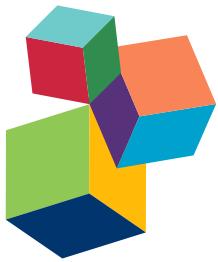


IS THE RECENT BURST OF THERAPEUTIC ANTI-TUMOR ANTIBODIES THE TIP OF AN ICEBERG?

EDITED BY: Leonor Kremer and Jose A. Garcia-Sanz

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IS THE RECENT BURST OF THERAPEUTIC ANTI-TUMOR ANTIBODIES THE TIP OF AN ICEBERG?

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Iceberg type representation of the anti-tumor therapeutic antibodies field. The currently approved formats of anti-tumor therapeutic antibodies are shown on the surface of the iceberg. These include naked antibodies, antibodies linked to a toxin or a radioactive isotope, and bispecific antibodies. The antibodies undergoing pre-clinical development are below sea level. Downwards there is a wide amalgam of undefined shaped antibodies representing all the recently selected or characterized antibodies with potential to become therapeutic agents.

Image: Isabel Corraliza Gorjón (CNB-CSIC).

The high effectiveness of antibodies as anti-tumor therapeutic agents has led to a burst of research aiming to increase their therapeutic applications by the use of antibodies against new targets, new antibody formats or new combinations. In this e-book we present relevant research depicting the current efforts in the field.

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Editorial: Is the Recent Burst of Therapeutic Anti-tumor Antibodies the Tip of an Iceberg?

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Editorial on the Research Topic

Is the Recent Burst of Therapeutic Anti-tumor Antibodies the Tip of an Iceberg?

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To summarize in a single research topic, all the useful information regarding the generation, characterization, and applications of antitumor therapeutic antibodies, including basic research, preclinical and clinical phases, turns out an impossible task. Therefore, our humble aim was to gather manuscripts from a group of researchers with expertise in the area, who through a set of 18 original research or review articles provide relevant and up-to-date information on several relevant aspects of this topic, exemplifying the ways in which antibody therapies will progress through technological advances in the generation of antibodies, the discovery of new targets, or new clinic applications that are being explored, as well as highlighting the main problems that remain to be solved.

The usefulness of antibodies for the treatment of infections, inflammatory diseases, and cancer cannot be questioned nowadays, and is evidenced by the large number of antibodies approved for their clinical use by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Corraliza-Gorjón et al.).

The first four articles of this research topic describe improved engineering approaches used to optimize antibody functions and the structural and functional advantages of different antibody formats. The review by Almagro and coworkers focuses in advances and problems on the design and development of antibodies for cancer therapy. It describes the methods used for the discovery, engineering, and optimization (for specific therapeutic uses) of FDA approved antibodies. It emphasizes on the development of antibodies with higher human content and less immunogenicity, compiling genetic engineering approaches for isotype switching and Fc fragment modifications allowing to modulate effector functions and bioavailability (half-life), as well as the technologies for Fv fragment engineering (Almagro et al.). Ilieva and coworkers describe on an original research article a novel approach to generate rapidly and with a high yield Fc mutant mAbs and ascertain their functional features. The approach includes coupling on a single expression vector antibody cloning together with simultaneous Fc region point mutagenesis and high-yield transient expression in human mammalian cells. The authors engineered antibody panels recognizing the cancer antigens HER2/neu and chondroitin sulfate proteoglycan 4 (CSPG4). Antibody variants with Fc mutations, affecting antibody-natural killer (NK) cell interactions, were generated in a few days from design to purified material. This strategy can facilitate the generation of antibodies with defined effector functions and potentially enhanced efficacy against tumor cells (Ilieva et al.). Bannas et al. comprehensively review the potential of nanobodies (Nbs) and Nb-based human heavy chain antibodies to overcome many of the limitations of the conventional, high molecular weight IgG antibodies. Nbs have small size, high

solubility, high stability, and excellent tissue penetration *in vivo*. The authors discuss recent developments and perspectives for applications of Nb and Nb-based human heavy chain antibodies as antitumor drugs, describing methods and applications that use Nb linked genetically to other proteins or conjugated chemically at specific sites on other molecules to have additional properties. Iezzi et al. summarize how the unique properties of single-domain antibodies (sdAb), mainly their small size and high stability, make them suitable for image diagnostics and cancer treatment. Their contribution also describes the diversity of platforms available to generate new sdAb and some interesting examples of their therapeutic application.

Three contributions focus on the use of antibodies that target inhibitory immune checkpoints, an approach with demonstrated efficacy for the treatment of solid tumors (1–3). Aris et al. summarized clinical trials using immunomodulatory antibodies in combination with other strategies such as vaccines, in patients with cutaneous melanoma. Most of these studies include anti-CTLA-4 and anti-PD-1 mAbs and were designed aiming to obtain a durable antitumor immune response. The authors distinguish between immunotherapeutic strategies that “push” the tumor immunoreactivity from those which “release” inhibitory mechanisms of immune regulation. The original contribution from Taylor and Rudd focuses on the mechanisms of action of anti-PD-1 mAbs and their implications for immunotherapy. Their results demonstrate that glycogen synthase kinase 3 (GSK-3) plays a central role for priming CD8⁺ cytolytic T cells (CTL) in anti-PD-1 immunotherapy, as the inactivation of GSK-3 during priming can substitute for CD28 co-stimulation potentiating CTL function. Xu-Monette et al. discuss in their review the complexity of the PD-1–PD-L1 axis immunologic regulatory network, which can drive T cells to an irreversible dysfunctional state that cannot be rescued by blockade of this axis. The authors review the effectiveness of PD-1/PD-L1 blockade in many functional and clinical studies and the development of therapeutic strategies to overcome the resistance mechanisms, clearly improving our understanding on the mechanism(s) responsible for their efficacy.

Six contributions deal with the generation and use of antibodies for the treatment of hematological malignancies either generating antibodies against new tumor targets or by selecting mAbs with improved functions against known targets. Marshall et al. extensively review the history of the development of clinically approved anti-CD20 antibodies, since rituximab, the first mAb approved by the FDA in 1997, until the new generation of anti-CD20 mAbs with enhanced effector functions. The authors discuss strategies to overcome mechanisms of resistance to the therapy and the use of combinations of anti-CD20 mAbs with other agents. Cuesta-Mateos et al. focus on antibodies evaluated in clinical trials for treating hematological malignancies, emphasizing on studies using mAbs directed against non lineage-specific antigens, including membrane surface glycoproteins, oncogenic receptors, chemokine receptors, antigens within the tumor niches, and immune checkpoint components. Schmitt et al. describe the use of a new generation of humanized antibodies targeting KIR3DL2, a member of the killer cell immunoglobulin-like receptor (KIR) family, for the treatment of cutaneous T-cell lymphomas, discussing aspects of KIR3DL2

on the functions of CD4⁺ T cells and highlighting preliminary clinical studies using the anti-KIR3DL2 mAb IPH4102 for the treatment of cutaneous T-cell lymphomas. The original contribution by Somovilla-Crespo et al. describes the generation and characterization of a novel anti-CCR9 mAb (92R), able to inhibit the growth of human CCR9⁺ leukemia in NSG mice xenografts. This chemokine receptor has been proposed as an attractive target for the treatment of human CCR9⁺ T cell acute lymphoblastic leukemia, since its normal expression is restricted to a low percentage of circulating immune cells. Ilieva et al. review the potential of antibodies directed to the Chondroitin Sulfate Proteoglycan 4 (CSPG4), overexpressed in several malignant diseases, with apparently restricted and low expression in normal tissues, and examine their direct antiproliferative/metastatic and immune activating mechanisms of action. Santamaria et al. discuss the possibilities of using therapeutic antibodies targeting the cancer stem cell (CSC) subpopulation, in particular since the CSC is responsible for tumor maintenance and metastasis generation.

Another group of contributions focuses on the advantages and challenges of treatments using combinations of antibodies with other therapeutic agents. Corraliza-Gorjón et al. summarize the use of antibodies in combinations with other biologicals for cancer treatment, describing their main characteristics, advantages, and challenges raised by the combinations. Muntasell et al. describe the main advances in the use of anti-HER2 antibodies in combination with harnessing NK cell responses for the treatment of HER2⁺ breast cancers. These approaches include the use of immune checkpoint blocking/stimulatory antibodies, cytokines, and toll-like receptor agonists.

Two contributions focus on how to design antibodies that can reach antigens or body sites that are normally inaccessible for these molecules. Trenevská et al. highlight that the target repertoire of actually approved antibodies is limited to tumor cell surface or soluble antigens. They describe how peptides from intracellular proteins that are presented on the cell surface in the context of major histocompatibility complex class I molecules can be targeted by antibodies known as T-cell receptor mimic or TCR-like antibodies. They summarize multiple approaches for targeting intracellular antigens, discussing their advantages and disadvantages and the potential to advance their therapeutic use into the clinic. Razpotnik et al. summarize new approaches to target brain tumors with therapeutic antibodies, especially malignant gliomas, as well as their potential drawbacks. They describe the properties of an antibody necessary to efficiently penetrate the blood-brain barrier; summarize studies demonstrating the successful brain delivery of single-chain fragment variable bispecific antibodies or cell-based systems, together with a summary of the most recent progress of related clinical trials.

The complexity of the use of therapeutic antibodies, alone or in combination keeps growing, as shown in the original research report from Marini et al. demonstrating the potential of using stable transfected human cells for the *in situ* expression of antibodies for cancer therapy. The authors generated a murine mesenchymal stem cell line stably secreting a dimeric EGFR-specific diabody single-chain TRAIL, demonstrating its therapeutic activity upon peritumoral injection in a Colo205 xenograft tumor model. These results support the potential of developing well-characterized

stocks of stable drug-producing human MSC lines to establish standardized protocols of cell-based therapy broadly applicable in cancer treatments.

Most of the mAbs developed for tumor therapy are directed either against the tumor (tumor cell antigens, stromal cells, or secreted molecules) or against the host immune system (able to modify the immune response). These antibodies are used either alone, in combination, naked, as drug conjugates, as bivalent antibodies, or even as fusion proteins with cytokines/cytokine receptors. From our point of view, this line of research will expand generating new mAb, or Ab with non-conventional formats (including Nbs, multipecific Ab, etc.) against new tumor antigens. In addition, we believe that there will be a burst on the use of combinations of immunomodulatory antibodies with either mAbs against the tumor or with tumor-derived antigens administered as vaccines (in multiple forms, i.e., tumor cell extracts, plasmids, virus, peptides, or purified proteins). The aim in both cases is to overcome the mere usage of mAbs as passive immunotherapy and use them for active immunotherapies, either to restore the effector activity of the immune system or to generate a new immune response against non-self antigens (tumor antigens), bypassing the hijacking of the immune system by the tumor. The other area that will grow on the near future will be the use of mAbs combined with adoptive cell therapies (harnessed T- or NK-cells, cells carrying expression vectors allowing them to express antibodies, tumor antigens, or chimeric antigen receptor T cells).

These contributions summarize the recent progress on therapeutic use of antitumor antibodies, providing new and important information for the understanding of how cancer treatments using antibodies might evolve, highlighting many unresolved issues and controversies. Taken together, these contributions should

allow to gather a representative view of the enormous potential of antibodies and antibody-based molecules as effective tools for cancer immunotherapy. We hope that this information will be useful for a wide audience, including researchers, immunologists, oncologist, and students of biomedical sciences.

AUTHOR CONTRIBUTIONS

LK and JG-S designed and wrote this editorial article.

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Progress and Challenges in the Design and Clinical Development of Antibodies for Cancer Therapy

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The remarkable progress in engineering and clinical development of therapeutic antibodies in the last 40 years, after the seminal work by Köhler and Milstein, has led to the approval by the United States Food and Drug Administration (FDA) of 21 antibodies for cancer immunotherapy. We review here these approved antibodies, with emphasis on the methods used for their discovery, engineering, and optimization for therapeutic settings. These methods include antibody engineering *via* chimerization and humanization of non-human antibodies, as well as selection and further optimization of fully human antibodies isolated from human antibody phage-displayed libraries and immunization of transgenic mice capable of generating human antibodies. These technology platforms have progressively led to the development of therapeutic antibodies with higher human content and, thus, less immunogenicity. We also discuss the genetic engineering approaches that have allowed isotype switching and Fc modifications to modulate effector functions and bioavailability (half-life), which together with the technologies for engineering the Fv fragment, have been pivotal in generating more efficacious and better tolerated therapeutic antibodies to treat cancer.

Keywords: therapeutic antibodies, oncology, humanization, chimerization, phage display, Fc engineering, transgenic mice

INTRODUCTION

The hybridoma technology developed in the mid-1970s by Köhler and Milstein (1) proved to be an efficient means to isolate single specificity antibodies and produce them in unlimited amounts. This seminal achievement paved the way to effectively generate antibodies for a diverse array of therapeutic applications (2). Due to their exquisite specificity and high affinity, monoclonal antibodies have been considered particularly attractive molecules for diagnosis and/or therapy of multiple diseases, and currently antibody-based drugs represent the fastest-growing segment of all the therapeutic proteins in the biotechnology industry (3).

The first approved monoclonal antibody by the United States Food and Drug Administration (FDA) in 1985 (4) for therapeutic settings was muromonab-cluster of differentiation 3 (CD3) (Orthoclone OKT3®). This mouse monoclonal IgG2a antibody developed using the hybridoma

technology, blocks CD3-mediated activation of T cells and was instrumental in the prevention of organ rejection after transplantation (5). Nonetheless, patients who were given Orthoclone OKT3® developed a significant percentage of anti-drug antibodies, also known as a “human anti-mouse antibody” (HAMA) response (6). The HAMA response leads to the inactivation and elimination of the murine antibody (7). It also prevents the use of multiple administrations of the antibody that is required for the therapy of cancer. These issues, along with the fact that murine monoclonal antibodies can be associated with the generation of severe allergic reactions further hampered the use of antibodies of murine origin in human therapy (7).

Additionally, murine antibodies poorly interact with the human immune effector system. Relevant antibody effector functions mediated by the mouse fragment crystallizable (Fc), such as antibody-dependent cell-mediated cytotoxicity (ADCC), are decreased or absent in humans (8). This also applies to the interaction with the neonatal receptor (FcRn), also known as the Brambell or “salvage receptor”, which could result in a very short half-life of murine antibodies when used for human therapy (9). Hence, the multiple drawbacks of murine monoclonal antibodies as biotherapeutics in humans motivated efforts to make them more human-like molecules.

To engineer more human-like antibodies and, thus, increase efficacy while decreasing immunogenicity, non-human variable (V) domains were combined with human constant (C) domains to generate molecules with 70% or more human content. This method called chimerization was developed at the beginning of the 1980s (10) and led to the approval in 1997 of first chimeric therapeutic antibody to treat cancer, **rituximab** (Rituxan®). **Rituximab** has been a tremendous medical and commercial success, currently being the fourth best-selling innovative drug of any kind (3).

In parallel to the clinical development and success of **rituximab**, other technology platforms emerged in the 1980s and 1990s aiming to generate more human-like V domains. These technology platforms have been perfected during the last three decades and include humanization (11), selection of fully human antibodies from Fv and Fab phage-displayed libraries (12), and the development of transgenic animals capable of generating fully human antibodies (13, 14). Moreover, the ground-breaking work on chimerization (10) also highlighted the possibility of linking any V fragment to diverse human Fc isotypes to increase or decrease cytotoxicity. Since antibody effector functions such as ADCC have been considered important mechanisms of action (MOA) for cancer immunotherapy, human IgG1 was the isotype of choice for therapeutic development of the first approved oncology therapeutic antibodies (15). More recently, other isotypes such as human IgG2 and IgG4 have increasingly been used for therapeutic development in oncology. The first human IgG2 approved in 2006 to treat cancer was **panitumumab** (Vectibix®) (16). The MOA of **panitumumab** mostly relies on the target blockade rather than engaging immune effector killing mechanisms such as ADCC.

Discovery and optimization platforms to generate highly specific V regions with a higher human content for therapeutic settings combined with Fc engineering have enabled the approval

of 21 antibodies to treat cancer (**Table 1**). This review focuses on these antibodies, lessons learned from their engineering and clinical development, as well as challenges and prospects to generate more efficacious therapeutic antibodies. We first provide an overview of the IgG molecule, the therapeutic format of the currently approved naked antibodies. Second, we briefly review the oncology targets for which there is more than one approved therapeutic antibody. Third, we discuss the human content of the approved antibodies and the technology platforms used to engineer their V regions. Finally, we provide a summary of the variations of effector functions and bioavailability (half-life) of human IgG isotypes and the approaches used to modify them. Since the Fc engineering field has achieved significant progress in the last few years, beyond the development and approval of the currently marketed antibodies, we also expand on Fc variants in study and/or clinical development.

It should be noted that in addition to naked antibodies, other therapeutic modalities to treat cancer based on the antibody molecule have been gaining momentum in recent years. Such modalities include antibodies conjugated to cytotoxic organic compounds, also known as antibody-drug conjugates (ADCs) (17) as well as antibodies conjugated to radionuclides (18), protein toxins (19), and immunomodulators such as cytokines (20). Other modalities known as bispecific antibodies (21), combining two specificities in a single molecular entity, have also shown increased efficacy and/or a novel MOA when compared to the combination of the two naked antibodies binding individual targets used as the source to engineer the bispecific molecule.

In fact, the relatively recent FDA approval of three ADCs (17) and two bispecifics (21) has fueled the engineering and clinical development of these modalities. The first FDA-approved ADC was gemtuzumab ozogamicin (Mylotarg™) for the treatment of acute myeloid leukemia (AML). Mylotarg™ was voluntarily withdrawn in 2010 in the United States market but, due to the critical unmet need of treatment for patients with AML, it has recently been reintroduced in the United States with different dosing and administration schedules. The two other FDA-approved ADCs are brentuximab vedotin (Adcetris®) and trastuzumab emtansinem (Kadcyla®), which have proven to be highly efficacious with limited toxicity (17). The two approved bispecific antibodies are catumaxomab (Removab®) and blinatumomab (Blinacyto®). These antibody-based drugs have shown to be a breakthrough in the field of cancer immunotherapy. Both bispecifics bind CD3 on T-cells with one arm of the molecule. With the other arm, catumaxomab and blinatumomab bind cancer cells expressing epithelial cell adhesion molecule (EpCAM) or cluster of differentiation 19 (CD19), respectively. Simultaneous binding of CD3 and EpCAM or CD19 bring in the close proximity cancer cells with T-cells leading to a specific and highly efficacious killing process of the cancer cells (21). Although ADC and bispecific modalities are not reviewed here due to the vast amount of information published in this field, compounded with space limitations, it should be highlighted that the methods for discovery and optimization of V regions and modifications of the Fc to tailor the effector functions to a given MOA, are common and can be applied to all antibody-based modalities.

TABLE 1 | United States FDA-approved therapeutic antibodies to treat cancer as of July 30, 2017.

International non-proprietary names (INN)	Commercial name	Company	Approval date	Type	Isotype	Target	Indication
Rituximab	Rituxan®	Genentech	11/26/1997	Chimeric	IgG1	CD20	B-cell non-Hodgkin lymphoma
Trastuzumab	Herceptin®	Genentech	9/25/1998	Humanized	IgG1	HER2	Metastatic breast cancer
Alemtuzumab	Campath®	Genzyme	5/7/2001	Humanized	IgG1	CD52	B-cell chronic lymphocytic leukemia
Cetuximab	Erbtux®	ImClone Systems	2/12/2004	Chimeric	IgG1	EGFR	Metastatic colorectal carcinoma
Bevacizumab	Avastin®	Genentech	2/26/2004	Humanized	IgG1	VEGF	Metastatic colorectal cancer
Panitumumab	Vectibix®	Amgen	9/27/2006	Fully human	IgG2	EGFR	Metastatic colorectal cancer
Ofatumumab	Arzerra®	Glaxo Grp	10/26/2009	Fully human	IgG1	CD20	Chronic lymphocytic leukemia
Ipilimumab	Yervoy®	Bristol-Myers Squibb	3/25/2011	Fully human	IgG1	CTLA-4	Metastatic melanoma
Pertuzumab	Perjeta®	Genentech	6/8/2012	Humanized	IgG1	HER2	Metastatic breast cancer
Obinutuzumab	Gazyva®	Genentech	11/1/2013	Humanized	IgG1	CD20	Chronic lymphocytic leukemia
Ramucirumab	Cyramza®	Eli Lilly	4/21/2014	Fully human	IgG1	VEGFR2	Gastric cancer
Pembrolizumab	Keytruda®	Merck	9/4/2014	Humanized	IgG4	PD-1	Metastatic melanoma
Nivolumab	Opdivo®	Bristol-Myers Squibb	12/22/2014	Fully human	IgG4	PD-1	Metastatic melanoma
Dinutuximab	Unituxin®	United Therapeutics	3/10/2015	Chimeric	IgG1	GD2	Pediatric high-risk neuroblastoma
Daratumumab	Darzalex®	Janssen Biotech	11/16/2015	Fully human	IgG1	CD38	Multiple myeloma
Necitumumab	Portrazza®	Eli Lilly	11/24/2015	Fully human	IgG1	EGFR	Metastatic squamous non-small cell lung carcinoma
Elotuzumab	Empliciti®	Bristol-Myers Squibb	11/30/2015	Humanized	IgG1	SLAMF7	Multiple myeloma
Atezolizumab	Tecentriq®	Genentech	5/18/2016	Humanized	IgG1	PD-L1	Bladder cancer
Olaratumab	Lartruvo®	Eli Lilly	10/19/2016	Fully human	IgG1	PDGFRA	Soft tissue sarcoma
Avelumab	Bavencio®	EMD Serono	3/23/2017	Fully human	IgG1	PD-L1	Metastatic Merkel cell carcinoma
Durvalumab	Imfinzi®	AstraZeneca	5/1/2017	Fully human	IgG1	PD-L1	Urothelial carcinoma

The table was generated by parsing the information on approved antibodies compiled by The Antibody Society (<http://www.antibodysociety.org/news/approved-antibodies/>), CenterWatch (<http://www.centerwatch.com/drug-information/fda-approved-drugs/>), and contrasted with recent reviews on the state of the art in therapeutic antibodies for treatment of cancer cited in this review. The antibodies are listed chronologically in the table in the order of approval date. The INN are highlighted in bold in the Table and text of the article.

THE IgG MOLECULE

Five different antibody classes exist in humans, determined by the nature of the C regions of the heavy chain (HC). These classes are designated by lower-case Greek letters: γ for IgG; δ for IgD; ϵ for IgE; α for IgA, and μ for IgM (22). The IgG is the most prevalent class of antibodies in blood and the most common molecular format used as therapeutic. **Figure 1** shows that the IgG is assembled with two identical HCs and two identical light chains (LCs), classified in two types, κ and λ . The LC has a single variable (V_L) domain and a single constant (C_L) domain, whereas, the HC consists of a single variable (V_H) domain, a hinge region, and three constant (C_{H1} , C_{H2} , and C_{H3}) domains. The C_{H3} domain is located at the C-terminus of the IgG. In the N-terminus, the pairing of the LC and the Fd fragment (V_H and C_{H1}) from the HC forms the fragment antigen binding (Fab), where the antigen-binding site is located. The heterotetrameric structure of IgG is held together covalently by disulfide bonds between the C_L and C_{H1} domains and between the hinge (inter-domain) region of the two HCs.

The diversity of the antigen-binding site, and hence the capacity of antibodies to bind virtually any target, comes from diverse germline gene repertoires (24). The IGLV and IGLJ germline genes encode the V_L domain, whereas the V_H domain is encoded by the repertoires ofIGHV, IGHD, andIGHJ germline genes (25). Additional amino acid variation in the antigen-binding site occurs through somatic mechanisms, such as somatic hypermutation in humans and mice, and gene conversion in other species such as chickens and rabbits (26, 27).

The germline and somatic amino acid variability is concentrated in the complementarity-determining regions (CDRs).

Three CDRs in V_L : CDR-L1, CDR-L2, and CDR-L3, and three in V_H : CDR-H1, CDR-H2, and CDR-H3, alternate with conserved regions called framework regions (FRs), four in V_L : FR-L1, FR-L2, FR-L3, and FR-L4, and four in V_H : FR-H1, FR-H2, FR-H3, and FR-H4. The six CDRs are brought together by folding and non-covalent association of the V domains in the Fv (**Figure 1**) at the tip of the Fab.

Two Fabs are linked to one Fc via the hinge region that provides flexibility to the antibody molecule to interact with diverse configurations of the targets. The Fc is formed by the non-covalent association of C_{H2} and C_{H3} domains, with critical residues in the hinge and C_{H2} determining the immune effector functions of the IgG antibody via interaction with the Fc gamma family of receptors (FcγRs) and the complement component C1q. Engagement of FcγRs on immune effector cells activates cellular responses such as ADCC and antibody-dependent cell-mediated phagocytosis (ADCP). Complement fixation, starting with the interaction of the antibody and the complement component C1q, induces activation and formation of the membrane attack complex (MAC), finally resulting in complement-mediated cytotoxicity (CDC).

The human IgG has four subclasses: IgG1, IgG2, IgG3, and IgG4, also known as isotypes (28). These isotypes have evolved different Fc sequences with differential capacity to elicit effector functions (**Table 2**). Isotype-specific engagement of such immune functions is based on selective Fc receptor interactions on distinct immune cell populations such as natural killer (NK) cells, neutrophils, and macrophages, as well as the ability to bind C1q, an initial protein in the complement pathway leading to a “cascade” of events that results in the formation of the MAC and the induction of tumor cell killing.

