogenous T cells in patients (15), while anti-CD19 CAR T cells are genetically engineered to be specifically activated against CD19 expressing target cells when infused in patients (22). Despite exceptional responses associated with these targeted therapies, some patients relapse and in many cases loss of CD19 antigen is reported. Duffner et al. reported a patient who was diagnosed with B-ALL associated with MLL-gene rearrangements but with no evidence of mixed lineage phenotype. Although blinatumomab therapy led to the complete disappearance of leukemic B cells, the patient relapsed with a more aggressive monocytic AML, which was negative for typical lymphoid markers such as CD19 (20). Similarly, CD19-specific CAR-T cell therapy could achieve complete response in all seven MLL-rearranged B-ALL patients. However, two of the seven patients relapsed with clonally related AML with no expression of B lymphoid antigens (21). Interestingly, both patients who showed lineage switch also had the presentation of cytokine release syndrome (21). Interleukin-6 (IL6), a key mediator of cytokine release syndrome, has also been shown to induce lymphoid to myeloid dedifferentiation *in vitro* (23) and *in vivo* (24). Although this is an indication of myeloid dedifferentiation of the original lymphoid blasts as an indirect effect of CAR-T cell therapy, it is also possible that myeloid clone is already present along with the lymphoid blasts, albeit below detection level, and is selected following the lymphoid-directed therapy. Ruella et al. recently described the presence of a small CD19-negative population in B-ALL patients before the administration of anti-CD19 CAR-T cell therapy (CTL019). Although there were no cases involving lineage switch, patients relapsed with CD19-negative B cell tumor following the CAR-T cell therapy (CTL019) and, as proposed by the authors, was most likely due to the selective outgrowth of the original CD19-negative subclone (13, 25). Beyond targeted immunotherapies, the phenomenon of lineage switch has also been observed following chemotherapy. As reported by Park et al., four patients of childhood B cell lineage ALL were treated with chemotherapy and were later presented

with the relapse of clonally related AML (one patient) or a novel AML clone (three patients) (19).

While the link between treatment and lineage switching is not clear, the precise mechanism of antigen loss following mAb therapy is identified in several B cell malignancies. Rituximab, a chimeric antibody against CD20, has become a standard therapeutic option for various B cell ($CD20^+$) malignancies including non-Hodgkin lymphoma (NHL), follicular lymphoma, diffuse large B cell lymphoma, and CLL (26, 27). The loss of CD20 antigen following rituximab therapy has been observed for follicular lymphoma (27), B cell NHL (28), and CLL (29). Two main mechanisms have been reported for CD20 loss from the CLL cells following rituximab (anti-CD20 mAb) treatment. While CD20 internalization by malignant B cells plays a minor role, the majority of CD20, along with the bound rituximab, is removed by the Fc γ receptor-expressing monocytes and macrophages in a process called as trogocytosis or shaving (30–32). This does not only result in the rapid clearance of rituximab following the infusion but also leads to selection of CD20-negative CLL cells that are resistant to anti-CD20 therapy. Similarly, CD19 internalization is also reported by anti-CD19 antibody XmAb5574 in CLL (33). Interestingly, Jones et al. reported the loss of CD19 from the CLL cells during the shaving (trogocytosis) of anti-CD20 rituximab. It was shown that CD19 was also transferred from B cells to monocytes in Fc receptor-dependent manner (34). Moreover, antigen loss in CLL is not only associated with the mAb therapy, for example, decrease in the cell surface expression of CD20 is observed by an immune modulating agent lenalidomide (26) or following the long-term *in vitro* coculture with mesenchymal stromal cells (29).

THERAPEUTIC STRATEGIES TO COMBAT THE ANTIGEN LOSS VARIANTS

As most tumor relapses involving antigen loss have been observed following antigen-specific therapies, one plausible solution is to use therapeutic approaches that are more general in their specificity and do not depend upon a particular tumor antigen. Immunotherapy with cytokine(s) such as IL2, IL12, and IL15 act via enhancing NK and T cell-mediated immune response against tumor (35). Although side effects associated with cytokines (e.g., IL2 and interferons) greatly limit their current use in the clinics, this approach still holds promise especially at lower doses and in combination with other anti-cancer therapies (35). Alternatively, checkpoint blockade involves blocking of the inhibitory receptors on immune cells to reverse the immune suppression by tumor cells (36, 37). Recent success in blocking of inhibitory receptors on T cells such as CTLA-4 and PD-1 by FDA-approved antibodies (checkpoint inhibitors) has led to the development of novel checkpoint inhibitors blocking NK cell inhibitory receptors KIR (lirilumab, Innate Pharma) and CD94/NKG2A (IPH2201, Innate Pharma) (36–38). The advantage is that such immune-modulatory approaches aim to promote an overall antitumor environment and are predicted to be less susceptible to the limitations associated with tumor heterogeneity and antigen loss (39). However, treatment options with no specificity for tumor are less likely to be curative as mono-agents and are often associated with the

systemic side effects as observed in the form of immune-related adverse events following the checkpoint blockade approach (40).

Another strategy is to broaden the specificity of the current targeted therapies that have already shown promise in the clinics. CAR-T cells with dual specificities have been developed to improve T cell targeting of tumor cells even when one of the antigens is lost from the cell surface. A prototype CAR T cell with two distinct antigen-specific scFvs in tandem (TanCAR) retained T cell activity against antigen loss variants (41). The treatment of B-ALL patients enrolled in the pediatric CTL019 trial (the University of Pennsylvania/Children's Hospital of Philadelphia) with CD19-specific CAR-T cells led to the outgrowth of CD19-negative malignant clone, which retained the expression of an IL3 receptor α chain (CD123) (25). Taking advantage of this, Ruella et al. developed CD19/CD123 CAR-T cells and proved its ability to completely eradicate the primary B-ALL blasts ($CD19^+CD123^+$ and $CD19^-CD123^+$) and to prevent the CD19 antigen loss relapse in an immunodeficient (NSG) mouse model (25). Despite the encouraging progress with the dual-specific CAR-T cell approach, major safety concerns typically associated with CAR-T cell therapy such as “on-target, off-tumor toxicity” and “cytokine release syndrome” would demand an equal attention (42).

Alternatively, NK cells, unlike T cells, express a diverse array of activating and inhibitory receptors to sense for the presence of stressed, virally infected or malignant cells. Moreover, there are multiple ligands for some of the activating receptors on NK cells (43). For example, the natural killer group 2 member D (NKG2D), an activating receptor on NK cells, can induce NK cell effector functions upon binding to any of the natural ligands such as UL16-binding proteins (ULBP1–6) and MHC-I-related chains (MICA/B) (43). This makes NK cells unlikely to succumb to the tumor heterogeneity and antigen loss provided that malignant cells remain visible to the NK cell scanning. However, the ligands for the NK cell-activating receptors, including NKG2D, are occasionally lost from the surface of leukemic cells in order to evade NK cell immunity (2, 3, 44). Of note, as shown by the recent work of Deng et al., soluble MULT1, a murine NKG2D ligand, played an indirect role in promoting NK cell immunity suggesting that soluble ligands may be more than inhibitory for overall NK cell activity (45). Our group has developed a therapeutic strategy to resensitize leukemic cells for NKG2D-dependent NK cell attack. To this end, we have developed and tested several bi- and trispecific recombinant immunoligands containing an NKG2D ligand ULBP2 fused to the various tumor antigen-specific scFvs (46–48). The idea is that these immunoligands will bind specifically to the tumor antigens and will coat the tumor cells with ULBP2 ligand. This will turn the otherwise NK cell-resistant tumor cells visible to NK cells for the attack. This was recently tested for the trispecific immunoligands (triplebodies) against CLL and MLL cells, which showed successful NK cell-mediated killing of leukemic cells in both, *in vitro* and *in vivo* settings (47).

The ability of a dual-targeting triplebody ULBP2-aCD19-aCD33 to target antigen loss variants is showed in the present report. The term “dual-targeting triplebody” represents a trispecific immunoligand targeting two distinct antigens such as CD19 and CD33 in the case of ULBP2-aCD19-aCD33 against a B-cell precursor leukemic cell line BV173. The rational of this approach

is that ULBP2-aCD19-aCD33 would coat not only the CD19- and CD33-positive target cells such as leukemic cells with MLL phenotype but also any existing or newly emerging clones that lost one of the antigens (**Figure 1**). ULBP2-aCD19 and ULBP2-aCD33, the bispecific immunoligands either targeting CD19 or CD33, would fail in this regard. To mimic the antigen loss variants of BV173 cell line, CD19 and/or CD33 antigens were preblocked using molar excess of CD19- or CD33-specific scFv moieties (aCD19scFv or aCD33scFv) that lacked an ULBP2 ligand. This has previously shown to completely abolish binding of the immunoligands and subsequent killing of target cells in an antigen-specific manner (47). As shown in **Figure 2**, when both antigens were accessible on BV173 (CD19⁺CD33⁺), all three immunoligands significantly enhanced the NK-cell-dependent killing of BV173 cells, albeit depending upon the expression level of the respective antigen. Of note, the surface expression of CD19 on the BV173 is several fold higher compared to CD33 (47, 49). When CD19 antigen was blocked (CD19^{block}CD33⁺) by preincubation with aCD19scFv construct, BV173 killing induced by ULBP2-aCD19 was completely abolished while ULBP2-aCD33 and ULBP2-aCD19-aCD33 retained their toxic effects. Similarly, CD33 blocking on BV173 (CD19⁺CD33^{block}) by aCD33scFv could

abolish killing by ULBP2-aCD33 but not by ULBP2-aCD19 and the triplebody. Only, simultaneous blocking of both CD19 and CD33 antigens could abolish the killing induced by the dual-targeting triplebody ULBP2-aCD19-aCD33. This prototype immunoligand can also be modified to target a different combination of antigens such as CD19 and CD20 in case of CLL. Theoretically, it is also possible that tumor clones that have lost the expression of both antigens preexist within the heterogeneous tumor population and can be further selected even after dual-targeting approach. Moreover, this is also relevant in the context of antigen loss following targeted therapy as simultaneous loss of CD19 and CD20 antigens has been noted following rituximab therapy (34). Therefore, clinical success of the dual-targeting strategy will require careful selection of the tumor antigen pair and combination therapies should be considered in the case of double antigen loss.

Although this study focused on NK cell-dependent effects, NKG2D is also a shared activating receptor on γ/δ T cells and a coactivating receptor on CD8⁺ T cells. NKG2D-dependent antitumor effector functions of both of these T cell populations have been reported by us and others. Therefore, we believe that NKG2D targeting would facilitate a more dynamic immune

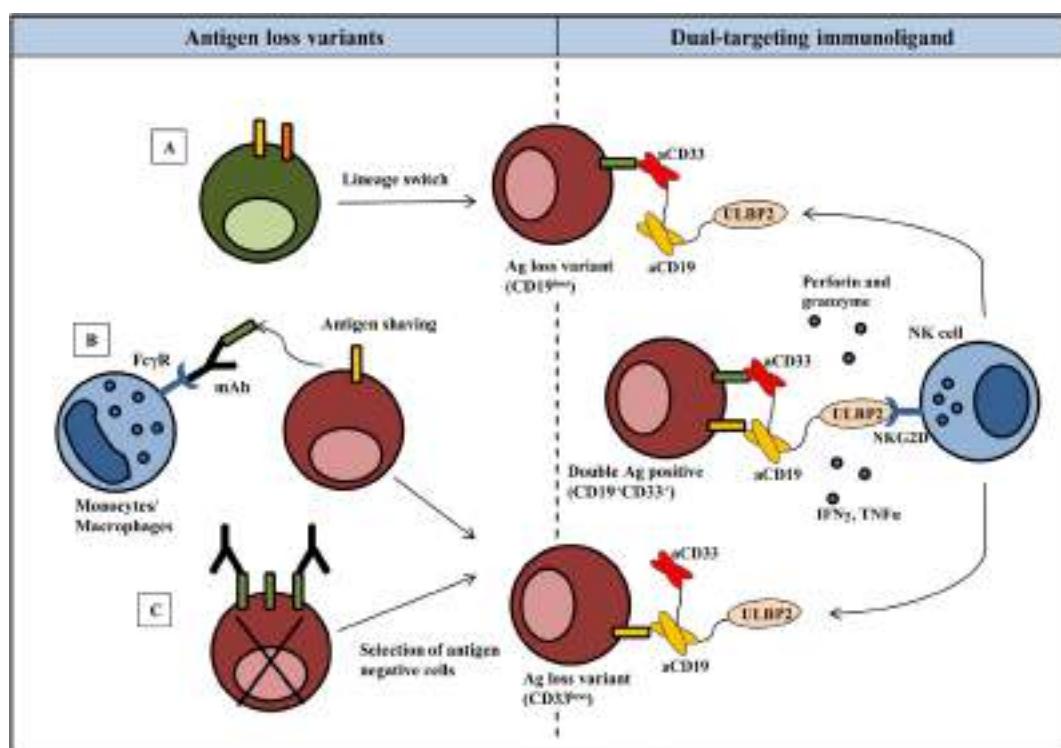


FIGURE 1 | Harnessing NK cells to control antigen loss variants: rational for the dual-targeting immunoligand approach. Emergence of antigen loss variants in most cases is seen following targeted therapy and can be associated with lineage switching (**A**), shaving or trogocytosis of antigen–antibody complexes from the tumor cells (**B**) or selective outgrowth of antigen-negative cells (**C**). NK cell activating dual targeting immunoligand (triplebody) consists of two scFVs against distinct antigens on tumor cells and a natural ligand to activate NK cells. As an example, ULBP2-aCD19-aCD33 (dual targeting triplebody) binds not only to the double antigen-positive (CD19⁺CD33⁺) target cells but also to the antigen loss variants. ULBP2, now coated on the target cells, activates NK cell effector functions via NKG2D receptor resulting in the killing of tumor cells by perforin and granzymes and secretion of IFN γ and TNF α . For simplicity, cross-linking is only shown between CD19⁺CD33⁺ target cells and NK cell; however, identical NK cell targeting is possible in response to antigen loss variants.

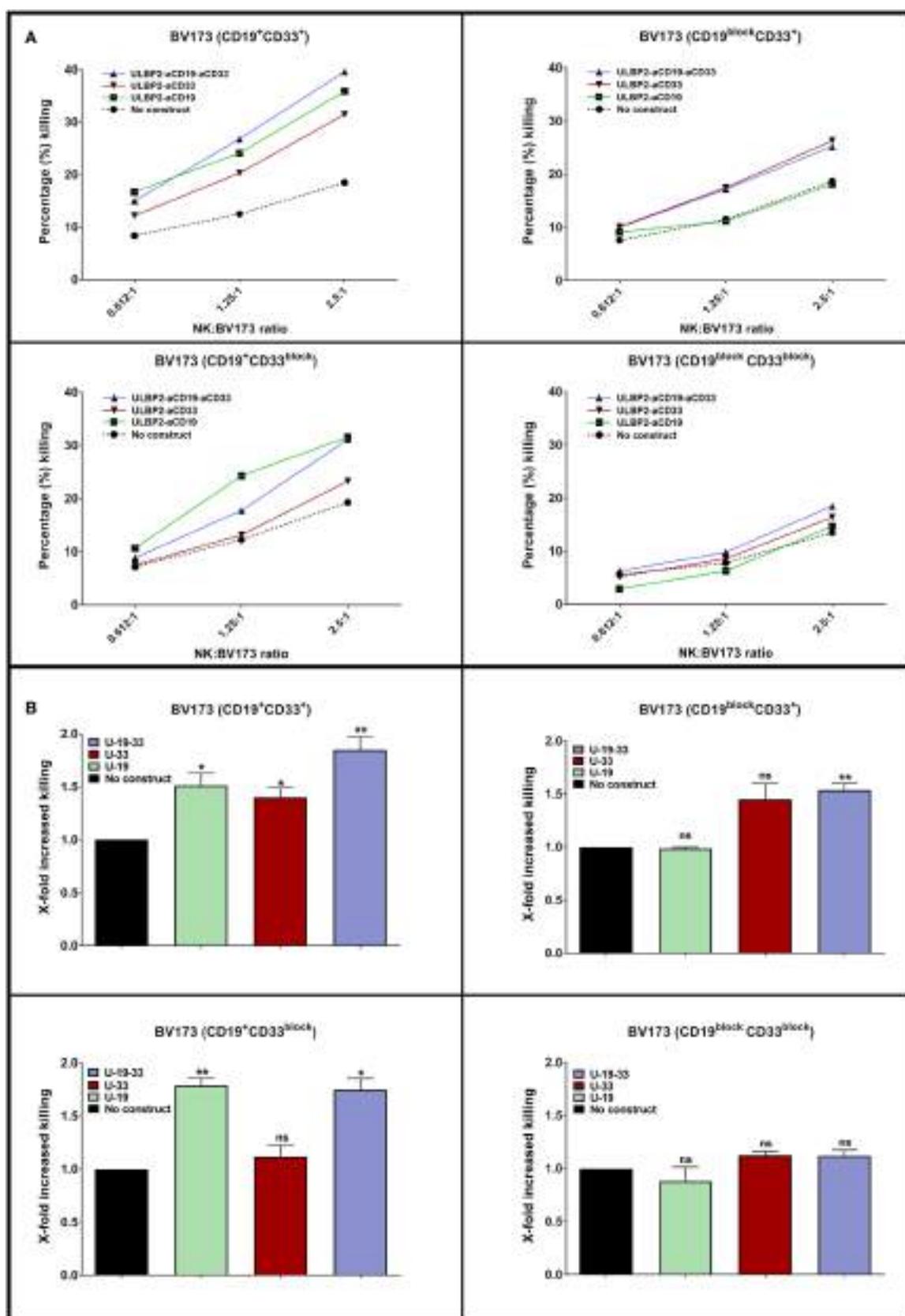


FIGURE 2 | Continued

FIGURE 2 | Continued

A dual targeting triplebody ULBP2-aCD19-aCD33 mediates NK cell-dependent killing of antigen loss variants. (A) NK cells were purified from healthy donor by negative selection and were primed by IL2 (200 U/ml) + IL15 (10 ng/ml) cytokines for 15–18 h (overnight). Next day, primed NK cells were incubated with DiR dye-labeled BV173 cells at indicated effector to target (E:T) ratio for 3 h. The incubation was continued either alone (No construct) or in the presence of 100 nM of immunoligand (U-19: ULBP2-aCD19, U-33: ULBP2-aCD33, U-19-33: ULBP2-aCD19-aCD33). After incubation, 7-AAD was added and 7-AAD-positive cells within DiR-positive gate indicated dead BV173 cells. One representative toxicity assay is shown. (B) Cumulative analysis of four independent toxicity assays at 2.5:1 (E:T) ratio ($N = 4$; each N represents an independent healthy NK cell donor). Error bars indicate SEM and statistical analysis by one-way ANOVA.

reaction involving both, innate and adaptive arms. In human and mice, chronic stimulation of NKG2D receptor by membrane bound ligands leads to the reduced surface expression of NKG2D receptor (50, 51). However, ULBP2 is not as effective as MICA in causing downmodulation of NKG2D receptor (51), and we do not anticipate that the recombinant protein will be retained in the body fluids for a relevant period to cause significant downmodulation of NKG2D receptor.

Taken together, incorporating additional tumor specificity to the current mono-targeting T and NK cell-based therapies appears to be a promising approach to prevent or treat antigen loss relapse. Their ultimate clinical benefits may be more accurately predicted by addressing whether there are any additional adverse effects that are particularly associated with dual specificities.

ETHICS STATEMENT

The collection of and the experiments with human NK cells from healthy volunteers were approved by the local ethics committee of the University of Cologne under reference number

11-140. Donors provided written consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MV and ES contributed to the design and analysis of the experiment. MV performed the experiments. MV, RM, and ES participated in writing and reviewing of the manuscript.

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Patient's Natural Killer Cells in the Era of Targeted Therapies: Role for Tumor Killers

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Natural killer (NK) cells are potent antitumor effectors, involved in hematological malignancies and solid tumor immunosurveillance. They infiltrate various solid tumors, and their numbers are correlated with good outcome. The function of NK cells extends their lytic capacities toward tumor cells expressing stress-induced ligands, through secretion of immunoregulatory cytokines, and interactions with other immune cells. Altered NK cell function due to tumor immune escape is frequent in advanced tumors; however, strategies to release the function of NK infiltrating tumors are emerging. Recent therapies targeting specific oncogenic mutations improved the treatment of cancer patients, but patients often relapse. The actual development consists in combined therapeutic strategies including agents targeting the proliferation of tumor cells and others restorating functional antitumor immune effectors for efficient and durable efficacy of anticancer treatment. In that context, we discuss the recent results of the literature to propose hypotheses concerning the potential use of NK cells, potent antitumor cytotoxic effectors, to design novel antitumor strategies.