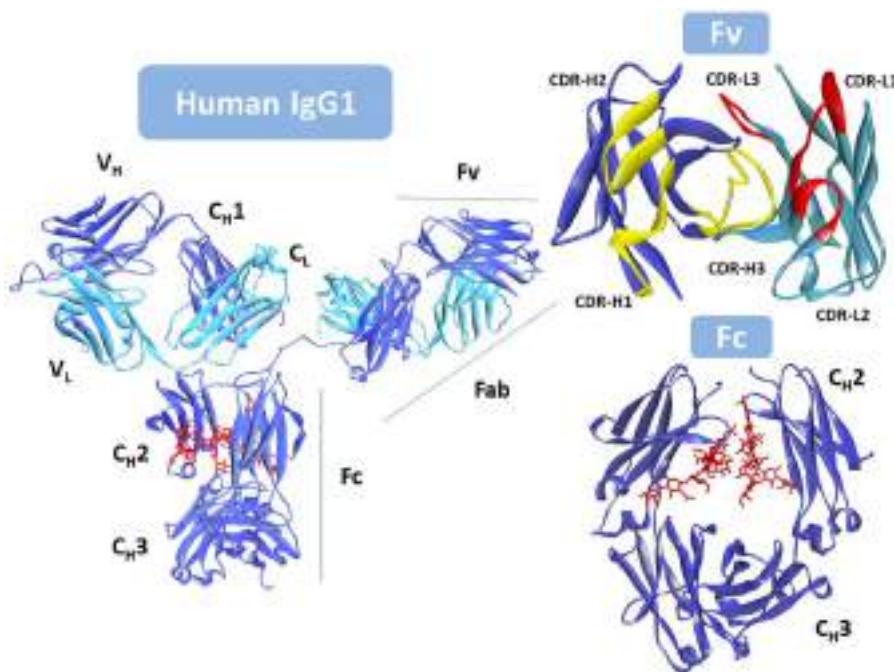


FIGURE 1 | Intact human IgG1, Protein Data Bank (PDB) ID: 1HZH (23). Heavy chain is shown in blue. Light chain in cyan, and the *N*-glycan in red. Fv (top right) with the antigen-binding site seen from the antigen perspective. V_L complementarity-determining regions (CDRs) in yellow; V_H CDRs in red. Fc (bottom right) rotated with respect to the antibody to better show the location of the *N*-glycan (in red). Notice that one of the hinge peptides is missing in the figure. Due to its flexibility it was not solved since coordinates for this region are not available in the PDB file. This figure was generated using Discovery Studio.

Additionally, IgG antibodies contain an *N*-glycosylation site at asparagine-297 (N297) in the C_H2 domain. Modification of this *N*-linked glycan affects the Fc-mediated effector functions. Furthermore, specific residues located near the C_H2–C_H3 junction engage the major histocompatibility complex (MHC) class I related receptor, known as the FcR_n that largely determines the blood half-life of antibodies.

THERAPEUTIC ANTIBODIES APPROVED FOR THE TREATMENT OF CANCER

The current FDA-approved antibodies to treat cancer (**Table 1**) target 13 molecules including membrane proteins such as cluster differentiation 20 (CD20) and epidermal growth factor receptor (EGFR), soluble protein ligands for instance vascular endothelial growth factor (VEGF), and a disialoganglioside (GD2). These antibodies aim at different MOAs (**Figure 2**), which result from an interplay of the biology of the target, affinity of the antibody for the target, and/or effector functions such as ADCC, ADCP, and/or CDC that are elicited. The MOAs may also include blockade of oncogenic pathways with inhibition of malignant cell proliferation and/or induction of apoptosis, blockade of the formation of new blood vessels, and enhancement of the antitumor cytotoxic T cell (CTL) immune response to target tumor cells by inhibiting the immune cell checkpoint resulting in their activation.

The most relevant targets in terms of number of approved therapeutic antibodies are CD20, EGFR, its paralog human EGFR2 (HER2), and programmed cell death protein 1 (PD-1)

TABLE 2 | Functional properties of the human IgG isotypes.

Properties	IgG1	IgG2	IgG3	IgG4
Approximate molecular weight (kDa)	146	146	165	146
Hinge length (number of amino acids)	15	12	62	12
Antibody-dependent cell-mediated cytotoxicity	+++	+/-	++	+/-
Antibody-dependent cell-mediated phagocytosis	+	+	+	+/-
C1q binding	+	+/-	+++	-
Complement-mediated cytotoxicity	++	+/-	++	-
FcR _n binding	+	+	+/-	+
Plasma half-life (days)	21	21	5–7.5	21
Approximate average plasma concentration (mg ml ⁻¹)	9	3	1	0.5

Adapted from Strohl and Strohl (29) and Bruggemann et al. (30).

and its ligand 1 (PD-L1). A brief description of these targets and the interaction with the therapeutic antibodies follows. A recent review on the above targets, other targets, and their interaction with therapeutic antibodies and MOAs has been published (31).

Anti-CD20 Antibodies

Targeting CD20 with antibodies led to the approval of **rituximab** back in 1997, the first therapeutic antibody approved to treat cancer. CD20 is highly expressed on B cells throughout their development, but is absent on the hematopoietic stem cell (32). Although the physiological function of CD20 remains unclear, several lines of evidence suggest a role for CD20 in calcium signaling of B-cell antigen receptor activation. It has also been suggested that CD20 exists predominantly as a tetramer on the cell surface. CD20 is not shed or internalized upon antibody binding, which facilitate

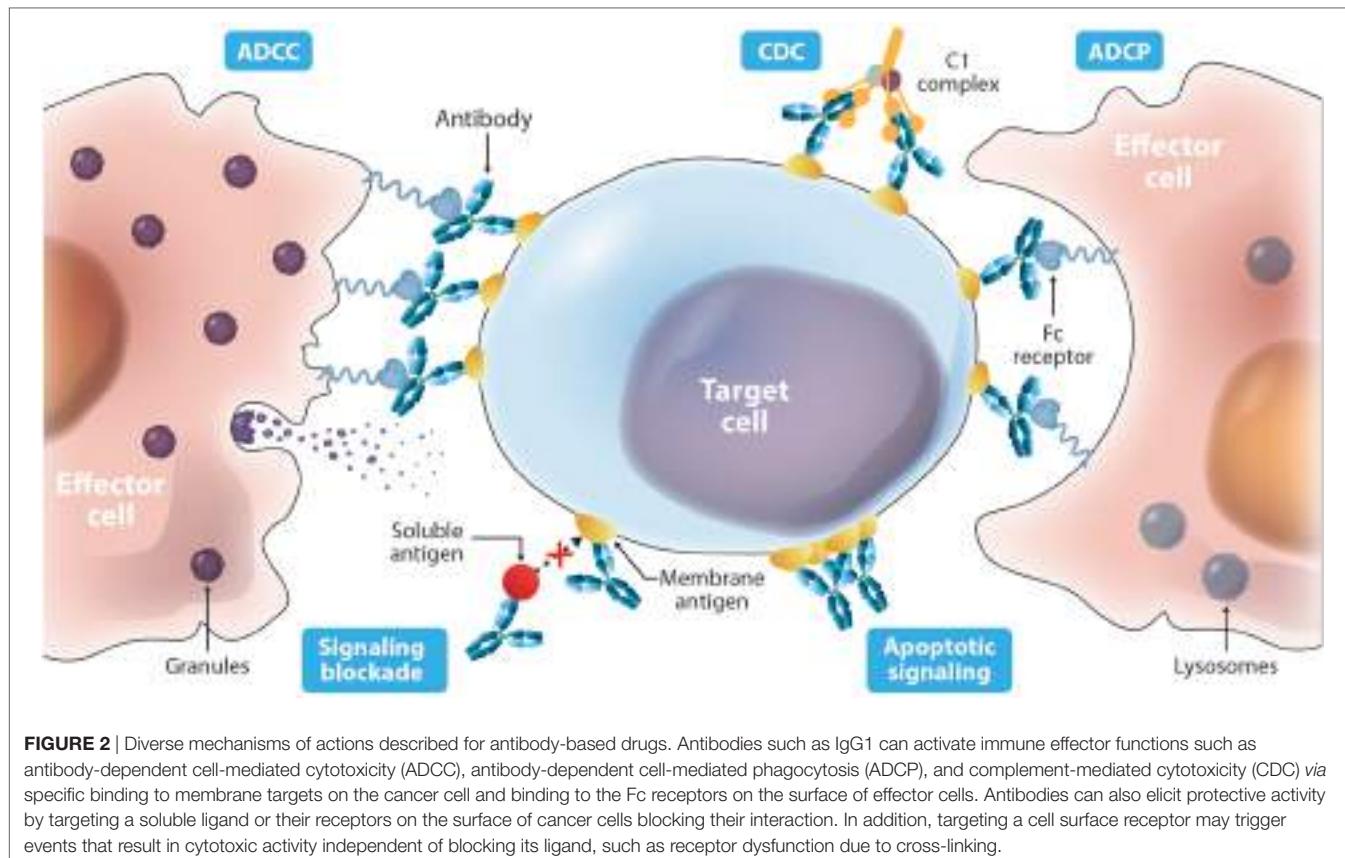


FIGURE 2 | Diverse mechanisms of actions described for antibody-based drugs. Antibodies such as IgG1 can activate immune effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-mediated cytotoxicity (CDC) via specific binding to membrane targets on the cancer cell and binding to the Fc receptors on the surface of effector cells. Antibodies can also elicit protective activity by targeting a soluble ligand or their receptors on the surface of cancer cells blocking their interaction. In addition, targeting a cell surface receptor may trigger events that result in cytotoxic activity independent of blocking its ligand, such as receptor dysfunction due to cross-linking.

the recruitment of immune effector cells and mediate sustained immunologic activity as relevant MOA (33).

CD20 has four transmembrane domains with two extracellular loops, one large loop of 45 amino acids, and a short loop of nine residues. Anti-CD20 antibodies are classified as Type I or Type II according to their interaction with CD20 and primary MOA (32). **Rituximab** and **ofatumumab** are Type I antibodies, whereas **obinutuzumab** is a Type II. Among other characteristics, Type I antibodies have full binding capacity, high CDC, and moderate direct cell death induction. Type II antibodies have half binding capacity, low CDC, and stronger direct cell death induction.

Peptide scanning and mutagenesis studies have shown that **rituximab** binds the large CD20 loop (34). Although **obinutuzumab** is a Type II antibody, its epitope overlaps with that of **rituximab**, but is shifted toward the C-terminus of the large CD20 loop. X-ray crystallography (35) of the extracellular large loop in complex with **rituximab** or **obinutuzumab** Fabs have confirmed that while these antibodies bind partially overlapping epitopes, they differ in their interaction with the large CD20 loop.

Ofatumumab is a Type I antibody like **rituximab** but binds the small CD20 extracellular loop (32). Binding of **ofatumumab** seems to influence the large loop conformation but does not interact with the critical residues of CD20 determining the epitope of **obinutuzumab** and **rituximab**. The differences in primary MOA of **ofatumumab**, **obinutuzumab**, and **rituximab** suggest that in addition to the different epitopes they bind, other factors such as orientation of the antibodies when bound to CD20 are important in their therapeutic efficacy (32).

Anti-EGFR Antibodies

EGFR and HER2 were among the first receptors to be identified and associated with human tumors (36, 37). Physiologically, EGFR (also known as HER1/ErbB1) and the EGFRs 2, 3, and 4 (HER2/ErbB2, also known as the *neu* oncogene, HER3/ErbB3, and HER4/ErbB3) are involved in cell growth control and differentiation. Several crystal structures of EGFR and HER2 and complexes with therapeutic antibodies are now available (38). The extracellular domain (ECD) of EGFR is composed of four domains I–IV, which are arranged in two conformations: an “extended” active form and the alternative inactive form, which is folded over or “tethered.” In the inactive form, domain II interacts with domain IV, while domains I and III are far apart. The active extended dimeric form is induced by the ligand, epidermal growth factor (EGF), in which domains I and III are closer together.

All three approved anti-EGFR therapeutic antibodies bind domain III and block the interaction with EGF. The X-ray crystal structures of **necitumumab** and **cetuximab** Fabs in complex with EGFR indicate that these antibodies bind a very similar surface on EGFR but, having different CDRs, do so through a set of different interactions (39). In fact, **necitumumab** was isolated from a human antibody phage-display library by competition with **cetuximab**. The similarity in the epitopes of **necitumumab** and **cetuximab** suggested that the former would have similar properties to the chimeric antibody **cetuximab**, but with the benefits of a fully human antibody (39).

The epitope recognized by **panitumumab** also overlaps with **cetuximab** (40). However, screening of peptide phage-display libraries and mutagenesis studies have shown that although these antibodies bind overlapping regions on EGFR, some amino acids in the epitope are critical for **cetuximab** binding, whereas others are specific for **panitumumab**. The relevance of these specific interactions in clinical settings emerged from studies in a patient with colorectal cancer who acquired a point mutation under treatment with **cetuximab** and developed resistance to treatment with this antibody, whereas treatment with **panitumumab** was still effective in this patient (41). This mutation seemed to abrogate **cetuximab** binding to the mutated EGFR, while **panitumumab** binding remained unaffected. Thus, differences in the functional epitopes of **panitumumab** and **cetuximab** could have clinical relevance as they may be instrumental in the selection of patients and decisions regarding their treatment.

Anti-HER2 Antibodies

The structure of HER2 is similar to that of EGFR (42), but HER2 does not bind a ligand, functioning primarily *via* heterodimerization with ligand-bound partners of the EGFR family, mostly HER3. Comparison of several X-ray crystal structures (38) indicate that HER2 ECD adopts an extended conformation due to two non-conservative key mutations in the domain IV residues, which replace glycine 563 (G563) and histidine 565 (H565) in EGFR by proline (P) and phenylalanine (F) in HER2. These mutations prevent the contacts of domain II–IV, rendering in HER2 the extended active conformation seen in EGFR but without ligand binding in HER2. Moreover, HER2 ECD does not homodimerize in solution, perhaps due to conformational differences between the extended ECD module of HER2 and the EGFR dimeric conformation (38).

Trastuzumab binds domain IV, close to the membrane, and its MOA involves disruption of HER2 homodimerization and prevention of cleavage of the ECD, which leads to the active truncated receptor p95HER2 (43). This truncated form of HER2 maintains kinase activity and can migrate to the nucleus to act as oncogenic nuclear factor. **Pertuzumab** binds domain II and prevents heterodimerization of HER2 with HER3 and EGFR, blocking growth of HER2-amplified breast cancer (44). Of note, the combination of **pertuzumab** and **trastuzumab** in breast cancer therapy has been shown to be more efficacious than the treatment with the single therapeutics (45).

Anti-PD-1/PD-L1 Antibodies

Programmed cell death protein 1 (PD-1) and its ligand (PD-L1) are immune checkpoints that inhibit CTL activity (46, 47). PD-L1 is constitutively expressed on a subset of macrophages, but may be rapidly upregulated in different tissue types and by tumors in response to interferon-gamma (IFN- γ) and other inflammatory mediators. Importantly, many cancer cells express PD-L1 as a mechanism of immune evasion. Thus, targeting PD-1 and PD-L1 with antibodies has demonstrated significant therapeutic benefits in clinical trials, especially resulting in activation of the antitumor CTL response, a phenomenon known as immune checkpoint blockade (48).

The structure of PD-L1 in complex with PD-1 has been extensively studied (49). Several X-ray crystal structures are now available, including human PD-L1 alone, mouse PD-1 complexed with human PD-L1, and human PD-1 complexed with human PD-L1 or antibodies. These structures have shown that **pembrolizumab** and **nivolumab** epitopes on PD-1 overlap with part of the PD-L1 binding site. The affinity of these antibodies for PD-1 is in the low picomolar range (50). This is several orders of magnitude stronger than the affinity of PD-L1 for PD-1, estimated in the nanomolar range (51), which suggests that the MOA of **pembrolizumab** and **nivolumab** is through outcompeting PD-L1 for binding to PD-1. Furthermore, **pembrolizumab** and **nivolumab** have been engineered with IgG4 isotypes, which has an important influence in their MOA by reducing toxicity of these antibodies. IgG4 lacks effector functions, such as ADCC and CDC, which may be potentially harmful to the immune cells expressing PD-1 when targeting this ligand with antibodies.

On the PD-L1 side, **atezolizumab**, **durvalumab**, and **avelumab** bind distinct epitopes but all interfere with PD-1 binding (52), preventing the PD-L1/PD-1 interaction. These three checkpoint inhibitors are of the IgG1 class, but the Fcs of **atezolizumab** and **durvalumab** have been modified to eliminate antibody effector functions. **Atezolizumab** is an aglycosylated antibody, whereas **durvalumab** is a Fc-modified triple mutant variant. **Avelumab** is reported to be a non-modified IgG1. Therefore, like with the anti-PD-1 therapeutic antibodies, the MOA of **atezolizumab**, **durvalumab**, and **avelumab** is an interplay between affinity, epitope, and Fc variants.

IMMUNOGENICITY AND HUMAN CONTENT

Immunological reactions to biotherapeutics involve a complex combination of diverse components not fully yet understood, including product-, disease- and patient-specific factors (53). The lack of standardization in the terminology and approaches used for collecting, analyzing, and presenting immunogenicity data also makes it difficult to find a consensus on immunogenicity results (54).

Nonetheless, pioneering work on the specificity of the immune reactions to peptides (55), prior to determining the amino acid sequence of a protein or its three-dimensional (3D) structure, suggested that the phylogenetic distance between two species is an important factor in eliciting antibodies. Subsequent determination of the amino acid sequence of the first proteins in the early 1960s indicated that the phylogenetic distance between two species is imprinted in the amino acid sequences (56). These differences play a key role in launching specific immune responses against non-self proteins. In fact, diverse *in silico* predictive methods to assess potential immunogenic spots based on the amino acid sequence have been developed and are part of the toolbox used to engineer therapeutic proteins (57). Thus, engineering antibodies with lower immunogenicity has been driven in part by generating amino acid sequences that are as human as possible.

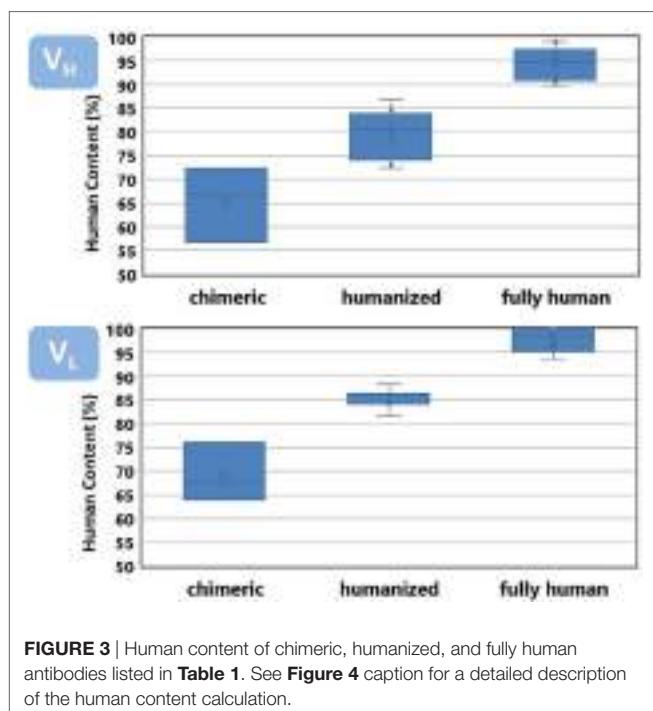
The diversity of antibodies comes from the recombination of diverse germline gene repertoires (25) and somatic mutations generated during the hypermutation process (26). Since

the somatic mutations are specific to an immune response and some mutations may be immunogenic in other individuals with a different immunological history and background, it can be assumed that the ideal human antibody from an immunogenicity standpoint should be identical to the genes encoded by the germline repertoire.

The physical maps of the human IGH and IGL genes loci were elucidated in the 1990s, together with an initial estimation of number and germline gene sequences encoding the functional human antibodies (25, 58–60). In the last two decades, more human germline genes from diverse populations have been sequenced and studied. More recently, the application of next-generation sequencing methods to the study the antibody repertoires from diverse individuals have led to the characterization of an increasing number of alleles (61, 62). This information is compiled and curated at the international ImMunoGeneTics information system (IMGT[®])¹, with alleles “01” representing the oldest germline genes. The “01” alleles have also been identified in several individuals, thus, perhaps representing the most common human antibody germline genes.

Figure 3 shows a comparison of the percentage of identities of the V_H and V_L regions of the antibodies listed in **Table 1** with respect to the closest match in the repertoire of human antibody germline genes compiled at the IMGT. Both V_H and V_L of the chimeric antibodies have an average around 70% identities with respect to the human germlines, also defined as human content. Chimeric antibodies have a wider variation in the percentage of identities with respect to the human germline genes as well. Humanized antibodies reach an average 85%, whereas, fully human antibodies show 90% or more human content.

¹<http://www.imgt.org>.



Overall, the V_H region has a slightly less human content than V_L. V_H leads the interaction of the antibody with its specific target and tends to accumulate more somatic mutations (24), diverging faster from the germline configuration. It poses a higher challenge for antibody engineers to increase the human content of the therapeutic antibodies while preserving the specificity and affinity of the parental, non-human antibody.

The departure from 100% human content observed in fully human antibodies roughly corresponds with the frequency of somatic mutations observed in the antibody human sequences studied by several research groups (63–65). It has been reported that mutations in V_H and V_L follows an exponential distribution, with as much as 15–20% of the V regions showing no mutations at the amino acid level. Following these sequences in the germline gene configuration, fewer and fewer sequences have an incremental number of mutations. The average number of somatic mutations per human V region observed in diverse samples of sequences product of immune responses has been estimated in around eight and five mutations for V_H and V_L, respectively.

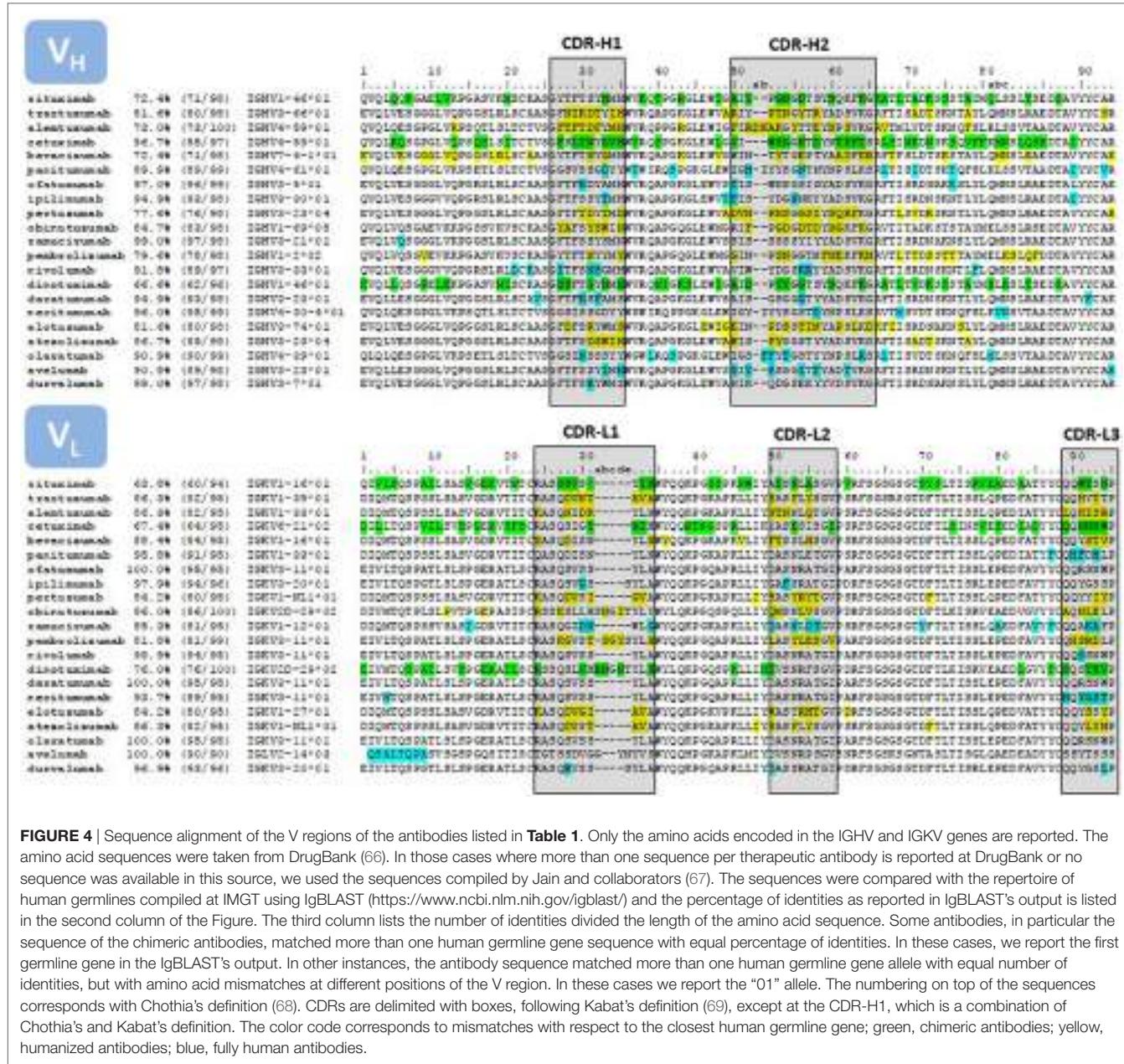
The placement of mutations with respect to the closest germline gene match is shown in **Figure 4**. Chimeric antibodies show amino acid differences all along the V regions, with a high number of non-human residues in the FR-3 of V_H and FR-1 of V_L. In contrast, the mutations of the humanized antibodies are mostly clustered in the CDRs. Fully human antibodies show very few mutations, with some V regions being in the germline configuration.

Chimeric Antibodies

After the FDA approval of **rituximab**, two additional chimeric antibodies, **cetuximab** and **dinutuximab**, reached the market for oncology indications. **Cetuximab** was generated by immunizing mice with purified EGFR and replacing the mouse constant domains of the mouse antibody 225 with those of human IgG1. The chimeric molecule, named C225, showed around five-fold higher affinity and increased tumor growth reduction than the parental mouse antibody (70). **Dinutuximab** was developed from a murine antibody specific for GD2 (71). The chimeric molecule (ch14.18), also a human IgG1, showed identical binding as the murine IgG2a antibody but, the ADCC was 50-fold to 100-fold higher than the parental mouse antibody when using human effector cells (72). Therefore, in addition to rendering less immunogenic molecules, chimerization overcame some of the drawbacks of the early murine monoclonal antibodies by generating therapeutic molecules with the same or improved affinity than the parental mouse antibodies but with enhanced effector functions.

Humanized Antibodies

Although chimeric antibodies were more efficacious and less immunogenic than mouse antibodies, they still elicited a “human anti-chimeric antibody” (HACA) response (73). Thus, to further increase the human content of therapeutic antibodies, in the second half of the 1980s Winter's group at the Medical Research Council (11) showed that by grafting the CDRs from an antibody into FRs of another antibody, the specificity and affinity of CDR donor antibody can be transferred to the antibody providing



the FRs. It was first applied to engineer **alemtuzumab** (74). The parental antibody was a rat IgG2a, called YTH 34.5HL. Its CDRs were grafted into the human V_H and V_L FRs of the known antibody structures at the time NEW (75) and REI (76), respectively.

In an alternative approach, Queen and collaborators (77) at Protein Design Labs (PDL) humanized daclizumab (Zenapax[®])—not discussed here as it has no indication in cancer. Daclizumab was humanized by CDR grafting, but the human FRs were selected by maximizing homology between the murine antibody sequence providing the CDRs and the human antibody donating the FRs. In addition, a computer model of the mouse antibody, guided the identification of several murine amino acids in the FRs that interacted with the CDRs or antigen and back mutated those amino acids into the CDR-grafted antibody, thus, improving binding of

the final product. Together with the successful humanization of **alemtuzumab** by Winter's group, these pioneering works laid the foundations for humanization *via* CDR grafting, the humanization method used to engineer all of the humanized antibodies listed in **Table 1**.

For instance, Carter and collaborators (78) humanized the murine antibody mumAb4D5, which had potential for human therapy due to its anti-proliferative (cytostatic) effect against human breast and ovarian cancer cell lines overexpressing HER2. The CDRs of mumAb4D5 were grafted in consensus human FRs and several mouse residues were incorporated into the FR aiming to retain the affinity of the parental mumAb4D5 antibody. One of the humanized versions of this antibody named humAb4D5-8, later named **trastuzumab**, showed a four-fold increase in HER2

binding affinity compared to the parental mumAb4D5, similar cytostatic activity, and more efficient ADCC against HER2 overexpressing cancer cells. Thus, this study demonstrated that humanization technologies can be helpful not only to increase the human content of Fv region but also to enhance the binding properties of the antibody and its therapeutic efficacy.

Bevacizumab (79) and **pertuzumab** (80) were engineered using a similar method. The other humanized antibodies for oncology indications, e.g., **pembrolizumab**, **atezolizumab**, **obinutuzumab**, and **elotuzumab** have also been obtained by grafting non-human CDRs into human FRs, with designed back-mutations, which replace human residues by the original mouse residues in the FR to stabilize the CDR conformations and, hence, preserve or improve the affinity of the parental non-human antibody. The number of backmutations varies depending on the source of the FR, namely: mature antibodies (77), consensus FRs (78) and more recently the use of human germline genes (81, 82). The FR selection method, sequence similarity between the parental non-human donor and human acceptor sequences, as well as the affinity for the target are all contributing factors to obtaining humanizing antibodies with higher human content (83).

Although humanized antibodies have more human content than chimeric antibodies they still do not eliminate the possibility of the induction of a “human anti-human antibody” (HAHA) response (84). However, humanization is still broadly used due in part to the accessibility of hybridoma technology to academic laboratories and small biotech companies. Also, the relatively recent expiration of the dominant humanization patents (85, 86) and diversification of humanization methods (83) have contributed to the widespread use of the technology.

While the HAMA response can in principle be directed against the entire antibody and the HACA response against the V regions, the HAHA response is even more focused against the CDRs (see **Figure 4**). Thus, replacing non-human amino acids in the CDRs with human amino acids has been undertaken by several companies such as Xencor (87), Facet Biotech Corporation (88), and more recently Pfizer (89, 90). For instance, Townsend and collaborators (89) generated libraries of binary substitutions at the CDRs by combining the parental non-human residues with human germline residues at each position and screened the libraries for clones with restored antigen-binding capacity. The resulting antibodies increased the human content by 17–29%, rendering molecules indistinguishable from fully human antibodies. Apgar and collaborators (90) followed a rational approach based on the structure of the antigen-antibody complex and were able to replace 11 out of 26 non-human residues in the CDRs. Thus, it could be expected that due to the accessibility and low cost of the hybridoma technology and commoditization of CDR grafting compounded with CDR humanization rendering antibodies indistinguishable from fully human antibodies, humanized antibodies with a higher human content will reach clinical development in the near future.

Fully Human Antibodies

Fully human therapeutic antibodies emerged in the 1990s with the development of two technology platforms: human antibody Fv or Fab phage-display libraries (12) and transgenic animals

bearing the human antibody repertoire (14). Eleven antibodies listed in **Table 1** have been discovered using these platforms. Of note, only three: **necitumumab**, **ramucirumab**, **avelumab** were obtained using phage display. Although phage display was developed prior to transgenic animals, the latter requires less optimization and thus shorter timelines to reach clinical development (91). In addition, since enriching technologies such as phage display are based on *in vitro* selection, the antibody fragments coming out of the selection and screening processes do not undergo the *in vivo* selection process and tend to carry developability liabilities (discussed below).

Two transgenic mice produced the fully human antibodies approved for oncology indications, e.g., Medarex (14) and Abgenix (16). The first therapeutic antibody developed by one of these platforms was **panitumumab**. This therapeutic antibody was obtained using the XenoMouse® (92). **Durvalumab** was discovered using the same platform (93), whereas, the other five antibodies listed in **Table 1** were discovered using the Medarex technology. Interestingly, four of the Medarex antibodies share the same V_L, IGKV3-11*01, with three of them in the germline gene configuration and one (**nivolumab**) with only one mutation at the CDR-L3 (**Figure 4**). Although the Medarex mouse has only a small fraction of the complete human V_H and V_L repertoires, it demonstrated that even with limited diversity, the plasticity of V_L to pair with diverse V_H chains can generate specific and high affinity therapeutic antibodies against unrelated targets. Other transgenic mice have been developed in the last three years including the Kymouse (94) and the Trianni mouse (95). These platforms rely on more diverse repertoires of human antibody genes, which enable the selection of highly diverse human antibodies and circumvent some of the limitations of the early transgenic mice.

Despite the success of transgenic mice as a source of therapeutic antibodies, immunization does not always lead to antibodies with the desired antibody affinity and specificity (96, 97). This is particularly true for conserved epitopes between human and mouse orthologs. Transgenic rats (98) and more recently chickens (OmniChicken) (99) may partially mitigate this limitation. Nonetheless, toxic targets and selection of proteins with specific active conformations in environments of interest for a given MOA are not well suited for an immunization approach and require an alternative solution for antibody discovery.

Phage display opened the possibility of designing and manipulating the repertoire of antibody genes to be used as source of antibodies (24), thus, leading to selection of fully antibodies *in vitro*. Since the discovery process *via* phage display is performed *in vitro* one can also choose the optimal conditions to select for desired biophysical and biochemical properties, target pre-defined epitopes locked in specific conformations, avoid immunodominant epitopes by masking them with other known antibodies and/or focus the selection of rare or cross-reactive epitopes.

For instance, **ramucirumab** was developed starting from three antibodies with identical V_H sequence isolated from de Haard and collaborators' Fab display library (100). These antibodies bound specifically the VEGF receptor 2 (VEGFR2), blocked the VEGF/KDR interaction, and inhibited VEGF-induced proliferation of human endothelial cells and migration of KDR leukemia

cells. A new library was built by combining the single V_H with a repertoire of naïve V_L chains, and diverse and specific V_L chains for VEGFR2 were selected. Then, a consensus V_H : V_L pair, termed 1121, was identified after selection tailoring the stringency of the panning conditions to obtain picomolar binders (101).

Necitumumab was also isolated from de Haard and collaborators' library by using A431 carcinoma cells, which express high levels of EGFR. Competition with **cetuximab** for binding to the cell surface generated one clone, termed 11F8, which displayed a dose-dependent inhibitory effect on EGF-stimulated EGFR activation in A431 cells. A comparison of the structures of Fab11F8 with the Fab derived from **cetuximab** (FabC225) both in complex with EGFR, indicated that the epitope of the two Fabs was remarkably similar, but the antibodies having different CDRs, bound EGFR through a set of different interactions (39). **Necitumumab**, the new fully human antibody was thus developed, had similar biological properties to **cetuximab**, but without the disadvantages of a chimeric antibody.

Until relatively recently, phage display technology was controlled by a few companies, holding their technology patents (102). These patents expired in Europe and the United States, thus, allowing the free use of the antibody discovery methodology *via* phage display by academic laboratories and small biotech organizations. As phage display technology has become a commodity, several companies such as BioRad using HuCAL® (103), Distributed Bio *via* SuperHuman synthetic libraries,² and GlobalBio/ADL by means of semi-synthetic ALTHEA Gold Libraries^{TM3} are licensing phage display libraries and/or offering discovery services at a relatively low cost without royalty payments.

In addition to phage, other display platforms have been developed, including ribosome (104), bacteria (105), yeast (106), and mammalian (107) display. Each of these platforms has advantages and disadvantages. One of the advantages of yeast display, which is the most commonly used, over phage display is the screening using fluorescence-activated cell sorting. This advantage has proven to be an efficient means to isolate antibodies with very high affinity, e.g., in the low femtomolar range (108). In addition, while phage is limited to the display of antibody fragments such as scFvs or Fabs, yeast enables the display of full IgG antibodies with glycosylation. Since the end therapeutic product is commonly an IgG and its efficacy and toxicity are an interplay between target epitope, affinity, Fc isotype and/or variants and glycosylation, yeast display has become a suitable platform for efficient therapeutic discovery and development (109).

DEVELOPABILITY

As more antibodies have reached the market, and more importantly, many failed to perform in preclinical development and clinical trials, the term "developability" emerged in the 2010s (110, 111). Developability encompasses a set of design principles and experimental methods to assess the potential of

antibodies to be further developed or manufactured, formulated, and stabilized to achieve the desired therapeutic effect. For instance, post-translational amino acid modifications such as deamidation of asparagine (N), oxidation of methionine (M), and isomerization of aspartic acid, have been identified as potential developability liabilities (112). Modifications of these amino acids can lead to heterogeneities in the drug and/or lack of potency if these amino acids are involved in the interaction with the target. Other amino acids such as tryptophan (W) can induce aggregation and, thus, immunogenicity or lack of solubility at concentrations required for the therapeutic indication, which impairs the further development of the product. Hence, identification of these amino acids and removal when possible during the early discovery process are now part of the antibody engineering process to increase the success rate of preclinical and clinical development.

On the experimental side, recently Jain and collaborators (67) have assessed the limits of developability of 137 FDA-approved therapeutic antibodies (including those listed in **Table 1**) as well as those in advanced stages of clinical development, i.e., clinical phase I, II, and III. More favorable biophysical properties were found in approved antibodies. Hence, the biopharma industry has progressively been implementing experimental assessment of biophysical properties in early stages of the discovery campaign to progress molecules that would perform well in preclinical development. This is particularly important for therapeutic antibodies generated *via* phage display, related enriching technologies, and humanized antibodies where the selection process proceed *in vitro* without the filters imposed *in vivo* that tend to select well-behaved molecules when used as therapeutics.

MODULATING Fc-DEPENDENT EFFECTOR FUNCTIONS

As reviewed above, choosing the right IgG isotype is key to achieve the desired MOA. All antibodies compiled in **Table 1** are IgG1 except three: **panitumumab**, **nivolumab**, and **pembrolizumab**. The human IgG1 elicits strong effector functions such as ADCC and CDC (**Table 2**), which have been shown to be an important mechanism to kill cancer cells and has been broadly used in development of anticancer antibodies. However, the importance of the other IgG isotypes such as IgG2 and IgG4, as well as mutations of the Fc region (**Table 3**) have had a significant impact in the success of cancer-targeting antibodies, and are now an essential part of designing and testing therapeutic antibodies. The current approved Fc-engineered antibodies and further developments in the field of Fc engineering are reviewed in the following sections.

Human IgG Isotypes and Mutations to Alter Effector Functions

ADCC occurs when an antibody simultaneously binds its cognate antigen on the surface of the malignant cell and the Fc region of the antibody binds activating Fc gamma receptors (FcγR) on the surface of an effector cell. This stimulates a signaling cascade

²<http://www.distributedbio.com>.

³<http://www.globalbioinc.com/Services/>

TABLE 3 | Examples of human Fc mutations for functional modification.

Function	Effect	Class	Application	Mutations or changes	Reference			
ADCC	Enhanced	IgG1	Cancer	S298A/E333A/K334A	(113, 114)			
				S239D/I332E	(114, 115)			
				S239D/A330L/I332E	(114, 116, 117)			
				S298A	(113)			
				D280H	(118)			
				K290S	(118)			
	Diminished	IgG1	Cancer	F243L/R292P/Y300L	(119)			
				F243L/R292P/Y300L/V305I/P396L	(119)			
				G236A	(120)			
				K326W/E333S	(121)			
ADCP	Enhanced	IgG1	Cancer	C130S/C136S/C139S/P148S	(122)			
				C226S/C229S/E233P/L234V/L235A	(123)			
				S298N, S298V, or S298D	(118)			
	Diminished	IgG1	Cancer	D265A	(113)			
				ID, RA, Cancer	(124)			
				M252Y/S254T/T256E				
			IgG3	L234A/L235A/P329S	(125)			
			IgG4	L235A/G237A/E318A	(126)			
			Cancer	G236A	(120)			
			Cancer	S239D/I332E	(114)			
CDC	Enhanced	IgG1	Cancer	S239D/A330L/I332E	(114)			
				C226S/C229S/E233P/L234V/L235A	(123)			
				K326W	(121)			
				E333S	(121)			
				T256N/A378V/N434Y ^a	(127)			
	Diminished	IgG1	Cancer	T256N/A378V/S383N/N434Y ^a	(127)			
				P228L/T256N/A378V/N434Y ^a	(127)			
				P230S/N315D/M428L/N434Y ^a	(127)			
				K320E/Q386R ^b	(128)			
			IgG2	K326W/E333S	(121)			
Half-life	Increased	IgG1	ID, RA, Cancer	S239D/A330L/I332E	(114)			
				C226S/C229S/E233P/L234V/L235A	(123)			
				D270A	(129)			
				K322A	(129)			
				P329A	(129)			
				P331A	(129)			
				T307A/N315D/A330V/E382V/N389T/N434Y	(127)			
				N315D/A330V/N361D/A378V/N434Y	(127)			
				E294Del/T307P/N434Y ^c	(127)			
				M252Y/S254T/T256E	(127)			
Decreased	Decreased	IgG1	Cancer, AID	IgG3	P329S	(125)		
				ID252Y/S254T/T256E	(124)			
				T250Q/M428L	(130)			
				N434A	(131, 132)			
				L235A/G237A/E318A	(126)			
				T307A/E380A/N434A	(131)			
				M428L/N434S	(133)			
				T307A/N315D/A330V/E382V/N389T/N434Y	(134)			
				T256N/A378V/N343Y	(134)			
				N315D/A330V/N361D/A378V/N434Y	(134)			
^a Produced in YB2/0 cells to yield afucosylated antibodies to enhance ADCC.								
^b This construct also includes the T299L mutation that leads to an glycosylated antibody with reduced ADCC activity.								
^c E294Del: residue 294 is deleted in this construct.								
AID, autoimmune disease; ID, infectious diseases; RA, rheumatoid arthritis.								
EU numbering is used in all cases.								

^aProduced in YB2/0 cells to yield afucosylated antibodies to enhance ADCC.^bThis construct also includes the T299L mutation that leads to an glycosylated antibody with reduced ADCC activity.^cE294Del: residue 294 is deleted in this construct.

AID, autoimmune disease; ID, infectious diseases; RA, rheumatoid arthritis.

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within the effector cell that results in the release of cytotoxic granules (see **Figure 2**) that kill the targeted tumor cell. The activating Fc γ Rs are the high affinity Fc γ RI (CD64) that is expressed on immune cells such as macrophages, neutrophils, and dendritic cells; the intermediate affinity Fc γ RIIa (CD32a) that is expressed on macrophages, neutrophils, and Langerhans cells; and the low-affinity Fc γ RIIa (CD16a) that is expressed on NK cells, macrophages, and neutrophils (137).

ADCC has been shown to play an important role in the efficacy of many antibodies that target cell surface proteins for cancer therapy (138, 139), especially through the interaction with the Fc γ RIIIa receptor. The allotype (158V) of Fc γ RIIIa binds IgG with a higher affinity and shows increased ADCC activity compared to the low-affinity allotype (158F) (140). For instance, a correlation exists between the clinical efficacy of **rituximab**, **trastuzumab**, and **cetuximab** administered to cancer patients with a homozygous allotype 158V (141–144). ADCP is a similar effector function that results in phagocytosis instead of the release of granules from the effector cell. Effector cells that are capable of phagocytosis, such as macrophages, can mediate both ADCP and ADCC against targeted tumor cells (145). Increasing the ability of a therapeutic antibody to elicit ADCC and ADCP is advantageous in many applications for cancer therapy where ADCC and ADCP are known to play important roles in eliminating the tumor. An IgG1 named 3F2-3M that is specific for the receptor tyrosine kinase EphA2 and contains three mutations (S239D/A330L/I332E) in the Fc domains demonstrated enhanced ADCC activity against a panel of three EphA2-expressing malignant cells regardless of the Fc γ RIIIa allotype of the peripheral blood mononuclear cells that were used as effector cells (116). No ADCC was observed against a malignant cell line not expressing EpHA2. These same mutations in a **trastuzumab** variant showed increased ADCC compared to wild-type **trastuzumab** irrespective of HER2 expression levels and Fc γ RIIIa allotype (114). A different triple mutant S298A/E333A/K334A of **trastuzumab** showed similar effects (113). These studies are important because they suggest that these mutant antibodies can be used effectively in all patients, not just those with certain allotypes. Furthermore, the S239D/A330L/I332E triple mutant of **trastuzumab** and **rituximab** also showed enhanced ADCP (114).

CDC is initiated when the Fc region of an antibody bound to a cancer cell binds the C1q protein, which triggers a cascade of events that culminates in the formation of the MAC that forms transmembrane channels in the cell membrane of the malignant cell leading to cell death. CDC has also been shown to be a mechanism of action of some therapeutic antibodies (138, 139). In these cases, Fc engineering to enhance the CDC activity of an antibody can be advantageous. For example, **rituximab** with single mutations (K326W or E333S) has shown increased CDC (121). It is important to note that an increase in CDC was not always observed with mutations of K326 to other amino acids besides tryptophan (W). This serves as a cautionary note that the amino acid substitution can be critical and should be considered carefully.

For antibodies targeting checkpoint inhibitors such as PD-1/PD-L1, the induction of ADCC, ADCP and/or CDC would

potentially lead to the destruction of normal immune cells. For this specific application, the therapeutic antibody functions to block the inhibitory signal leading to the induction of an immune response. For instance, **nivolumab** is a human IgG4 isotype with an S228P mutation, which replaces a serine (S) residue in the hinge region with a proline (P) that prevents Fab arm exchange with endogenous IgG4 antibodies, while retaining the low affinity for activating Fc receptors associated with wild-type IgG4 antibodies. In fact, no *in vitro* ADCC or CDC activity was observed with **nivolumab** in assays using PD-1-expressing activated T cells as target cells (146).

Human IgG1 has also been modified to attenuate the effector functions. For instance, abatacept (Orencia[®]) is an FDA-approved fusion protein consisting of the external domain of human CTLA-4 and the Fc region of human IgG1 that contains four mutations: C130S/C136S/C139S/P148S. Abatacept does not induce ADCC against the human B cell line PM-LCL that expresses CD80 and CD86, which interact with CTLA-4 (122). Note that in case of abatacept binding to the targeted cells occurs *via* CTLA-4 and not *via* a variable region of an antibody. These constructs are known as immunoadhesins because they have an adhesive molecule linked to an antibody Fc fragment. Another example is an Fc-engineered anti-CD70 IgG1 that contains five mutations (C226S/C229S/E233P/L234V/L235A) and shows decreased ADCP activity (123). Furthermore, a human IgG3 targeting the transferrin receptor 1 (TfR1) containing only two mutations (L234A/L235A) showed decreased ADCC activity against TfR1 expressing target cells, an effect that was increased by the addition of a third mutation P329S (125). However, the P329S single mutant showed no effect on ADCC (125).

Similar to ADCC and ADCP, there are instances where the induction of CDC may be harmful. For example, CDC has been associated with injection site reactions (147). Additionally, it has been reported that complement activation may interfere with the induction of ADCC (148). Therefore, for antibodies that have CDC-related toxicities, eliminating the ability of the antibody to elicit CDC would be advantageous. For example, the abatacept fusion protein containing the four mutations C130S/C136S/C139S/P148S that lacks ADCC activity as discussed above, also does not induce CDC against a human B cell line, PM-LCL (122). However, for antibodies in which ADCC is a major antitumor mechanism, a mutant that elicits ADCC but not CDC may be the most efficacious. The C1q binding “epicenter” of human IgG1 has been localized to D270, K322, P329, and P331 (129). Point mutations at any one of these sites in rituximab decreased CDC activity, but not ADCC or binding to FcRn (129). In a human IgG3 targeting TfR1, the P329S mutation abolished CDC activity against TfR1 expressing target cells, but no impaired ADCC was detected with this single mutation (125).

Glycoengineering

Glycosylation of an antibody can also alter its function. Asparagine-297 (N297) in the C_H2 domain is conserved among the IgG subclasses (149). Glycosylation at this residue stabilizes the Fc region and keeps it in an open conformation (150). This glycosylation is critical for binding to the activating Fc γ Rs and

the induction of ADCC. However, if glycosylation is completely eliminated at this site, the C_H2 domains collapse inward and binding to Fc_γRs is lost (149). For example, mutation of this residue to alanine (N297A) eliminates glycosylation and results in an antibody that is unable to bind activating Fc_γRs (151). Mutation of the nearby residue threonine 299 to leucine (T299L) leads to a similar effect since T299 is considered to be part of the “glycosylation motif” that is important for glycosylation of this residue (N297) (128). Antibodies containing the T299L mutation are unable to elicit ADCC against cancer cells (128).

Modulation of the specific carbohydrate composition at N297 can have the opposite effect and enhance the ADCC activity of the antibody. The affinity of an antibody for the activating Fc_γRs depends on the composition of the N297 N-linked oligosaccharide (152). There are 32 different possible combinations of oligosaccharides that can occur at this site (150). Naturally occurring human IgG and those produced by hybridomas or other common expression systems (including murine myeloma cells such as SP2/0-Ag14, P3X63Ag8.653, and NS0/1; CHO cells; and HEK cells) are usually composed of *N*-acetylglucosamine (GlcNAc) and three mannose residues that form a core carbohydrate. This core is attached to two additional GlcNAc groups to form biantennary branches (150). The addition of galactose at each branch can occur as well as the terminal addition of sialic acid to these galactose molecules. Fucose is often part of the core GlcNAc. This fucose, through steric hindrance, obstructs the interaction of the antibody with the Fc_γRIIIA (149, 150). Thus, elimination of this fucose molecule while maintaining other forms of glycosylation at this site increases the binding of the antibody to the activating Fc_γRs, enhancing its ability to elicit ADCC and ADCP (152, 153).

Elimination of the fucose at N297 can be achieved by glycoengineering through various methods to produce afucosylated antibodies that have enhanced ADCC capabilities. Examples are shown in **Table 4**. The use of an expression system that is unable to attach fucose molecules to the antibody is a common way to produce afucosylated antibodies. *FUT8* encodes the fucosyltransferase enzyme that is responsible for the addition of fucose during protein synthesis. Thus, cells that lack or express low levels of this enzyme produce proteins that lack fucosylation. Rat YB2/0 cells are commonly used for this purpose and many different wild-type and Fc-mutated antibodies that show increased ADCC activity have been produced in these cells (127, 134, 154, 155).

Alternatively, siRNA can be used to knock down the expression of this enzyme in commonly used expression systems or exogenous expression of various other glycosyltransferases can force a specific type of glycosylation at N297, both leading to afucosylation at this residue. For example, overexpression of the glycosyltransferase β (1,4)-*N*-acetylglucosaminyltransferase III (GnTIII), which catalyzes the addition of bisecting GlcNAc, in CHO cells yielded an IgG with reduced core fucosylation and enhanced ADCC (161). This technology is now known as GlycoMab (162). **Obinutuzumab**, produced using the GlycoMab Technology, was approved by the FDA in 2013 as part of a combination therapy for previously untreated patients with chronic lymphocytic leukemia and later in 2016 as part of a combination therapy for patients with follicular lymphoma that are refractory to or have relapsed after treatment with **rituximab** (168, 169). Metabolic interference with host biosynthesis pathways through the addition of kifunensine, an inhibitor of the *N*-linked glycosylation pathway, to the growth medium during production can

TABLE 4 | Glycoengineering examples to enhance ADCC.