Keywords: tumour immunosurveillance, natural killer ligands, immune checkpoint inhibitors, BRAF inhibitor, AMLMDS, melanoma

INTRODUCTION

Natural killer cells have been known and actively studied for more than four decades. They were first described as large granular lymphocytes cytotoxic for various tumor cells without prior stimulation (1, 2). In addition to their cytolytic activity against neoplastic and virus-infected cells, NK cells also display immunomodulatory functions by their ability to release cytokines, like interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α), and chemokines. NK cells represent 5–15% of blood lymphocytes. They are present in the bone marrow, liver, uterus, spleen, lungs, in mucosa-associated lymphoid tissues, thymus, and secondary lymphoid tissues (SLT) and are recruited in inflamed sites. In SLT, NK cells provide an early source of IFN γ and interact with dendritic cells to promote T helper cell type 1 responses (3).

Natural killer cells are now grouped in the system of innate lymphoid cells (4). These populations, mostly tissue resident and characterized by their capacity to produce high amounts of cytokines, constitute innate homologs of T helper cell (CD4) and cytotoxic T cell (CD8) subsets. ILCs are implicated in tissue homeostasis and autoimmune diseases. Their distribution and capacity to produce cytokines suggest that they may also be involved in the development or evolution of cancer. NK cells

are considered as cytotoxic counterparts of ILC1, both depending on the T-bet transcription factor for their development.

Human NK cells, defined as CD45⁺/CD3⁻/CD56⁺ cells (5), are classically subdivided in two subsets based on the relative membrane expression of CD56 and CD16, the low-affinity receptor for the Fc portion of IgG (FcγRIIIA): CD56^{dim} NK cells that express high levels of CD16 mediate antibody dependent cell cytotoxicity (ADCC), whereas CD56^{bright} NK cells express no or low levels of CD16. These two subsets are present in different proportions in the different tissues. CD56^{dim} NK cells represent 90% of blood and splenic NK cells, while CD56^{bright} NK cells predominate over CD56^{dim} in the SLT [lymph nodes (LN) and tonsils] representing up to 90% of NK cells and also constitute the major NK subset in tissues. It is accepted that CD56^{bright} NK cells are less mature than CD56^{dim} NK cells and display an immunoregulatory function, secreting high amounts of IFNγ and TNFα. CD56^{dim} NK cells represent mature NK cells with a high cytotoxic activity (6).

The activation of NK cells is tightly regulated by a balance between activating and inhibitory signals delivered through engagement of numerous activating and inhibitory receptors with ligands on the target cell. Natural cytotoxicity receptors (NCRs), such as NKp46 and NKp30, are expressed by resting NK cells while NKp44 is induced after activation by cytokines, such as IL-2 and IL-15 (7, 8). The NCRs are implicated in the lysis of various tumor cells (9). The activating NK group 2 member D (NKG2D) receptor is expressed by most circulating NK cells and binds the stress-induced MHC-class I polypeptide-related sequence (MIC)-A/B molecules and UL16-binding proteins 1–6 (ULBP1–6) (10). DNAX accessory molecule-1 (DNAM-1) binds Nectin family molecules CD155 and CD112.

Natural killer cell activation is efficiently controlled by specific inhibitory NK receptors binding human leukocyte antigen of class I (HLA-class I) molecules. The C-type lectin CD94/NKG2A receptor binds HLA-E molecules (11) sensing the global HLA-class I molecules on the target while killer Ig-like receptors (KIRs) bind classical HLA-class I molecules, including HLA-C, HLA-Bw4, and some HLA-A alleles.

NK CELLS IN TUMOR IMMUNOSURVEILLANCE

A link between NK cell function and cancer development was reported in a Japanese 11-year follow-up study including 3,625 patients in which cancer incidence was negatively correlated with blood NK-mediated cytotoxicity (12). Authors further showed that individuals with particular NKG2D haplotypes, HNK1/HNK1 haplotype (correlated with high NK activity) had a decreased risk of cancer compared to those with an LNK1/LNK1 haplotype (correlated with low NK activity) (13).

Additional results including ours showed the impact of NCR transcripts in the evolution of melanoma, lung cancers, and gastrointestinal stromal tumors (GIST) patients (14–16). High NKp46 correlated with better survival in metastatic melanoma patients and particular profiles of NKp30 isoforms was associated with better outcome and response to treatment in GIST patients.

The cancer immunoediting process (17) resumes cancer progression in three phases. In the elimination phase, immune cells and among them NK cells eradicate developing tumor cells. During the equilibrium phase, the immune system may select tumor variants with less immunogenicity gradually leading to the tumor escape phase and tumor progression. It is considered that most tumors at diagnosis are in the phase of immune escape associated with functionally altered tumor infiltrating NK cells (18). Tumor immunoediting selecting variants with decreased expression of stress-induced ligands provide tumor escape to NK cell-mediated lysis through activating receptors NKG2D or NKp46 (19, 20).

The challenge is thus to overcome tumor immunosuppression and restore NK cell activities. To this aim, understanding the mechanisms that lead to NK cell defects in tumor is required.

NK CELLS IN HEMATOLOGICAL MALIGNANCIES

Numerous studies showed that severe quantitative and qualitative alterations of NK cells are associated with different hematological malignancies, particularly in myeloid disorders. In chronic myelogenous leukemia patients, low numbers of NK cells are associated with defects in their proliferation, and weak NK cell cytolytic functions in comparison with healthy donor blood NK cells (21). Furthermore, profound alterations in the activating receptors profile have also been reported including downregulation of NKp30 and NKp46 as well as DNAM-1, 2B4, and NKG2C on NK cells from acute myeloid leukemia (AML) patients. Decreased NKp30 and NKp46 expression was correlated with reduced NK cell killing and poor leukemia prognosis (22–25). Recently, Khaznadar et al. analyzed by cell imaging the lytic NK immunological synapse following interaction with AML cells and showed defective lytic granule polarization in NK cell-AML conjugates leading to impaired NK cell cytotoxic function (26).

Importantly, the intimate relationship between immune pressure and leukemogenesis has been suggested in two recent studies. Stringaris et al. described an immunoediting process induced by AML blasts that limits NK cell control of leukemia. They showed that abnormal NKG2A expression and TNFα production predict a poor response to chemotherapy in AML patients (27). Conversely, Khaznadar et al. showed that NK cell defects in AML patients at diagnosis could be associated with a specific transcriptional program in AML blasts and with patient's outcome including relapse occurrence (28).

Furthermore, the beneficial role in the graft-versus-leukemia (GvL) of allogeneic NK cells for leukemic patients receiving allogeneic hematopoietic stem cell transplantation (HSCT) is well documented (29). Several studies showed that NK cells have a potent GvL effect in both KIR/HLA-class I-mismatched and -matched donor-recipient combinations after allogenic HSCT in AML patients (30–32). Moreover, rapid NK recovery after HSCT is also associated with a greater GvL effect and improved outcome in AML patients (33).

NK CELLS IN SOLID TUMORS

In situ detection of NK cells infiltrating various human tumors/tissues was carried out, leading sometimes to divergent results due to the disparity of NK cell markers used (CD57, CD56, NKp46, double CD3/CD56 staining). However, several reports showed that NK cells can infiltrate clear-cell renal cell carcinoma (34), melanoma (35), non-small cell lung cancer (NSCLC) (36), breast cancer (BC) (37), GIST (38), and colorectal carcinoma (CRC) (39) although NK cells were mainly localized at the tumor's periphery. In several tumors, infiltrations by NK cells were reported to have a prognostic value. Increased overall survival was associated with a high NK cell infiltrate within the tumor or tumor stroma in lung adenocarcinoma (40), metastatic renal carcinoma (41), and lung metastasis of renal cancer (42). Elevated number of NK cells was associated with reduced risk of cancer progression in prostate cancer (43), with a reduced risk of death in squamous cell lung cancer (44), and a better prognosis in gastric carcinoma (45) and CRC (46). In addition, the number of NKp46⁺ NK cells was found inversely correlated with metastasis occurrence in patients with GIST (47). Furthermore, a positive association between a high numbers of tumor infiltrating CD56⁺ NK cells with a regression of melanocytic lesions was observed (48).

In most tumor types studied, *ex vivo* tumor-infiltrating NK cells displayed severe phenotypic and functional alterations compared to blood NK cells and more interestingly compared to NK cells present in adjacent normal tissues. Those alterations affected the expression of activating receptors including NKp30, CD16, DNAM-1, and ILT2 on NK cells from patients with non-invasive and invasive BC (49) or NSCLC (36). A concomitant-increased expression of the inhibitory molecule NKG2A was also observed in BC (49). This deficient phenotype was associated with impaired functions including decreased cytotoxicity against tumor cells (36, 49) and reduced IFN γ production (36). Recently, Carrega et al. reported that lung and BC tissues were highly enriched in CD56^{bright} perforin^{low} NK cell subset compared to matched normal tissues (37). It is of note that comparison between NK cells from tumor and normal adjacent tissue is required for better understanding of the effect of the tumor environment on their activation.

Interestingly, our team recently identified in tumor draining LN from melanoma and BC patients, the presence of a CD56^{bright} CD16⁺ NK-cell subset that displays higher expression of activating receptors, perforin molecules, and performs ADCC (50). We found that different NK receptors regulate the two LN-NK cell subsets in melanoma and BC (personal communication) and that NK-infiltrating LN recapitulate the alterations reported in the primary tumors. The presence of CD16⁺ NK cells in certain tumors (51) and metastatic LN emphasizes the interest for ADCC function of such NK cells.

Alterations in Blood NK Cells from Patients with Solid Tumors

Alterations in blood NK cells from patients with solid tumors were also reported, but in a lesser extent than in tumor infiltrating NK cells. Compared to healthy donors, a downregulation

of NKG2D and an increase of the inhibitory receptor CD158b expression were correlated with impaired NK cell function (52–54) in metastatic melanoma patients. Our group showed a progressive decrease of NKp46 expression on blood NK cells with the disease progression in melanoma patients (55). In BC patients with invasive tumor, blood NK cells display altered expression of activating receptors NKp30, NKG2D, DNAM-1, 2B4, and CD16 and an upregulation of the inhibitory receptors NKG2A and CD85j. This phenotypic change was correlated with decreased NK cell cytotoxicity function and cytokine production (IFN γ and TNF α) (49). Blood NK cells from soft-tissue sarcoma patients displayed reduced proportions of CD56^{dim} NK cells. Low percentages of blood NK cells associated with a reduced NKp30, NKp46, and NKG2D expression were reported in patients with invasive squamous cervical cancer (56).

NK CELLS: A POTENTIAL PARTNER FOR TARGETED THERAPIES

The advent of targeted therapies that counteract a vital cellular process within the tumor cell greatly improved cancer treatment strategies. Thus, mitogen-activated protein kinase (MAPK) inhibitors that control the mutation-driven oncogenic pathway present in most cancers are new efficient players in the arsenal of therapies for cancer patients. In addition, monoclonal antibodies (mAbs) that recognize tumor-associated antigens have been established as one of the most successful therapeutic strategies for both hematologic malignancies and solid tumors. These mAbs may activate antibody-dependent cell-mediated cytotoxicity involving NK cells.

Combining targeted therapies and methods to stimulate patient's immune players is actively evaluated and represents a promising and natural evolution in cancer treatment as this could ally immediate efficiency, specificity, and long-term antitumor efficacy.

It is of note that targeted therapies also display off-target effects, connecting oncogenesis to immunosurveillance. We discuss below the interest of NK cell-based therapies in the context of such tumor-targeted therapies (Figure 1).

Effect of Cancer Treatment on NK Cells

Most melanoma patients (65%) bear a BRAF-mutated tumor and receive specific inhibitors targeting mutated BRAF^{V600E} alone or in combination with MEK inhibitors, upstream of ERK (57). These inhibitors may exert bystander effects on certain immune cells that depend on MAPK for their activation and/or proliferation. BRAF inhibitors do not affect NK cell phenotype *in vivo* and *in vitro*, but blood NK cell numbers were increased in vemurafenib-treated patients (58, 59). MEK inhibition alters the expression of the main NK receptors and the function of cytokine-activated NK cells, but the combined BRAF and MEK inhibitors did not (60).

In addition, targeted therapies may interfere with the NK/target interactions through modulation of NK ligands on cancer cells. We have shown that a BRAF inhibitor modulates the expression of MICA and ULBP2 (ligands of NKG2D), changing

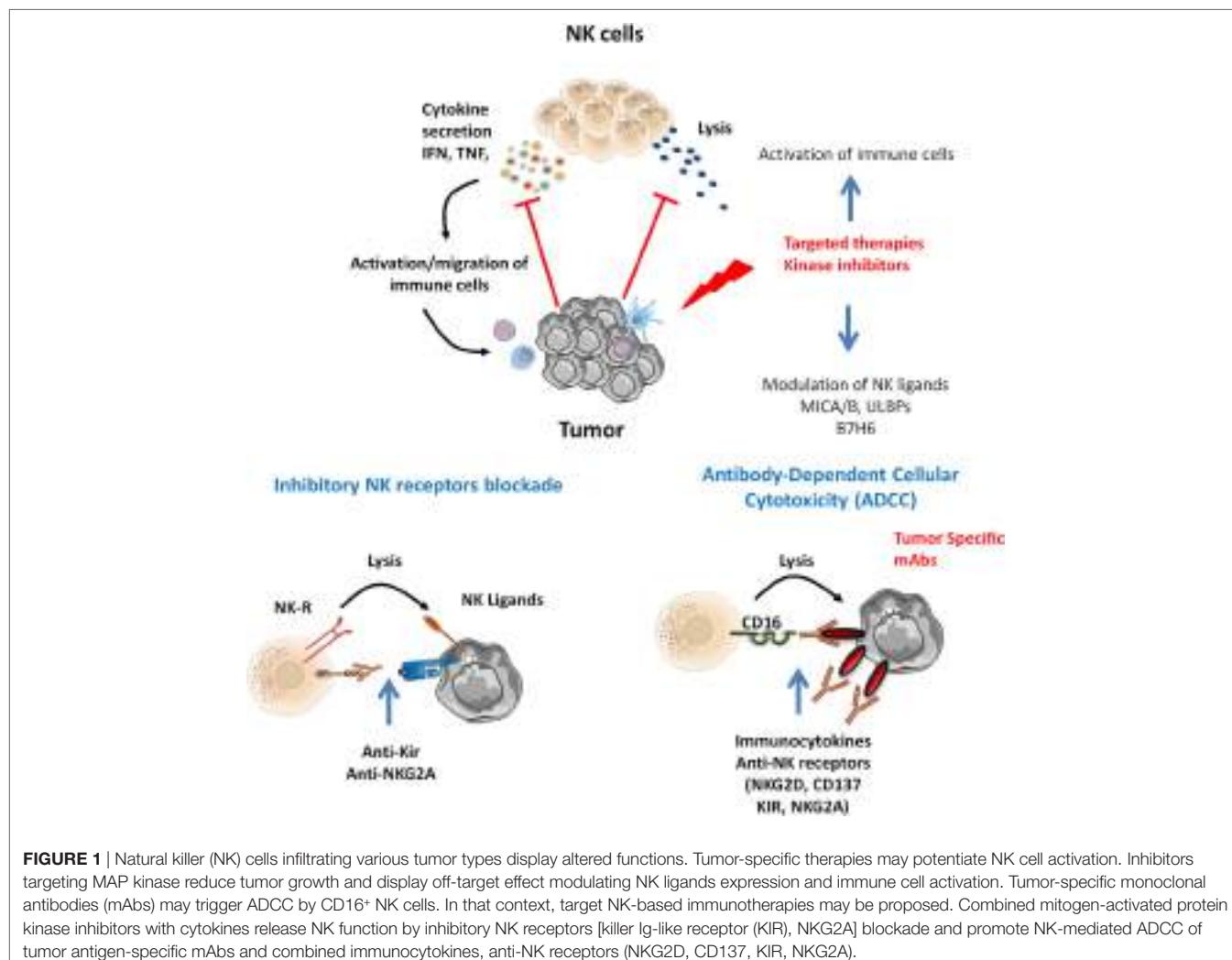


FIGURE 1 | Natural killer (NK) cells infiltrating various tumor types display altered functions. Tumor-specific therapies may potentiate NK cell activation. Inhibitors targeting MAP kinase reduce tumor growth and display off-target effect modulating NK ligands expression and immune cell activation. Tumor-specific monoclonal antibodies (mAbs) may trigger ADCC by CD16⁺ NK cells. In that context, target NK-based immunotherapies may be proposed. Combined mitogen-activated protein kinase inhibitors with cytokines release NK function by inhibitory NK receptors [killer Ig-like receptor (KIR), NKG2A] blockade and promote NK-mediated ADCC of tumor antigen-specific mAbs and combined immunocytokines, anti-NK receptors (NKG2D, CD137, KIR, NKG2A).

the ratio between membrane expression and soluble form, and increases B7H6 (ligand of NKp30) expression and HLA-A,B,C and HLA-E molecules expression that engage inhibitory receptors (KIRs, NKG2A), thus interfering with NK cell-mediated lysis (in revision). Resistance to a BRAF inhibitor is accompanied by higher NK ligands expression (personal communications).

Our findings and recent results from the literature emphasize that therapeutics designed to limit cancer cell growth by acting through kinase inhibitors should also be considered in terms of their impact on immunosurveillance (61). In a murine model of BRAF-mutated melanoma, host NK cells and perforin were required for the effect of a BRAF inhibitor (62) and correlated with the reduction of tumor growth, and an increased NK and T cell infiltration of the tumors (63).

Combining specific MAPK inhibitors with immunotherapies to increase response rates is evaluated leading to yet discordant results. BRAF inhibition augments melanoma antigen expression and maintains T cell function (64). However, inhibition of BRAF in a murine model of human melanoma was associated with decreased tumor-resident lymphocytes and resistance to CTLA-4 mAb (65). MEK inhibitors increased antigen-specific T cell

within the tumor sparing their cytotoxicity and combined with anti-PD-L1 mAb they exerted a synergic effect of tumor growth inhibition (66). Other kinase inhibitors such as those targeting Jak involved in the signaling cascade of cytokine receptors may influence NK (67).

A better understanding of off-target efficacy of MAPK inhibition affecting tumor-host interactions is required to develop strategies aimed at facilitating antitumor immune responses. The emerging findings indicate a potential synergy between targeted therapies, which change the balance between ligands of activating and inhibitory NK receptors, and NK-based immunotherapies, opening new interesting opportunities for the design of clinical trials.

Anti-KIR/Anti-NKG2A mAbs: Increasing NK Function by Blocking Negative Signaling

One promising approach is to release NK cell function with anti-KIR or anti-NKG2A mAbs as NK cells are strictly controlled by receptors specific for HLA-class I molecules. Fully human anti-KIR mAbs, 1-7F9 mAb, and then lirilumab (recombinant

version with a stabilized hinge) were generated (68). They prevent the binding of KIR2DL1, KIR2DL2, and KIR2DL3 receptors to their HLA-C ligands and blocking their inhibitory signaling. *In vitro* and *in vivo* studies showed that anti-KIR mAbs augmented NK cell-mediated lysis of HLA-C⁺ tumor cells, including autologous AML blasts and autologous CD138⁺ multiple myeloma cells (68–71). In addition, transient increases of TNF α and MIP-1 β serum concentrations and CD69 expression on NK cells were observed from treated patients (72). In a clinical trial, Benson et al. showed that 1-7F9 mAb is safe in patients with multiple myeloma and enhances *ex vivo* patient-derived NK cell cytotoxicity against tumor cells (73).

Other immune receptors highly expressed by NK cells are in development, such as anti-NKG2A (monalizumab).

Targeting inhibitory pathways in NK cell/tumor interactions may be complementary to small-molecule inhibitors for the treatment of advanced tumors such as melanoma. The prospect of combining NK cell-based immunotherapy with approaches to target the immunosuppressive tumor microenvironment or immune checkpoints, such as KIR blockade, is especially relevant to the treatment of solid tumors (74, 75) and particularly for tumors refractory to targeted therapies.