Cell line	Species and cell type	Description	Reference
YB2/0	Rat hybridoma (B lymphoblast)	Low natural <i>FUT8</i> expression levels	(127, 134, 154)
Ms704	Hamster ovary (CHO/DG44 variant)	<i>FUT8</i> knock out	(156)
LEC13	Hamster ovary (CHO variant)	Deficient in GDP-mannose 4,6 dehydratase (GMD)	(157)
CHO	Hamster ovary	Fucosyltransferase-deficient (Biowa Potelligent Technology)	(158)
CHO	Hamster ovary	siRNA knockdown of α 1,6 fucosyltransferase	(159)
CHO	Hamster ovary	Bisected, afucosylated carbohydrates by exogenous co-expression of β 1,4- <i>N</i> -acetylglucosaminyltransferase III and Golgi α -mannosidase II	(160)
CHO	Hamster ovary	Overexpression of GnTIII (GlycoMab Technology)	(161, 162)
HEK293F	Human embryonic kidney	Addition of kifunensine to growth medium to inhibit the <i>N</i> -linked glycosylation pathway	(163)
HEK293-EBNA	Human embryonic kidney	Exogenous transient expression of chimeric protein, a fusion between the catalytic domain β 1,4- <i>N</i> -acetylglucosaminyltransferase III and the localization domains of Golgi-resident enzymes	(164)
Strains B1868/4 and B1868/7	<i>Tetrahymena thermophile</i> (ciliate)	Altered glycosylation pattern including lack of fucose	(165)
Lemna Expression System (strain 8627)	<i>Lemna minor</i> (plant)	siRNA α 1,3-fucosyltransferase and β 1,2-xylotransferase	(166)
Strains YAS309	<i>Pichia pastoris</i> (yeast)	Expression of <i>Kluyveromyces lactis</i> UDP-GlcNAc transporter, α 1,2 <i>Mus musculus</i> Mnsl, β 1,2 GlcNAc transferase I, β 1,2 <i>Rattus norvegicus</i> GlcNAc transferase II, <i>Drosophila melanogaster</i> Mnsl, <i>Schizosaccharomyces pombe</i> Gal epimerase, <i>Drosophila melanogaster</i> UDP-Gal transporter, <i>Homo sapiens</i> β 1,4 galactosyl transferase	(167)

CHO, Chinese hamster ovary; HEK, human embryonic kidney.

also result in antibodies with low fucose levels and enhanced ADCC activity. Other eukaryotic expression systems can also be glycoengineered to produce antibodies with low fucose levels (165–167).

Mutations to Alter Half-Life

Altering the interaction between FcRn and antibodies may lead to the development of antibodies with higher efficacy due to altered pharmacokinetic and pharmacodynamic properties. Binding of human IgG to FcRn is pH dependent where binding occurs within a pH range of 6–6.5 and release occurs at pH 7.0–7.5 (170–172). IgG is taken up by endothelial cells through pinocytosis, binds the FcRn in endosomes where the pH is 6.0–6.5, and is recycled back to the cell surface where the IgG is released due to the neutral pH of blood. If an antibody does not bind the FcRn, it is routed to the lysosomal pathway where degradation occurs. Thus, binding of IgG to the FcRn is important to prolong the half-life since this interaction rescues the antibody from degradation. For this reason, the FcRn is known as the “salvage receptor.”

The serum half-life of human IgG1, IgG2, and IgG4 is 21 days, while that of IgG3 is 5–7.5 days (Table 2). Human IgG3 differs from human IgG1 at residue 435. IgG3 contains arginine (R), while IgG1 contains histidine (H) at this position. Both isotypes show pH-dependent binding to A375 human melanoma cells expressing the human FcRn α chain and that transport of both classes is equally efficient in an *in vitro* transport model using these cells (135). However, when both subclasses are present, IgG1 inhibits IgG3 transport leading to its degradation, which can help to explain its shorter half-life. An R435H variant of IgG3 shows similar half-life to human IgG1 (135).

Increasing the serum half-life of IgG1 can also be advantageous to reduce the frequency of administration of the treatment and enhance efficacy. Residues I253, S254, H435, and Y436 play a relevant role in binding of IgG to FcRn since single alanine (A) substitutions at any of these residues substantially decreases binding to the FcRn (113, 173). Using random mutagenesis and a pH-dependent phage display for selection, numerous mutations have been identified that showed increased binding to human FcRn and increased persistence in human FcRn transgenic mice (127). An N434A mutant of a humanized IgG1 antibody showed increased half-life in cynomolgus monkeys (*Macaca fascicularis*) compared to the wild-type IgG1 antibody targeting the B-cell surface receptor (132). This mutant had increased binding affinity to human and monkey FcRn at pH 6, but negligible binding to FcRn at pH 7.4. Mutant N434W had increased binding at both pH and did not show this increase in half-life, demonstrating the pH-dependent binding of IgG to FcRn (132). The triple mutant M252Y/S254T/T256E (YTE) of an anti-respiratory syncytial virus IgG1 also shows pH-dependent binding including increased binding to human and cynomolgus monkey FcRn at pH 6.0 and efficient release at pH 7.4, which results in a four-fold increase in serum half-life in cynomolgus monkeys (124). Furthermore, this YTE mutant showed increased serum half-life in healthy humans (174).

There may be cases in which a reduction in serum half-life may be advantageous. For example, if the antibody is conjugated to a toxic compound, a longer half-life may lead to more unwanted side effects. For example, the I253A mutant of trastuzumab did

not bind FcRn *in vitro* and showed enhanced clearance in human FcRn transgenic mice (131). However, it should be noted that other human IgG1 mutants P257I/Q311I, P257I/N434H, and D376V/N434H specific for tumor necrosis factor-alpha (TNF- α) that showed increased *in vitro* binding to human, cynomolgus, and mouse FcRn at pH 6 and no binding at pH 7.4, paradoxically had increased clearance in CD-1 and C57BL/6 mice (136). The serum half-life of these mutants (P257I/N434H and D376V/N434H) was similar to that of the wild-type IgG1 in cynomolgus monkeys (136).

CONCLUDING REMARKS AND FUTURE DEVELOPMENTS

In the last 40 years, after the seminal work by Köhler and Milstein, academic laboratories and the biopharmaceutical industry have achieved remarkable progress in the engineering and clinical development of therapeutic antibodies, leading to the approval of 21 antibodies for diverse indications in oncology. V region discovery and engineering platforms have evolved from selecting and developing mouse and rat monoclonal antibodies to engineering chimeric antibodies by joining rodent V regions with human C regions, to humanized antibodies by rodent CDR grafting into human FRs, to fully human antibodies developed *via* phage display or transgenic animals. Fully human antibodies have a higher human content, with some antibodies being in the germline gene configuration and, thus, in principle having minimal immunogenicity.

Although these technology platforms have matured, additional incremental but important improvements are still in progress. For instance, humanization of the CDRs is leading to humanized antibodies indistinguishable from fully human antibodies. Better and more diverse phage display antibody libraries are now available. These libraries are built with variable regions with high expression levels in production cells, highly soluble and more stable than the initial naïve libraries composed of the entire repertoire of human antibodies, some of which may not be developable. Importantly, humanization platforms and phage display methodology are now off patent thus, becoming commodities, which make them accessible to academic laboratories and small biotech companies at a lower cost, fostering more innovation and further exploration of diverse and novel targets. In addition, other platforms such as yeast display have been developed, which allows for efficient selection of high affinity antibodies and full IgG with diverse Fc isotypes and glycosylation. More efficient transgenic mice and transgenic species other than the mouse, such as the chicken, have been generated, which can tackle some of the limitations of the early transgenic mice and expand the possibilities of obtaining antibodies targeting conserved epitopes in human and murine orthologs.

On other hand, in the last 15 years, as more antibodies have been approved for therapeutic settings and many failed to perform in preclinical development and clinical trials, a set of design principles and experimental methods have been implemented to ensure further development or manufacturing of antibodies with therapeutic potential. This has been particularly important for therapeutic antibodies generated *via* phage display and related

enriching technologies. Perfecting predictive algorithms to spot in the design phase potential developability issues and applying robust developability experimental methods as early as possible in the antibody discovery phase to select the molecules to further develop should reduce costs and enable more companies to advance fully human antibodies faster and with a higher probability of success in clinical development.

In parallel with the advances in the V region engineering, the Fc has been extensively modified to enhance or attenuate ADCC, ADCP, and/or CDC and thus, tailor the effector functions of therapeutic antibodies to diverse MOAs. Modifications in the residues interacting with the FcRn to extend the half-life of antibodies have been also reported. These modifications are having an impact on dosage and cost of goods, with the ultimate benefit for the treatment of patients. Several of the Fc modifications are being validated in preclinical development and clinical trials and hence it is expected that more therapeutic antibodies with engineered Fc mutations will be approved soon. Beyond IgG, the class of choice for all the approved therapeutic antibodies used for oncologic applications, other classes of antibodies such as IgA (175) and IgE (176) are emerging as new options for cancer therapy. These new options, together with the outstanding

progress in the development of antibody engineering methods to modify the V regions should lead to a profound impact in the therapy of cancer in the near future.

AUTHOR CONTRIBUTIONS

JA and MP devised the concept of the review and contributed to the overall direction of the manuscript including the general writing, preparation of the figures, and editing processes. JA contributed the sections on antibody engineering. TD-W contributed to the writing and editing of the manuscript with an emphasis on the Fc-engineering section. SP-T and JA contributed the section on the targeted molecules.

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Functionally Active Fc Mutant Antibodies Recognizing Cancer Antigens Generated Rapidly at High Yields

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Monoclonal antibodies find broad application as therapy for various types of cancer by employing multiple mechanisms of action against tumors. Manipulating the Fc-mediated functions of antibodies that engage immune effector cells, such as NK cells, represents a strategy to influence effector cell activation and to enhance antibody potency and potentially efficacy. We developed a novel approach to generate and ascertain the functional attributes of Fc mutant monoclonal antibodies. This entailed coupling single expression vector (pVitro1) antibody cloning, using polymerase incomplete primer extension (PIPE) polymerase chain reaction, together with simultaneous Fc region point mutagenesis and high yield transient expression in human mammalian cells. Employing this, we engineered wild type, low (N297Q, NQ), and high (S239D/I332E, DE) FcR-binding Fc mutant monoclonal antibody panels recognizing two cancer antigens, HER2/neu and chondroitin sulfate proteoglycan 4. Antibodies were generated with universal mutagenic primers applicable to any IgG1 pVitro1 constructs, with high mutagenesis and transfection efficiency, in small culture volumes, at high yields and within 12 days from design to purified material. Antibody variants conserved their Fab-mediated recognition of target antigens and their direct anti-proliferative effects against cancer cells. Fc mutations had a significant impact on antibody interactions with Fc receptors (FcRs) on human NK cells, and consequently on the potency of NK cell activation, quantified by immune complex-mediated calcium mobilization and by antibody-dependent cellular cytotoxicity (ADCC) of tumor cells. This strategy for manipulation and testing of Fc region engagement with cognate FcRs can facilitate the design of antibodies with defined effector functions and potentially enhanced efficacy against tumor cells.

Keywords: antibodies, cancer immunotherapy, mutants, cloning, expression, ADCC, HER2, chondroitin sulfate proteoglycan 4

INTRODUCTION

Monoclonal antibodies for cancer therapy engender a range of anti-tumor functions. These may be defined by Fab region recognition of target antigen epitopes and by engagement of the Fc region with cognate Fc receptors (FcR) on a variety of effector cell subsets. Anti-tumor functions can include direct tumor cell killing through Fab-mediated antagonistic activity (1–6), or modulating the behavior of cytotoxic T cells by checkpoint blockade (7, 8). Furthermore, Fc-mediated effects such as complement-dependent cytotoxicity (CDC) (6, 9, 10), antibody-dependent cellular cytotoxicity (ADCC) (5, 6, 11, 12) and phagocytosis (ADCP) (13) constitute important functions of a large proportion of clinically used antibodies in oncology. As many antibodies can exert multimodal anti-tumor activities influenced by both Fab- and Fc-mediated effects (6, 14), engineering antibodies with defined attributes and delineating their mechanisms of action is important for the generation of effective therapeutic agents.

The Fc portion of the antibody and the nature and affinity of its interactions with FcRs are critical in determining antibody serum and tissue half-life, bio-distribution (15), recruitment, and activation of immune effector cells such as NK cells and macrophages against specific target cells (16). Even though monoclonal antibodies are potent anti-cancer therapeutics, their potency in restricting disease progression may be limited by low affinity for activating FcRs, competition with endogenous serum antibodies, low half-life in tissues and interactions with the inhibitory Fc γ receptor, Fc γ RIIB, which restricts effector cell functions (17, 18). On the other hand, agonistic antibodies specific for the TNFR family molecules have been reported to be significantly more efficacious if they have higher affinity for the inhibitory receptor Fc γ RIIB and lower affinity for activating FcRs (19, 20). This further highlights the crucial importance of assessing the contributions of the Fc region in different antibody immunotherapy approaches for cancer and the unmet need for novel Fc-engineered antibody variants to serve the purposes of specific strategies. Protein engineering methods utilizing structural bioinformatics and computational design have identified amino acid residues that can play key roles in enhancing antibody FcR-binding and Fc-mediated functional capabilities (21). Alternatively, abrogation of antibody binding to FcRs and abolition of Fc-mediated effector functions could be a useful approach for the design of antibodies with direct agonistic or antagonistic activities where effector cell engagement may not be desirable (22–25). Developing a strategy to easily manipulate antibody Fc regions can help to dissect the mechanisms of action of anti-cancer antibodies, allowing analysis of Fab-mediated functions in the presence or absence of Fc-mediated activities. Such approaches could find a broad applicability in therapeutic antibody engineering and translation (26–28).

In vitro and *in vivo* potency and mechanistic evaluations of engineered antibodies and their downstream applications in cancer research are heavily dependent on the availability of sufficient quantities of high quality functional material generated from expression systems such as human embryonic kidney (HEK293), Chinese hamster ovary (CHO), and mouse myeloma

(SP2/0, NS0) cells (29–31), mostly utilizing variable regions derived from hybridoma (32, 33) or phage display technologies (34). Current approaches largely rely on the generation of stable expressing cell lines, and do not include efficient built-in tools for sequence engineering and mutagenesis, which may be lengthy and labor-intensive (35).

We previously reported the design and implementation of a single dual expression vector system combined with efficient insertion of any antibody variable and constant regions through polymerase incomplete primer extension (PIPE) cloning. We showed that this can facilitate antibody production by human embryonic kidney (HEK293F) cells (36, 37). In this study, by employing a novel cloning approach based on PIPE combined with simultaneous point mutagenesis, we generate monoclonal antibodies specific for tumor-associated antigens with modified Fc domains designed to alter interactions with immune effector cells. Most well established mutagenesis cloning methods require a two round PCR method or cannot be applied to large plasmids without increasing the risk of random amplification error (37–40). Our study represents an improvement of traditional PCR mutagenesis methods by offering efficient mutagenesis (requiring one round of PCR only), combined with enzyme-free cloning for the generation of large expression-ready constructs (over 8,000 kb). We also designed this system to allow generation of different versions of the same antibody construct. This could find wide applicability for functional and translational studies and could be applied to any IgG1 antibody due to the universal nature of the mutagenesis approach we are employing. To our knowledge, this is the first antibody production platform that combines generation and functional validation of high yields of specific Fc mutant antibodies. With this strategy, we aim to design agents with defined effector functions in a substantially shorter timeframe, employing small culture volumes and at significantly higher yields.

MATERIALS AND METHODS

Isolation of Human Immune Cells

Peripheral blood was obtained through the UK National Health System (NHS) Blood and Transplant system from anonymous donor leukocyte cones. NK cells were isolated using RosetteSep™ Human NK Cell Enrichment Cocktail (STEMCELL™ Technologies), according to the manufacturer's instructions.

Cell Culture

All tumor cell lines were sustained at 37°C in a humidified atmosphere in 5% CO₂, unless otherwise specified. Cell culture medium was supplemented with 10% fetal calf serum (FCS, Thermo Fischer Scientific), unless otherwise specified. Adherent cells were detached using 0.25% Trypsin-EDTA except for cancer cell lines expressing the trypsin sensitive antigen chondroitin sulfate proteoglycan 4 (CSPG4), which were detached using 5 mM EDTA solution in phosphate buffered saline (PBS). The cell lines BT-474 (invasive ductal carcinoma, primary site derived), SK-BR-3 (invasive ductal carcinoma, metastasis

origin) MDA-MB-231 (invasive ductal carcinoma, metastasis origin), and Hs 578T (breast carcinoma, primary site derived) were purchased from the American Tissue Culture Collection (ATCC) and cultured in DMEM GlutaMAX™ (Thermo Fischer Scientific). The cell lines HCC1954 (invasive ductal carcinoma, primary site derived), MDA-MB-231 HTB-26 (human breast adenocarcinoma, metastasis origin), ZR-75-30 (invasive ductal carcinoma, metastasis origin) and BT-549 (invasive ductal carcinoma, lymph node metastasis origin) were purchased from ATCC and cultured in RPMI GlutaMAX™ (Thermo Fischer Scientific). MDA-MB-231-CSPG4⁺⁺⁺ cells were generated in-house by knocking in the coding sequence of the full-length tumor-associated antigen CSPG4 (GenBank accession number BC172576) and cells were cultured as the wild type (WT) cells. Expi293F cells (Thermo Fischer Scientific) were grown on a Stuart orbital shaker (model SSL1) (41), at 125 rpm in 8% CO₂ in serum-free Expi293 expression medium (Thermo Fischer Scientific).

Generation of Fc Mutant Antibodies

The amino acid sequences of the trastuzumab heavy and light variable regions were obtained from the DrugBank database (www.drugbank.ca), translated in nucleotide sequences and manually codon optimized for a human expression host. Optimized sequences were synthesized using GeneArt Gene Synthesis (Thermo Fischer Scientific). The variable region fragments of trastuzumab were then cloned into pVitro1-hygro-mcs vector as previously described (36). PIPE PCR was then performed using the ready cloned WT antibody pVitro1 constructs as a template and mutagenic PIPE primers to generate four linear fragments of the construct with 5' PIPE overhangs ("sticky ends" for PIPE cloning) and the desired point mutations (Table 1). The PCR reagents are listed in Table S1 in Supplementary Material. The cycling conditions used for execution of the mutagenic PCRs were as described before (36), but with varying extension times (Table 2), depending on the length of the fragment, optimized to favor the incomplete extension. The PCRs were performed on a ProFlex 3 × 32-well PCR System thermal cycler (Thermo Fischer Scientific). The amplified DNA fragments were subjected to DpnI (New England Biolabs) digestion for 2 h at 37°C. Following the digestion, the four PCR products were diluted three times with deionized water, then mixed unpurified in a ratio of 1:1:1:1, incubated at room temperature for 30 min to overnight and between 4 and 10 µL of the mixture were transformed into One Shot TOP10 chemically competent *E. coli* cells (Thermo Fischer Scientific), according to the manufacturer's instructions. Successful cloning was confirmed by Sanger sequencing (Source BioScience Sanger Sequencing Service, UK).

Production of Monoclonal Antibodies

Expi293F cells were transfected with the pVitro1-hygro-mcs antibody construct using the ExpiFectamine293 Transfection kit (Thermo Fischer Scientific) as per manufacturer's instructions. Transfection was carried out in Expi293 Expression Medium (Thermo Fischer Scientific). Supernatants of Expi293F cells transfected with an indicated antibody construct were

harvested, spun down at 300 × g for 5 min and then at 3,100 × g for 40 min and filtered using a 0.45 µm membrane. For comparative studies, anti-CSPG4 WT IgG1 antibody (anti-CSPG4 WT) was produced using the previously described HEK293-F method (36), as follows: starting with transient transfection and using 30 mL cultures in 125 mL shaker flasks for 2 weeks under Hygromycin B (Thermo Fischer Scientific) selection, those were subsequently scaled up to 500 mL in 1 L shaker flasks. The

TABLE 1 | PCR primers for generation of anti-HER2 and anti-CSPG4 Fc mutant antibodies.

Primer name	Sequence 5'→3'	Mutation	Amplified fragment
F-DE-1	CAGCACCTGAACTCCTGGGGGG ACCG <u>GAC</u> GTCTTCCTCTCCCC	S239D	DE F1
R-DE-1	GGGGCTGCCCTTGGCTTGAGATG GTTTCTC <u>TC</u> GGGGGCTGG	I332E	DE F1
F-DE-2	CCAGCCCC <u>GAA</u> GAGAAAACCATC TCCAAAGCCAAGGGCAGCCCC	I332E	DE F2
R-pVitro-1-Kappa	ACCGCGGCTAGCTGGAACCC AGAGCAGCAGAAACCCAATG	–	DE F2
F-pVitro-VL-Univ	CATTGGGTTCTGCTGCTGCTG GGTCCAGCTAGCCGGGT	–	DE F3
R-DE-3/ R-NQ-3	GGGGGAAGAGGAAGACGTCGG TCCCCCAGGAGTTCAGGTGCTG	–	DE F3; NQ F3
F-DE-4/ F-NQ-4	GTTGCTTGTATTACAACACTGGA GAGAAATGCAGCATGTTGCTGATT	--	DE F4; NQ F4
R-DE-4	GGGGGAAGAGGAAGAC <u>GTCC</u> CG GTCCCCCAGGAGTTCAGGTGCTG	S239D	DE F4
F-NQ-1	<u>CAG</u> AGCACGTACCGGGTGGT CAGCGTCTCACCGTCTGCACCAG	N297Q	NQ F1
R-NQ-1	TGATCTACCCGCGCTCAGCCC TGGGGCATGCTCTCGCGCTGTC	–	NQ F1
F-NQ-2	CGAGGAGGATGCGCCCAAGGG CTGAGCGCGGGTAGATCAGAGCACA	–	NQ F2
R-NQ-2	TACAAAGTGTACCCCTCTAG ACCTGGAAAGACCAGGCGGAGTT	–	NQ F2
F-NQ-3	GCCTGGCTTCCAGGTCTAG AGGGGTAACACTTGTACTGCGTT	–	NQ F3
R-NQ-4	AGGACGGTGAGGACGCGTGACCACC CGGTACGTGCT <u>CT</u> GGTACTGC	N297Q	NQ F4

Underlined nucleotides depict mutated codons; sequences in red font indicate mutated nucleotides.

TABLE 2 | PIPE PCR fragments with accompanying specific PCR extension times for fragment amplification.

Fragment	Approximate size (bp)	Extension time (s)
NQ F1	~2,000	28
NQ F2	~2,000	28
NQ F3	~2,000	28
NQ F4	~2,600	35
DE F1	~350	5
DE F2	~3,150	42
DE F3	~2,800	35
DE F4	~2,400	35

supernatants were harvested after 2 weeks of stable antibody expression without antibiotic selection. Monoclonal antibodies were purified using Pierce™ Protein A Columns, 1 mL (Thermo Fischer Scientific). Antibodies were eluted using acetate elution buffer (0.58% glacial acetic acid, 0.15 M Sodium chloride) and neutralized with 120 µL neutralization buffer (1 M Tris, pH = 9). Purified antibodies were stored in PBS.

Aleuria Aurantia Lectin (AAL) Western Blot

Purified antibody samples (500 ng) were mixed with 4× Laemmli buffer (Bio-Rad) containing 10% 2-mercaptoethanol and incubated at 95°C for 10 min. Subsequently, the reduced samples were run on 4–15% precast polyacrylamide gels (Bio-Rad) and semi-dry blotted using the Bio-Rad Trans-Blot® Turbo™ Blotting System, according to the manufacturer's instruction. The membrane was then cut into two just above 35 kDa to separate the antibody heavy chain (50 kDa) and light chain (25 kDa), using the PageRuler prestained protein ladder (Thermo Scientific) as a reference. Next, the membrane containing proteins above 35 kDa was blocked using Carbo-Free™ Blocking Solution (Vectorlab) and the one containing proteins below 35 kDa—in 5% bovine serum albumin (BSA) solution in PBS 0.05% Tween 20 (PBST). For probing, the over-35 kDa membrane was incubated with 0.2 µg/mL biotinylated AAL in Carbo-Free™ Blocking Solution for 1 h at room temperature and subsequently washed in PBST 3× for 10 min. Next, the membrane was incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fischer Scientific, 1:30,000) for one hour, washed and developed with Enhanced Chemiluminescence (ECL, GE Healthcare). The under-35 kDa membrane was probed with rabbit anti-human-kappa light chain antibody (Abcam, 1:4,000) in PBST 5% BSA for 1 h at room temperature, washed and incubated with anti-rabbit-IgG HRP antibody (Cell Signaling Technology, 1:2,000), washed again, and developed as above. The results were analyzed using the ImageJ software.

Western Blot Semi-Quantitative Analysis

The AAL signal values of the different protein bands (peak area) were normalized to the anti-kappa signal values (LC kappa). The AAL/LC kappa ratio values of anti-HER2 antibody variants were presented as a proportion (percentage) of the value of trastuzumab. Similarly, anti-CSPG4 antibody variant values were presented as a proportion of anti-CSPG4 WT.

Evaluations of Antibody Yields by ELISA

IgG antibody expression by Expi293F cells was monitored using a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, Maxisorp 96-well plates (Thermo Fischer Scientific) were coated overnight with 0.5 µg/mL mouse anti-human IgG1 antibody (Bio-Rad) in carbonate buffer. On the next day, the plate was washed with PBS 0.5% Tween 20 (PBST) and unspecific binding was blocked using 2% skim milk in PBST (blocking buffer). Trastuzumab (Herceptin®, Roche) was used at as a standard at a starting concentration of 3 µg/mL. All samples were diluted (1:1,000) in blocking buffer and incubated on the

plate overnight. The plates were developed on the next day, following an incubation with anti-human IgG-HRP (Sigma) at a dilution of 1:5,000, using o-Phenylenediamine dihydrochloride (Sigma) as the HRP substrate. Optical density was measured using Fluostar® Omega Spectrophotometer (BMG Labtech), at 492 and 650 nm, the latter used to subtract background absorbance. Standard curves were constructed on the Fluostar® Omega analysis software using a 4-parameter fit.

Assessment of Antibody Binding to Cells by Flow Cytometry

Recognition of FcγRs on fresh peripheral blood NK cells and of the target antigen on tumor cells by different anti-HER2 and anti-CSPG4 antibody variants were assessed through flow cytometric binding assays. Cells were detached, as described above, re-suspended in staining buffer (PBS, 2% FCS) and incubated in 96-well round-bottom plates (0.2×10^6 cells per well) on ice in the presence of the serially diluted antibody variants (concentration range 0.008–5 µg/mL) for 30 min on ice. The cells were subsequently washed and incubated with 1 µg per test FITC-conjugated goat anti-human IgG (Vector Laboratories Ltd.) on ice, for another 30 min. After one wash, cells were analyzed on a BD LSRFortessa™ (BD Biosciences), using the High Throughput Sampler (HTS) option. The BD Cytofix/Cytoperm™ (Becton Dickinson) kit was used according to the manufacturer's instructions for Expi293F cell permeabilization and intracellular staining to detect antibody production. The results were analyzed using FlowJo software 7.6.5.