NK Cell-Mediated ADCC Using Tumor-Specific mAb

Natural killer cells express activating low-affinity FcgRIIIa (CD16) and are key mediators of antibody-dependent cellular cytotoxicity. The relevance of ADCC in tumor control using therapeutic mAbs was evaluated in several cancers. The contribution of ADCC to the clinical efficacy of a therapeutic mAb has been observed in non-Hodgkin's lymphoma patients treated by anti-CD20 (rituximab) (76). Other therapeutic mAbs likely inducing NK cell-mediated ADCC are anti-CD19 in patients with B malignancies, anti-GD2 in neuroblastoma patients, and anti-HER2 mAbs (trastuzumab) in metastatic breast and gastric cancer patients (76–78). Anti-EGFR mAb (cetuximab) was shown to increase ADCC-mediated lysis of colon tumor cells by blood NK cells from colorectal cancer patients that display altered natural cytotoxic activity (51).

Several modifications of the antibody structure, such as class switching, humanization, and point mutations to reduce complement interaction/activation, are developed to engineer mAbs with increased NK cell ADCC function and limit their toxicity. Thus, humanized anti-GD2 mAb (hu3F8-IgG1) exerts reduced toxicity compared to other anti-GD2 mAbs, by leveraging ADCC over complement-mediated cytotoxicity (79). Higher FcyRIIIa-binding affinity of anti-CD19 antibody significantly increased NK cell-mediated ADCC, leading to malignant B-cell clearing in non-human primates (78, 80). Other strategies to enhance the

effect of ADCC include the coadministration of cytokines, IL-12 with anti-HER2/neu (trastuzumab) (81) to stimulate IFN γ production by NK cells and T cells and promote the CD56^{dim}CD16⁺ NK cell differentiation to mediate ADCC (82). Co-infusion of anti-CD20 (rituximab) and TLR9 agonist (CpG) that is known to raise the membrane expression of CD20 on malignant B cells enhances ADCC (83). The infusion of immunocytokines, cytokines linked to the Fc terminus of humanized Abs, is also evaluated to potentiate ADCC. In preclinical study, Buhtoiarov et al. demonstrated that the humanized anti-GD2 immunocytokine hu14.18-IL-2 exerts higher antitumor effect than the reagents given separately (84).

Combining tumor-specific mAbs and mAbs targeting NK receptors (NKG2D, costimulatory molecule CD137) is another option. Anti-CD137 coadministered with rituximab led to a subsequent stimulation of these NK cells and enhanced rituximab-dependent cytotoxicity against the lymphoma cells (85). Furthermore, combination of rituximab with antibodies that block KIR2DL1 significantly improved NK cell-mediated lysis of tumor targets (86).

CONCLUSION

Restoring NK cell functions in addition to administration of tumor-specific therapies with kinase inhibitors or tumor-specific mAbs may benefit patients. It would increase the control of residual tumor cells, enhance mAbs efficiency, and promote the adaptive immune response necessary for long-lasting protective immunity. In that context, cytokines, blockade of inhibitory NK receptors (KIRs, NKG2A), or transfer of alloreactive NK cells are promising NK-based therapies.

ETHICS STATEMENT

The study protocol was approved by an ethic committee “Ile de France” (CPP: 2834), and the Declaration of Helsinki protocols were followed.

AUTHOR CONTRIBUTIONS

MM, ND, and AC wrote the manuscript; MM did the figure; AF, PG, and AT read and corrected the manuscript.

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Restoring Natural Killer Cell Immunity against Multiple Myeloma in the Era of New Drugs

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Transformed plasma cells in multiple myeloma (MM) are susceptible to natural killer (NK) cell-mediated killing via engagement of tumor ligands for NK activating receptors or “missing-self” recognition. Similar to other cancers, MM targets may elude NK cell immunosurveillance by reprogramming tumor microenvironment and editing cell surface antigen repertoire. Along disease continuum, these effects collectively result in a progressive decline of NK cell immunity, a phenomenon increasingly recognized as a critical determinant of MM progression. In recent years, unprecedented efforts in drug development and experimental research have brought about emergence of novel therapeutic interventions with the potential to override MM-induced NK cell immunosuppression. These NK-cell enhancing treatment strategies may be identified in two major groups: (1) immunomodulatory biologics and small molecules, namely, immune checkpoint inhibitors, therapeutic antibodies, lenalidomide, and indoleamine 2,3-dioxygenase inhibitors and (2) NK cell therapy, namely, adoptive transfer of unmanipulated and chimeric antigen receptor-engineered NK cells. Here, we summarize the mechanisms responsible for NK cell functional suppression in the context of cancer and, specifically, myeloma. Subsequently, contemporary strategies potentially able to reverse NK dysfunction in MM are discussed.

Keywords: multiple myeloma, immunotherapy, natural killer cells, killer immunoglobulin-like receptors, cytokines, immune checkpoint inhibition, daratumumab, elotuzumab, IDO inhibitors, chimeric antigen receptor

INTRODUCTION

Multiple myeloma (MM) is a B-cell malignancy characterized by an abnormal growth of malignant plasma cells which derive from a post-germinal B-cell of the lymphoid cell lineage. The treatment paradigm for MM has undergone a dramatic evolution in the past decade given a considerable improvement in the understanding of disease pathogenesis. Despite the development of novel therapeutic agents such as proteasome inhibitors—bortezomib, carlfizomib—and immunomodulatory drugs—lenalidomide, pomalidomide—which target not only MM cells but also their interplay with the microenvironment, MM remains an incurable disease and the prognosis of patients

with relapsed/refractory MM remains very poor. A number of factors concur to make MM a hard-to-treat hematologic malignancy. Drug resistance remains a major concern. MM is a highly heterogeneous disease with pathogenic processes that may greatly differ among newly diagnosed patients and others that may arise during the disease course. In recent years, several studies have focused on mechanisms of drug resistance even though many are not yet completely understood. It is widely assumed that cytogenetic and epigenetic abnormalities, deregulated signaling pathways, the MM bone marrow (BM) microenvironment, and the MM stem cell itself are all elements which play significant roles in drug resistance. Deletion 17p13 is one of the most relevant chromosomal abnormalities present in approximately 10–15% of newly diagnosed patients and observed more frequently in refractory-relapsed patients. It has been associated with resistance to new agents such as bortezomib and lenalidomide (1, 2). Aberrant drug transport processes and anti-apoptosis mechanisms have also been correlated with drug resistance (3, 4). Moreover, a pivotal role is played by the intense cell-cell crosstalk between the BM microenvironment and MM cells and their interplay with the extracellular matrix (5). All the abovementioned mechanisms make MM very challenging to eradicate with single-agent or combination modalities. Thus, an urgent need exists for new therapeutic strategies to overcome resistance to current therapies. MM is also characterized by a gradual and progressive immune dysregulation with impairs functions of B and T cell immunity, natural killer (NK) cells, and antigen-presenting/dendritic cells that allow malignant plasma cells to escape immunosurveillance. The combination of an “immunosuppressive” microenvironment and clonal evolution activate signaling pathways that invariably promote disease survival and progression. Several immunotherapies have recently been proposed and, among others, they have included monoclonal antibodies, antibody-drug conjugates, chimeric antigen receptor T cell therapy (CAR-T cells), tumor vaccines, and immune checkpoint inhibitors. This review provides an overview of the biological functions and potential clinical role of NK cells as a form of immunotherapy that may improve MM clinical outcomes.

PHYSIOLOGY OF NK CELLS AND THEIR RECEPTORS

Missing-Self Recognition and Inhibitory NK Cell Receptors

In the early 1970s, immune effectors isolated from mice and humans were found to display *in vitro* antitumor cytotoxicity without prior immunization by tumor antigens *in vivo* (6–9). These cells were functionally defined as *N-cells* or *NK cells* and were believed to belong to the lymphoid lineage, but to be distinct from B and T cells (10–13). Mechanisms regulating NK cell-mediated target recognition and killing remained obscure for more than a decade after natural cytotoxicity was first described. In 1986, Karre et al. reported that resistance of mice lymphoma cells to NK cell-mediated rejection was dependent on major histocompatibility complex (MHC) class

I antigen expression on cancer surface (14). This observation led to the assumption that NK cell would possess receptors able to transduce negative signals upon MHC class I engagement, thus sparing putative targets. Lack of MHC class I would instead trigger NK cell activation, a phenomenon known as *missing-self* recognition (15).

In humans, the NK cell inhibitory receptors able to recognize HLA class I are type I transmembrane structures belonging to the immunoglobulin (Ig) superfamily, known as killer immunoglobulin-like receptors (KIR). Inhibitory KIR share a long (L) cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs that can process signals through the recruitment and activation of the SH2-domain-containing tyrosine phosphatase 1 protein (16–20). Three inhibitory KIR engaging HLA class I ligand groups are critical regulators of NK cell function: KIR2DL1, specific for HLA-C2 group antigens (sharing Asn at position 77 and Lys at position 80 of the HLA-Cw heavy chain); KIR2DL2/3, specific for HLA-C1 group antigens (sharing Ser at position 77 and Asn at position 80 of the HLA-Cw heavy chain) (21, 22); and KIR3DL1, specific for the HLA-Bw4 epitope (located at position 77–83 of the heavy chain of certain HLA-B and HLA-A alleles) (23–25).

In the last two decades, multiple additional inhibitory NK cells receptors have been identified, leading to the currently accepted notion that NK cell effector function is dependent on the overall balance of signals transduced by multiple inhibitory and activating receptors recognizing cognate ligands on virally infected and cancer cells. Examples of non-KIR inhibitory NK receptors include the c-type lectin-like CD94/NKG2A (CD159a) heterodimer and ILT2 (LILRB1, CD85j), respectively, engaging HLA-E and various HLA class I antigens (26, 27); NKR-P1A (CD161) recognizing the lectin-like transcript 1 (28, 29); and the carcinoembryonic antigen-related cell adhesion molecule 1 (CD66a) recognizing the CD66 ligand (30–32).

Activating NK Cell Receptors

Activating NK cell receptors are also described. Among them, NKG2D (CD314) has ligand specificity for a wide range of stress-induced cell surface ligands (NKG2D-L), including the MHC-related ligands MICA and MICB (33) and the human cytomegalovirus glycoprotein (UL16)-binding proteins ULBP1–6 (33, 34). Natural cytotoxicity receptors (NCRs) NKp46 (NCR1, CD335) (35, 36), NKp44 (NCR2, CD336) (37), and NKp30 (NCR3, CD337) (38) are potent activating receptors almost exclusively restricted to NK cells. Ligands for NCR are currently incompletely characterized. NKp46 and NKp44 are known to bind several viral hemagglutinins (39, 40), while NKp30 recognizes the HLA-B-associated transcript 3 (BAT3) (41) and B7-H6, a member of the B7 immunoreceptor family (42). CD94/NKG2C (CD159c) binds the non-classical HLA-E, similar to its inhibitory CD94/NKG2A counterpart (25). CD16 (Fc γ RIIIA) (43) is the low-affinity IgG receptor, strongly expressed on mature NK cells, mediating antibody-dependent cellular cytotoxicity (ADCC) (44). Other important activating receptors include the SLAM-related 2B4 (CD244) (45) engaging the pan-leukocyte surface antigen CD48 (46) and the adhesion molecule DNAM-1 (47) involved in recognition of PVR (CD155) and nectin-2 (CD112) (48).

NK CELL IMMUNITY DYSFUNCTION IN MM

Tumor-Induced Microenvironment Transformation

Accumulating evidence indicates that microenvironment transformation may significantly impair NK cell effector function in MM (49). Plasma cells and T regulatory (T_{reg}) cells from patients with MM secrete high levels of TGF- β (50, 51), a potent immunosuppressive cytokine known to downregulate multiple NK-activating receptors and to impair NK cytotoxicity (52–54). IL-10 and IL-6 are increased in MM (55–57) and independently act as powerful growth factors for malignant plasma cells (58, 59). IL-10 inhibits production of pro-inflammatory IFN- γ and TNF- α (60, 61) and promotes development of NK-resistant tumor phenotypes (62), although it may also enhance NK cytotoxicity in response to IL-15 exposure *in vitro* (63). IL-6 has been shown to impair NK cell activity in experimental models, human disease, and when administered to patients with advanced cancer (64–66). Altered levels of IFN- γ may also contribute to NK cell dysregulation in MM. In two studies, serum IFN- γ levels were found to be significantly lower in subjects with MM than in normal controls (55, 56), potentially affecting NK cell activity. Besides cytokines, other soluble factors are known to suppress NK-mediated antitumor capabilities. Prostaglandin E2 inhibits activating signals transduced by NCR, NKG2D, and CD16 (67) and has been shown to be actively produced in cultures of BM from patients with MM (68). Indoleamine 2,3-dioxygenase (IDO) promotes cancer cell immune escape through potent immuno-regulatory effects on antigen-presenting cells *via* enzymatic degradation of L-tryptophan (69) (see IDO inhibitors). Della Chiesa et al. described that IDO-mediated immunosuppression also involves NK cells *via* L-kyureunine (Kyn), a L-tryptophan (Trp) degradation product impairing NKp46/NKG2D-specific lysis (70). Interestingly, interaction between CD28 on MM cells and CD80/86 stimulates IDO production by stromal dendritic cells (71), in agreement with the observation that CD28 expression on MM plasma cells is a marker correlating with poor disease outcome (72).

Additional microenvironmental factors may contribute to blunted NK cell cytotoxicity and cytokine production in MM. Among them, myeloid-derived suppressor cells (MDSCs) have been found to be expanded in MM (73, 74) and to directly contribute to downregulation of NK cell responsiveness *via* the NKp30-activating receptor (75), membrane-bound TGF- β (76), and TIGIT-mediated inhibitory signaling (77). Furthermore, reduced oxygenation described in MM BM (78, 79) may inhibit NK cell anti-myeloma responsiveness (80).

Effect of Soluble Ligands on NK Cell-Mediated Immunity in MM

MICA and MICB (collectively named MIC) are stress-inducible NKG2D ligands frequently overexpressed in response to malignant transformation (81). When bound to tumor surface, they act as markers of “abnormal self” and may trigger NK cell cytotoxicity *via* NKG2D signaling. Conversely, cleavage of

membrane-bound MIC is a strategy employed by MM and other tumors to evade NK cell immunosurveillance (82–85). In individuals with MIC⁺ tumors, soluble MIC (sMIC) ligands induce internalization of surface NKG2D (but also NCR and chemokine receptors) and substantial impairment of NK effector functions (86–88). In addition, sMIC has been shown to promote the accumulation of MDSC and macrophages with an immunosuppressive phenotype (89), potentially contributing to NK cell suppression. Not surprisingly, presence of sMIC is associated with poor cancer survival (90–92). In MM, shedding of MIC may result from exposure of MM cells to the genotoxic agents, doxorubicin and melphalan (93). Proteolytic cleavage by ADAMTS10 has been described to mediate this phenomenon, suggesting that the combination of metalloproteinase inhibitors with chemotherapy would exert a protective effect against escape of MM cells from NK-mediated recognition (93). Similar to NKG2D-L, NCR-specific soluble ligands may in some instances induce NK cell functional impairment. For example, circulating BAG6/BAT3 may inhibit NK cell cytotoxicity by inducing NKp30-specific hyporesponsiveness (94). Shedding of these ligands in the context of MM has not been investigated.

Effect of Cell Contact on NK Cell-Mediated Immunity in MM

Derangement of NK cell effector functions may be further amplified by tumor ligand surface expression patterns favoring dominance of inhibitory NK signals. Ligands recognized by NK-activating receptors are often poorly expressed in cancer. Downregulation of membrane-bound NKG2D-L is common in multiple tumors, resulting in impaired NKG2D-dependent NK cell cytotoxicity (95–97) and unfavorable clinical outcomes (97). In the context of monoclonal gammopathy, expression of MICA is known to decrease upon transition from pre-cancerous monoclonal gammopathy of undetermined significance (MGUS) to MM (84). Of note, various pharmacological interventions may counter NKG2D-L downregulation in MM: vincristine, *via* p38 MAPK pathway activation (98); doxorubicin, melphalan, and bortezomib as a result of oxidative stress, DNA damage, and tumor senescence (99, 100) the heat shock protein-90 (HSP90) chaperone protein inhibitors 17-allylaminogeldanamycin and radicicol (101); and inhibition or degradation of bromodomain and extra-terminal proteins (102). Exposure to therapeutic agents with activity on MM has similarly been shown to induce upregulation of PVR (an activating ligand for DNAM-1) on malignant plasma cells (98, 100, 103). Besides NKG2D-L, surface expression of the B7-H6 ligand, engaging the NKp30 NCR, has been found to be downregulated in cell lines generated from multiple cancers, including MM, resulting in NKp30-dependent NK cell functional impairment (104).

Upregulation of tumor-bound HLA class I antigens is another mechanism of protection against NK cell immunosurveillance. Malignant plasma cells obtained from the BM of early-stage myeloma patients display low HLA class I expression potentially favoring NK-mediated killing (105). In contrast, high HLA class I levels are observed on plasma cells derived from pleural effusions of patients with advanced MM (105). HLA-E is a non-classical

HLA class I antigen frequently upregulated on cancer cells, a phenomenon correlating with poor prognosis (106). In MM primary cells, high HLA-E expression results in restrained *in vitro* degranulation of NK cell subsets expressing the HLA-E-specific inhibitory NK receptor NKG2A (107).

Surface overexpression of ligands for inhibitory NK receptors is not restricted to HLA class I antigens. Notably, the CD200 glycoprotein is also commonly overexpressed on cancer surface, specifically in myeloid and lymphoid leukemias, where it is a marker of poor prognosis (108, 109). Leukemia blasts overexpressing CD200 escape NK-mediated immunosurveillance by dampening NK cell cytolytic capabilities and NKp44/NKp46 receptor expression (110), a phenomenon that can be reversed by CD200 blockade (111). CD200 is also frequently expressed in patients with MM, where it adversely affects clinical outcomes following stem cell transplantation (112).

Numerical, Phenotypic, and Functional Characteristics of NK Cells in MM

Multiple reports describe numerical, phenotypic, and functional NK cell alterations in MM. Subjects with MGUS and untreated, early-stage MM have been generally found to have higher (113–115) or similar (116–118) numbers of circulating and BM NK cells than healthy donors. Upregulation of CD57 and CD16 on NK cell surface is also observed (119, 120), suggesting the emergence of terminally differentiated subsets with high-cytotoxic potential. While these findings suggest efficient response to malignant clones subject to NK-mediated immunosurveillance, several lines of evidence favor the view that such early anti-MM effects are rather to be interpreted as a sign of immunological stress resulting in poor disease control. In fact, the effector function of expanded NK cells from MM subjects has been unexpectedly found to be similar to that of NK cells obtained from healthy donors (114), and NK cells obtained from untreated or previously treated MM patients show a lower increase in cytotoxicity to the K562 cell line in response to pre-incubation with IFN- γ (121). Moreover, NK cell effector functions positively correlate with presence of adverse prognostic factors, including anemia, low albumin, high β 2-microglobulin, and renal failure (115), suggesting a “stressed” immunoresponse under the pressure of an aggressive clonal expansion (115). Notably, NK cells from patients with MM display an “exhausted” phenotype signature that includes downregulation of multiple activating receptors and upregulation of programmed death receptor-1 (PD-1). Surface expression of activating 2B4 is reduced in both PB (122) and BM (123) NK cells obtained from untreated subjects with MM, potentially preventing killing of plasma cells despite low HLA class I expression (105). NKG2D and NCR are also downregulated in MM, but preferentially in the BM (122, 123), supporting the concept that downregulation of certain activating NK cell receptors is both dependent on soluble ligands and direct cell-cell contact. Negative signaling from PD-1 is a well-established marker of exhaustion on T cells, but can also disrupt NK cell cytotoxicity and cytokine production (124). In MM, both expression of PD-1 on NK cells and of its ligand PD-L1 on plasma cells has been described (125, 126). PD-1/PD-L1 interactions may therefore promote NK cell functional exhaustion

in MM, a phenomenon potentially reversible by checkpoint blockade inhibition (see Inhibitors of the PD-1/PD-L1 Pathway; Figure 1).

Natural killer cell-mediated immunity further deteriorates in advanced MM. Compared to MGUS and untreated MM, PB NK cell numbers are substantially reduced in advanced disease (113). Altered distribution of NK cell subsets in human BM may be likewise hypothesized based on studies in mice demonstrating selective decrease of KLRG1⁺ NK cells during MM progression (127). Evolving phenotype editing further promotes tumor escape from NK cell-mediated immunosurveillance. Furthermore, the activating receptor DNAM-1, expressed on NK cells from healthy donors and with MM in complete remission, is downregulated on NK cells from patient with active MM (128). This phenomenon is particularly relevant for late-stage cancer immune escape, as killing of malignant plasma cells is in certain circumstances critically dependent on DNAM-1 engagement of PVR and nectin-2 (128, 129). In line with these findings, NK cell activity in advanced MM is significantly impaired (130). Taken together, these data

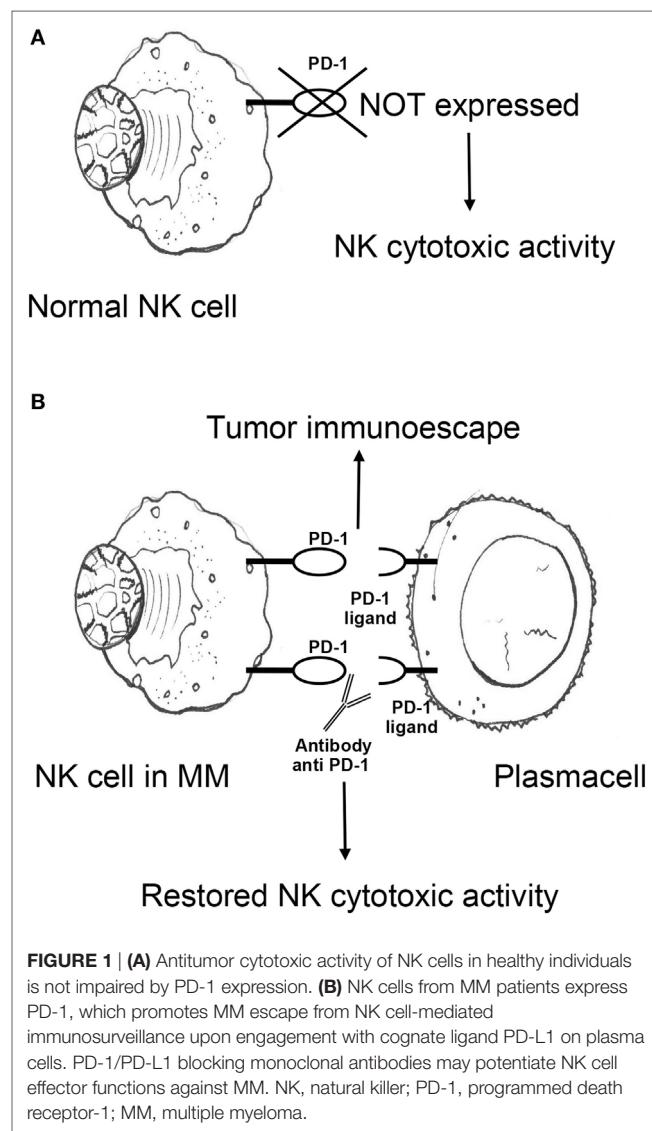


FIGURE 1 | (A) Antitumor cytotoxic activity of NK cells in healthy individuals is not impaired by PD-1 expression. **(B)** NK cells from MM patients express PD-1, which promotes MM escape from NK cell-mediated immunosurveillance upon engagement with cognate ligand PD-L1 on plasma cells. PD-1/PD-L1 blocking monoclonal antibodies may potentiate NK cell effector functions against MM. NK, natural killer; PD-1, programmed death receptor-1; MM, multiple myeloma.

indicate that NK cell immunity alterations, already detectable in early myeloma, progress in a clinical stage-dependent manner and that immunotherapy modalities based on efficient NK cell effector function such as (i.e., mAbs) are likely to exert a more effective anti-myeloma effect when used in early-stage disease.

Factors promoting NK immunity suppression in MM are summarized in **Table 1**.

IMMUNE CHECKPOINT BLOCKADE OF NK CELLS

Inhibitors of the PD-1/PD-L1 Pathway

Programmed death receptor-1 is a transmembrane protein expressed on the surface of antigen-activated T and B cells. It has two ligands, PD-L1 and PD-L2. PD-L1 is expressed on both antigen-presenting cells/dendritic cells and a wide spectrum of non-hematopoietic cells. PD-1/PD-L1 interactions physiologically counter T cell stimulatory signals and allow T cell homeostasis and self-tolerance by suppressing activation and proliferation of autoreactive T cells. PD-1/PD-L1 binding delivers an inhibitory costimulatory signal that induces a state of T cell exhaustion that prevents activation and proliferation of T cells. Unlike NK cells from healthy donors, NK cells from MM patients express PD-1 (**Figure 1A**), suggesting that NK cells from healthy donors do not

express PD-1 (**Figure 1A**), however, NK cells from MM patients do. This may show that a functional change in NK cells in response to MM may cause an immunosuppressive microenvironment for MM to grow. In the light of these observations and the broad expression of PD-1 and its ligands in the MM microenvironment, the PD-1/PD-L1 pathway may play a pivotal role in the immune evasion of MM cells (**Figure 1B**).

A role for the PD-1/PD-L1 signaling pathway in the NK cell immunoresponse against MM and of the anti-PD1 antibody CT-011 was first shown by Benson et al. (125). CT-011 was demonstrated to enhance human NK cell function against autologous, primary MM cells by affecting NK cell trafficking, immune complex formation with MM cells, and cytotoxicity toward MM cells expressing PD-L1 while sparing normal cells (**Figure 1B**).

It was also shown that lenalidomide had the ability to down regulate PD-L1 on primary MM cells and, by so doing, increase NK cell functions against MM. Thus, targeting the PD-1/PD-L1 pathway may become a feasible clinical strategy in MM, especially in patients with persistent residual disease (131).

One preliminary phase I study reported on 17 patients treated with pembrolizumab, a PD-1 inhibitor, in combination with lenalidomide and dexamethasone (132). Overall response and very good partial response rates were 76 and 23%, respectively. Some 75% of patients achieved stable disease. Many patients were

TABLE 1 | Microenvironment alterations potentially promoting natural killer (NK) immunity suppression in multiple myeloma.

Factors	Function	Effect of TM	Impact on NK cell immunity	Reference
Soluble				
TGF- β	Anti-inflammatory cytokine	↑	Reduced NK effector functions Downregulation of activating receptors	Castriconi et al. (52); Lee et al. (53); Mamessier et al. (54)
IL-10	Anti-inflammatory cytokine	↑	Resistance to NK cytotoxicity Reduced NK cytokine production	Tsuruma et al. (62); Sharma et al. (55); Zheng et al. (56)
IL-6	Pro-inflammatory cytokine	↑	Reduced NK effector functions	Bataille et al. (57); Scheid et al. (66)
IFN- γ	Pro-inflammatory cytokine	↓	Reduced NK effector functions	Sharma et al. (55); Zheng et al. (56)
PGE2	Prostaglandin	↑	Reduced NK effector functions Inhibition of positive intracellular signaling	Lu et al. (68); Martinet et al. (67)
sMIC	NKG2D ligand	↑	Reduced NK effector functions Downregulation of NK activating receptors	Groh et al. (86); Jinushi et al. (84); Xiao et al. (89)
Cell bound				
mMIC	NKG2D ligand	↓	Resistance to NK cytotoxicity	Jinushi et al. (84)
B7-H6	NKp30 ligand	↓	Resistance to NK cytotoxicity	Fiegl et al. (104)
HLA class I	KIR/NKG2A ligands	↑	Resistance to NK cytotoxicity	Carbone et al. (105); Bossard et al. (106); Sarkar et al. (107)
CD200	Membrane glycoprotein	↑	Reduced NK effector functions Downregulation of NK activating receptors	Moreaux et al. (112); Coles et al. (110)
2B4	Activating receptor	↓	Reduced NK effector functions	Fauriat et al. (122); Costello et al. (123)
NKG2D	Activating receptor	↓	Reduced NK effector functions	Fauriat et al. (122); Costello et al. (123)
NCRs	Activating receptors	↓	Reduced NK effector functions	Fauriat et al. (122); Costello et al. (123)
DNAM-1	Activating receptor	↓	Reduced NK effector functions	El-Sherbiny et al. (128)
PD-1	Immune checkpoint receptor	↑	Reduced NK effector functions	Benson et al. (125); Gorgun et al. (126); Beldi-Ferchiou et al. (124)
KLRG1	Co-inhibitory receptor	↑	Reduced NK effector functions	Ponzetta et al. (127)

TM, tumor microenvironment; PGE2, prostaglandin E2; sMIC, soluble MIC; mMIC, membrane-bound MIC; KIRs, killer immunoglobulin-like receptors; NCRs, natural cytotoxicity receptors; PD-1, programmed cell death protein 1/programmed cell death protein ligand 1; KLRG1, killer cell lectin-like receptor subfamily G member 1.

↑ denotes increase; ↓ denotes decrease.

heavily pretreated with other lines of therapy. Almost all patients, however, experienced at least one adverse event with anemia, neutropenia, thrombocytopenia, fatigue, hyperglycemia, and muscle spasms being the most common. Two other recent studies with nivolumab showed acceptable toxicity but no objective responses (133, 134). Efficacy assessment of nivolumab, alone or in combination, is ongoing.

More recently, a novel subpopulation of human NK cells expressing high levels of PD-1 have been identified in ovarian cancer, characterized by low proliferative responses, and impaired antitumor activity that can be partially restored by antibody-mediated disruption of PD-1/PD-L1 interaction (135).

Future studies to evaluate the real therapeutic role of anti-PD-1 antibodies, maybe in combination with other agents with potent anti-myeloma activity such as lenalidomide, are warranted.

KIR-Specific Immune Checkpoint Inhibition

The role of NK cells as graft-vs.-myeloma effectors was first investigated in preclinical models. Frohn et al. described for the first time the killing ability of NK cells against three different MM cell lines. The mean NK cell killing ability on MM samples ranged from 23 to 34.5% (136). Moreover, KIR-ligand mismatch in T cell-depleted allogeneic stem cell transplantation reduced the relapse incidence in MM recipients. The impact of KIR-ligand mismatch was assessed in a cohort of 73 MM patients who received reduced-intensity unrelated donor transplants. KIR-ligand mismatch in the graft-vs.-host disease direction was significantly associated with lower risk of relapse (HR: 0; $p < 0.0001$) (137).

To exploit this pathway, Romagné et al. generated an IgG monoclonal antibody, 1-7F9, against three different KIRs (KIR2DL-1, KIR2DL-2, and KIR2DL-3) to enhance the NK cells antitumor effect. This checkpoint inhibitor augmented NK cell-mediated lysis of HLA-C-expressing tumor cells without interfering with normal peripheral blood (PB) mononuclear cells (138) (**Figure 2A**). The therapeutic potential of 1-7F9 was then demonstrated in preclinical mouse models, providing the platform for translational studies in humans (139).

The drug IPH2101, formerly 1-7F9, was tested in a phase I trial in 32 patients with relapsed/refractory MM. IPH2101 was administered for up to four 28-day cycles, in 7 dose-escalated cohorts (0.0003–3 mg/kg). Only one patient developed severe toxicity, characterized by grade 4 acute renal failure with hyperkalemia and hyperuricemia. From a biological point of view, the drug determined the full saturation of NK inhibitory KIRs (140). Furthermore, lenalidomide and IPH2101 were investigated as a novel, steroid-sparing, dual immunotherapy in 15 MM patients: the biological endpoint of full KIR occupancy was achieved, 5 patients had a response, and 5 severe adverse events were reported (141).

In an open-label, single arm two-stage phase II trial, IPH2101 was employed at the dose of 1 mg/kg every other month for six cycles in nine patients with smoldering MM. Despite the promising results from preclinical and phase I studies, the trial was terminated before planned second stage due to lack of patients meeting the primary objective (50% decline in M-protein) (142).

A recombinant version of IPH2101 was developed with a stabilized hinge (lirilumab). A phase I study of the safety and tolerability of lirilumab with elotuzumab in myeloma patients is currently in progress. Of note, lirilumab recognizes both the inhibitory KIR2DL1, -L2, and -L3 and the activating KIR2DS1-2. Therefore, lirilumab-mediated modulation of intracellular signals is expected to vary according to patient's HLA class I genetic background and KIR receptor repertoire.

In vitro experiments showed that KIR2D molecules are removed from NK cells surface by trogocytosis. This phenomenon culminated in a strong reduction of NK cell cytotoxic function correlating with the loss of free KIR2D surface molecules (143). These data favor future protocol designs where lirilumab is administered in combination with other NK cell-activating agents, rather than as single agent.

IMMUNOMODULATORY DRUGS AND MONOCLONAL ANTIBODIES

Lenalidomide

Lenalidomide, a thalidomide analog, is an immunomodulatory drug with multiple mechanisms of action in MM. It is currently approved in both EU and USA in association with dexamethasone for the maintenance treatment of patients with newly diagnosed MM who have undergone an autograft. Four pivotal phase III studies have associated lenalidomide with improved progression-free survival and better overall response rates (144–147). Although lenalidomide has also been associated with increased risk of a second primary cancer, the overall survival benefits outweigh the risk (148).

Due to failure of single-agent anti-KIRs in phase II studies, researchers from multiple institutions investigated possible combined therapies. *In vitro*, the immunomodulatory agent lenalidomide was responsible of NK cell expansion and activation associated with malignant cells apoptosis (149). On this platform, Benson et al. tested the cytotoxicity of IPH2101 in combination to lenalidomide against MM cell lines U266 and K562 (139, 140). Healthy donor NK cells pretreated with lenalidomide or IPH2101 alone and combined showed increased IFN- γ production against primary MM cells compared to controls ($p < 0.05$). Furthermore, NK cells pretreated with both lenalidomide and IPH2101 led to the highest IFN- γ peak. The statistical interaction of p -value was 0.0182, suggesting a synergistic effect between the two drugs. Then, healthy donor PB mononuclear cells (PBMCs) incubated as control or with lenalidomide and/or with IPH2101 were used as effectors against U266 MM cell targets. Lenalidomide increased the specific release, a surrogate for cytotoxicity, by around 1.39-fold relative to control ($p < 0.01$). IPH2101 increased the specific release by 1.48-fold ($p < 0.01$). The two drugs combined increased the specific release by 2.09-fold relative to control ($p < 0.001$), which means a significantly higher cytotoxic effect than either lenalidomide or IPH2101 alone. Patient-derived NK cell cytotoxicity against autologous MM targets was enhanced by the combination of lenalidomide plus IPH2101 (128 ± 9 spots/well) compared with control conditions (81 ± 7 spots/well). Based on *in vitro* results, the authors

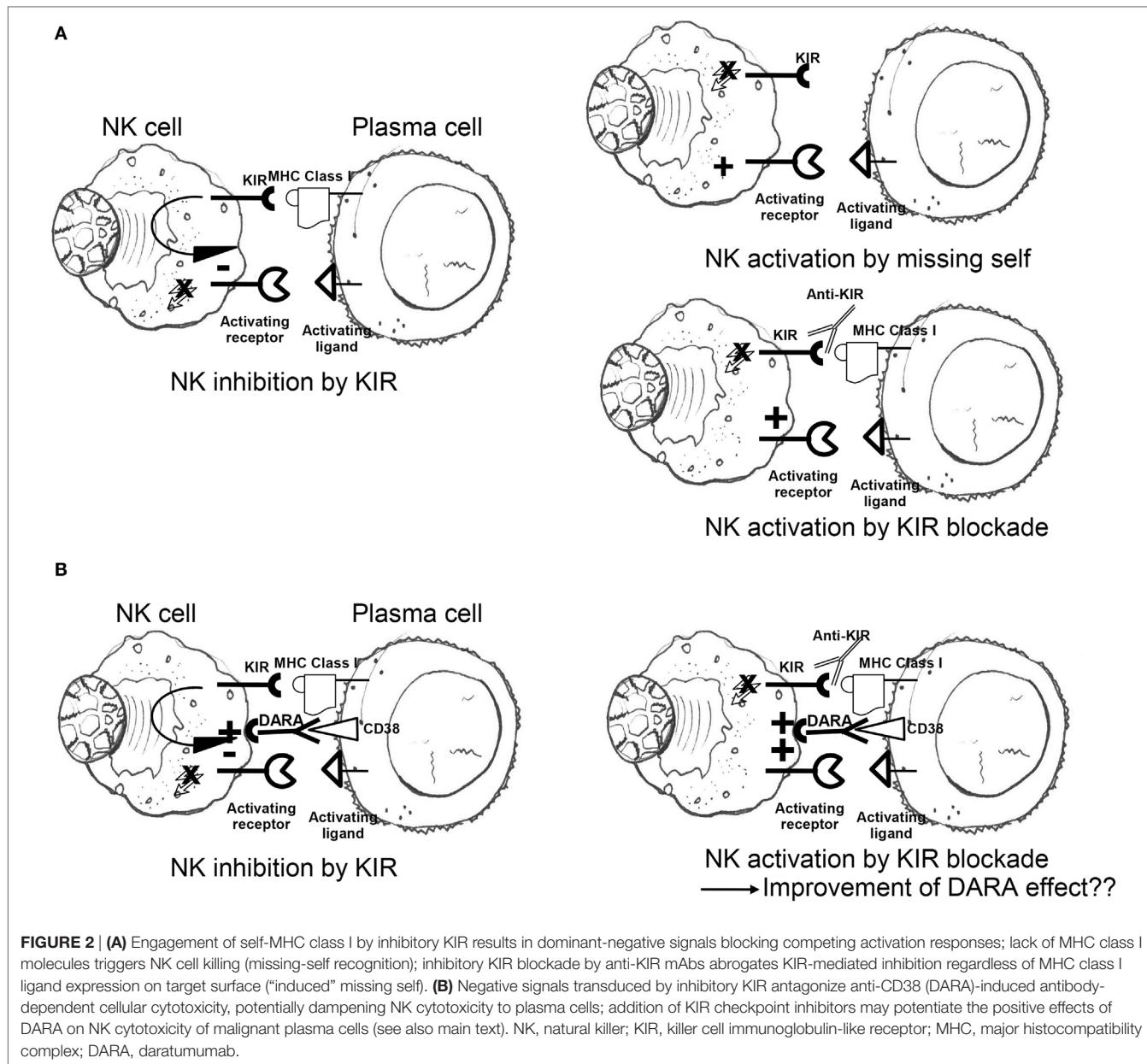


FIGURE 2 | (A) Engagement of self-MHC class I by inhibitory KIR results in dominant-negative signals blocking competing activation responses; lack of MHC class I molecules triggers NK cell killing (missing-self recognition); inhibitory KIR blockade by anti-KIR mAbs abrogates KIR-mediated inhibition regardless of MHC class I ligand expression on target surface ("induced" missing self). **(B)** Negative signals transduced by inhibitory KIR antagonize anti-CD38 (DARA)-induced antibody-dependent cellular cytotoxicity, potentially dampening NK cytotoxicity to plasma cells; addition of KIR checkpoint inhibitors may potentiate the positive effects of DARA on NK cytotoxicity of malignant plasma cells (see also main text). NK, natural killer; KIR, killer cell immunoglobulin-like receptor; MHC, major histocompatibility complex; DARA, daratumumab.

evaluated the efficacy of the anti-KIR 5E6 in lenalidomide pre-treated mice. The tumor burden was significantly reduced when the combination of 5E6 and lenalidomide was employed, in comparison to controls ($p < 0.005$). These data provide the basis for the translation of IPH2101 and lenalidomide combination in phase I and II studies.

Daratumumab (DARA)

Daratumumab is an IgGk monoclonal antibody targeting CD38, a cell surface protein that is overexpressed on MM cells (150, 151). Preclinical studies have shown that DARA induces MM cell death through several mechanisms, including complement-dependent cytotoxicity (152), ADCC (153), antibody-dependent cellular phagocytosis (154), and apoptosis (155). The drug showed efficacy as single agent in heavily pretreated MM patients or in

combination with bortezomib and dexamethasone (156). When combined to lenalidomide, the DARA cell-mediated MM cell clearance was enhanced due to lenalidomide-dependent NK cell activation. In the light of preclinical results of lenalidomide in combination with anti-KIR agents, Nijhof et al. hypothesized that the NK cell-mediated cytotoxicity induced by DARA could be enhanced by anti-KIRs (Figure 2B). The effect could be further improved through the association with lenalidomide which stimulates the proliferation of NK cells and activates them (157), overcoming NK cells depletion induced by DARA itself (158).