Cell Proliferation Assay

To determine the effect of anti-HER2 antibody variants on cell proliferation, 5,000 (BT-474) or 1,000 (SK-BR-3, MDA-MB-231, HCC1954) cells per well were plated in a 96-cell well tissue culture plates in complete medium and treated with the serially diluted antibodies (concentration ranges 0.0016–25 µg/mL). Cell proliferation was assessed after 96 (BT474, MDA-MB-231) or 120 h (SK-BR-3, HCC1954), using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega), according to the manufacturer's instructions. Cell densities were measured using Fluostar® Omega Spectrophotometer (BMG Labtech).

Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay

ADCC assays were performed using the HER2-overexpressing BT-474 cells as target cells and fresh peripheral blood NK cells as effector cells. NK cells and cancer cells were mixed at E:T ratio 10:1 (0.2×10^6 NK cells: 0.02×10^6 target cells) in RPMI GlutaMAX™ (Thermo Fischer Scientific) containing 2% FCS (Thermo Fischer Scientific). They were incubated in 96-well round bottom plates for 4 h (Thermo Fischer Scientific) in the presence of serially diluted anti-HER2 antibody variants or isotype control antibodies (at concentrations ranging from 64 pg/mL to 5 µg/mL). Cellular cytotoxicity was assessed

using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fischer Scientific), according to the manufacturer's instructions. Absorbance read out was measured at 490 and 680 nm using

Fluostar® Omega Spectrophotometer (BMG Labtech) and % cytotoxicity was calculated as per manufacturer's instructions using the following formula:

$$\% \text{ Cytotoxicity} = \left(\frac{\text{Experimental value} - \text{Effector Cells Spontaneous Control} - \text{Target Cells}}{\text{Target Cell Maximum Lysis Control} - \text{Target Cells Spontaneous Control}} \right) \times 100$$

Half maximal effective concentration (EC50) values at which different anti-HER2 Fc variant antibodies induced ADCC were calculated using the GraphPad Prism software (version 6, GraphPad Software Inc., USA). The data were normalized to a maximal (detergent induced) and minimal (isotype control) cell lysis and analyzed using a non-linear regression model.

Calcium Flux Assay

Freshly isolated human NK cells were incubated with 1 nM Indo-1 calcium indicator (Thermo Fischer Scientific) for 30 min at 37°C in serum-free medium and subsequently washed and rested for 30 min in complete medium (42). Cells (1×10^6 cells per test) were incubated with anti-HER2 and anti-CSPG4 antibody variants at 15 µg/mL for 30 min at 37°C, as above, and re-suspended in 500 µL RPMI GlutaMAX™ (Thermo Fischer Scientific). Before acquisition each sample was warmed up to 37°C for exactly 5 min. The sample was acquired for 30 s to measure basal fluorescence, then the FcRs on the surface of the NK cells were crosslinked by adding 5 µg goat anti-human IgG F(ab')2 (Thermo Fischer Scientific). Each sample was acquired for 5 min on BD LSRIFortessa™ (BD Biosciences) with the violet laser (405 nm) turned off to avoid interference. Ionomycin at 1 µg/mL was used as a positive control. Results were analyzed using FlowJo software version 8.7.

Human Specimens

Blood cone samples from anonymized donors were purchased from the National Health Service Blood and Transplant Service, United Kingdom. Sample processing was supported through a local ethical framework conducted in accordance with the Helsinki Declaration and approved by the NHS Research Ethics Committee, Guy's and St. Thomas' NHS Trust ("Immunopathogenesis and Molecular Biology in Breast Cancer Subtypes," REC reference number: 13-LO-1248).

RESULTS

Antibody Fc Region Manipulation Design

First, we devised a cloning strategy that allows the manipulation of monoclonal antibody Fc regions in order to influence FcRs binding properties and consequently antibody effector functions. Alongside WT equivalents, we generated Fc-mutant variants designed either to have diminished or enhanced binding to FcRs on human immune effector cells, and we exemplified this strategy by engineering panels of antibody clones that recognize two cancer-associated antigens.

We initially employed WT IgG1 antibody heavy and light chain fragments incorporated in pVitro1-hydro-mcs vectors (Figure 1A). We then utilized these vectors as templates in four separate PIPE PCRs in order to generate linear fragments carrying specific Fc region point mutations (Figures 1B,C). Point mutations were introduced using mutagenic PIPE primers (Table 1; Table S1 in Supplementary Material). Mutations were designed to either reduce [N297Q (NQ), Figure 1B] or enhance [S239D/I332E (DE), Figure 1C] binding of the antibody Fc to FcγRs (Fc gamma receptors) on human effector cells. Since Asn297 is a conserved glycosylation site for the human IgG1 Fc domain, we mutated this to Gln. This is expected to lead to aglycosylation and a conformational change which can impair binding to FcγRs (26, 28). By contrast, we designed S239D/I332E (DE) mutated IgG1 Fc regions to have increased binding to the FcγRIII compared to WT IgG1 antibodies.

To generate the N297Q mutant antibody variants designed to have reduced binding to FcRs (Figure 1B), we linearized the pVitro1 WT constructs through PIPE PCR (see Table 1 for primer pairs), yielding Fragments 1, 2, 3, and 4. Fragment 1 was generated to carry CAG (Q) instead of AAC (N) at positions 889–891. Shortened extension times and lack of final extension step favored the PIPE process during the PCR. Therefore, the Fragment 1 final product had 5' PIPE overhangs ("sticky ends") but also carried the desired CAG codon. Similarly, we generated Fragment 4 with 5' overhangs and the desired mutated codon. The other two fragments, Fragment 2 and Fragment 3, were as well amplified with 5' PIPE overhangs but without point mutations.

The S239D/I332E mutants designed to yield antibodies with enhanced FcR binding were generated in a similar manner (see Table 1 for primer pairs). The pVitro1 WT constructs were linearized through PIPE PCR to favor the occurrence of sticky ends for PIPE cloning (Figure 1C). All fragments were amplified with PIPE 5' overhangs; however, Fragment 1 carried both the GAC (D) codon instead of the WT TCA (S) codon, and the GAA (E) codon instead of the WT ATC (I) codon (positions 877–879 and 994–996, respectively); Fragment 2 carried the GAA (E) codon instead of ATC (I) codon; and Fragment 4 carried GAC (D) codon instead of TCA (S) codon.

The primers used for the generation of both N297Q (NQ) and S239D/I332E (DE) Fc mutants are designed to be universal and can be used for the generation of NQ and DE mutant versions of any IgG1 antibody cloned into pVitro1-hydro-mcs (see Table 2 for lengths of the pVitro1 PIPE fragments and extension times for amplifications).

Following amplification, linear pVitro1 fragments were treated with DpnI enzyme to digest any remnants of the pVitro1 WT construct used as a template in the PCR (Figure 1D). All four fragments were then mixed together unpurified, thus eliminating

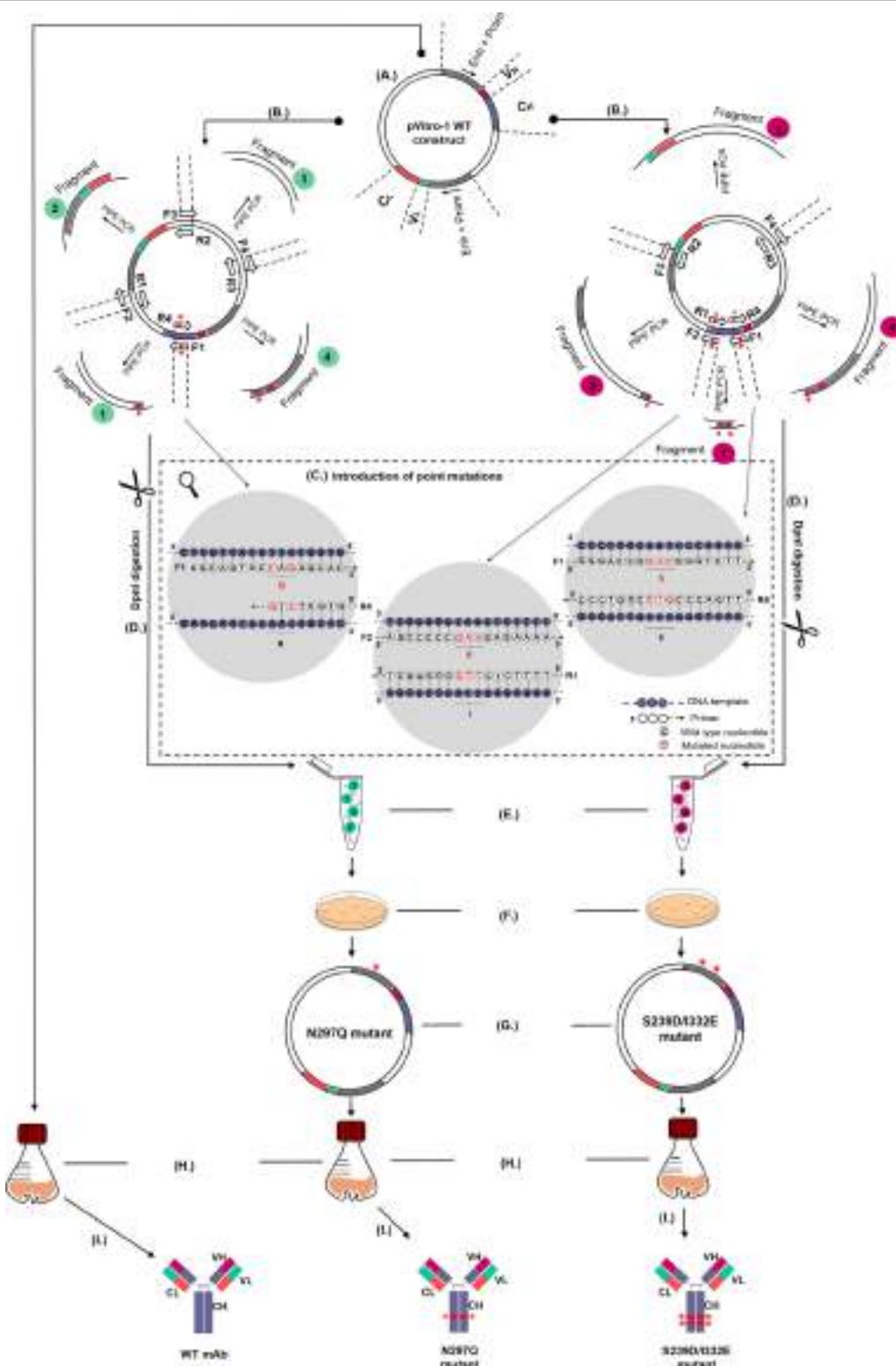


FIGURE 1 | Schematic representation of the pipeline for generation and production of wild-type (WT) and Fc mutant IgG antibodies. **(A)** WT antibody construct in pVitro1-hygro-mcs. **(B)** Polymerase incomplete primer extension (PIPE) PCR linearization and mutagenesis of the WT construct to generate pVitro1 DNA fragments carrying the N297Q (left, fragments 1 and 4) or S239D/I332E (right, fragments 1, 2, and 4) mutations. Mutations indicated by “**”. **(C)** Introduction of mutations in WT constructs through mutagenic PIPE primers. **(D)** DpnI digestion. **(E)** Enzyme-independent assembly of the linear pVitro1 fragments. **(F)** Bacterial transformation of the assembled constructs. **(G)** Confirmation of the insertion of desired mutations. **(H,I)** Recombinant expression in Expi293F cells **(H)** and purification **(I)** of antibody WT and mutant variants.

the need to perform PCR clean up or to use ligation enzymes. The mixtures were incubated at room temperature (**Figure 1E**) and transformed into competent bacteria (**Figure 1F**). Correct transformants were verified through Sanger sequencing (**Figure 1G**). Expi293F cells were transfected with the pVitro1 mutant constructs in 30 mL working volumes (**Figure 1H**) and antibodies were secreted in culture supernatants (**Figure 1I**).

Overall, this strategy facilitates the manipulation of monoclonal antibody Fc regions to allow the generation of mutant versions of any IgG1 antibody clone.

Generation of Anti-HER2 and Anti-CSPG4 N297Q and S293D/I332E IgG1 Mutant Constructs

Using our pipeline, we generated WT, N297Q (NQ), and S293D/I332E (DE) mutant antibodies recognizing two cancer antigens (i) the human epidermal growth factor receptor 2 (HER2/neu) (3), known to be overexpressed by 25% of breast carcinomas and (ii) the CSPG4, reported to be overexpressed in 70% of melanomas,

a proportion of triple-negative breast cancers, and other solid tumors (41, 43, 44). In order to produce anti-HER2 IgG1 WT antibody, we used the variable regions of the 4D5-8 therapeutic antibody clone (trastuzumab) (2, 4, 45). The sequence was manually codon-optimized (Figure S1 in Supplementary Material), and cloned into the pVitro1-hydro-mcs vector (36). The anti-CSPG4 IgG1 WT construct was derived using the variable regions of a murine antibody (46) and cloned into pVitro1-hydro-mcs (36). The pVitro1 WT constructs were used as templates for the generation of the corresponding mutant variants. The DNA products of the mutagenic PIPE PCRs were visualized using agarose gel electrophoresis (**Figure 2**), confirming that the fragments were of the expected sizes.

All four mutant constructs were successfully cloned on first attempt and between 1 and 15 colonies were analyzed for the different constructs (Table S2 in Supplementary Material). The desired mutations were present in all the screened plasmids (each purified from a single colony) and no background contamination with the WT construct was observed. These findings suggest high mutagenesis efficiency for this cloning approach.

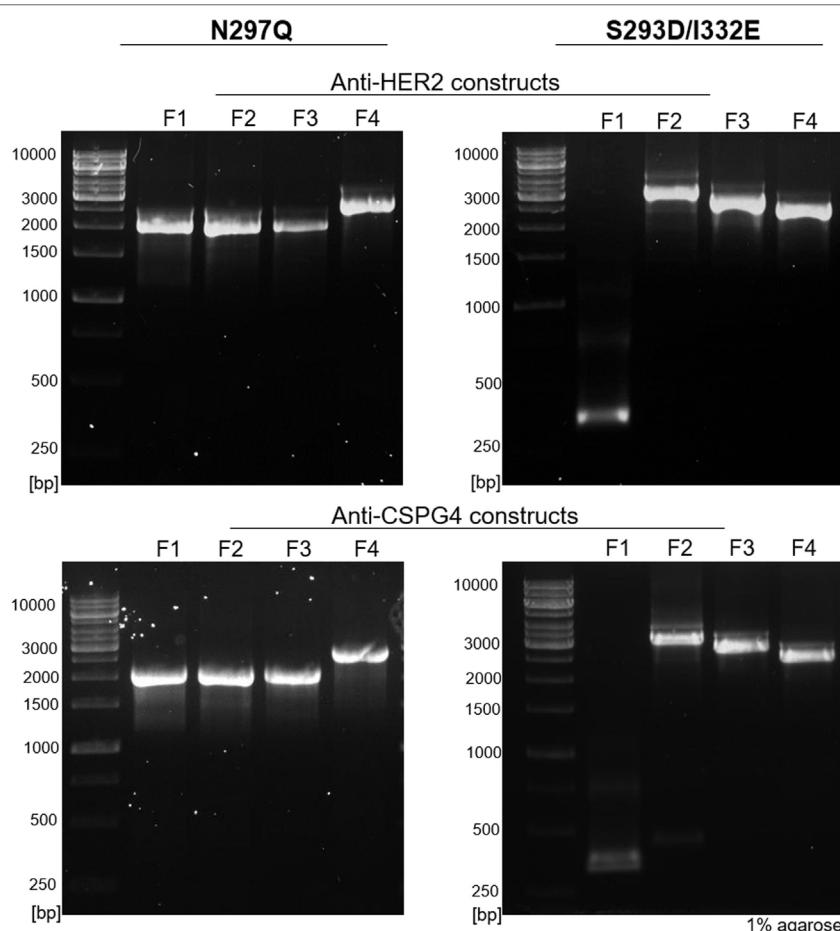


FIGURE 2 | Agarose gel electrophoresis of polymerase incomplete primer extension (PIPE) PCR fragments for the generation of anti-HER2 and anti-CSPG4 N297Q (NQ) and S293D/I332E (DE) Fc mutant IgG1 antibodies. O'Generuler™ 1 kb DNA ladder (Thermo Fischer Scientific) was used as a molecular weight marker. The lanes labeled F1–4 represent the NQ and DE PIPE PCR fragments 1–4, depicted in **Figure 1**. The expected molecular weights (in bp) of each fragment are listed in **Table 2**.

Optimized Antibody Production with the Expi293F Expression System

We produced monoclonal antibodies both by using a previously described HEK293-F system as well as by employing the cloning approach described herein with the human embryonic kidney derivative cell line Expi293F. Antibody production with the previous method in HEK293-F cells was initiated using 30 mL shaker flask cultures under selection for 2 weeks, followed by a 2-week scale up to 500 mL shaker flask cultures. With the previous platform, after 4 weeks, we obtained an average of 13 µg/mL of anti-CSPG4 WT (by ELISA). By contrast, using the present transient expression Expi293F platform, we produced an average of 130 µg/mL anti-CSPG4 WT and 160 µg/mL anti-HER2 WT antibodies using only 30 mL shaker flask cultures volumes within 5–7 days post-transfection (detected by ELISA) (Figure 3A).

To evaluate the rate of transfection efficiency, we used flow cytometry to analyze the expression of IgG by permeabilized Expi293F cells 3 days after transfection with the anti-HER WT, DE, and NQ constructs (Figure 3B). A large proportion ($\geq 98\%$) of transfected Expi293F cells was positive for human IgG, indicating a high transfection efficiency rate. To investigate whether the addition and timing of antibiotic selection (Hygromycin B) to Expi293F cell cultures could affect antibody yields, we expressed anti-HER2 WT antibody with or without the addition of Hygromycin B at different times following transfection and found that addition of Hygromycin B did not affect purified anti-HER2 antibody yields (Figure 3C). To then determine the optimal time to harvest the supernatants, we monitored the concentrations of anti-HER2 WT and anti-CSPG4 WT antibodies over time by ELISA. For both antibodies, the maximum production yields were reached between 5 and 7 days post-transfection (Figure 3D).

We then measured total antibody yields by Expi293 cells after purification of anti-HER2 and anti-CSPG4 variants. We obtained an average of 56 µg/mL anti-HER2 DE, 62 µg/mL anti-HER2 NQ (Figure 4A, left), 44 µg/mL anti-CSPG4 DE, and 73 µg/mL anti-CSPG4 NQ (Figure 4A, right). We then confirmed that Fc mutations did not impair the binding of antibodies to protein A used for antibody purification: no significant differences in protein yields of either anti-HER2 DE (Figure 4B, left) or anti-HER2 NQ variants (Figure 4B, right) were measured after purification by protein A column, or by KappaSelect column, which binds antibody kappa light chains. We concluded that none of the mutations could alter antibody binding to either matrix and, thus, the purification method did not appear to influence antibody yields.

Next, the integrity and purity of different anti-HER2 and anti-CSPG4 antibody variants were confirmed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing (Figure 4C, top) and reducing (Figure 4C, bottom) conditions. All antibodies migrated according their expected approximate molecular weights of ~150 kDa (non-reducing conditions) and no other contaminating proteins or antibody fragments were observed. Under reducing conditions, the heavy chains of the WT and DE variants of both antibodies migrated according to the expected molecular weight of 50 kDa. Anti-HER2 and anti-CSPG4 NQ variants exhibited increased mobility compared with the WT and DE analogs, in accordance

with previous reports (28). The light chains of all the variants were found to be of the expected size of 25 kDa.

To ascertain whether different Fc mutations influence glycosylation and to simultaneously evaluate the fucosylation patterns of the anti-HER2 and anti-CSPG4 variants, we probed purified antibody samples with AAL (fucose specific, Figure 4D). We used an anti-human kappa chain antibody as a loading control (Figure 4D, bottom strips). We observed no AAL signal with the anti-HER2 and anti-CSPG4 NQ variants, confirming that the introduced N297Q amino acid substitution has resulted in lack of core glycosylation (Figure 4D, top panel). We performed semi-quantitative analysis of the AAL Western blot data using the ImageJ software (Figure 4E). The values obtained with the NQ variants were close to background. No significant differences were observed between WT and DE mutant antibodies (both anti-HER2 and anti-CSPG4 variants). These findings suggest that mutating the Fc region of IgG1 at position 297 disrupts a conserved glycosylation site, most likely eliminating core glycosylation, while mutations at positions 293 and 332 do not affect the decoration of the core glycan with fucose.

Therefore, we confirmed the implementation of an optimized Expi293F expression platform with additional features and which outperforms that previously reported. Key attributes are five-fold: namely (i) provision for generation of mutant antibody variants; (ii) high mutagenesis efficiency; (iii) high transfection efficiency (eliminating the need for antibiotic selection); (iv) up to 10-fold higher antibody yields in ~17-fold smaller (30 mL) culture volumes; and (v) 3-fold reduced time frame (12 days) for implementation from concept to purified antibody (Table 3).

Fc Mutants Retain Fab-Mediated Binding to Antigens but Exhibit Differential Fc-Mediated Receptor Recognition Compared with WT Antibodies

We next investigated the target antigen recognition and binding properties of engineered antibody variants against different cancer cell lines expressing cell surface HER2 (Figure 5A; Figure S2 in Supplementary Material) or CSPG4 (Figure 5B). All HER2 variants recognized HER2 on BT-474 (top left), SK-BR-3 (top right), ZR-75-30 (bottom left) and HCC1954 (bottom right) breast cancer cell lines, and bound in a dose-dependent manner and with comparable kinetics to the commercially produced trastuzumab (Herceptin®, Roche). Similarly, for the engineered antibodies recognizing CSPG4 (Figure 5B), no differences were observed in their binding characteristics to the CSPG4-overexpressing CSPG4 knock-in MDA-MB-231 (top left), and natural lower-expressing MDA-MB-231, HTB-26 (top right), Hs 578T (bottom left), and BT549 (bottom right) breast carcinoma cell lines.

We then investigated the binding characteristics of anti-HER2 and anti-CSPG4 Fc variants to FcγRIII, the Fcγ receptor expressed by NK cells (47) (Figure 5C). Fluorescence intensities observed for human peripheral blood NK cells incubated with anti-HER2 (top) and anti-CSPG4 (bottom) NQ variants were similar to those measured for the secondary anti-human kappa chain APC-conjugated antibody control at all concentrations tested, suggesting that the NQ mutation resulted in impaired binding to FcγRIII. Binding of the WT anti-HER2 antibodies, including

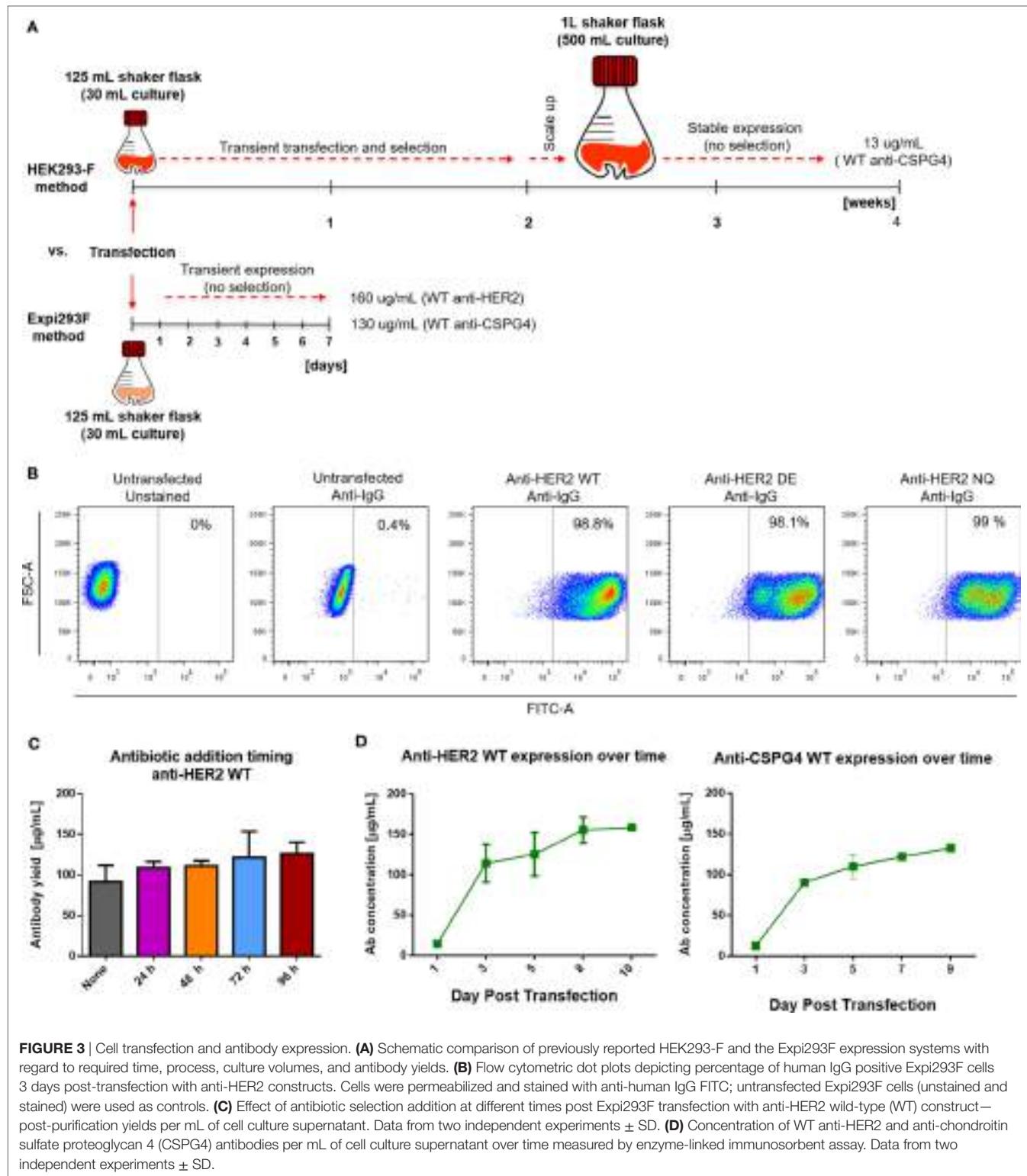


FIGURE 3 | Cell transfection and antibody expression. **(A)** Schematic comparison of previously reported HEK293-F and the Expi293F expression systems with regard to required time, process, culture volumes, and antibody yields. **(B)** Flow cytometric dot plots depicting percentage of human IgG positive Expi293F cells 3 days post-transfection with anti-HER2 constructs. Cells were permeabilized and stained with anti-human IgG FITC; untransfected Expi293F cells (unstained and stained) were used as controls. **(C)** Effect of antibiotic selection addition at different times post Expi293F transfaction with anti-HER2 wild-type (WT) construct—post-purification yields per mL of cell culture supernatant. Data from two independent experiments \pm SD. **(D)** Concentration of WT anti-HER2 and anti-chondroitin sulfate proteoglycan 4 (CSPG4) antibodies per mL of cell culture supernatant over time measured by enzyme-linked immunosorbent assay. Data from two independent experiments \pm SD.

the commercially available trastuzumab (Herceptin[®]) to human NK cells were detected only at the highest concentrations tested (1 and 5 μ g/mL). Similarly, the anti-CSPG4 WT antibody only bound to NK cells at high (5 μ g/mL) concentrations. By contrast,

the anti-HER2 and anti-CSPG4 DE variants displayed up to 7-fold (anti-HER2) and 17-fold (anti-CSPG4) higher binding to Fc γ RIII on NK cells compared with binding of WT antibodies at 5 μ g/mL concentrations to Fc γ RIII (Figure 5C).