Elotuzumab

Initially, Hsi et al. described a humanized antibody, HuLuc63, which specifically targeted CS1 (CCND3 subset 1, CRACC, and SLAMF7), a cell surface glycoprotein that had not previously been

associated with MM cells. By flow-cytometry, HuLuc63 showed specific staining of CD138⁺ myeloma cells, NK cells, NK-like T cells, and CD8⁺ T cells. HuLuc63 showed significant *in vitro* ADCC against primary myeloma cells as targets and allogeneic or autologous NK cells as effectors. The authors concluded that HuLuc63 could eliminate MM partly through NK-mediated ADCC and targeting CS1 with HuLuc63 could become a novel treatment strategy (159). Tai et al. also showed that HuLuc63 was effective in inducing ADCC against primary MM cells resistant to novel therapies such as bortezomib and HSP90 inhibitor. Moreover, pre-treatment with conventional or novel anti-MM agents enhanced HuLuc63-induced MM cell lysis (160). Collins et al. also hypothesized that elotuzumab may have other mechanisms of action. A number of findings clearly suggested that elotuzumab may enhance NK cell function beyond ADCC. Elotuzumab was shown to induce NK cell activation by binding to CS1 which promotes cytotoxicity against CS1⁺ MM cells but not against autologous CS1⁺ NK cells. Moreover, NK cell activation was shown to be dependent on differential expression of the signaling intermediary EAT-2 which is present in NK cells but absent in primary, human MM cells (161). Therefore, HuLu63 enhances NK cell cytotoxicity to MM via a dual mechanism (**Figure 3**). The synergy between current anti-CS1 antibody elotuzumab, formerly known as HuLuc63, and bortezomib was also shown by van Rhee et al. (162). Elotuzumab was approved by FDA in 2015 for the treatment of MM, specific for signaling lymphocytic activation molecule-F7 (SLAMF7, or CS1) (163). As previously mentioned, SLAMF7 is a member of the Ig gene superfamily, almost universally expressed (>95%) on the surface of marrow MM cells, but not on normal tissues, with restricted expression on specific lymphocytes including NK cells. SLAMF7 determines activating or inhibitory effects on NK cells depending on the expression or not of EAT-2, an adapter protein (**Figure 3**). Given that MM cells lack EAT-2, the molecular mechanism by which SLAMF7 mediates inhibition in NK cells was investigated

by Guo et al. It was shown that the inhibitory effects of SLAMF7 in EAT-2⁻ NK cells was mediated by a mechanism implicating lipid phosphatase SHIP-1, Src kinases, and protein tyrosine phosphatase CD45. Coupling of SLAMF7 to SHIP-1 was highly compromised in MM cells. This correlated with a lack of CD45, which is required to activate Src family kinases in hematopoietic cells and was needed to initiate SLAMF7 inhibitory signals. This defect may explain why elotuzumab eliminates MM cells by an indirect mechanism that involves NK cells activation (164, 165). An elegant preclinical model clearly showed that elotuzumab activates NK cells and promotes myeloma cell death in healthy donor PB lymphocyte (PBL)/myeloma cell cocultures (166). Moreover, the combination of elotuzumab plus lenalidomide demonstrated higher anti-myeloma activity on established *in vivo* MM xenografts and in *in vitro* PBL/myeloma cell cocultures than either agent alone. In the same study, it was interestingly shown that the increased NK cell anti-myeloma functions were also due to increased secretion of IL-2 and production of TNF- α that combined to enhance NK cell activation and MM cell killing. All these findings supported the clinical application of combination strategies. Elotuzumab initially showed activity in combination with lenalidomide and dexamethasone in a phase I and a phase Ib-2 clinical studies in relapsed/refractory MM (167, 168). In a subsequent randomized study, patients with relapsed/refractory MM received either elotuzumab with lenalidomide and dexamethasone, or lenalidomide and dexamethasone alone. Patients who received a combination of elotuzumab, lenalidomide, and dexamethasone had a significant relative reduction of 30% in the risk of disease progression or death (169). Finally, Jakubowiak et al. reported on a phase II study in relapsed/refractory MM patients where combined elotuzumab/bortezomib/dexamethasone were compared with bortezomib/dexamethasone until progression or unacceptable toxicity. Overall, elotuzumab appeared to provide clinical benefit without clinically significant toxicity when combined with bortezomib (170).

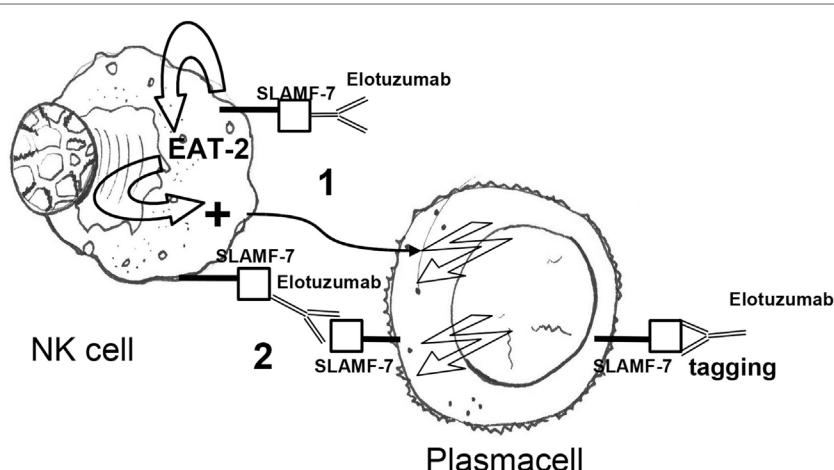


FIGURE 3 | Elotuzumab activates NK cells via (1) an indirect mechanism, i.e., binding of the extracellular portion of SLAMF7 and recruitment of the EAT-2 adaptor protein and (2) a direct mechanism, i.e., antibody-dependent cellular cytotoxicity in response to SLAMF7 tagging on plasma cells. Owing to the absence of EAT-2 in plasma cells, elotuzumab engagement does not cause activation of plasma cells. NK, natural killer; EAT-2 Ewing's sarcoma-associated transcript 2; Fc, fragment crystallizable; Fab, fragment antigen binding.

IDO Inhibitors

Indoleamine 2,3-dioxygenase plays a pivotal role in the metabolic cascade that converts the essential amino acid L-tryptophan (Trp) into L-kynurenine (Kyn). Moreover, IDO has also been shown to be involved in the establishment and maintenance of peripheral tolerance. This function may partly be due to IDO1 capacity to restrict the microenvironmental availability of Trp and to increase the accumulation of Kyn and derivatives. The expression of IDO on neoplastic lesions may help cancer to escape immunosurveillance. IDO inhibitors (i.e., 1-methyl-tryptophan) have therefore become a new class of anti-cancer agents. Current models imply that IDO limits both innate and adaptive immunoresponses by depleting immunoeffector cells of Trp (171, 172) and by promoting the accumulation of Kyn and its derivatives 3-dydroxykynurene and 3-hydroxyanthranilic acid (173, 174). These derivatives have been shown not only to exert cytostatic and cytotoxic effects on several immunoeffectors including CD8 T cells, NK cells, and invariant NKT cells (173–175) but also to inhibit TH17 cells and to promote the differentiation of naive CD4 T cells into T_{reg}s and tolerogenic activity of dendritic cells (174–181). Interestingly, Bonanno et al. investigated IDO expression in 25 symptomatic MM and in 7 with either MGUS or smoldering MM (182). IDO-driven tryptophan breakdown was correlated with the release of hepatocyte growth factor (HGF) and with the frequency of T_{reg} cells and NY-ESO-1-specific CD8 T cells. Kyn was increased in 75% of MM patients and correlated with the expansion of T_{reg} cells and the contraction of NY-ESO-1-specific CD8 T cells. *In vitro*, primary MM cells promoted the differentiation of allogeneic CD4 T cells into T_{reg} cells and suppressed IFN- γ /IL-2 secretion while preserving IL-4 and IL-10 production. Both T_{reg} expansion and inhibition of Th1 differentiation were partly reverted by D,L-1-methyl-tryptophan, an inhibitor of IDO. Of note, HGF levels were higher within the marrow microenvironment of patients with IDO⁽⁺⁾ MM as compared with patients with IDO⁽⁻⁾ MM. The antagonism of MET receptor for HGF with SU11274, a MET inhibitor, prevented HGF-induced AKT phosphorylation and resulted in reduced IDO protein levels and functional activity. These findings suggest that IDO expression may contribute to immunosuppression in patients with MM. IDO inhibitors are currently being tested either as single agent or in combination with other anti-cancer drugs in a number of solid tumors even though this class has not yet been evaluated in MM.

NK CELLS AND THEIR ROLE IN ALLOGRAFTING: LESSONS FROM ACUTE LEUKEMIAS

Some of the most convincing proofs of the potential of NK cells as immunotherapeutic tools derive from evidences accumulated over the past two decades in the setting of allogeneic hematopoietic stem cell transplantation (allo-HSCT). The interest in NK cell immunobiology stemmed mainly from two observations: (a) NK cells are the first lymphocyte subset to recover after transplantation, often reaching percentages and absolute counts superior to those commonly observed in healthy subjects (183, 184),

and (b) since KIRs and HLA ligands are encoded on different, independently inherited, chromosomes (chromosome 19 and 6, respectively), the KIR gene repertoire transferred from the donor into the host is often different posing the issue whether, and how, an efficient NK cell functionality can be achieved (185–187).

Two hallmark studies came from the Stanford group (188, 189) where it was demonstrated that after a variable number of months following HLA-matched, KIR-mismatched allo-HSCT, the NK cell repertoire is dominated by lymphocytes displaying an immature phenotype (CD56^{bright} and CD94/NKG2A⁺) and then it stabilizes and becomes similar to that of donor origin. Moreover, these studies highlighted significant differences in the repertoire recovery kinetics among patients, and clearly correlated impaired NK cell reconstitution with occurrence of post-transplant complications. The development of a HSCT platform which allowed to safely infuse HLA-haploididentical grafts set the stage for some of the most exciting discoveries in transplant biology and translational potential of NK cells.

Starting from preclinical studies on the tolerogenic potential of “stem cell megadoses” (190), the Perugia team developed a protocol which combined a highly immunosuppressive myeloablative conditioning regimen with the infusion of high doses of extensively T cell-depleted HLA-haploididentical hematopoietic stem cells (HSCs). Full donor engraftment of the partially incompatible HSCs was successfully achieved, and despite the absence of post-transplant pharmacological prophylaxis, neither acute nor chronic GvHD ensued (191). This elegant and technologically advanced HSCT platform offered the opportunity to investigate the metrics of NK cell reconstitution in a partially HLA-mismatched host and in the absence of confounding factors such as alloreactive T cells or immunosuppressive drugs. This highly favorable environment further boosted the early expansion of NK cells that had been already described in other transplant settings, and, importantly, led to the appearance of donor-derived NK cells with alloreactivity against the host (192).

Velardi et al. brilliantly described the principles by which NK cell alloreactivity developed and designed an algorithm to easily predict it. Based on this model—later defined as “ligand-ligand” or “KIR-ligand mismatch” model—post-transplant alloreactivity is unleashed when the donor carried one or more KIR ligands (i.e., HLA class I alleles encompassing the Bw4, C1, or C2 motifs) absent in the host. In this setting, inhibitory KIRs expressed on the surface of donor-derived NK cells—which, in the host, are continuously engaged by their respective ligands in the pre-transplant phase—do not find their cognate HLA molecules on host cells and tissues leading to a perception of “missing self” that activates an alloreactive response (193, 194).

One of the most striking observations by the Perugia group was that NK alloreactivity did not result in clinical GvHD, but, conversely, led to a potentially eradicating mechanism of residual leukemic cells reducing relapse incidence and risk of graft failure and GvHD (195, 196). Over the following years, several studies confirmed and consolidated the evidence that in T cell-depleted haploididentical HSCT NK cell alloreactivity represents the main driver of the graft-vs.-leukemia effect and a major predictor of overall clinical outcomes in both adults (197–199) and children (200, 201). In addition, these studies were a major drive for the

development of cell therapy protocols in which haploidentical KIR ligand-mismatched NK cells were infused in leukemic patients after lymphodepleting chemotherapy with highly promising results (202–204).

In more recent years, several new platforms of haploidentical HSCT have been developed, mainly with the aim at improving T cell immune reconstitution and at reducing post-transplant infectious complications (205). In this “new era,” studies on the impact of KIR-ligand mismatches on transplant outcomes reported some conflicting results. For instance, it was shown that infusion of unmanipulated BM grafts or of donor post-transplant T cell add-backs may mask, or blunt, the effectiveness of NK cell alloreactivity (206–208). By contrast, other recent haploidentical HSCT platforms based on the selective depletion of $\alpha\beta$ T cells or on the infusion of balanced doses of conventional and regulatory T cells appeared to better preserve the positive effect of KIR ligand mismatches (209, 210).

In partially HLA-mismatched unrelated donor HSCT, either from adult volunteers or cord blood (CB) units, the potential role of NK alloreactivity has also been a matter of debate. Some studies supported a positive role of KIR ligand mismatches (211–213) and others found no significant advantage or even adverse effects (214–216).

To overcome these inconsistencies, several alternative immunogenetic models have been developed to better predict NK cell-driven effects on transplant outcomes. In particular, Cooley et al. focused on the donor genetic repertoire and demonstrated in a number of independent studies that donors with a KIR gene asset enriched in activating receptors—group B KIR haplotypes—can provide a superior relapse-free survival after unrelated HSCT for leukemias (217–219). Another model which takes into account both donor activating KIR asset and donor/recipient HLA typing has been proposed and validated by Venstrom et al. In an analysis on more than 1,200 unrelated HSCTs, the authors observed that the presence of donor-activating receptor KIR2DS1 and of HLA-C1 ligands provided a significant protection from relapse, further enhanced in case of recipient HLA-C1 positivity (220).

Despite the multiplicity of models proposed over the years in the setting of allo-HSCT, not all the immunogenetic mechanisms that regulate NK cell interactions and alloreactivity have fully been understood. However, it is widely assumed that NK cell alloreactivity is instrumental in control and eradication of hematological malignancies.

NK CELL THERAPIES

Expanded NK Cells for MM Treatment

Expansion of NK cells from PBMC of patients with MM has been achieved using a culture system supplemented with IL-2 and OKT3 (221). NK cells could be extensively propagated (average 1,625-fold expansion in 20 days) and displayed increased levels of activating receptors as well as cytotoxicity to the NK-susceptible K562 line and to autologous MM cells (222). Another NK cell expansion strategy for MM immunotherapy is based on the artificial feeder K562 transfected with CD137L and membrane-bound IL-15. This technique allowed extensive *in vitro* NK cell

propagation (average 447-fold, range 20–10,430 on harvest day, i.e., days 10–14). Transfer of these cells into a xenogeneic model of high-risk MM resulted in myeloma growth inhibition and protection against osteolysis (223). The same group tested the safety, persistence, and activity of expanded NK cells in seven heavily pretreated patients with high-risk relapsed myeloma: no serious adverse events related to NK cell infusion was observed. Moreover, the infusion of fresh, rather than cryopreserved, cells resulted of fundamental importance for their *in vivo* expansion. Two/seven patients showed some responses which lasted for at least 6 months (224). More recently, human studies were performed with allogeneic, KIR ligand-mismatched NK cells from haploidentical family donors. NK cells were cytotoxic to K562, the myeloma line U266, and recipient primary MM cells. Fifty percent of the patients with advanced MM achieved near complete remission when these cells were infused prior to autologous SCT (225). Another phase I clinical trial (NCT02481934) evaluated safety and efficacy of multiple infusions of activated and expanded NK cells in combination with lenalidomide- or bortezomib-based regimens (226). Five heavily pretreated refractory/relapsed patients were enrolled. NK cells were activated and expanded for 3 weeks with K562mb15-41BBL cells. Patients received four cycles of new drug-based treatment with two infusions of 7.5×10^6 /kg NK cells. Four patients showed stable disease while on NK cell treatment, two showed a 50% reduction in BM plasma cell infiltration and one obtained a response >1 year. No major toxicities were reported. Expanded NK cells showed a highly cytotoxic phenotype and *in vitro* killing and were detected in both BM and PB of treated patients. While efficacy and safety of multiple NK cell infusions need further assessment, these data suggest that repeated transfer of *in vitro* activated and expanded NK cells into MM patients is feasible and may result in clinical benefit when combined with anti-myeloma drugs.

CB NK Cells

Umbilical CB represents a promising source of allogeneic NK cells. However, GMP-grade large scale *ex vivo* expansion is indispensable to generate CB-derived NK (CB-NK) cell doses that may be used in the clinical setting. Shah et al. recently described a strategy for the expansion of NK cells from cryopreserved CB units (227). By co-culturing for 14 days CB units using artificial antigen-presenting feeder cells (aAPC), a highly expanded cell product (average 1,848- and 2,389-fold in 14 days from fresh and cryopreserved samples, respectively) of 95% purity for CB-NK cells and less than 1% CD3⁺ cells was obtained. Despite differences in the expression of certain cytotoxicity receptors, aAPC-expanded CB-NK cells were phenotypically very similar to CB-NK cells expanded with IL-2 alone. Most importantly, aAPC-expanded CB-NK cells clearly showed cytotoxicity against both *in vitro* MM targets and *in vivo* anti-myeloma activity in a xenogenic mouse model. The same group investigated the mechanisms of CB-NK-mediated cytotoxicity against MM cells (228). Interestingly, a mechanism of transmissible cell death between cells induced by lipid-protein vesicles transferred from CB-NK to MM cells was described. Moreover, these vesicles were capable of migrating from recipient MM cells to neighboring MM cells enhancing cytotoxicity of CB-NK. Altogether, these findings

supported the development of CB-NK-based cellular therapies for the treatment of MM. An encouraging first-in-human study of CB-NK cells for MM patients undergoing high dose chemotherapy and autologous transplantation was recently conducted (229). Patients received lenalidomide at a dose of 10 mg from day -8 through -2, standard melphalan at 200 mg/m² on day -7. CB-NK cells were infused on day -5 and the autograft performed on day 0. Twelve patients were treated with different dose levels. Most patients were heavily pretreated and had high-risk cytogenetics. Overall CB-NK cells with an activated phenotype (NKG2D⁺/NKp30⁺) were detected *in vivo* in six patients. Importantly, no signs/symptoms of GVHD were observed. Eight patients achieved at least near complete remission and two additional patients a very good partial response. After a median follow-up of 21 months, four patients relapsed or experienced progressive disease.

TABLE 2 | Summary of current treatments with novel agents for multiple myeloma (MM) potentially affecting natural killer (NK) cell activity.

Agent	Mechanism of action on NK cells	Clinical trials	Reference
PD-1/PD-L1 checkpoint inhibitors	<i>Block of the recognition of PD-L1 by PD-1 on NK cells</i> PD1 blockade may neutralize competitive negative signals resulting in enhanced trafficking, immune complex formation, and cytotoxicity of NK cells (Figures 1A,B)	Phase I trial of pembrolizumab with lenalidomide and dexamethasone. Two Phase I trials involving nivolumab showed acceptable tolerability. Efficacy assessment of nivolumab, alone or in combination is ongoing.	Benson et al. (125); San Miguel et al. (132); Suen et al. (133); Lesokhin et al. (134)
HLA-KIR checkpoint inhibitors	<i>Prevent inhibitory KIR recognition of cognate HLA class I ligands</i> Blockade of KIR-HLA interactions may neutralize negative signals transduced by inhibitory KIR2DL1/2/3 (Figure 2A)	Anti-KIR monoclonal antibody IPH2101 (1-7F9) determined the full saturation of NK inhibitory KIR in a phase I trial enrolling patients with RR MM. Full KIR occupancy was also achieved in a study combining lenalidomide and IPH2101. In this study, 5 (33%) patients had a response. In a single arm two-stage phase II trial, IPH2101 was employed in 9 patients with smoldering MM. The study was stopped before planned second stage due to lack of patients meeting the primary objective (50% decline in M-protein). A phase I study combining elotuzumab with lirilumab, a recombinant version of IPH2101, is currently in progress	Frohn et al. (136); Benson et al. (139); Benson et al. (140); Benson et al. (141); Korde et al. (142); Carlsten et al. (143)
Daratumumab (DARA)	<i>ADCC to CD38⁺ MM cells</i> Cytolytic activity to MM cells triggered by CD16 signaling upon recognition of antibody tagged to CD38 antigen. NK cell-mediated cytotoxicity induced by DARA could be enhanced by lenalidomide and KIR blockade. Other mechanisms: complement-dependent cytotoxicity, antibody-dependent cellular phagocytosis, and apoptosis (Figure 2B)	DARA was tested in combination with bortezomib and dexamethasone in RRMM. The primary end point was progression-free survival. DARA in combination with bortezomib and dexamethasone resulted in a significantly longer progression-free survival than bortezomib and dexamethasone alone	Palumbo et al. (156)
Elotuzumab	<i>Direct effect: ADCC to MM cells expressing SLAMF7</i> Indirect effect: activation of SLAMF7 ⁺ NK cells Dual mechanism of action: (1) NK cell activation via SLAMF7 binding and recruitment of the EAT-2 adaptor proteins; (2) NK-mediated ADCC to SLAMF7 ⁺ MM cells (Figure 3)	Elotuzumab showed activity in combination with lenalidomide and dexamethasone in a phase I and a phase IIb-II clinical studies in RRMM. In a phase III study, patients with RRMM patients were treated with either elotuzumab with lenalidomide and dexamethasone, or lenalidomide and dexamethasone alone. Patients treated with the combination of elotuzumab, lenalidomide, and dexamethasone had a significantly reduced risk of disease progression or death. In a phase II study in RRMM patients, elotuzumab showed clinical benefit without significant toxicity when combined with bortezomib	Lonial et al. (167); Lonial et al. (169); Jakubowiak et al. (170)
IDO inhibitors	<i>Inhibition of L-tryptophan degradation</i> Reversal of NK immunosuppression by increased availability of L-tryptophan and reduced accumulation of L-kynurenine	IDO inhibitors are currently used as single agent or in combination in a number of solid tumors. This class has not yet been evaluated in clinical trials in myeloma patients	Uyttenhove et al. (172); Fallarino et al. (173); Bonanno et al. (182)

PD-1/PD-L1, programmed cell death protein 1/programmed cell death protein ligand 1; KIRs, killer immunoglobulin-like receptors; RR MM, relapsed/refractory MM; ADCC, antibody-dependent cellular cytotoxicity; SLAMF7, signaling lymphocytic activation molecule family 7; IDO, indoleamine 2,3-dioxygenase.

CAR-NK for Myeloma

The impressive clinical results obtained in patients with B cell malignancies with the infusion of T cells genetically modified to express synthetic chimeric antigen receptors (CARs) against the lineage-specific surface antigen CD19 represented a turning point in the history of cancer immunotherapy (230–236). Intriguingly, T cells engineered with an anti-CD19 CAR were capable to induce complete remission also in a patient with MM. However, given that the large majority of malignant plasma cells do not express CD19, studies to understand the mechanism that underlie this unexpected observation are currently in progress (237). More recently, a number of CARs have been developed to specifically target surface antigens expressed by pathological plasma cells, including CD38 (238, 239), CD138 (240), B cell maturation antigen (241, 242), κ light chains (243), SLAMF7

(244), and CD44v6 (245). However, despite their tremendous efficacy, CAR-T cells have also raised concerns on their short- and long-term toxicities, in particular the development of life-threatening cytokine release syndrome and the risks of prolonged aplasia of the healthy counterparts of the target tumor—“off tumor/on target toxicity”—and in case of allogeneic CAR-T cells the development of GvHD (246, 247).

To address these issues, genetic modifications with CARs of cells belonging to the innate immune system, and of NK cells in particular, may yield several potential advantages. For instance, most innate cells recognize and eliminate tumors by stereotyped patterns and have been infused into allogeneic recipient without excessive toxicities and with some promising intrinsic antitumor efficacy. Moreover, the short-lived persistence of innate immunocells in an allogeneic host, considered up to now one of the major limitations, may become an added value in case of CARs targeting antigens that are shared with mature cell types for which prolonged aplasia may be a concern (i.e., memory B cells, monocytes, or plasma cells) (248, 249).

Genetic modification of the human NK cell lines NKL and NK-92 by means of a lentiviral vector encoding for anti-SLAMF7 and anti-CD138 CARs has proven feasible. This did not substantially modify the expression profile of transduced cells and conferred selectivity for the target and the ability to kill human malignant plasma cells both in *ex vivo* and in an orthotopic xenograft models (250, 251). Overall, several steps to optimize and validate CAR-modified NK cells should be taken before their possible clinical use. In particular, the choice of the most appropriate NK cell source to be modified is a matter of intense debate (252). Whether freshly isolated NK cells may represent the most physiological choice to achieve sufficient cell doses and transduction efficiency remains unknown. Conversely, NK cells expanded from PB or from progenitor cells may be more easily modified even though their expression profile and functional competence may be negatively affected by prolonged *ex vivo* culture. Finally, immortalized human NK cell lines, such as NK-92, can be very efficiently transduced and expanded in desired numbers even for “off-the-shelf” use even though their cell surface expression of activating receptors is lower than in freshly isolated or expanded NK cells. Moreover, the need to irradiate the cell product before infusion would further limit their *in vivo* persistence (252, 253). A new modality that exploits the combination of the anti-CR38 monoclonal antibody DARA with CD38⁽⁺⁾ NK cells armed with CS1 CAR has very recently been described by Wang et al. to treat relapsed MM (254). Given that both CS1 and CD38

are MM-associated antigens, their simultaneous targeting may prevent progression. The same authors previously showed that DARA induces apoptosis in CD38⁽⁺⁾ NK cells but not in CD38⁽⁻⁾ NK cells. It was then hypothesized that DARA in combination with CD38⁽⁻⁾ CS1-CAR NK cells may show a synergistic effect and possibly lead to MM eradication. Long-term follow-up of clinical outcomes of this study are eagerly awaited.

FUTURE PERSPECTIVES

The potent crosstalk between malignant plasma cells and their BM microenvironment plays a central role in MM progression and resistance to current therapies. Novel forms of immunotherapy against MM represent a rapidly developing area in cancer therapy. They include treatment strategies that may be delivered either alone or in combination with currently employed therapy lines such as IMiDs and proteasome inhibitors as well as newer agents (Table 2). Moreover, immunotherapy may attenuate the systemic toxicity of cytotoxic chemotherapy. A robust body of evidence has clearly shown that enhancing host anti-myeloma immunity within the BM microenvironment may lead to a more efficient disease control. NK cells play a pivotal role in the intricate network of cells and signaling pathways that may prevent immune escape mechanisms. NK cells were clearly shown to have potent *in vivo* antileukemia activity in patients undergoing allografting. Recent observations on NK cell functions in MM have become promising immunotherapeutic strategies. New avenues of research have included expansion of NK cells from PB as well as CB, and the generations of specific CAR-NK cells against myeloma-specific antigens. Moreover, MM NK cells express PD-1 whereas NK cells from healthy individuals do not. This phenotypic characteristic may indicate that immunocheckpoint blockade of NK cells may be an area to fully explore given the remarkable results obtained with anti-PD 1 inhibitors in cancer treatment. Altogether, the studies reported in this review show that NK cells hold promise in changing the natural course of MM and that may help restore immunity to MM and thereby improve survival outcomes.

AUTHOR CONTRIBUTIONS

GP and BB contributed to the initial conception and designed of the manuscript. CB, MF, DM, and LG provided study materials and critically reviewed the manuscript. GP, LV, MF, and BB wrote the manuscript. All authors gave the final approval to the manuscript.

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Modeling Natural Killer Cell Targeted Immunotherapies

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Animal models have extensively contributed to our understanding of human immunobiology and to uncover the underlying pathological mechanisms occurring in the development of diseases. However, mouse models do not reproduce the genetic and molecular complexity inherent in human disease conditions. Human immune system (HIS) mouse models that are susceptible to human pathogens and can recapitulate human hematopoiesis and tumor immunobiology provide one means to bridge the interspecies gap. Natural killer cells are the founding member of the innate lymphoid cell family. They exert a rapid and strong immune response against tumor and pathogen-infected cells. Their antitumor features have long been exploited for therapeutic purposes in the context of cancer. In this review, we detail the development of highly immunodeficient mouse strains and the models currently used in cancer research. We summarize the latest improvements in adoptive natural killer (NK) cell therapies and the development of novel NK cell sources. Finally, we discuss the advantages of HIS mice to study the interactions between human NK cells and human cancers and to develop new therapeutic strategies.

Keywords: humanized mouse models, innate lymphoid cell, natural killer cells, cancer immunotherapy, natural killer cell immunotherapy

INTRODUCTION

Since the generation of the first inbred mouse strains in the early 20th century, mice have served as model organisms to study mammalian biology. This approach has given birth to some of the most important scientific breakthroughs and discoveries that, in many cases, led to the development of successful treatments for previously untreatable diseases (e.g., acute promyelocytic leukemia) (1). However, *Mus musculus* and *Homo sapiens* have been evolving divergently for 85 million years, adapting to very different environments and undergoing selection for many traits, from the circadian rhythm to our body size (2). Thanks to the genome decoding, we can now appreciate that the one fifth of the genetic divergence between mice and humans is enriched in regions implicated in the immune system, metabolic processes, and stress responses (3). It is, therefore, not surprising that only less than 8% of the cancer studies in animal models reach clinical trials and that more than 80% of these eventually fail when tested in humans (4). The increasing knowledge of the molecular differences between mice and humans should allow us to evaluate the degree in which animal models may be suitable for translational research and when this is not the case, to then search for better systems.

With this aim, mice have been “humanized” by introducing human genes or genomic regions and by transferring human tissues or cells to study various aspects of human biology. The engraftment

of human blood cells or blood-forming cells and organs into immunodeficient mice has opened a new era for translational immunology and the improvement of immunotherapies against human cancer and infectious diseases caused by pathogens with exclusive human tropism, such as HIV, HBV, and HCV.

DEVELOPING HUMAN IMMUNE SYSTEM (HIS) MICE

Since the discovery of the nude athymic mutations in the 1960s, our knowledge of the host immune system and its ability to reject xenografts have led to the development of several mouse strains that permit long-term “take” and function of the human tissue grafts (5). Experiments performed in the 1980s with severe combined immunodeficient (SCID) mice (that lacked functional mouse adaptive lymphocytes due to mutations in the DNA-dependent protein kinase *Prkdc*) showed that these mice could be reconstituted with human peripheral blood mononuclear cells (PBMCs) or hematopoietic stem cells (HSCs) (6, 7). However, some residual adaptive (leakiness) and an essentially intact innate immunity in SCID mice limited the complete reconstitution of all human immune subsets. Moreover, SCID mice failed to engraft human tumor xenografts, thereby limiting the development of preclinical cancer models. An alternative system with analogous immunodeficiency was obtained by mutating the recombinant activating genes (*Rag1*, *Rag2*) loci that avoided genetic “leakiness” and, in contrast to SCID mice, did not result in host radiosensitivity (8, 9). Additional genetic modifications followed to further the immunodeficiency of host mice in order to promote tolerance to human cells. Two breakthroughs have remarkably boosted the advancement of the field. First, Greiner and colleagues found that the NOD strain supported an enhanced tolerance compared to other strains and, several years later, Takenaka’s team revealed that the molecular basis for this lies in the signal regulatory protein alpha (*Sirpa*) allele polymorphism (10–13). Contrarily to other strains, SIRP α from NOD mice binds to human CD47 ligand triggering a negative signal in mouse macrophages that prevents their phagocytosis (13, 14). This finding prompted the generation of transgenic mice expressing the human or NOD strain *Sirpa* allele thus conferring enhanced human cell engraftment (15–17). The second turning point for achieving a successful xenotransplantation was the common cytokine receptor gamma chain (γ_c , encoded at *Il2rg*), which leads to complete impairment of natural killer (NK) cell development and dendritic cell (DC) dysfunction (18, 19). Mice carrying *Il2rg* mutations were developed in various genetic backgrounds [NSG or NOG (both NOD *Prkdc*^{SCID} *Il2rg*^{-/-}) and BRG (Balb/c *Rag2*^{-/-} *Il2rg*^{-/-})] allowing robust, long-lasting *de novo* multilineage development of the HIS, including human thymopoiesis, and are the basis for most of the currently used models (20–23). From that point forward, a number of model variants have been developed to address specific questions or improve particular aspects of immunity, either by genetic manipulation, engraftment of additional human tissues, or exogenous administration of human factors. This is the case of the recently described Balb/c *Rag2*^{-/-} *Il2rg*^{-/-} *Flt3*^{-/-} (BRGF) model with specific boost of conventional and plasmacytoid DCs after exogenous Flt3 ligand

treatment. This model offers a great platform for screening of immune adjuvants and DC targeting therapies (24).

HUMAN CANCER MODELS IN “HUMANIZED” MICE

Immunodeficient mice allow great flexibility for the study of human tumor immunobiology. Human tumors can be generated in NSG, NOG, BRGS, and other strains using established tumor cell lines, after transplantation of human primary tumors or following *de novo* induction of hematological neoplasms (Figure 1). These different models provide systems that better reflect the complexity of the disease. In order to allow human tumor to engraft and grow in mice, the host immune system is generally compromised leading to tumor kinetics that may not reflect the true patient situation. As discussed earlier, human immune components can be generated *in vivo* from human HSCs or other progenitors and “supported or potentiated” later on or infused once the tumor is established. These approaches provide “mixed” systems in which human immune cells and human tumors can co-exist allowing the dissection of immune deviation as well as studying immunotherapy.

A wide range of established tumor cell lines from different origins (brain, colon, breast, melanoma, ovarian, prostate, etc.) have been engrafted in immunocompromised mice and have greatly contributed to drug development and the preclinical assessment of potential therapies. However, the gradual accumulation of genetic and phenotypic aberrations in these cells due to their long-term culture impacts the surface markers and the tumorigenicity of the malignancy (25). These limitations have set aside these models to preliminary studies addressing specific questions like the ability of a potential therapy to target a certain molecule that has been overexpressed in the cell line. In recent years, the field has been, therefore, switching toward the engraftment of patient-derived primary tumors (PDX, patient-derived xenografts) that retain the phenotypic and genetic complexity observed in clinical samples thus better predicting drug efficacy and clinical translatability (26, 27). These include tumor stromal cells and tumor-associated lymphocytes that contribute greatly to tumor growth and metastasis and, therefore, to the therapeutic response. These PDX-HIS mouse models can engraft the tumor as efficiently as the non-humanized mice, they respond to standard chemotherapeutic drugs similarly to patients and they have proven to be responsive to newly derived immune modulators.

One of the better-characterized PDX models is the AML that has contributed to the identification of leukemia stem cells (LSC) by transplanting different stem-like cell fractions and analyzing the leukemia-initiating activity of each in SCID mice (28–31). The discovery of the concept of cancer stem cell (CSC) has been a breakthrough in cancer biology due to the clinical benefits for the long-term disease-free survival. CSC presence has been identified in numerous other malignancies through transfer into immunocompromised mice and, interestingly, markers associated to CSCs have been correlated to the tumorigenic potential (32, 33). Recent improvements in HIS mouse models by the transgenic expression of certain factors, like the NSG-SGM3 expressing human

	Mouse strain	Origin human immune cells	Type of tumor xenograft	Origin of tumor xenograft	References
	NSG	HSC	Rhabdomyosarcoma	Cell line	(38)
	NSG	HSC	Acute leukemia	Oncogene induced	(39)
	NOD	HSC	Pancreatic carcinoma	Cell line	(40)
	NSG	HSC+NKT	Neuroblastoma	Primary	(41)
	NSG	HSC+FT	Leukemia	Oncogene induced	(42)
	NSG	HSC+FT	Melanoma	Cell line	(43)
	NSG	HSC+FT+liver	Oral squamous carcinoma	Primary	(44)
	NSG-SGM3	HSC+FT+liver	Melanoma	Primary	(45)
	NOD	PBMC	ATLL	Primary	(46)
	BRG	PBMC	B-cell lymphoma	EBV-induced cell line	(47)
	NSG	PBMC	Pancreatic adenocarcinoma	Cell line	(58)
	NSG	Tumor-derived	Lung tumor (non-disseminated)	Primary	(49)
	NSG	NK cells	Ovarian carcinoma	Cell line	(50)
	NSG	NK cells	Glioblastoma	Primary	(51)
	NOD/SCID	BM-T cells	Breast carcinoma	Primary	(52)
	NSG	T cells + DCs	Prostate carcinoma	Cell line	(53)
	SCID	EBV-specific T cells	B-cell lymphoma	EBV-induced cell line	(54)
	NSG	CAR-T cells	Pleural mesothelioma	Cell line	(55)
	BRG	$\gamma\delta$ T cell	AML	Primary	(56)
	BRGS	UCB-NK cells	Colonctal carcinoma	Cell line	(37)

FIGURE 1 | Human immune system (HIS) mouse models used in cancer research. PBMC, peripheral blood mononuclear cells; NSG, NOD *Prkdc*^{SCID}/*Il2rg*^{-/-}; BRG, Balb/c *Rag2*^{-/-}/*Il2rg*^{-/-}; BRGS, Balb/c *Rag2*^{-/-}/*Il2rg*^{-/-} *Sirpa*^{NOD}; HSC, hematopoietic stem cell; FT, fetal thymus; BM, bone marrow; CAR, chimeric antigen receptor; UCB, umbilical cord blood; ATLL, T-cell leukemia/lymphoma; AML, acute myeloid leukemia; EBV, Epstein–Barr virus (37–56).

SCF, GM-SCF, and IL-3, have further ameliorate the engraftment and growth of human leukemia allowing the study of the tumor initiating cells (34). The demonstration that HIS mouse models reproduce the heterogeneity and behavior of human tumors creates great expectation on the better phenotyping of these tumor-initiating cells and the identification of drugs targeting this key population (35).

One of the advantages of modeling cancer in humanized mice is that we can study the systemic environment and the contribution of nearly all the immune cells to the pathogenesis. In this regard, a NSG model of treatment-refractory B-cell leukemia revealed that infiltration of leukemia cells into the bone marrow rewrites the tumor microenvironment to inhibit engulfment of antibody-targeted tumor cells. This resistance could be overcome by combination regimens involving therapeutic antibodies and chemotherapy that lead to macrophage infiltration and phagocytic activity in the bone marrow improving the efficacy of targeted therapeutics (36).

TARGETING NK CELLS FOR CANCER IMMUNOTHERAPY

To date, most immunomodulatory strategies have focused on agents or cell therapies targeting T cell immunity. In contrast,

innate immune cells, such as NK cells, have been less exploited. Nevertheless, the fundamental role for these cells has been for long justified by the higher cancer incidence in individuals with defective NK function (57). Furthermore, a number of mouse models lacking or deficient for NK cell function have corroborated their importance in tumor immunosurveillance (58).

Natural killer cells exert an immediate cytotoxicity when encountering a malignant cell and they do so without a specific antigen priming but instead, by the integrated signal of an array of activating and inhibitory receptors. Among the first group, the C-type lectin-like receptors CD94/NKG2C and NKG2D and the natural cytotoxicity receptors NKP30, NKP44, and NKP46 as well as the Ig-like receptor DNAM-1 (CD266) mediate NK cell activation when they recognize tumor cells. On the other hand, polymorphic inhibitory killer cell immunoglobulin-like receptors (KIRs) with their cognate human–leukocyte–antigen (HLA) ligands as well as CD94/NKG2A with the non-classical class I molecule HLA-E as ligand provide inhibitory signaling. In addition to the contact mediated regulation of the activity, NK cells also respond to cytokines like IL-2, IL-12, IL-15, IL-18, and IL-21, as well as toll-like receptor ligands that shape their differentiation, proliferation, and activation status (59). Cytotoxicity activity is triggered through activation of the low-affinity activating receptor FcγRIIIa (CD16) that binds the Fc portion of immunoglobulin

G1, which has been exploited in monoclonal antibody immunotherapies. NK cells kill virus-infected and tumor cells using a cargo of perforin and granzymes contained in cytotoxic granules and less efficiently by a mechanism dependent on FAS ligand, TNF, or TNF-related apoptosis-inducing ligand (60).

Given that NK cells in HSC-derived humanized mice express all the afore-mentioned receptors and respond similarly to the same cytokines (61, 62), these *in vivo* models represent a powerful platform to explore the pivotal role of NK cells in cancer immunosurveillance (63–65) (Lopez-Lastra et al., in revision). Additionally, environmental components such as inhibiting factors (TGF- β , IL-10, prostaglandin E2...) and immunosuppressive cells (Tregs, MDSCs) that influence the NK cell antitumor activity have also been described in HIS mice, enabling the evaluation of therapeutic strategies targeting the suppression of NK cells (66).

Although chemotherapy is still the core of the current clinical anticancer treatments, immunomodulators have now regained expectations after the revolutionary discovery of the CTLA-4 and PD-1 checkpoint inhibitors targeting T-cell activation (67). Humanized mice have proven to recapitulate the therapeutic effect of those antibodies as well as the side effects and have began to provide insights about the mechanism behind and possible strategies to improve them (68–70). The expression of these receptors on human NK cells suggests that they could also be targeted by checkpoint molecules and, therefore, contributes to the outcome of the therapy (71, 72). Indeed, mouse studies on a glioma model treated with activated NK cells preincubated with an anti-PD-1 blocking antibody showed an enhancement of the survival suggesting a role that must be explored in a human system (73).