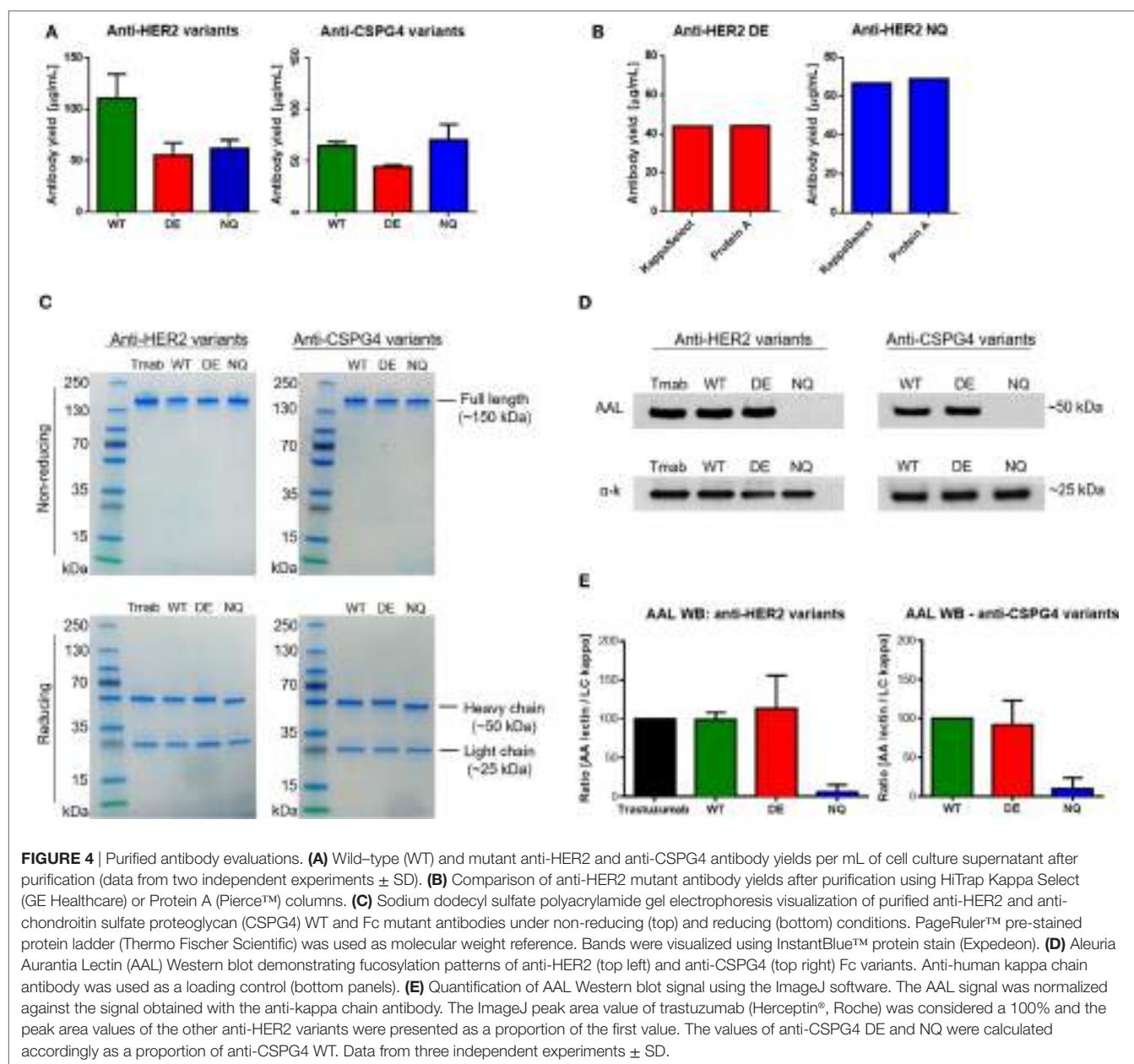


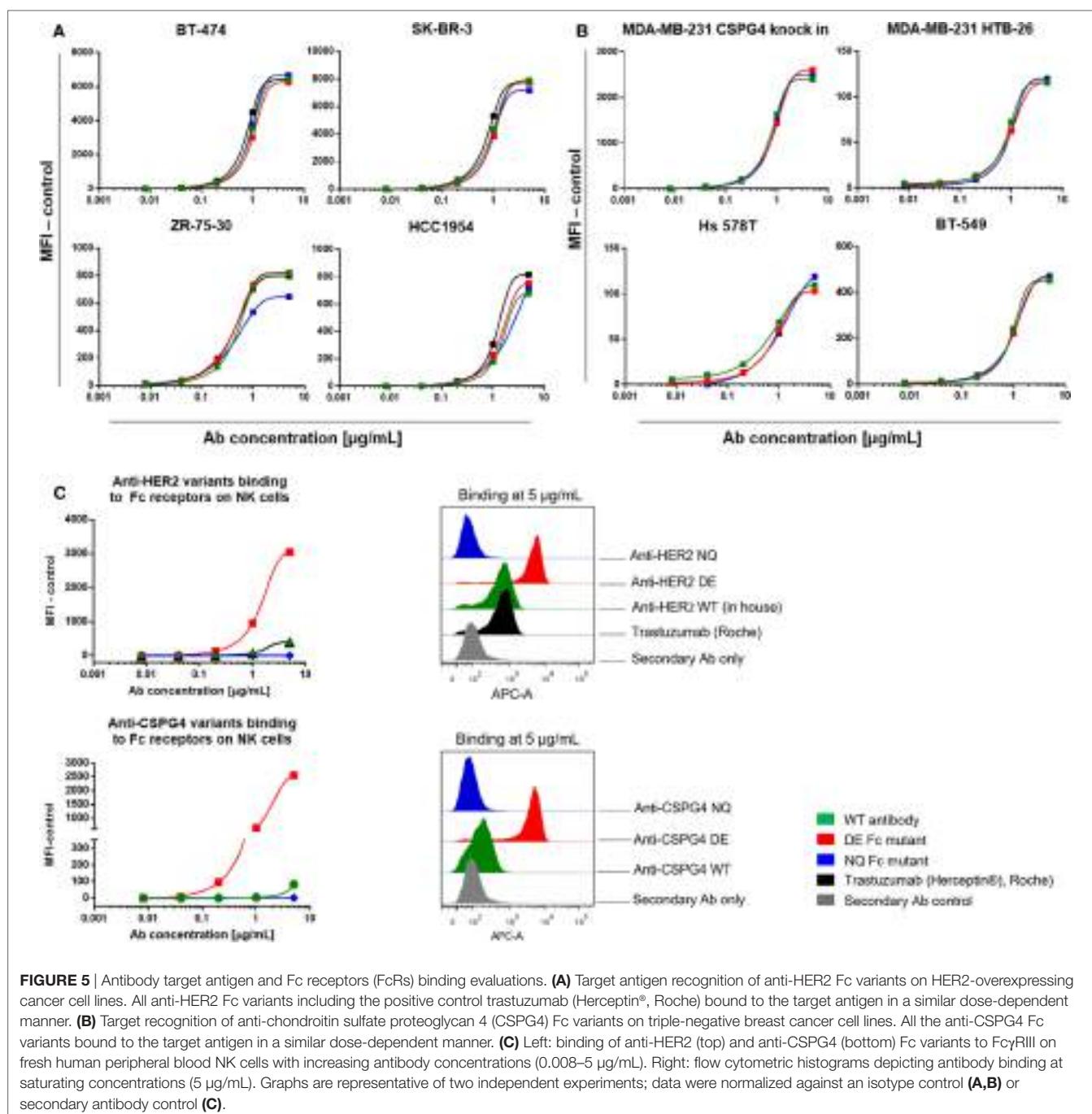
TABLE 3 | Characteristics of the Expi293F vs. the HEK293-F cloning/expression platforms.

Expression host	Expi293F	HEK293-F
Wild-type (WT) antibody polymerase incomplete primer extension (PIPE) cloning	Yes	Yes
Generation of Fc mutants	Yes	No
Expression culture conditions	Serum free	10% FBS
Expression system type	Transient	Stable
Antibody concentration (sup)	130 µg/mL (anti-CSPG4 WT) (anti-CSPG4 WT)	13 µg/mL (anti-CSPG4 WT) (anti-CSPG4 WT)
Working culture volume (expression)	30 mL	500 mL
Time required for cloning/sequencing verification	3 days	3 days
Time required for large scale plasmid DNA production/transfection	2 days	2 days
Time required for expression/purification	1 week	4 weeks
Total time required (whole platform)	12 days	33 days

These findings suggest that while Fc-mutated antibodies retain Fab-mediated recognition of target antigen-expressing cancer cells, DE mutant antibodies have enhanced, and NQ mutants have diminished binding to FcγRIII on human primary NK cells compared with their WT equivalents.

DE Mutant Antibodies Have Enhanced, While NQ Mutants Have Diminished Ability to Induce Fc-Mediated Activation of NK Cells Compared with WT Antibodies

To further assess the ability of antibody variants to engender NK effector cell activation through engagement of FcγRIII, we measured calcium ion influx by cross-linking with polyclonal anti-IgG antibodies to mimic immune complex formation.



No calcium flux was detected in cells not treated with cross-linker (**Figure 6A**, top). The levels of calcium flux following cross-linking of WT antibodies [anti-HER2 WT, trastuzumab (Herceptin®), anti-CSPG4 WT] and NQ antibodies (anti-HER2 NQ and anti-CSPG4 NQ) were all comparable to background levels of control samples given cross-linker only to engage endogenous IgG (**Figure 6**). On the other hand, cross-linking of either anti-HER2 DE or anti-CSPG4 DE antibody variants triggered higher intracellular calcium mobilization compared with all other antibodies (**Figure 6**). These findings, confirmed with NK cells from different human donors (Figure S3 in Supplementary Material),

are consistent with the higher binding attributes of DE mutant variants to FcγRIII on NK cells (**Figure 5C**).

Anti-HER2 Variants Retain Their Ability to Inhibit HER2-Overexpressing Cancer Cell Proliferation but Differ in Their Potency to Trigger NK Cell-Mediated Anti-Tumor Functions

Trastuzumab (Herceptin®) has been reported to directly inhibit the proliferation of HER2-over-expressing cell lines (1, 2, 4, 48).

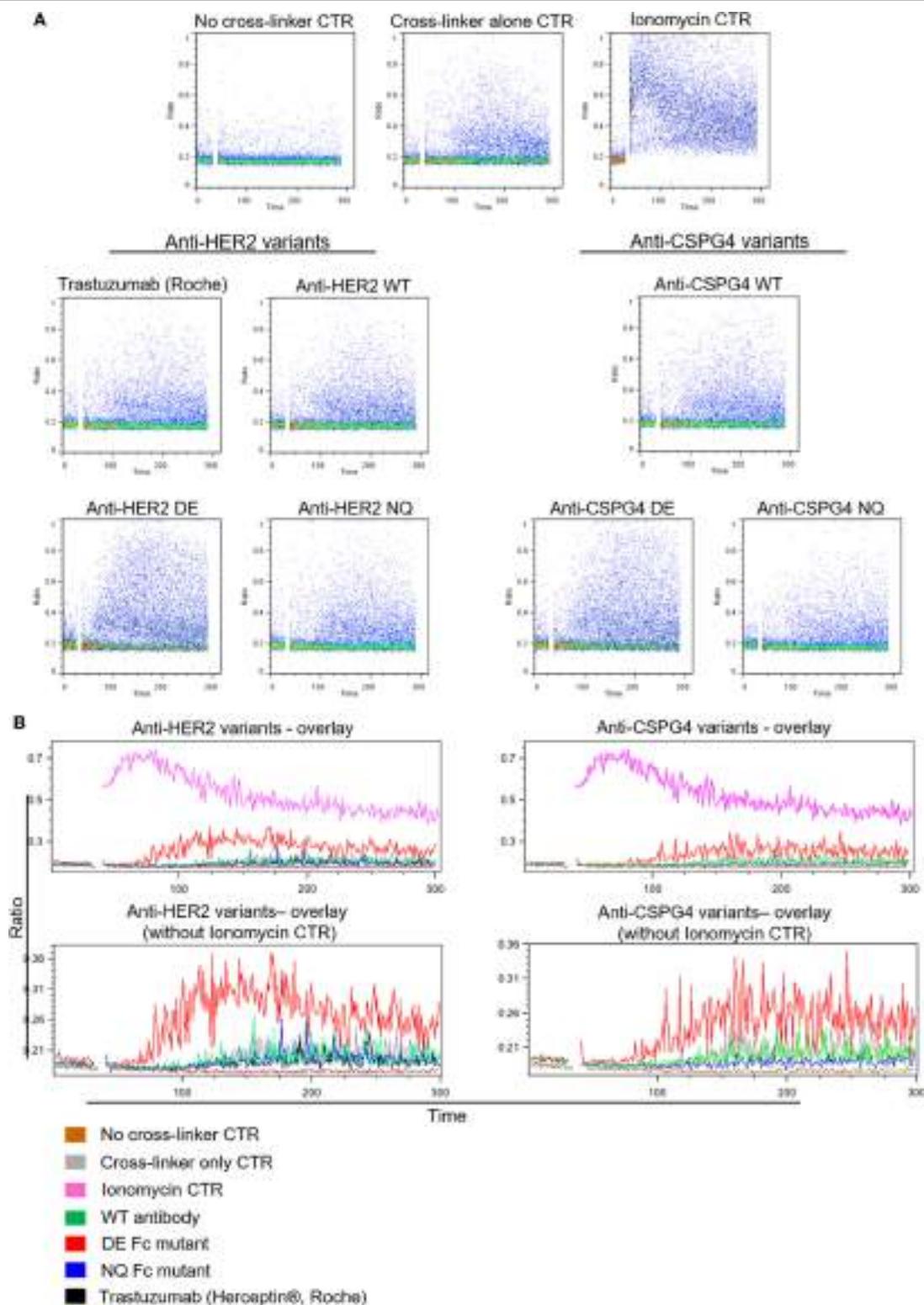


FIGURE 6 | Antibody Fc-mediated calcium mobilization of human NK cells. **(A)** Flow cytometric dot plot graphs of Ca^{++} flux assay measurements showing activation of NK cells pre-incubated with different anti-HER2 Fc (left) and anti-chondroitin sulfate proteoglycan 4 (CSPG4) (right) Fc variants after cross-linking with a polyclonal anti-IgG antibody. Ca^{++} flux into the cells was visualized through the increase in the DAPI/Indo-1 (blue) ratio over time. **(B)** Histogram overlay demonstrating the differences in Ca^{++} influx between different anti-HER2 (left) and anti-CSPG4 (right) antibody variants depicted as the changes in the DAPI/Indo-1 Blue fluorescence ratio over time. The top overlays include all antibody variants and controls and the bottom exclude the ionomycin control to more clearly demonstrate the differences between the antibody variants on a smaller scale. Data representative of three independent experiments.

We, therefore, examined whether these attributes are retained by the anti-HER2 Fc variants. WT and mutant anti-HER inhibited the proliferation of the HER2-overexpressing BT-474 and SK-BR-3 breast cancer cells in a dose-dependent manner, and with comparable potency to those of the clinically available trastuzumab (Herceptin®). None of the variants could reduce the proliferation of the low HER2-expressing triple-negative MDA-MB-231 or of the trastuzumab-resistant HCC1954 breast cancer cell lines (**Figure 7A**) (49). We, therefore, concluded that Fc-engineered antibodies can retain the direct Fab-mediated effects of the original unmodified clone.

A known anti-tumor mechanism of action of trastuzumab (Herceptin®) is the ability to trigger antibody-dependent cellular cytotoxicity (ADCC) of HER2-expressing cancer cells mediated by NK cells (11, 50). Therefore, we studied the potency of different anti-HER2 Fc variants to induce tumor cell cytotoxicity (ADCC) of the HER2-overexpressing BT-474 cells (target cells) mediated by fresh peripheral blood NK cells from three different donors (effector cells) (**Figure 7B**) (51–53). The in-house produced anti-HER2 WT antibody and trastuzumab (Herceptin®) showed similar tumor cell killing potencies mediated by NK cells from all three human donors. Anti-HER2 DE induced higher tumor cell killing at significantly lower concentrations than WT antibodies. All antibodies showed similar levels of ADCC at saturating concentrations (5 µg/mL). In line with diminished binding to FcγRIII on the surface of NK cells, the anti-HER2 NQ mutant IgG1 failed to induce any tumor cell killing above controls (EC₅₀ of variants for each independent experiment, **Figure 7C**).

Furthermore, we tested the potency of anti-HER2 variants to trigger NK cell-mediated ADCC against cancer cells that express low levels of the target. We chose the triple-negative breast cancer cell line MDA-MB-231 known to express very low levels of HER2/neu (**Figure 7D**), and the HER2-overexpressing cell line BT-474 to compare the effects of the target expression on ADCC levels induced by NK cells from the same donors (**Figure 7D**, Donors 4 and 5). We observed no or very low ADCC against MDA-MB-231 with all the variants (**Figure 7**, top panels), while against BT-474, anti-HER2 DE, WT, and trastuzumab induced dose-dependent tumor cell lysis (**Figure 7D**, bottom panels), consistent with the findings depicted in **Figure 7B**.

Therefore, while antibody Fab-mediated direct effects are not affected, Fc-modified antibody variant engineering allows the manipulation of antibody binding kinetics to effector cells, and can alter antibody functional capacity and potency to mediate immune effector cell activation and NK cell-mediated cytotoxicity of tumor cells.

DISCUSSION

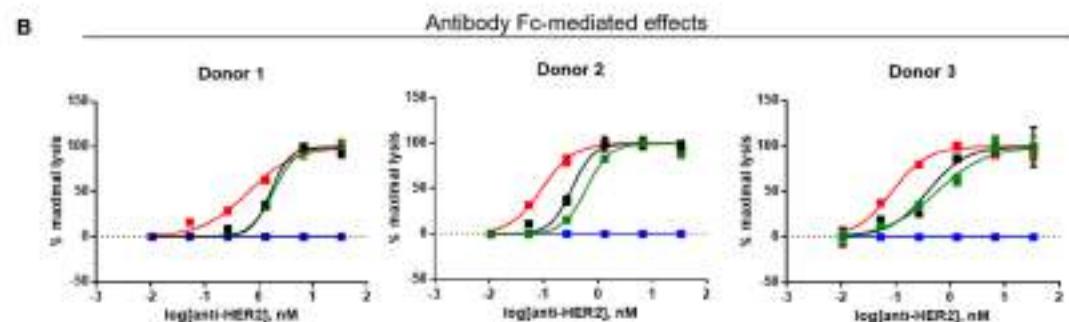
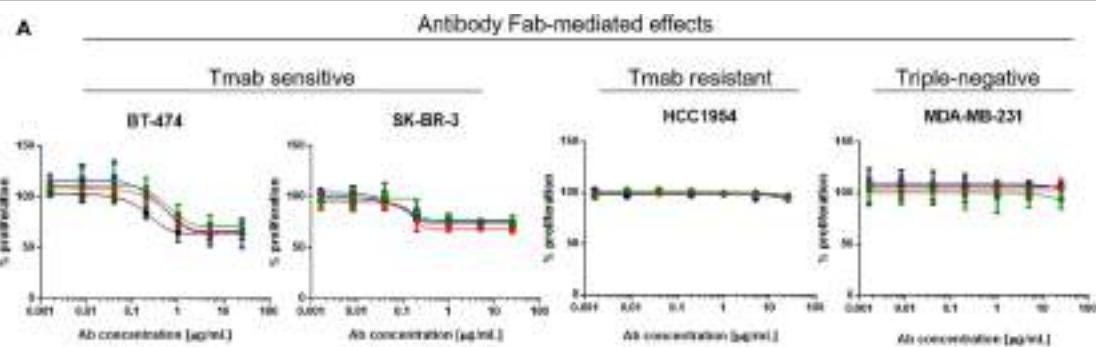
With monoclonal antibody therapies firmly established as part of the standard care of treatment for solid tumors and hematological malignancies (54–56), designing antibodies to engender well-defined Fab-mediated and Fc-mediated anti-cancer properties is highly desirable. Specifically, Fc-mediated effects can have a major impact on the ability of antibodies to engage cells of the immune system and can influence their anti-tumor functions and efficacy.

Here, we designed and implemented a novel seamless cloning and expression platform for the generation of a panel of anti-cancer monoclonal antibodies with modified human Fc regions. We evaluated the functional impact of antibody engineering on antibody Fab- and Fc-mediated functions. This strategy allows the generation of WT and Fc mutant antibodies of any specificity at high mutagenesis and transfection efficiency, short time frames for cloning and production, and high yields of pure material. Compared to WT antibodies produced using a previous cloning approach, this represents 10-fold increased yields at a 3-fold reduced time frame of 12 days from design to purified antibody material (**Table 3**). This approach permits the generation of Fc-modified variants of any antibody clone and may find wide applications in antibody engineering and functional screening.

Compared with existing mutagenesis cloning methods, our approach is unique in combining the following features: (i) high efficiency; (ii) no need of restriction or ligation enzymes; (iii) requirement for only a single step PCR; (iv) designed for mutagenesis/cloning of large expression ready plasmid constructs; (v) employs universal primers which could be applied to any IgG1 antibody construct; and (vi) applicability for the generation of different versions of the same antibody construct (36, 37, 40, 57). These features, combined with rapid, small volume, and high yield transient expression in mammalian Expi293F cells constitute a unique and novel methodology which can facilitate selection of therapeutic antibody candidates with the most suitable attributes by employing mechanistic and potency evaluations.

While all antibody variants retained recognition of target antigens expressed on tumor cells, engagement of FcγRs on human primary NK cells was affected in Fc-mutated variants. The DE and NQ mutants showed enhanced and diminished binding properties, respectively, compared with binding of WT antibodies, consistent with previous findings (58). Here, binding studies of the Fc variants were conducted by flow cytometry on natively expressed FcRs and in the presence of endogenous immunoglobulins on the surface of fresh human NK cells. Hence, our 7- to 17-fold increased affinity of the DE variants compared with native antibodies may underestimate absolute increased affinities, previously measured with recombinant FcγRIIIa (58). Furthermore, although we engineered our antibody panels for each clone to be of the same isotype, with identical Fc region sequences, we observed that DE mutations improved the binding of anti-CSPG4 to NK cells more than the same mutations improved the binding of anti-HER2 enhanced DE variant. It is possible that these differences may depend on the antibody Fab regions and that mutating Fc regions in an identical way could still result in different Fc-mediated properties for different antibodies and merits further study. We also found differences in enhanced binding between DE and WT antibodies with different donors. It is, therefore, possible that enhanced kinetics would also be subject to donor-dependent effects and this requires investigation.