ADOPTIVE TRANSFER OF NK CELLS FOR CANCER THERAPY

The potential of NK cells as innate effectors in cancer has been studied by the adoptive transfer of *ex vivo* expanded and/or activated NK cells in immunodeficient mice. Mice treated with adoptively transferred human NK cells show NK-mediated rejection of the engrafted human tumor and further administration of cytokines, such as IL-2 and IL-15 greatly improve the NK cell pool and their cytotoxic activity against transformed cell. These observations initially made in mice laid the foundation for the autologous NK cell infusion therapies started in the 1980s for metastatic cancers (74). Preclinical assessment of cytokine regimens in other cancer models, such as the low-dose IL-2 in the spontaneous EBV-associated B-cell lymphoma in PBL-SCID mice, demonstrated reduction of the tumor load and survival prolongation (75), and preceded a number of clinical trials for both hematological and solid tumors (76–79).

The discovery that inhibitory KIRs binding to MHC-I mediate inhibition of NK cells opened a new path on NK cell immunotherapies. NOD/SCID cancer models served as a platform to confirm the higher efficacy of alloreactive NK cells for the treatment of leukemia. Contrarily to T cells, NK cell do not provoke graft-versus-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT) contexts but, instead, protect the patient against it and eliminate leukemia relapse and graft rejection (80).

Later on, safety and efficacy of alloreactive NK cell infusion was confirmed in the clinic by Miller and colleagues in non-HSCT settings with patients suffering from metastatic melanoma, renal cell carcinoma, Hodgkin's lymphoma, and refractory AML (81). For many years, allogeneic NK cell infusions have been tested in the clinic with positive results and rare cases of mild toxicity (82). Strikingly, a recent pediatric clinical study has reported some patients suffering from acute GVHD after infusion of *ex vivo* expanded donor NK cells in HLA-matched HSCT (83), rising the necessity to perform more robust preclinical testing in humanized models. One strategy to do so was illustrated in a recent study performed in NSG mice, in which an alloreactive NK cell subpopulation expressing KIR2DS2 but lacking inhibitory KIR-HLA mismatch had dominant functional activation advantage to kill patient-derived glioblastoma cells (84). The regulation of the activity on infused NK cells has been classically based on HLA-KIR matching; however, other inhibitory receptors are implicated on the inhibition of NK cell cytotoxicity. A recent study in NSG mice engrafted with human HSC has shown that anti-NKG2A antibodies can stimulate human NK cell killing in AML and ALL models bypassing the need to search for NK cell alloreactive donors (85). *In vitro* experiments have also pointed to an increased NK cell-mediated lysis of lymphoma and myeloma cells with allogeneic NK cell infusion in combination with monoclonal antibodies blocking inhibitory KIRs but this effect need to be confirmed *in vivo* (86, 87).

Another strategy to increase NK cell activity without aggravating the side-effects is the expression of chimeric antigen receptors (CARs) directed against tumor antigens. Preclinical evaluation of CD20 targeting primary NK cell infusion in humanized mice has led to a clinical trial on B-lineage acute lymphoblastic leukemia currently undergoing (88). Other preclinical trials using CAR-engineered primary human NK cells are now being performed in lymphoma, leukemia, carcinomas, and neuroblastoma mouse models.

Natural killer cells are often infused in combination with immunomodulators that boost their antitumor effects or regulate their activity. CD16 receptor is targeted by many of those modulators since it mediates antibody-dependent cellular cytotoxicity (ADCC) when it recognizes an antibody on a tumor cell, leading to target cell lysis. This mechanism has been exploited by using monoclonal antibodies targeted tumor antigens thus stimulating the endogenous or adoptive NK cells. Evidences of NK cell-mediated ADCC and mild to moderate toxicity were observed in preclinical models and then confirmed for some cases in the clinical setting. Malignancies such as non-Hodgkin lymphoma with rituximab (anti-CD20), metastatic breast cancer with trastuzumab (anti-HER2) or metastatic colorectal, and squamous cell carcinoma of the head and neck have been treated with monoclonal antibodies together with NK cell infusions or in combination regimes extending the disease-free survival and overall survival of thousands of patients (89–91).

As mentioned before, CSCs are emerging as necessary targets to achieve cancer cures since current treatments eliminate the bulk of the tumor cells but rare resistant CSCs persist and lead to later tumor relapse (92). The upregulation of stress-induced antigens together with the ability of NK cells to target non-proliferating

cells suggest that NK cells could effectively eliminate CSCs. Indeed, recent studies in pancreatic carcinoma-bearing NSG mice demonstrated the capacity of activated transferred NK cells to reduce intratumoral CSCs and tumor burden (93–95).

NOVEL NK CELL SOURCES FOR ADOPTIVE THERAPY OF CANCER

Two of the parameters to consider when evaluating the safety of NK cell products in clinical applications are the cell source and the culture conditions before the infusion. GM-CSF mobilized PBMCs, bone marrow, or umbilical cord blood (UCB) are the main sources of NK cells. With GM-CSF effects on NK function still to determine and BM being logically difficult to obtain, UCB derived NK cells have been revealed as the best source of human material. Researchers are working on improving the expansion yield and purity as well as to enhance the activity of UCB derived NK cells before infusion in the patients. NSG mice demonstrated the capacity of these cells to migrate to BM, spleen, and liver and the inhibition of leukemia growth and prolongation of mice survival when combined with low-dose IL-15 (96). This preclinical result prompted a phase I clinical study in elderly AML patients that confirmed the safety and capacity of these cells to migrate and repopulate BM even in the absence of cytokine administration (97). This NK cell product aims at overcoming the major limitation of NK cell therapies in solid tumors, the delivery of high enough numbers of activated NK cells to the tumor site, and it is now under preclinical evaluation in the context of cervical and colorectal carcinomas (37, 98).

Alternative sources for NK cell therapy include embryonic stem cell (hESC)- or induced pluripotent stem cell (iPS)-derived NK cells, which are still under experimental development. Efficient generation of NK cells from hESC and iPS cells has been achieved, showing *in vitro* functional cytolytic activity against tumor cells, IFN- γ production, and expression of functional receptors (99). Very few reports are available regarding the *in vivo* activity of these products, with the most encouraging being in a NOD/SCID mouse model in which hESC-derived NK cells efficiently cleared a leukemia cell line tumor (100). Nevertheless, feeder-free conditions of NK cell generation need to be improved and the stability and safety of these NK cells products should be further proved in preclinical humanized models.

Finally, there is great prospect in NK cell lines as a potentially unlimited “pure” NK cell source. A clonal NK cell line NK-92 has shown the highest and most consistent cytotoxicity due to the combination of activating receptors it expresses and the absence of inhibitory KIRs (101). AML, myeloma, and melanoma are some of the numerous malignancies that have been partially eliminated from SCID mice after infusion of NK-92 (102–104). Clinical trials have further confirmed the safety and efficacy of this cell line in both solid and hematologic malignancies (105, 106). One further advantage of NK-92 is the ease of transfection with non-viral vectors allowing them to express IL-2 (required for their proliferation), thus representing a powerful “off-the-shelf” cell therapeutic (107). Additionally and inspired by the remarkable responses obtained by CAR-T cells and the early results in primary NK cells, NK-92 can be very easily transfected with

a gene that expresses a tumor-CAR (108). The first preclinical tests in NSG mice have shed very optimistic results in leukemia models after CD19- or CD20-specific NK-92 infusions as well as in patient-derived glioblastoma with EGFR-specific NK-92 (109, 110). Still, these cellular therapies retain safety concerns including on-target/off-tumor effects and unregulated cytotoxicity. As such, suicide genes (including herpes-simplex-thymidine-kinase and inducible caspase-9) have been integrated into these cell products thus allowing their subsequent selective destruction (111, 112).

The latest of the NK cell therapeutic strategies was developed by Vallera and colleagues with the bi- or tri-specific killer cell engagers, BiKEs and TriKEs that are small molecules containing two or three single chain variable fragments from antibodies of different specificities (113). These are generated to bind CD16 on NK cells and one or two tumor antigens such as CD19 and CD20 (B-cell non-Hodgkin's lymphoma) (114), CD33 or CD33 and CD123 (AML) (115), CD30 (Hodgkin's lymphoma) (116), EGFR or EpCAM (EGFR/EpCAM overexpressing carcinomas) (117, 118), and many others. The initial preclinical evaluation in humanized mice proved very promising translational potential with results exceeding those of monoclonal antibodies, like in the case of CD16-CD19-CD20 TriKE versus rituximab, and also proved efficient for bypassing HLA-mediated inhibition in refractory AML blasts.

IL-15 is the master cytokine necessary for NK cell differentiation and survival and it is currently used in clinical trials alone or as an adjuvant for certain types of metastatic solid tumors to promote *in vivo* cell expansion and NK cell function (63, 119). Taking advantage of this, novel TriKE structures have been developed that use a human IL-15 as a modified cross-linker between the anti-CD16 and the antitumor antigen in order to promote *in vivo* NK cell proliferation. Assessment of the activity of a CD33 specific TriKE in an AML NSG model of NK cell adoptive transfer has shown *in vivo* persistence, high cytotoxic activity, and no toxicity to the construct (120). Clinical development is currently under progress and will probably obtain FDA approval in the upcoming months to be tested in patients.

MODELING VIRALLY INDUCED HUMAN TUMORS USING HIS MICE

While NK cell first identification was based on its antitumor activities, it is also a critical innate effector against pathogen invasions particularly viral infections. Human NK cells have been proven essential for the immune response against members of the herpesvirus, poxvirus, and papillomavirus families, as demonstrated by the predisposition of NK deficient individuals to suffer from these virus infections (121, 122). Remarkably, in one fifth of human cancers viral infection and oncogenesis are intimately linked. Viruses act on carcinogenesis either by directly promoting the initiation of the disease or by interacting in the immune response and/or immune evasion (123). Particularly, Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus, human T-cell lymphotropic virus, Kaposi's sarcoma herpesvirus, and Merkel cell polyomavirus account for the majority of tumor cases linked to viral infection. Humanized mice offer a platform to access the molecular

mechanisms behind that causal role and the receptor–ligand interactions occurring at the interface “NK cell–infected cell” that could eventually have therapeutic value. The engrafted human cells occupy relevant physiological sites, where they proliferate and function, and eventually interact with oncogenic viruses that spread and replicate to other cells or organs thus recapitulating the physiological human infection. Additionally, their unique susceptibility to infection by virus with exclusive human tropism and the possibility to manipulate the timing and dose of the infection render them indispensable for better understanding the virus–tumor interplay and disease progression as well as for developing therapeutic approaches.

Epstein–Barr virus is the most common human tumor virus worldwide (more than 200,000 associated malignancies every year) and is also the cause of infectious mononucleosis. It has been extensively studied in humanized mice modeling the different protein expression patterns of the virus that lead to latent infection 0, I, II, and III as well as low level lytic replication, although only latency III has been unequivocally demonstrated (124–129). Several studies have reported specific adaptive cellular and humoral immune response to EBV in humanized mice (128, 130, 131). Furthermore, transformation of B-cell *in vivo* has been also reported and this model has disclosed one of the viral genes (EBNA3B) responsible for tumor formation (125). Preclinical studies in HIS mice have been pivotal for the development of the therapeutic vaccines that are now undergoing clinical trials, including the EBV gp350 neutralizing antibody and infusion of EBV-specific T cells (132). The involvement of NK cells in EBV infection and disease progression was demonstrated by depletion of NK cells from EBV-infected NSG mice resulting in higher EBV DNA load in the spleen, exaggerated CD8⁺ T-cell responses to the virus and an increased risk of EBV-induced lymphoproliferation (65). Current investigations try to deepen our understanding of the NK cell-mediated control of primary EBV infection in HIS mice and will likely provide insight on the NK cell subset responsible for that viral control (133).

About 80% of hepatocellular carcinomas (HCC) are due to HBV or HCV infections. There are more than 250,000 new cases of HCC and an estimated half a million deaths due to this disease annually (123, 134). We and others have developed mouse models harboring both the immune system and human hepatocytes, allowing the natural course of acute infection and also chronic hepatitis, characterized by advanced liver disease and hepatocellular carcinoma genesis (17, 135, 136). In addition to the immunodeficiency, these mice have liver defects that allow engraftment and expansion of transplanted human hepatocytes. Several immune system–liver humanized models have been developed, including BRGS-uPA (BALB/c *Rag2*^{-/-}*Il2rg*^{-/-}*Sirpa*^{NOD}*uPA*^{g/g}) (17), uPA-NOG (uPA-NOD *Prkdc*^{SCID}*Il2rg*^{-/-}) (135), and FRGN (*Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} NOD) (136). These doubly humanized mice show high level of human liver chimerism and immune engraftment in primary and secondary lymphoid organs with reconstitution of myeloid and lymphoid populations at levels similar to the single HIS models. In BRGS-uPA mice, NK cells are present in spleen and liver in numbers even higher than BRGS mice and display the same NK receptor expression profile (unpublished data). Infection with HBV and HCV has

been achieved in these mice and human immune responses have been detected as well as associated liver diseases that resemble the human pathology. Furthermore, both mimic the clinical response upon treatment with anti-HBs neutralizing antibodies and IFN α -2an, respectively, and prevented the leukocyte infiltration and liver fibrosis (137, 138). Any in depth analysis of the NK cell response against the virus or the role in tumorigenesis has been so far performed in these mice, other than the detection of CD56⁺ cells, to our knowledge.

IMPROVING THE NK CELL COMPARTMENT IN HIS MICE

Given the central role of NK cells in immune responses in infection, malignancy and inflammation and the great therapeutic potential they hold, it is necessary to optimize the available models for understanding their biology and preclinically evaluate new therapies.

In previous sections, we discussed about two types of HIS mice for the study of NK cell biology, those in which the human immune cells develop *in vivo* from injected hematopoietic precursors and a second category that adoptively receive mature NK cells freshly isolated or derived from an *ex vivo* expansion or activation process, a cell line or an ES or iPS cell. The later have fewer requirements in terms of niche, cell–cell interactions, and soluble growth factors that are needed for NK cell development, and instead require cytokines for their survival and homeostatic proliferation. Common cytokine receptor γ cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) play critical roles. In particular, IL-15 is responsible for NK cell maintenance and homeostatic proliferation through IL-15R α presentation (139, 140), while IL-2 effect *in vivo* is oriented to the activation and induction of cytotoxicity through the regulation of the peripheral NK subsets. These humanized mice serve as platforms to understand the mechanisms underlying NK survival and function and provide preclinical information for the design of new therapeutics. Furthermore, they give valuable information about the cell migration capacity and synergistic effects with other cell types or immunomodulators.

As mentioned earlier, several immunodeficient hosts (NSG, NOG, BRG, BRGS) support multilineage development of human immune cells, including low levels of NK cells. In the BRGS model (16, 141), NK cells expressed CD56 and NKp46 as well as some level of CD16 and were able to degranulate moderately after stimulation with a cancer cell line. However, in both BRGS and NSG mice, NK cell displayed defects in maturation, functionality, and heterogeneity in comparison with the human counterparts due to a deficient cytokine signaling (142). The absence of human appropriate MHC class I expression on hematopoietic or stromal cells may result in the failure to “educate” or “license” developing NK cells in HIS mice. This could explain the abundance of immature NK cells (CD56^{bright}CD16⁻KIR⁻) and their functional defects. In line with this idea, recent publications showed improved NK cell licensing in a HIS models expressing diverse educating HLA alleles (143, 144). This approach may allow better definition of the mechanisms underlying human NK cell education *in vivo*.

Based on their cytokine requirements, IL-15 has been exogenously administered either alone or as a complex with IL-15R α resulting in an extensive NK cell proliferation and accumulation of CD16 $^+$ KIR $^+$ NK cells. Also, NK cell differentiation progressed from CD56 $^+$ to CD56 $^{\text{low}}$ CD16 $^+$, and finally to CD56 $^{\text{low}}$ CD16 $^+$ KIR $^+$ mimicking the human model (63). On the other hand, the constitutive high expression of the high-affinity heterotrimeric IL-2 receptor complex in CD56 $^{\text{bright}}$ CD16 $^{\pm}$ NK-cell subset and the effect of IL-2 in NK expansion and activation prompted the development of an IL-2 transgenic NOG mouse strain (145). When IL-2 $^{\text{tg}}$ NOG mice were engrafted with human HSC, CD56 $^+$ massively developed with a highly active phenotype including IFN- γ production and cytotoxicity against tumor cells. Interestingly, treatment of these mice with a therapeutic humanized anti-CCR4 Ab (mogamulizumab) suppressed the growth of a CCR4 $^+$ lymphoma, suggesting that the human NK cells in the mice exerted active Ab-dependent cellular cytotoxicity *in vivo*. These cells expressed various NK receptors, including NKp30, NKp44, NKp46, NKG2D, and CD94, as well as a diverse set of killer cell Ig-like receptor molecules at levels comparable to normal human NK cells from the peripheral blood (62). Nevertheless, there are several limitations in this model due to the supra-physiological levels of IL-2 and, therefore, the high activation status of the NK cells.

It is well known that NK homeostasis and function are regulated by the interaction with other immune cells, particularly macrophages, DCs, and T cells. In addition, soluble factors released by those cells, like NKG2D ligands, IL-2, IL-12, or IL-15, signal on NK cells leading to proliferation and activation. Based on these crosstalk events, others and we have developed humanized mice that through the enhancement of the myeloid compartment, NK cell development results improved. As it happens for other lineages, human myelopoiesis is driven by soluble factors normally present in the BM niche and periphery, which are from murine origin in HIS mice. Some of these mouse cytokines cross-react to some extent with the human cells but others, the species-specific cytokines, do not. In order to circumvent this deficiency, human cytokines have been administered to HIS mice either as recombinant proteins (63) by cytokine-encoding plasmids (146) or by insertion of the cytokines either as transgenes in the mouse genome or by knocking in the human gene to replace the mouse counterpart (147, 148). As mentioned before, transgenic models provoke supra-physiological levels of the cytokine in the periphery and in the case of pro-myeloid factors, such as TPO, IL-3, GM-CSF, or M-CSF, also lead to the exacerbated mobilization and HSC exhaustion limiting the utility of the system. Swapping mouse coding exons for M-CSF, IL-3/GM-CSF, TPO, and SIRP α with their human counterparts allowed for the creation of the MISTRG strain (149). This host expresses these human cytokines under control of mouse regulatory elements and show superior human myeloid cell engraftment. Subsequently, MISTRG HIS mice showed an increased number of functional NK cells, including higher expression of KIR, CD94, and CD161 receptors (149). Nevertheless, cellular and humoral immune responses in MISTRG HIS mice are poor and these mice develop severe anemia.

The transpresentation of IL-15 occurs mainly through the IL-15R α expressed by DCs, so efforts have been made to increase

specifically this cell population in order to increase the NK pool avoiding the overdevelopment of other myeloid subsets. In our laboratory, Flt3-deficient BRG mice (BRGF) were created and after reconstitution with human HSC, human Flt3L was administered to the mice. The result was a specific increase of all the DC subsets and the promotion of NK cell hematopoiesis, with enhanced CD94, CD16, and KIR receptor expression. The combination of this system with the expression of the *Sirpa*^{NOD} protein in the BRGSF model has led to further augmentation of NK cell numbers and also an enhanced functional competency as demonstrated by their degranulation capacity and cytokine production activity (unpublished data). This HIS model provides a unique platform to study NK cell development, crosstalk mechanisms with other immune cells, and the preclinical assessment of new immunotherapies targeting innate cells.

The combination of the protocols detailed in the previous sections for modeling human cancer or infection with the abovementioned strategies to boost the NK cells in HIS mice will raise the potential to understand how NK cell interact with malignant or infected cells. Moreover, HSC-HIS mice offer the possibility to study the tissue specific interactions, the reservoirs, the migration patterns, and the crosstalks within the immune compartment that may be important to develop combinatorial therapies that avoid metastasis, tumor relapse, and “relocation” of the viruses.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Therapies designed to induce or potentiate the immune response against tumors are an appealing strategy to control tumor growth and have been the object of intense research since their discovery in the 1970s. Despite representing the most promising cancer treatment since the emergence of chemotherapy, several cases of side effects or disappointing clinical results have downshifted the development of new immunotherapies. The better understanding of the tumor heterogeneity, the mechanisms of the immune response, and the interaction with the tumor microenvironment is a required step for the development of safe and effective therapies. Humanized mice have the potential to reproduce the HIS, the tumor growth and immune evasion, and the response to treatments targeting immune effector cells or immunomodulators. One of the most challenging aspects of tumor research has been to understand the variability within the same type of cancer among individuals and, therefore, the disparate responses and outcomes after therapy. Current efforts are being made to overcome these limitations by creating truly personalized HIS-PDX mouse models in which both the immune system and the tumor are derived from the same individual. These models will provide an invaluable bridge between immunotherapy discovery and the clinic, increasing the success rate of new therapies in human trials and improving the chances to beat cancer.

Natural killer cells have been for long time considered the only innate effectors of the lymphoid system but nowadays we appreciate that they belong to a larger family, the “innate lymphoid cells” (ILCs) (150). These recently described populations lack cytotoxic capacity but instead, they exert very potent cytokine production.

In recent years, there has been a rapid advance in our understanding of their development, phenotypic, and functional diversity, which has been nicely reviewed elsewhere (151–155). ILCs come in three groups mirroring the cytokine and transcriptional profile of CD4⁺ helper T cells (Th1, Th2, and Th17/22). Given the myriad of cytokines they produce, ILCs have been involved in the early orchestration of immune responses against a number of pathogens, in tumor immunosurveillance and in inflammatory diseases. Recent works in mice have proven the antitumor effects of ILC1s whereas ILC3s have been found to exert both beneficial and tumor-promoting effects depending on the circumstances (156–158). The multifaceted functions of ILCs suggest new alternatives for immunotherapeutic approaches against tumors that need to be explored in *in vivo* humanized models. The improvement of human helper ILCs in HIS mice could open new avenues

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Both authors have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Natural Killer Cell-Based Cancer Immunotherapies: From Immune Evasion to Promising Targeted Cellular Therapies

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Immunotherapies based on natural killer (NK) cells are among the most promising therapies under development for the treatment of so far incurable forms of leukemia and other types of cancer. The importance of NK cells for the control of viral infections and cancer is supported among others by the findings that viruses and tumors use a multitude of mechanisms to subvert and evade the NK cell system. Infections and malignant diseases can further lead to the shaping of NK cell populations with altered reactivity. Counter measures of potential therapeutic impact include the blocking of inhibitory interactions between NK cell receptors and their cellular ligands, the enhancement of activating receptor signals, and the infusion of large numbers of ex vivo generated and selected NK cells. Moreover, the specific cross-linking of NK cells to their target cells using chimeric antigen receptors or therapeutic bi-/trispecific antibody reagents is a promising approach. In this context, NK cells stand out by their positive effects and safety demonstrated in most clinical trials so far. Based in part on results of the recent EC-sponsored project “NATURIMMUN” and considering additional published work in the field, we discuss below new developments and future directions that have the potential to further advance and establish NK cell-based therapies at the clinics on a broader scale.

Keywords: immunotherapy, natural killer cells, immune evasion, cell therapy, checkpoint inhibitors, chimeric antigen receptors, bispecific antibodies

INTRODUCTION

Natural killer (NK) cells have been classically defined as part of the innate immune system providing immediate reactivity against their main targets, virally infected and tumor cells (1). This view has been substantially extended over the recent years based on the findings that NK cells are calibrated to provide self-tolerance, can develop a memory, and play a role in the regulation of the adaptive immune response (2–5). Furthermore, NK cells have turned out to be part of a larger family of innate lymphoid cells (ILCs) that include ILC1–3 (6).

Natural killer reactivity, including cytokine secretion and cytotoxicity, is controlled by a balance of several germ-line encoded inhibitory and activating receptors such as killer immunoglobulin-like receptors (KIRs) and natural cytotoxicity receptors (NCRs) (1, 5, 7, 8). Evidence for the anticancer efficacy of NK cells comes from allogeneic or haploidentical hematopoietic stem cell

(HSC) transplants that have been used in combination with chemotherapy in the treatment of different forms of leukemia (9). This has shown that NK cells formed from the transplant not only are efficient in killing of allogeneic leukemia cells but are also instrumental in reducing the incidence of graft versus host disease due to their killing activity for dendritic cells (10). Taken together with clinical NK infusion trials in leukemia patients, which have shown exciting antitumor activities and generally safety of the procedure (11–13), it appears that NK cells could be the cells of choice in cellular therapies of leukemia not displaying the critical graft versus host activities of T lymphocytes. Although it is currently less clear whether NK cells will be similarly active in solid cancers, this is a further important area of interest.

IMMUNE EVASION MECHANISMS AND SHAPING OF THE NK CELL COMPARTMENT

Given the importance of NK cells, it is not astonishing that viruses and tumors use a wide array of mechanisms to avoid recognition by NK cells. A paradigm is represented by the Herpes virus family. Many mechanisms such as expression of viral ligands for inhibitory receptors have been described (14). Important is further the downregulation of human stress-induced ligands recognized by the activating NKG2D receptor present on the majority of NK cells. Normally, these stress ligands appear on the cell surface whenever a cell is virally infected or undergoes oncogenic transformation. Whereas internalization and miRNA-mediated downregulation of several stress ligands have been shown previously (15), additional novel mechanisms have been recently identified within the EC-funded project NATURIMMUN. For example, in the case of HHV-6B the expression of stress ligands is suppressed by proteasomal degradation induced by the virus. Consequently, HHV-6B-infected cells can evade immune surveillance by NK cells (16). These various evasion mechanisms of Herpes viruses are reviewed (17) within this research topic (“Tailoring NK Cell Receptor-Ligand Interactions: an Art in Evolution”).

Extending the importance of NKG2D ligands to tumors, Schmiedel et al. have shown within the NATURIMMUN project that the stress ligand ULBP2 can be suppressed by an RNA-binding protein that is frequently overexpressed in tumor cells. By binding of this oncogenic protein to ULBP2 mRNA the stability of the mRNA is reduced and ULBP2 levels on the cell surface are downregulated. In consequence, the tumor cells are protected from NK cell recognition (18). This strongly supports that modulation of stress ligands is an important escape mechanism used by cancer cells to diminish NK cell recognition. Involving a different inhibitory receptor, another unexpected novel evasion mechanism could be shown by the same group for colon cancer. NK cell killing was inhibited by the presence of fecal bacteria in the tumor environment. Bacterial proteins interacted with the inhibitory TIGIT receptor on NK cells leading to the inhibition of NK cell cytotoxicity (19). Inhibition of NK cells can also occur by blocking of NKG2D via soluble forms of the stress ligand MICA

as shown for neuroblastoma as well as head and neck carcinoma. This tumor escape can be overcome in part by highly activated NK cells with upregulated NKG2D (20, 21).

Viruses and human cancers can further have profound effects on and shape the NK cell compartment. Human cytomegalovirus (HCMV), a herpes family member, can trigger an adaptive NK cell response leading to the expansion of NK cell subsets with specific receptor expression (22–24), e.g., the activating NKG2C receptor. The adaptive NKG2C NK cells have been implicated in improved survival of leukemia patients receiving a HSC transplant from HCMV-positive donors (23, 25). Given the potential higher antitumor reactivity of the NKG2C NK cells, this subset is of therapeutic interest and was investigated within the frame of the NATURIMMUN project. Obtained results support that different adaptive NK cell subsets develop in response to viral infection and this is influenced by the copy number of the NKG2C gene (26).

It has been established that certain forms of leukemia display a defective NK cell compartment (27) rendering these forms priority cases for the exploration of NK cell-based therapies. In regard of acute myeloid leukemia (AML), we investigated within the NATURIMMUN project NK cells in patients receiving a novel maintenance therapy with histamine plus IL-2. In this study, AML patients displayed diminished and partly defective NK cells. The therapy strongly induced the immunomodulatory CD56^{bright}CD16[−] and CD56^{bright}CD16^{low} NK cell subtypes and contributed to the restoration of the NK cell compartment (28). This is in line with the described positive effects of the therapy on disease-free survival of AML patients (29, 30). In addition, our cooperation partner S. Huenecke describes in this research topic that during immune reconstitution after HSC transplantation the degree of development of the two CD56^{bright} and the CD56^{dim} NK cell subpopulations can serve as prognostic marker for both graft versus host disease and viral infections (31).

MODULATION OF INHIBITORY NK RECEPTOR-LIGAND INTERACTIONS AND NOVEL LIGANDS OF ACTIVATING RECEPTORS

Unprecedented rates and durations of clinical responses have been recently achieved in cancer patients by the treatment with antibody reagents that block inhibitory “checkpoint receptors” (32). Whereas these therapies have so far been restricted to the blockade of inhibitory pathways acting on T lymphocytes, the inhibition of NK cells by the interaction of inhibitory NK cell receptors with MHC class I ligands can be regarded as typical checkpoint inhibition. In fact, efforts are currently been undertaken to evaluate blockade of the inhibitory NKG2A/CD94 receptor and of inhibitory KIRs to elicit NK reactivity to cancer cells. The company Innate Pharma has developed first-in-class monoclonal antibodies that target inhibitory NK cell receptors and these are currently in preclinical and clinical evaluation (33).

While the ligands for inhibitory NK cell receptors are well established, ligands bound by important activating receptors are still incompletely identified. This is the case for the activating

NKG2C/CD94 receptor, several activating KIRs, and the NCRs. In this regard, a group participating in NATURIMMUN has studied how HCMV stimulates NK cells *via* the activating KIR2DS1 receptor. The ligand was identified as a specific class I molecule, HLA-C2, which in its normal form is recognized by the related inhibitory KIR2DL1 receptor. Possibly, a conformational change in normal HLA-C2 triggered by HCMV was required for KIR2DS1-mediated NK cell activation (34). Other participants in NATURIMMUN have developed assay systems and have work in progress to identify virally induced and potentially tumor ligands for the activating NKG2C receptor (Pupuleku et al., manuscript in preparation for this research topic) and the NCRs. The clarification of the molecular nature and mechanism of action of the corresponding activating ligands on virally infected and tumor cells will allow novel pathways of NK cell activation to be triggered.

GENERATION OF LARGE-SCALE THERAPEUTIC NK CELLS AND TECHNOLOGY TO TARGET AND CROSS-LINK NK CELLS TO CANCER CELLS

Exploiting and strengthening the NK cell response is a highly promising approach for future successful immunotherapies of cancer. This could be achieved by infusion of *ex vivo* expanded and activated NK cells, by genetic modification of NK cells with chimeric antigen receptors (CAR), by multivalent reagents cross-linking NK cells to cancer cells, or by a combination of these methods (**Figure 1**).

In regard of *ex vivo* expansion of peripheral donor NK cells several groups have developed corresponding technologies and some were or are being applied in clinical trials of NK cell infusions (11, 12, 35). Important for broader availability of these therapies are commercial sources of the necessary equipment and reagents and further development of automated systems for

production of GMP-compliant clinical-grade NK cells. A pioneer in this regard is the company Miltenyi Biotec. In part as participant of NATURIMMUN, this company has further developed a protocol to expand peripheral NK cells using irradiated autologous peripheral blood mononuclear cells as feeder cells. NK cell isolation and expansion were further fully automated for future clinical applications (36, 37). NK cells generated by this procedure have been evaluated in detail (Delso-Vallejo et al., submitted to this research topic).

Another possibility is the generation of therapeutic NK cells from umbilical cord blood stem cells (UCBSC), which was pioneered by the company Glycostem (38). Within NATURIMMUN, NK cells differentiated in this system were characterized in detail and the procedure improved to yield more mature NK cells (39). Furthermore, an important role of the transcription factor ZNF683/HOBIT for NK cell differentiation could be shown supporting that the factor could be used to modulate NK cell generation [(40), this research topic]. This research topic. UCBSC-derived NK cells have been evaluated in a phase I clinical trial in elderly AML patients and found to be safe (41). Furthermore, recent evidence obtained in NATURIMMUN supports that the cells possess high cytotoxicity against metastatic colorectal cancer cells (42, 43) and could be used in the therapy of solid cancers [(44), this research topic].

An important topic in the field is to harmonize the manufacturing of GMP-compliant therapeutic NK cell products, which was initiated within NATURIMMUN and has been described in a summary of the worldwide experience obtained so far with allogeneic adaptive NK cell therapies (12). It is conceivable that expanded therapeutic NK cells could be stored frozen and be shipped on demand. These NK cells could, therefore, qualify as off-the-shelf-products, and to what extent this will be possible is a relevant question for future research.

It has been shown that expanded and cytokine-activated NK cells can be functional in certain cancer types. However, evidence suggests that specific targeting and cross-linking of

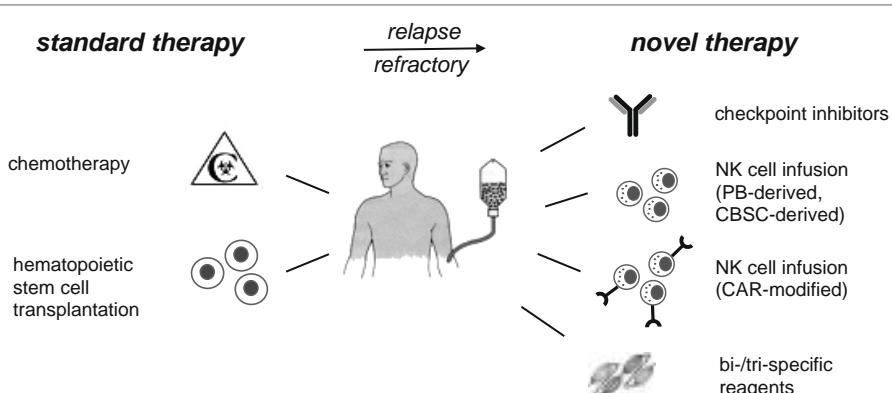


FIGURE 1 | NK cell-based immunotherapies. Standard therapy for high-risk leukemia includes high-dose chemotherapy, followed by hematopoietic stem cell transplantation. Patients, who do not reach remission or suffer from early relapse thereafter, have a poor prognosis and are in urgent medical need for advanced therapies. Current immunotherapeutic developments and phase I/II trials include checkpoint inhibitors for inhibitory NK receptors, infusion of expanded and activated autologous or allogeneic NK cells, and targeting of NK cells to cancer cells. The latter can be done by modification of NK cells with CAR or by application of multispecific reagents to cross-link NK cells with cancer cells. These immunotherapies should reduce relapse rates and constitute promising additional treatment options for high-risk patients. PB, peripheral blood; CBSC, cord blood stem cell; CAR, chimeric antigen receptor; NK, natural killer.

NK cells to cancer cells would strongly enhance their reactivity and the applicability of NK cell therapies. A paradigm in the field is currently the exciting successes of targeting of T lymphocytes to CD19 *via* genetic CAR modification (45, 46) or corresponding bispecific reagents (46). We believe that NK cells will provide important advantages to the use of T lymphocytes based on their comparable reactivity but much higher safety. We have achieved increased NK cell cytotoxicity against leukemia cells using transduction of NK cells with CAR constructs (47, 48) or by cross-linking with trispecific reagents (Kloess et al., submitted to this research topic). Furthermore, it is conceivable that procedures to achieve redirected primary human NK cells as an “off-the-shelf-immunotherapy” can be developed. For this, optimizing both the respective antigen binding and the triggering of the intracellular signaling cascade by the CAR will be desirable (49).

A possibility to target NK cells to cancer cells can be the use of monoclonal antibody therapeutics already approved for clinical application. Examples of these are the anti-CD20 antibody rituximab (50) for B cell leukemia and the anti-EGF receptor antibody cetuximab (51–53). The latter is in use for the therapy of colon carcinoma and head and neck cancer. It displays limited efficacy in colon with better activities in head and neck cancer. It is possible that synergistic activities could be gained by coapplication of NK cell infusions as these antibodies trigger ADCC *via* binding to the low-affinity Fc γ receptor present on NK cells. It could be shown within NATURIMMUN that NK cytotoxicity toward EGFR $^+$ colon and cervical cancer cells was strongly enhanced by cetuximab (42, 43). This provides a rationale to strengthen NK cell immunotherapy through a combination with cetuximab for metastatic colorectal cancer patients [(44), this research topic].

PRECLINICAL MODELS FOR EVALUATION OF HUMAN NK CELL-BASED CANCER THERAPIES

The preclinical evaluation of NK cell-based therapies in mouse models is hampered by the inherent problem that reagents designed to trigger human immune cell would not react at all or only partially with murine NK cells. Similarly, the evaluation of human NK cell infusions in mice does not provide a human immune cell compartment necessary for full functioning. This problem can be partly circumvented by mouse models with humanized immune system (HIS) in combination with xenotransplantation models of human cancers.

In this regard, a novel method to boost the inefficient human NK cell development in mice observed after engraftment of human HSC was recently developed. Normally, the differentiation of NK cells depends on the interplay with myeloid cells, and human myeloid cells are poorly reconstituted in available HIS mice due to competition with the murine cells (54). Therefore, a new model was developed in the NATURIMMUN project using mice that lack the Flt3 receptor (55) and display reduced murine myeloid differentiation. In these mice, human dendritic cells and consequently human NK cells could be successfully boosted by human Flt3 ligand providing a novel mouse model

with increased NK cell numbers [(56), this research topic]. This will be valuable for future evaluations of immunotherapies involving reagents designed for human cells as well as human NK cell infusions.

As an exemplary preclinical evaluation, we tested within NATURIMMUN the efficacy of NK cell infusions alone or in combination with the clinically approved cetuximab against human colon cancer. HIS mice were engrafted with a human colorectal carcinoma cell line and treated with cetuximab and infusions of PB-derived and UCBSC-derived NK cells. Then the tumor load and survival rate were monitored. Significant inhibition of tumor growth and improvement of survival rates were observed. These results provide a rationale for NK infusion therapies not only for leukemia but also for solid cancer treatment [(44), this research topic].

MAIN FUTURE DIRECTIONS TO ACHIEVE NK CELL-BASED CANCER IMMUNOTHERAPIES ON A BROADER SCALE

Collectively, the basic work on NK cells, their receptors, and NK evasion mechanisms have provided evidence for the importance of the NK cell system in the control of human cancers. Clinical trials of NK infusion therapies, performed mostly in different forms of leukemia, have uniformly shown safety of infused NK cells and in certain cases exciting effects on disease-free survival (11). This together underlines the feasibility and potential efficacy of NK cell-based immunotherapies. However, based on the currently available data a number of questions and major routes should be further explored in order for NK cell therapies to become clinically used on a broader scale. Among those are improved methods for the selection of the best donor NK cells to be able to optimally exploit the antitumor alloreactivity of NK cells (12). Then the question of best activation of NK cells by cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21 needs to be settled as reviewed within this research topic (57). In addition, the best expansion time points of clinical-scale NK cells have to be evaluated regarding both safety and efficacy with the overall goal to allow multiple adaptive NK cell application to the respective patients. The optimal application of the newly developed NK cell-directed checkpoint inhibitors needs to be explored. Further additional reagents for targeting and cross-linking of NK cells to cancer cells using bi-/trispecific antibody-based reagents should be developed to extend the range of targeted cancer cells. Similarly, additional CAR constructs for wider targeting should be derived and corresponding standard “off-the-shelf-procedures” developed for genetic modification of NK cells. Of special importance for NK infusion therapies, available technologies for NK cell generation need to be fully automated and harmonized protocols developed for large-scale GMP-compliant generation of clinical-grade therapeutic NK cells that have been recently classified as advanced therapy medicinal products in Europe. They are regulated accordingly either centralized or under hospital exemption by the member states [Regulation (EC) No 1394/2007; Directive 2001/83/EC and Regulation (EC) No 726/2004]. Given

the accessibility of the tumor cells the primary focus should be on leukemia as it is to be expected that progress will be more rapid in this area. But in light of the high need of new therapies for solid cancers these should also be pursued.

CONCLUDING REMARKS

The recent years have seen significant progress in immunotherapies of cancer based on novel checkpoint inhibitors and reagents and technology to boost T and NK lymphocytes. We propose that based on the available knowledge of NK cells, these cells will be much more amenable for therapeutic purposes based on their high cytotoxicity and generally demonstrated safety. Therefore, we suggest that a concerted effort in the development of NK cell-based immunotherapies has high potential to achieve novel therapies of hitherto untreatable and relapsed forms of leukemia and potentially also solid cancers. The development of broadly applicable NK cell-based therapies should extend the currently more restricted available T cell-based therapies and could thus boost the long-standing promise of cellular cancer therapies.

ETHICS STATEMENT

Described work at the Medical School Hannover was carried out in accordance with the recommendations of the Ethics Committee

of the Medical School Hannover with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Medical School Hannover (No 2159-2014).

AUTHOR CONTRIBUTIONS

EH prepared the outline, the article parts were jointly written with EH focusing on the more basic immunology parts and UK on the therapeutic aspects.

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