When we interrogated antibody Fc-mediated NK cell activation, we found enhanced calcium mobilization by mutant antibodies with high FcR-binding attributes. Furthermore, we observed low levels of Calcium influx triggered by endogenous IgGs on the surface of NK cells, which did not increase after the cells were incubated with WT antibodies. This may represent a



C

Ab variant	EC50 [nM]		
	Donor 1	Donor 2	Donor 3
Trastuzumab (Herceptin®)	1.655	0.3307	0.3828
Anti-HER2 WT	1.835	0.8106	0.5679
Anti-HER2 DE	0.633	0.09277	0.08523
Anti-HER2 NQ	N/D	N/D	N/D

Legend:

- WT antibody (green)
- DE Fc mutant (red)
- NQ Fc mutant (blue)
- Trastuzumab (Herceptin®, Roche) (black)

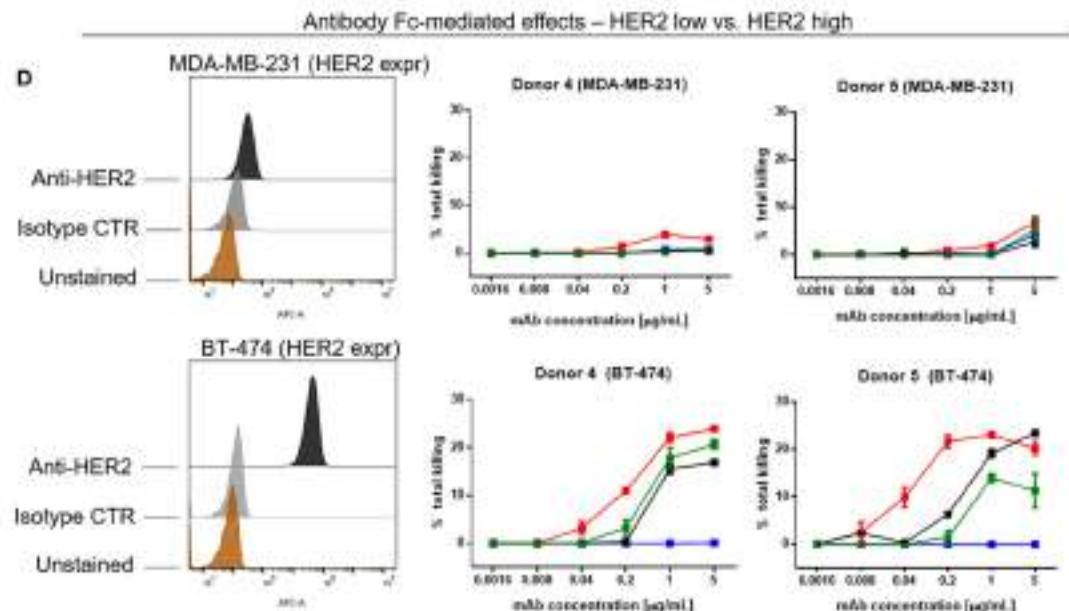
**FIGURE 7 | Continued**

FIGURE 7 | Continued

Assessments of direct and Fc-mediated effects of anti-HER2 Fc variants against breast cancer cells. **(A)** Effects of anti-HER2 antibody variants on the proliferation of trastuzumab-sensitive (BT-474, SK-BR-3), trastuzumab-resistant (HCC1954) and triple-negative (MDA-MB-231) breast cancer cell lines. Anti-HER2 variants inhibited the proliferation of BT-474 and SK-BR-3 cells in a similar dose-dependent manner, but did not affect the proliferation of MDA-MB-231 or HCC1954 cells. Graphs represent an average of two experiments \pm SD. **(B)** Human peripheral blood NK cell-mediated ADCC of BT-474 cancer cells induced by anti-HER2 variants measured by LDH release. Graphs are representative of independent experiments with three different human NK cell donors; data were normalized to minimal and maximal cell lysis. Error bars represent SEM values from technical replicates. N/D: not detected. **(C)** Effective concentration ([EC50] nM) measurements of ADCC by three human NK cell donors. **(D)** NK cell-mediated ADCC (measured by LDH release) of HER2 low (MDA-MB-231) and HER2 high (BT-474) breast cancer cells induced by anti-HER2 variants. The flow cytometric histograms on the left depict HER2 expression levels in MDA-MB-231 (top) and BT-474 (bottom) compared to unstained cells or cells stained with isotype control mAb. The graphs represent total cell killing levels of MDA-MB-231 cells (top) and BT-474 cells (bottom) mediated by NK cells from two different donors (Donors 4 and 5) at different concentrations of anti-HER2 variants.

picture close to the physiological conditions, where WT therapeutic antibodies would have to compete with endogenous IgGs with similar affinities for their cognate Fcγ receptors. This may be addressed through increasing the affinity of therapeutic antibodies to CD16 (59).

We demonstrated that while all anti-HER2 antibody variants retain their Fab-mediated direct effects in restricting HER2-expressing breast cancer cell proliferation, Fc-modified anti-HER2 antibodies engender differential anti-tumor cell cytotoxicity potencies by human NK cell effectors. Fc engineering may, therefore, provide a means of manipulating antibody binding kinetics to effector cells, and can alter the antibody's functional capacity and potency to mediate immune effector cell activation and cell-mediated cytotoxicity.

Even though there are conflicting reports about the ability of trastuzumab to trigger NK cell-mediated killing in low HER2-expressing cancer cells, in this study, we observed very low ADCC activity of anti-HER2 variants against cancer cells expressing low levels of the target antigen (51, 60). Although this may indicate the likely inability of Fc engineered antibodies to target low tumor antigen-expressing cancer cells, these findings could also be interpreted as favorable in terms of potential safety of clinical application of Fc-optimized anti-HER2 antibodies. By targeting high-expressing tumor cells but not low HER2-expressing normal tissues, such as cardiomyocytes, their application may engender anti-tumor effects in the absence of on-target, off-tumor toxicities (61).

We confirmed that our generated N297Q mutants completely lack fucosylation. The N297Q mutation most likely results in loss of the core glycan, previously reported to alter the "open" conformation of WT IgG1 antibodies in favor of a "closed" confirmation, leading to impaired ability to engage FcRs (26). On the other hand, loss of fucose from the N-linked core glycan can substantially improve IgG binding to FcγRIIIA and ADCC. Therefore, differences in the glycosylation of optimized Fc mutants may contribute to their ability to outperform their WT counterparts in FcR engagement and effector cell activation (62). In this study, we demonstrate that the enhanced FcR and Fc-mediated functional attributes of the S293D/I332E mutants are not linked to differential fucosylation. The exact mechanisms behind this functional superiority are currently unknown; unraveling these would require further in-depth structural, interaction, and functional studies.

The importance of engineering monoclonal antibodies with defined Fc functions is highlighted by findings of reduced clinical responses to monoclonal antibodies, such as cetuximab

(anti-EGFR), trastuzumab (anti-HER2), and rituximab (anti-CD20), in individuals who carry FcR polymorphisms that alter antibody binding affinities to immune effector cells (63–67). Different approaches have been developed to enhance the potency of monoclonal antibodies in activating effector cells against cancer (21). Optimization of IgG Fc domains through introduction of point mutations or glyco-engineering can influence binding kinetics to FcR. Fc mutant versions of trastuzumab (anti-HER2), alemtuzumab (anti-CD52), rituximab (anti-CD20), and others have shown significantly improved binding to FcγRs and enhanced effector functions (58, 68). Since removal of fucose from the N-linked core glycan can dramatically improve IgG-FcγRIIIA interactions and ADCC of tumor cells (62), two afucosylated antibodies (obinutuzumab and mogamulizumab) are approved for clinical use in hematological oncology (69, 70). An important reported advantage of certain Fc-optimized therapeutic antibodies is that they exhibit enhanced binding to FcγRIIIA even in the presence of polymorphisms that otherwise lower affinity for IgG (58, 64). In this aspect, a future development of our platform could include functional studies of the capacity of Fc-optimized variants to activate NK cells with defined FcγRIIIA genotype. Specific Fc mutations can also improve antibody pharmacokinetic characteristics through improved binding to the neonatal FcR, FcRn (21). Experimental approaches also entail switching the antibody isotype from the traditionally preferred IgG (mainly IgG1) to other isotypes, such as IgA or IgE, which could also provide advantages through alternative immune surveillance properties in specific tissues and through activating immune effector cell types that may be different to those engaged by IgG (18, 45, 71–74).

In this study, we also generated IgG1 antibody variants with vastly reduced FcR engagement and with impaired effector functions. Abrogation of antibody binding to FcRs and abolition of Fc-mediated effector functions is a useful approach for the design of antibodies with direct agonistic or antagonistic activities, antibody-drug conjugates or checkpoint blockade inhibitor antibodies. In some cases, effector cell engagement may not always be desirable, may impede Fab-mediated effects or bear safety hazards if intact Fc-mediated effector functions are retained (22, 23, 25, 75). Antibodies of the IgG2 or IgG4 isotypes are traditional choices in such drug design strategies. Nevertheless, IgG2 and IgG4 antibodies may not completely lack effector cell engagement (76). Furthermore, the latter may retain the potential to interact with other IgG Fc domains or undergo Fab arm exchange (FAE) which can lead to reduced activity or off-target effects (24). Efforts have, therefore, focused toward abolishing Fc-mediated functions

to avoid potentially dangerous immune-mediated toxicities. In addition, monoclonal antibodies used for cancer therapy are often associated with a range of adverse reactions including triggering cytokine storm or hypersensitivity responses (22, 23). Where the purpose of the therapy is to engender Fab-mediated functions (25), complete abrogation of Fc effector capabilities could often be a necessity for certain antibody therapeutic design applications.

Monoclonal antibodies of the same immunoglobulin isotype recognizing different cancer antigens often act through different immune-mediated mechanisms. The same Fc-modifications may engender different effector properties by antibodies with variable specificities and affinities to different antigens or antigenic epitopes. In this context, our approach provides a convenient tool to enable investigation of the effects of Fc point mutations on the function of monoclonal antibodies of any specificity. To exemplify this, we quickly generated Fc variants of monoclonal antibodies against two tumor-associated antigens, with which we were able to delineate antibody Fab-mediated and Fc-mediated mechanisms of action. This demonstrated the capability of the system to enable control of effector functions through introduction of point mutation capabilities. The universal nature of the Fc modification features built into our approach opens the door toward the generation of a range of Fc variants of antibodies of any specificity. Furthermore, serum-free production and quick high antibody yields could provide sufficient high quality material to facilitate early *in vitro* and *in vivo* screening, downstream translation to good manufacturing practice (GMP) pathways and facilitate potential clinical application.

In summary, with this design strategy, we are able to manipulate anti-cancer antibody Fc regions. We delineate the binding and functional attributes of WT and Fc-modified agents and evaluate their Fab-mediated and Fc-mediated functions and potency. This approach can find broad application in therapeutic antibody engineering and translation for cancer therapy and potentially in other areas beyond the cancer immunotherapy field.

ETHICS STATEMENT

Blood cone samples from anonymized donors were purchased from the National Health Service Blood and Transplant Service, United Kingdom. Sample processing was supported through a local ethical framework conducted in accordance with the Helsinki Declaration and approved by the NHS Research Ethics Committee (Van Cutsem et al.), Guy's and St. Thomas' NHS Trust

("Immunopathogenesis and Molecular Biology in Breast Cancer Subtypes," REC reference number: 13-LO-1248).

AUTHOR CONTRIBUTIONS

KI, SK, and AT conceived and designed the study. KI, JF-S, TD, SM, SC, PK, and IC helped with the development of the methodology. KI, JF-S, DA, TD, SM, SC, HB, AC, PK, and IC acquired the data or helped with the data analysis and interpretation. KI, JF-S, DA, SM, SC, HB, PK, MF, RM, DJ, AB, JM, JS, EJ-J, AT, and SK discussed and interpreted the data and edited the manuscript. SK and AT supervised the study. SK led and coordinated the project. KI and SK wrote 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.2017.01112/full#supplementary-material>.

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Nanobodies and Nanobody-Based Human Heavy Chain Antibodies As Antitumor Therapeutics

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Monoclonal antibodies have revolutionized cancer therapy. However, delivery to tumor cells *in vivo* is hampered by the large size (150 kDa) of conventional antibodies. The minimal target recognition module of a conventional antibody is composed of two non-covalently associated variable domains (VH and VL). The proper orientation of these domains is mediated by their hydrophobic interface and is stabilized by their linkage to disulfide-linked constant domains (CH1 and CL). VH and VL domains can be fused *via* a genetic linker into a single-chain variable fragment (scFv). scFv modules in turn can be fused to one another, e.g., to generate a bispecific T-cell engager, or they can be fused in various orientations to antibody hinge and Fc domains to generate bi- and multispecific antibodies. However, the inherent hydrophobic interaction of VH and VL domains limits the stability and solubility of engineered antibodies, often causing aggregation and/or mispairing of V-domains. Nanobodies (15 kDa) and nanobody-based human heavy chain antibodies (75 kDa) can overcome these limitations. Camelids naturally produce antibodies composed only of heavy chains in which the target recognition module is composed of a single variable domain (VHH or Nb). Advantageous features of nanobodies include their small size, high solubility, high stability, and excellent tissue penetration *in vivo*. Nanobodies can readily be linked genetically to Fc-domains, other nanobodies, peptide tags, or toxins and can be conjugated chemically at a specific site to drugs, radionuclides, photosensitizers, and nanoparticles. These properties make them particularly suited for specific and efficient targeting of tumors *in vivo*. Chimeric nanobody-heavy chain antibodies combine advantageous features of nanobodies and human Fc domains in about half the size of a conventional antibody. In this review, we discuss recent developments and perspectives for applications of nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics.

Keywords: nanobodies, heavy chain antibodies, antitumor therapeutics, nanobody-conjugates, nanobody fusion proteins, sortagging of nanobodies

INTRODUCTION

Monoclonal antibodies (mAbs) and antibody-derived biologics are essential tools for cancer research and therapy (1, 2). Antibodies can be used to inhibit tumor cell proliferation and as targeting moieties of effector domains. Many mAbs directed against tumor cell surface proteins interfere with the function of their target proteins, e.g., by blocking signaling *via* a growth factor receptor or by inducing apoptosis. By opsonizing the tumor cell, antibodies can also mark tumor

cells for attack by the complement system, NK cells and macrophages. Antibody engineering provides powerful technologies to improve antibody effector functions and to generate novel, bispecific biologics. The use of mAbs has revolutionized antitumor therapy, with impressive achievements in the treatment of both hematological malignancies and solid tumors (3).

However, certain inherent structural properties limit the applicability of mAbs and antibody-derived biologics for tumor therapy. The large size of mAbs (four polypeptide chains, 150 kD) can hamper access to tumor cells. Moreover, the nature of the antibody recognition module—a pair of variable domains non-covalently associated *via* a hydrophobic interface—poses obstacles to the development of bispecific biologics. These aspects illustrate the need for new antibody formats that provide the same binding specificity of mAbs but with better stability and *in vivo* pharmacodynamics.

The discovery of naturally occurring heavy chain antibodies (hcAbs, two polypeptide chains, 75 kD) containing a highly stable and soluble single antigen-binding V-domain—designated VHH or nanobody (15 kDa)—has opened the way for a new generation of antitumor therapeutics. Other excellent reviews describe the discovery and structure of nanobodies, their potential applications in oncology, infection, immunity, and other diseases (4–18). Here, we focus on the unique features of nanobodies and nanobody-based human heavy chain antibodies that underlie their huge potential as antitumor therapeutics. We provide insight into the current status, ongoing developments and future challenges toward successful implementation of nanobodies and nanobody-based human hcAbs as antitumor therapeutics.

THE ANTIGEN-BINDING MODULES OF CONVENTIONAL AND HEAVY CHAIN ANTIBODIES

Conventional mAbs are composed of two heavy and two light chains (Figure 1). Both chains contribute to two identical antigen-binding sites. Each target-binding site of a conventional antibody is composed of two non-covalently associated variable domains, designated VH and VL (Figure 2). The target specificity is mediated by three peptide loops at the tip of each V-domain, designated complementarity determining region (CDR). Together, these six CDR loops form the target-binding paratope or idiotype of an antibody. For proper target binding, the two V-domains need to pair up in the proper orientation in order for the CDR loops to jointly form a specific paratope. This is mediated by a hydrophobic interface between the VH and VL domain (illustrated by the black bars in Figures 1 and 2). In intact antibodies, the proper association of VH and VL domains is stabilized by the C-terminal linkage of each V-domain to a constant domain, i.e., CH1 and CL. In most antibodies, these two constant domains in turn are connected by a conserved disulfide bridge which provides further rigidity and stability to the target-binding module.

VH and VL domains can be fused genetically *via* a linker peptide into a small (30 kD) single polypeptide binding module, designated single-chain variable fragment (scFv) (Figure 1). In

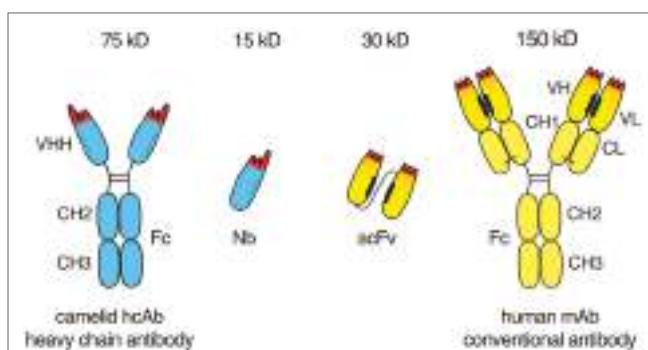


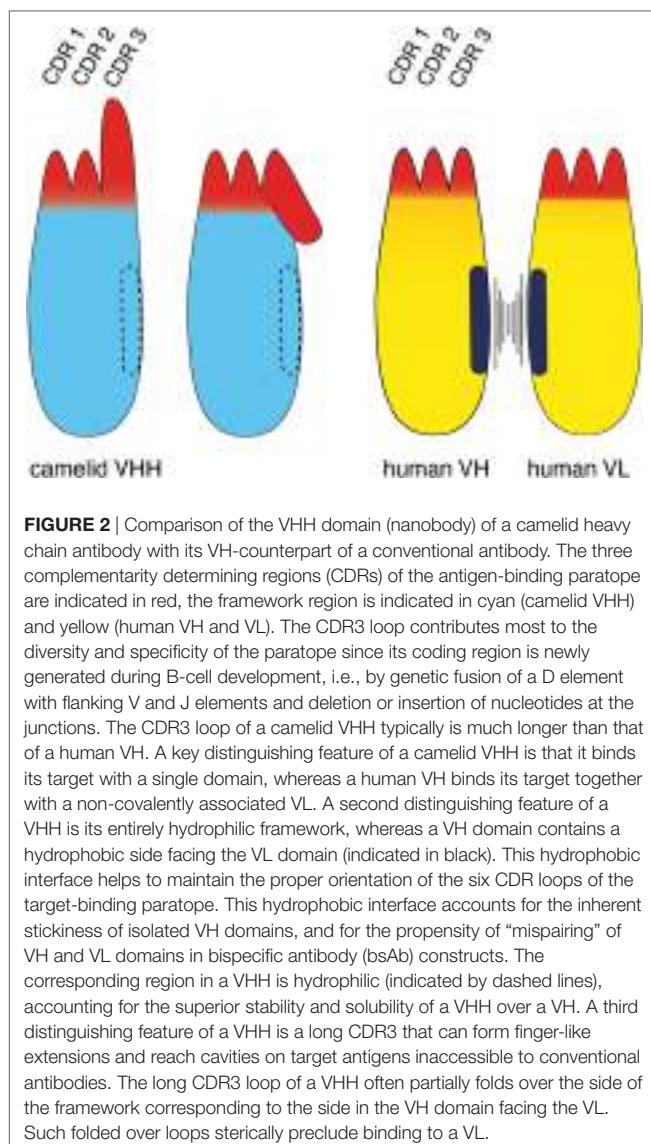
FIGURE 1 | Advantageous features of camelid heavy chain antibodies.

Heavy chain antibodies are composed of two heavy chains. The target-binding module is composed of a single VHH domain. A recombinant VHH domain, designated nanobody (Nb) is highly soluble and does not show any tendency to associate with other hydrophobic protein surfaces. Conventional antibodies are composed of two heavy and two light chains. The target-binding module is composed of two non-covalently associated variable domains VH and VL. In intact antibodies, the proper orientation of these domains is mediated by a hydrophobic interface (see Figure 1) and is further stabilized by the disulfide-linked CL and CH1 domains. A pair of VH and VL domains can be linked genetically into a single-chain variable fragment (scFv) in which the proper orientation of domains is mediated alone by the hydrophobic interface between the two V-domains.

an scFv, the proper association and orientation of the VH and VL domains to form the target-binding paratope is mediated almost entirely by the hydrophobic interface between the two V-domains. The hydrophobic faces of VH and VL domains can dissociate from one another and associate with other hydrophobic surfaces. This limits the solubility of scFvs and underlies their inherent instability and tendency to aggregate (19).

The VHH domains of camelid heavy chain antibodies have been shaped by more than 50 million years of evolution for high solubility and stability, independent of a partner VL domain. As recombinant proteins, VHH are designated single-domain antibodies or nanobodies (4) in reference to their small size in the nanometer range (20, 21). Importantly, nanobodies have a hydrophilic side (indicated by dashed lines in Figures 1 and 2) corresponding to the light chain interface of VH domains, do not bind light chains, and thus usually do not display any of the solubility and aggregation problems typical of VH domains of conventional antibodies.

A notable difference between the camelid VHH and the human VH domain is the length and orientation of the CDR3 loop (Figure 2). The CDR3 corresponds to the unique region of the antibody molecule that is encoded by a DNA element newly generated during B-cell development. Genetic recombination results in the fusion of a D-element with flanking V- and J-elements. During recombination further genetic diversity is generated by addition and/or deletion of nucleotides at the junctions. Thereby, the CDR3 loop provides the major contribution to antibody diversity and specificity. There is a much lower contribution to diversity by the CDR1 and CDR2 loops, since these loops are germline encoded by a limited number of different V-elements. The CDR3 loop of camelid VHHs shows a much broader distribution of lengths (3–28 amino acids) than



human VH domains (8–15 amino acids) (Figure 2) (4, 8). The long CDR3 of a VHH enlarges the potential interaction surface with the target antigen, thereby compensating in part for the missing VL-domain (4, 8). Often, the C-terminal part of a long CDR3 loop folds over onto the side of the VHH domain that corresponds to the side of a VH domain facing a VL domain. This accounts for the often skewed, sideways kind of binding of a VHH domain to its target as compared to the typical head-on binding of a VH–VL pair to its target. This is well illustrated, for example, in the recently reported crystal structure of a PD-L1-specific nanobody with the Ig-domain of PD-L1 and in two of three nanobodies co-crystallized with CD38 (22, 23). The partial folding over of the CDR3 loop onto the former VL interface sterically precludes binding of a VL domain and thereby also contributes to the independence of a nanobody from an associated VL domain. Interestingly, the longer CDR3 of a nanobody can form a finger-like extension that fits into a

cavity on the target protein. This allows nanobodies to bind to unique epitopes that are not accessible to conventional mAbs (4, 24, 25), whose antigen-binding interface generally is flat (26).

CONVENTIONAL AND HEAVY CHAIN ANTIBODIES AS ANTITUMOR THERAPEUTICS

The Fc-domain of conventional antibodies can activate the complement system and can serve as recognition module for Fc-receptors on natural killer cells and macrophages. However, the large size of mAbs (150 kDa) is a drawback, as this can limit penetration into tumors *in vivo* (27, 28). It has been estimated that only about 20% of administered mAbs take effect because of their poor pharmacokinetics and weak tissue penetration (29).

In 1993, naturally occurring heavy chain antibodies were discovered serendipitously while analyzing the serum of a dromedary in a practical biochemistry course at the University of Brussels (30). It was soon established that all extant members of the camelid family, i.e., dromedaries, camels, llamas, and alpacas, naturally produce antibodies composed only of heavy chains in addition to conventional antibodies (30, 31). These fully functional antibodies exhibit high specificity, high diversity, and binding capacities similar to those obtained by conventional mAbs, even though they lack the light chain and the CH1 domain of the heavy chain. In camelid hcAbs, a single variable domain, designated VHH, is linked directly to the hinge and Fc-domains of an IgG heavy chain. A heavy chain antibody is, thus, roughly only half the size (75 kDa) of a conventional mAb (150 kDa).

The use of animal-derived antibodies for antitumor therapy is limited, because the immune system typically mounts an antibody response against the foreign components of a therapeutic antibody (32, 33). Neutralization of an antitumor antibody by antibody-specific antibodies generally renders this therapeutic antibody useless for the patient producing anti-antibodies. The constant domains, in particular, are highly immunogenic across species barriers (26, 34, 35). Antibody responses to V-domains are much less frequent. This likely reflects the fact that V-domains undergo extensive somatic hypermutation in every physiological immune response. Somatic hypermutation can result in the substitution of 20 and more amino acid residues in each of the VH and VL domains (36). The human immune system is already tolerized at birth to a huge diversity of V-domains of maternal IgG antibodies that passed the placenta.

Some highly successful antitumor antibodies have been generated simply by replacing the constant domains of the parental mouse antibody with the corresponding domains of human IgG heavy chain and kappa or lambda light chains (Figure 3). Rituximab (anti-CD20) and cetuximab (anti-EGFR), for example, each carry the VH and VL domains of its parental mouse mAb fused to the constant domains of the human IgG1 heavy chain and the human kappa light chain. Analogous chimeric nanobody-human IgG heavy chain antibodies can be generated by fusion of the VHH encoding region to the hinge and Fc domains of a human IgG (37–39).

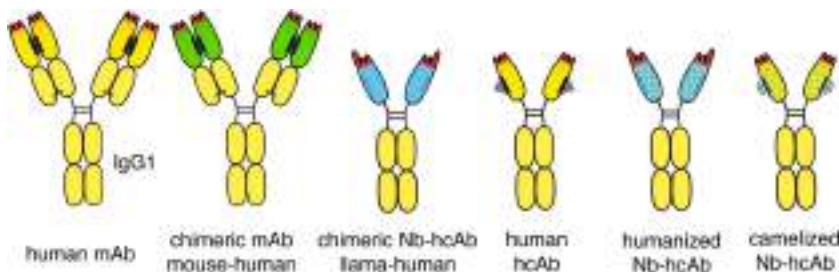


FIGURE 3 | Chimeric and humanized heavy chain antitumor antibodies. Second generation antitumor antibodies such as daratumumab are fully human antibodies, derived from human-Ig transgenic mice or synthetic libraries. Successful antitumor antibodies, such as rituximab and cetuximab, are chimeric antibodies composed of VH and VL domains from mouse monoclonal antibodies (mAbs, green) fused to the constant domains of human IgG1 and kappa, respectively. Chimeric antitumor heavy chain antibodies are easily generated by genetic fusion of a VHH domain (blue) to the hinge and Fc domains of human IgG1. Such chimeric heavy chain antibodies combine the advantageous features of a nanobody (Nb), i.e., high solubility and stability, with the effector functions of a human IgG. Fully human heavy chain antibodies often suffer from the poor solubility and stability of a partnerless VH domain with a vacant sticky hydrophobic side (indicated in black). By substituting divergent framework residues, camelid VHH domains can be “humanized” (yellow dots) and human VH domains can be “camelized” (blue dots) to reduce immunogenicity and to improve solubility, respectively.

With respect to immunogenicity, it is important to note that CDR3 loops likely contribute more to immunogenicity than framework residues of the V-domain (26). Indeed, a fraction of patients typically develop anti-idiotype antibodies even against fully humanized antitumor antibodies (33). In such cases, the particular antitumor therapeutic is rendered useless for the patient. As different antibodies against the same target become available, a therapeutic option for these patients will be to switch to a therapeutic antibody with a different idiotype.

VHH domains typically display a high sequence identity with human type 3 VH domains (VH3), likely accounting for their low immunogenicity (40). In addition, humanization of nanobodies can be performed to further minimize their immunogenicity (5, 8, 41). This is accomplished by substituting divergent framework residues with residues commonly found in human VH domains (indicated in Figure 3 schematically by yellow dots). Most divergent residues can indeed be “humanized” without affecting the specificity or solubility of the nanobody-heavy chain antibody. “Humanizing” hydrophilic residues at the side that corresponds to the interface with VL domains, however, can compromise the solubility of the antibody, i.e., render the antibody “sticky” and prone to aggregation.

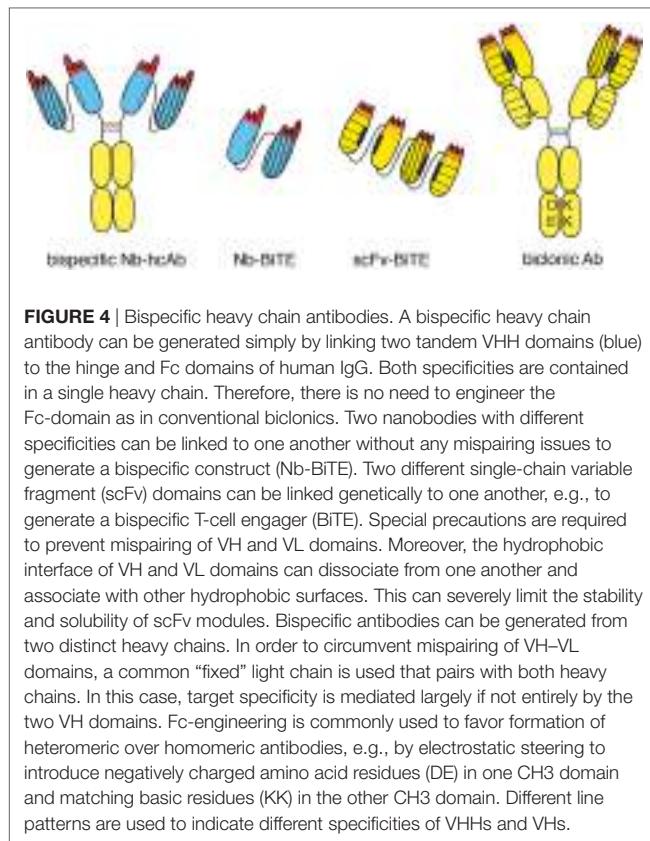
In order to reduce the residual immunogenicity of animal-derived V-domains in conventional antitumor antibodies, the six CDR loops have been successfully grafted from animal VH and VL domains onto human VH and VL domains (42). Campath-H1 (anti-CD52), for example, contains CDR loops from the parental rat mAb grafted onto the VH and VL domains of human IgG1 kappa (42). The majority of antitumor antibodies currently in clinical development are derived from fully human antibodies (43). Daratumumab (anti-CD38), for example, was generated from immunized human Ig-transgenic mice, necitumumab (anti-EGFR) was selected from a fully synthetic Fab display library (44).

Attempts have also been made to generate fully human heavy chain antibodies (45) (Figure 3). Such fully human heavy chain antibodies, however, suffer from poor solubility, likely due to the inherent tendency of the non-paired VH domain to bind to free light chains and to aggregate. Solubility of human VH domains

can be improved by “camelization,” e.g., by substituting residues in the hydrophobic interface with hydrophilic residues (indicated in Figure 3 schematically by blue dots) (46). This can ameliorate the aggregation and stickiness of human heavy chain antibodies. Further preclinical and clinical studies will show whether heavy chain antibodies based on “camelized” VH domains or on “humanized” VHH domains are better suited as antitumor therapeutics.

BISPECIFIC ANTITUMOR HEAVY CHAIN ANTIBODIES

Often, the target antigen of an antitumor antibody is expressed not only by tumor cells but also by healthy cells. In such cases, the target on healthy cells can act as a sink for the therapeutic. Cytotoxicity of the antitumor antibody to healthy cells can cause unwanted side effects. One strategy to improve the specificity of antitumor antibodies is to genetically link the target-binding modules of two distinct tumor-targeting antibodies into a single, bispecific antibody (bsAb) (47, 48) (Figure 4). The halves of two conventional antibodies can be combined to generate a bsAb. Catumaxumab, for example, contains an EpCAM-specific “half” antibody connected via disulfide bonds in the hinge region to a CD3-specific “half-mAb” (49). In this case, usage of antibodies from two distinct species, e.g., rat IgG2a and rat lambda on the one side and mouse IgG2b and mouse kappa on the other side, reduces mispairing of the VL and VH domains (50). When generating a bsAb from two distinct human mAbs, genetic engineering is usually required to ensure proper pairing of the two VH and VL domains. One strategy employs a common “fixed” light chain that can pair with both heavy chains. In this case, target specificity is mediated by the VH domains, while the common VL domain contributes only little if anything to target binding. Additional engineering—e.g., electrostatic steering or insertion of a “knob” in one CH3 domain and a “hole” in the other CH3 domain—is often used to favor heteromeric over homomeric pairing of heavy chains (51). For example,



the biclonic MCLA-128 (anti-HER2—anti HER3) contains a fixed human IgV kappa chain, and DE-KK Fc-engineered heavy chains (L351D L368E, L351K T366K) (52).

Since nanobodies have a completely hydrophilic surface and do not bind light chains, they can be easily linked into dimers and multimers without need for additional measures. Thus, a bispecific nanobody-based heavy chain antibody can be generated simply by genetically fusing a second nanobody *via* a linker peptide to the N-terminus of a heavy chain antibody (Figure 4). Since both specificities are contained in a single polypeptide in bispecific heavy chain antibodies, there is no need to engineer the Fc-domain as in conventional bicalonics. Such nanobody-based bispecific heavy chain antibodies routinely show excellent solubility, stability, and production yields akin to their parental heavy chain antibodies (our own unpublished observations).

A similar strategy, i.e., the genetic linkage of two target-binding modules, can also be used to attract and link mobile immune cells to cancer cells, e.g., with one of the modules binding to a tumor cell and the other to a T cell or an NK cell (53). For linking T cells or NK cells to tumor cells, the Fc domain is dispensable. Thus, two scFv domains can be linked genetically to one another, e.g., to generate a bispecific T cell engager (BiTE) or bispecific NK-cell engager (BiKE) (Figure 4). Blinatumumab, for example, contains a CD19-specific scFv linked to a CD3-specific scFv (54). When linking two or more scFvs, however, special precautions are required to prevent mispairing of VH and VL domains (55). In contrast, nanobodies can readily be fused into BiTEs or BiKEs with little, if any, solubility or stability issues.

Dimerization of two nanobodies or scFvs can also be achieved by genetic fusion to natural or synthetic dimerization domains (56). Fusion to the upper hinge region, for example, allows dimerization *via* formation of interchain disulfide bonds between cysteine residues in the hinge (57). Heterodimer-formation can be forced by fusion of two nanobodies or scFvs to distinct dimerization peptides or protein domains, e.g., fos-jun leucine zippers (58) or two distinct CH3 domains carrying electrostatic steering modules (L351D L368E, L351K T366K) (52) (as in Figure 4) or a hydrophobic “knob” (T366W) and a matching “hole” (T366S, L368A, and Y407V), respectively (51). In the context of tumor therapy, such nanobody dimers can be used as BiTEs or BiKEs to enhance the binding of cytotoxic lymphocytes to tumor cells.

NANOBODIES AS ANTITUMOR THERAPEUTICS

Antitumor therapeutics require a homogenous distribution within the entire tumor for successful tumor treatment. If only part of the tumor is exposed to the therapeutic, complete tumor eradication will not be achieved, leading eventually to tumor regrowth (14). In this regard, nanobodies are expected to outperform mAbs due to their small size and good tumor penetration *in vivo* (28, 59, 60). Nanobodies can readily be cloned into various formats by fusion to other proteins or effector domains, thereby tailoring their utility for specific therapeutic applications.

Antitumor nanobodies can be categorized into three types: naked monomeric or multimeric nanobodies, nanobodies genetically fused to effector domains, and as targeting moieties on liposomes or nanoparticles encapsulating a drug (10, 14). Below we describe and discuss the use of antitumor nanobodies according to these basic concepts.

“NAKED” MONOMERIC AND MULTIMERIC ANTITUMOR NANOBODIES

In oncology, “naked” nanobodies without a linked Fc domain have many interesting potential applications. Their high thermal stability, high refolding capacity, and good tissue penetration *in vivo* (28, 57, 61, 62) make nanobodies ideally suited for specific and efficient targeting of tumor antigens *in vivo*. Because of the modular and single-domain characteristic of nanobodies, molecular manipulation for generating multivalent or multispecific single-chain antibody molecules is relatively easy (Figure 5). Tandem cloning of two identical nanobodies connected by a linker peptide, e.g., a flexible glycine-serine linker, yields a bivalent molecule (30–35 kDa) with higher avidity for the antigen (57, 63). Similarly, tandem cloning of nanobodies that recognize two different epitopes of the same antigen yields a biparatopic molecule. The improved avidity of bivalent nanobodies leads to a reduced off-rate and a reduced release of the nanobody reagent from its target. Crosslinking of a target by a nanobody dimer can induce apoptosis and other signaling cascades (64) or internalization of the target molecule (65).

Fusion of an nanobody monomer or dimer to an albumin-specific nanobody increases the *in vivo* half-life of the reagent

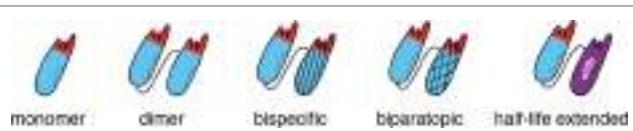


FIGURE 5 | Schematic representation of di- and multimeric antitumor nanobodies. Owing to their high solubility and stability nanobodies can readily be fused genetically to other nanobodies without the mispairing and solubility issues inherent to single-chain variable fragment-based dimers and trimers. Flexible glycine–serine linkers are commonly used to fuse nanobodies, e.g., one or more tandem modules of G4S composed of four glycine residues to provide maximal flexibility and a hydrophilic serine residue to improve solubility. Tandem fusion of two identical nanobodies yields a bivalent dimer, often with improved avidity over the respective monomer. Tandem cloning of two distinct nanobodies that recognize non-overlapping epitopes of the same antigen yields a biparatopic binder. Fusion of two nanobodies recognizing distinct cell surface proteins yields a bispecific binder. The *in vivo* half-life can be extended by fusing one or more antitumor nanobodies to an albumin-specific nanobody. Piggy-backing on albumin reduces the loss of antitumor nanobodies by renal filtration.

(64, 66–68). This is an elegant strategy to overcome the inherent disadvantage of the small size of nanobodies in the context of antitumor therapy *in vivo*. The size of monomeric, dimeric, and trimeric nanobodies is below the renal filtration sieve (approximately 60 kD). Thus, unbound nanobodies are rapidly cleared from the bloodstream by renal elimination. The resulting short *in vivo* half-life of 1–2 h reduces the time interval to bind to their target molecule within the tumor (28, 69, 70). Caplizumab, the first nanobody expected to be licensed for clinical use in 2018, consists of a dimeric nanobody (directed against von Willebrand factor) (71). A downside of dimeric and trimeric nanobodies is an increased size (30 and 45 kDa, respectively), resulting in a less efficient tumor penetration compared to monovalent nanobodies (15 kDa) (72).

In the context of cancer therapy, nanobodies have been developed against growth factor receptors and their ligands, chemokine receptors, death receptors (DR), and ecto-enzymes. Nanobodies against “classical” receptor targets can antagonize ligand binding and activation of signaling cascades by targeted tumor cells (14). For example, nanobodies against epidermal growth factor receptor (EGFR) (25, 64, 73–75), hepatocyte growth factor receptor (HGFR, c-Met) (76), human epidermal growth factor (HER2) (77, 78), and VEGFR (79) inhibit signaling by their respective ligands. Recently, new potential antitumor nanobodies have been developed against other membrane protein targets such as the DRs DR5 (63, 80) and survivin (81), the chemokine receptors CXCR4 (82) and CXCR7 (83), the ion channel P2X7 (84), and the ecto-enzyme CD38 (24, 85). Alternatively, antitumor nanobodies can be directed against receptor ligands, such as HGF (68), VEGF (86, 87), urokinase-type plasminogen activator (88), or CXCL11/12 (89). In a number of *in vivo* xenograft studies, treatment with bispecific or multivalent nanobodies resulted in delay of tumor growth (64, 68) and/or inhibition of angiogenesis (83). An example of a half-life extended antitumor nanobody is CONAN-1, a biparatopic anti-EGFR nanobody fused to an anti-albumin nanobody (64). Fusion to the anti-albumin nanobody increased the half-life of the antitumor nanobody from 1–2 h to

2–3 days. Importantly, in an *in vivo* model of athymic mice bearing tumor xenografts, CONAN-1 inhibited tumor outgrowth with a similar potency as the conventional mAb cetuximab, despite the fact that CONAN-1 is devoid of an Fc-domain that could mediate immune effector functions. CONAN-1 was also more potent than bivalent, monospecific nanobodies in inhibiting tumor growth (64). A recent study demonstrated the potential advantage of a bi-functional molecule, comprising an EGFR-targeted nanobody and DR-targeted ligand TRAIL (90). The results revealed that ENb-TRAIL has therapeutic efficacy in different tumor entities, which do not respond to either EGFR antagonist or DR agonist monotherapies.

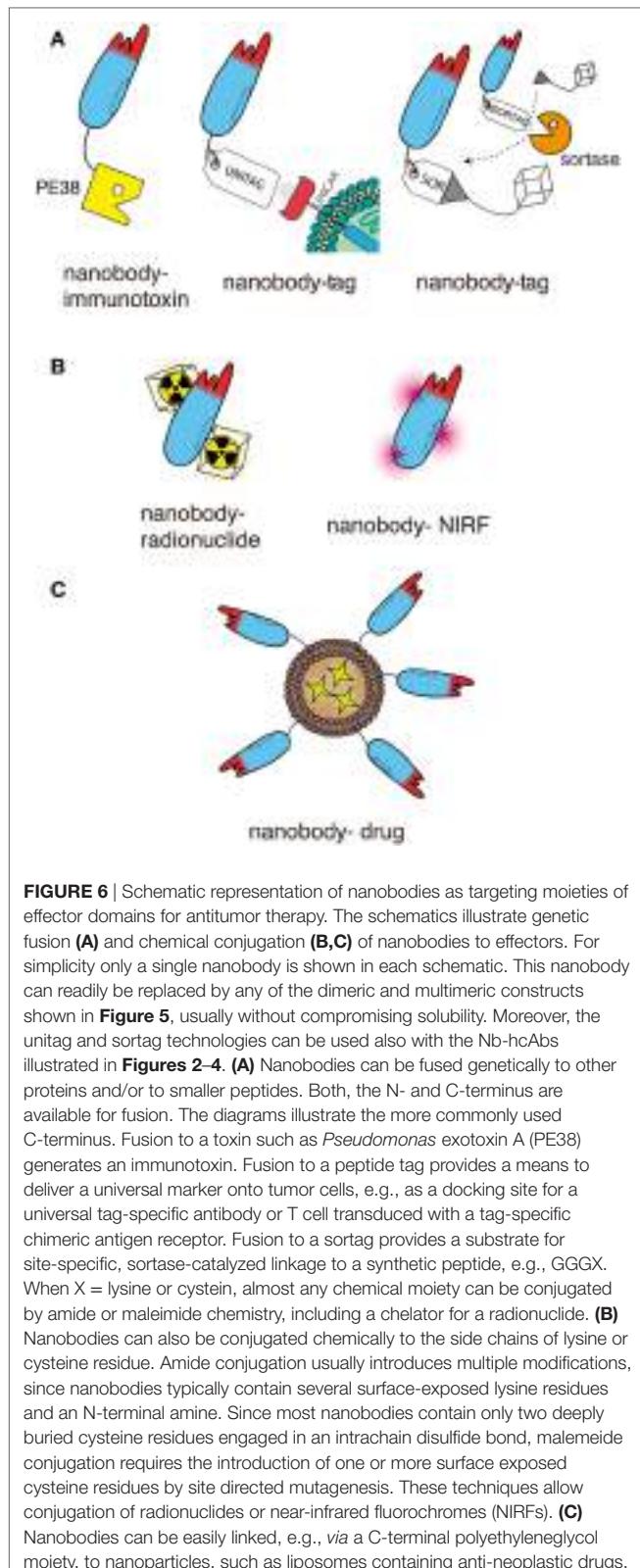
NANOBODIES AS TARGETING MOIETIES OF EFFECTOR DOMAINS

The antitumor effects of nanobodies can be enhanced by coupling nanobodies to protein, peptide, and chemical effectors (Figure 6). Effector-equipped nanobodies combine the advantages of high specificity of the nanobodies, their potential intrinsic therapeutic effects as antagonists, good tissue penetration, and specific accumulation within tumors, with the cytotoxic effects mediated by the effector. Lack of an Fc-domain may be advantageous in such cases, as Fc-mediated clearance may diminish delivery of the effector domain to the tumor (16). However, the attachment of foreign proteins, peptides, and chemicals also introduces potentially immunogenic epitopes and leads to an increase in size which may reduce the efficiency of penetration into the tumor (72).

Genetic fusion and chemical conjugation provide two, partially overlapping, options for equipping nanobodies with effectors. The following sections describe the combination of nanobodies with different effectors, including genetic fusion of nanobodies to protein toxins and peptides, and chemical conjugation to radionuclides, photosensitizers, and anti-neoplastic drugs.

NANOBODY-BASED IMMUNOTOXINS

Immunotoxins consist of a targeting moiety linked to a toxin and are designed to specifically kill targeted tumor cells. The toxin moiety (*Pseudomonas* exotoxin, *Diphtheria* toxin, ricin, cucurmosin) induces cell death, while the targeting moiety binds to antigens preferentially expressed on the cancer cell surface, minimizing cytotoxic side effects on normal cells. Nanobody-based immunotoxins typically are generated by genetic fusion of the protein toxin to the C-terminus of the nanobody, i.e., akin to fusion of an nanobody to the hinge and Fc domains described above (Figure 6A). For example, a dimeric anti-VEGFR2 nanobody fused to the truncated form of *Pseudomonas* exotoxin A (PE38) effectively inhibited the proliferation of VEGFR2-expressing cells *in vitro* (79). A similar fusion construct of a monomeric CD38-specific nanobody with PE38 resulted in highly selective cytotoxicity against multiple myeloma cell lines and patient-derived multiple myeloma cells (23). And CD7-specific nanobodies fused to PE38 showed cytotoxic efficacy in CD7-expressing T-cell acute



lymphoblastic leukemia *in vitro* and in a preclinical mouse model *in vivo* (91, 92). Genetic fusion of an anti-EGFR nanobody to cucurmosin, a pumpkin toxin from the family of type 1 ribosome

inactivating proteins induced cell death of EGFR-expressing cells lines *in vitro* (93).

A drawback of nanobody-based immunotoxins is the inherent immunogenicity of the foreign protein toxin (94). As in case of animal-derived constant Ig domains, the human immune system usually mounts a strong antibody response to the protein toxin. Consequently, nanobody-based immunotoxins should be considered for single use only, at least until reproducible tolerization strategies have been established. Immunotoxins, thus, represent targeted antitumor therapeutics, in particular for cancer patients in which standard treatment is no longer an option (95).

NANOBODY-PEPTIDE FUSIONS

C-terminal fusion of nanobodies to short peptide-tags is commonly used as a tool to facilitate the purification and detection of bound nanobodies, e.g., *via* a tag-specific antibody (**Figure 6A**). Peptide-tagged nanobodies can also be used to mark tumor cells for attack by tag-specific T cells (96). Peptide-tagging further provides an elegant means for site-specific conjugation of nanobodies to any chemical moiety (97).

Fusion of an antitumor nanobody to a peptide tag provides a tool to deliver a universal marker onto the tumor cells, e.g., as a docking site for a universal cytotoxic tag-specific antibody or for T cells transfected with a tag-specific chimeric antigen receptor (CAR). This has been demonstrated recently in an elegant proof of principle study with an EGFR-specific nanobody fused to the E5B9 peptide tag (the UniTag) (96). Opsonization of EGFR-expressing tumor cells with the nanobody-tag rendered the tumor cells highly sensitive for attack by human peripheral blood T cells that had been transduced to express a tag-specific CAR (UniCAR T cells).

Peptide-tagging can also be used as a tool for site-specific, enzyme catalyzed conjugation of a nanobody to virtually any desired chemical compound (**Figure 6A**). Genetic fusion of a nanobody to a C-terminal pentapeptide (Sortag) provides a substrate for site-specific, sortase-catalyzed C-terminal linkage of the nanobody to a small synthetic peptide, e.g., GGGX (97–100). “X” can be a lysine or cysteine residue conjugated by amide or maleimide chemistry to virtually any chemical moiety, e.g., a near-infrared fluorochrome (NIRF), a poly-ethylene-glycol tail, a chelator for a radionuclide, or tetrazine as a basis for click-reactions, e.g., for simple attachment of radioisotopes for PET imaging (97, 99, 100). In an elegant proof of principle study, sortagging was used to image tumor-infiltrating macrophages using ¹⁸F-labeled CD11c-specific nanobodies (97). The same group has recently reported the use of sortagging to attach a bi-functional tag to the C-terminus of a CD8-specific nanobody for imaging of tumor-infiltrating cytotoxic T cells (101). A chelator was used to install ⁸⁹Zr for PET imaging and an azide functionality for PEGylation. ⁸⁹Zr provided crisp PET images of lymphoid organs and CTL-infiltrated tumors, a 20-kD PEG moiety provide a much reduced accumulation of the labeled nanobody in the kidney compared to non-PEGylated nanobodies.

Akin to sortase-catalyzed transpeptidation of a nanobody fused to a sortag, BirA-catalyzed biotinylation of a specific peptide tag (Avi-tag) can be used to site-specifically biotinylate

nanobodies carrying an Avi-tag (102). BirA-catalyzed biotinylation can be achieved both *in vitro* with purified BirA or in cells by co-expression of BirA in the same cellular compartment as the Avi-tagged nanobody (103, 104). Biotinylation provides a universal anchor for high-affinity binding to Streptavidin-conjugates.

NANOBODY-TARGETED RADIONUCLIDES AND NIRFs

Nanobodies can readily be conjugated to radionuclides and fluorochromes, using either sortagging or classical chemical conjugation strategies (Figure 6B). Such nanobody-radionuclide and nanobody-NIRF conjugates are useful tools for imaging of tumors antigens or tumor-associated stromal cells, such as the mannose receptor of macrophages (MMR, CD206) (11, 16, 105–107). Moreover, such conjugates also have therapeutic potential, e.g., by local delivery of ionizing radiation to the tumor or by thermal cytotoxicity *via* a photosensitive, NIRF.

Conventional protein conjugation strategies use random conjugation to reactive side chains, most commonly amide conjugation to the amino group of lysine side chains or to the N-terminus of the protein (which, in case of V-domains, lies in proximity to the antigen-binding paratope). Random conjugation is difficult to control and may compromise the functionality of the nanobody, e.g., by sterically interfering with target binding, by sterically compromising tissue distribution, and by providing potentially immunogenic epitopes. Two elegant approaches have been developed to conjugate chemicals to nanobodies at a specific site. One involves the introduction of a cysteine residue at the C-terminus or at specific framework residues, providing a basis for site-specific maleimide conjugations (99, 102). The other method introduces a pentapeptide (LPXTG) that allows sortase-catalyzed transpeptidation (100) (see above).

Targeted radionuclide therapy is a systemic treatment that aims to deliver cytotoxic radiation to cancer cells and to cause at the same time minimal toxicity to surrounding healthy tissues. Radiopharmaceuticals consist of two components: a targeting moiety that specifically determines the accumulation of the radiopharmaceutical in the tumor and a radionuclide that delivers cytotoxic radiation through its decay (15). There is a growing interest in the use of nanobodies as targeting moieties for targeted radionuclide therapy (Figure 6B) (11). Nanobodies represent ideal candidates due to their high stability in harsh conditions, such as elevated temperatures and extreme pHs, offering the advantage to use a broader range of radiochemistry methods (15).

The utility of nanobodies as vehicles for targeted radionuclide therapy has been investigated in several preclinical models. An *in vivo* study demonstrated that ¹⁷⁷Lu-labeled anti-HER2 nanobodies efficiently targeted HER2-positive xenografts and prevented tumor growth, while keeping radioactivity levels low in normal organs (108). Another preclinical study in mice demonstrated that ¹⁷⁷Lu-labeled anti-idiotype nanobodies led to an inhibition of disease progression in multiple myeloma (109). However, radiolabeled nanobodies are characterized by fast clearance through kidneys, resulting in suboptimal absolute tumor

uptake but intense renal accumulation. Nephrotoxicity may be reduced by coadministration of gelofusin and lysine. This has been shown to reduce renal uptake of a ^{99m}Tc-labeled anti-EGFR nanobody by 45% in tumor xenografted mice (110).

Taken together, radiolabeled nanobodies are promising targeting moieties for targeted radionuclide therapy. Nanobody-based radionuclide therapy may be particularly beneficial in the treatment of micrometastatic and minimal residual disease, due to a highly specific deposition of radioactivity to tumor cells.

Photodynamic therapy induces cell death through light activation of a photosensitizer. NIRFs such as IRDye700DX can function as traceable photosensitizer (65, 100). For example, an EGFR-specific nanobody-photosensitizer conjugate rendered tumor cells sensitive to light induced death *in vitro* and in an orthotopic mouse tumor model *in vivo* (111).

NANOBODY-TARGETED NANOPARTICLES

Another approach for specific drug delivery is the generation of targeted nanoparticles (<200 nm), as encapsulation of drugs overcomes problems, such as poor solubility, limited stability, and rapid clearance (16) (Figure 6C). Nanoparticles used for this approach include liposomes (112, 113), micelles (114, 115), albumin-based nanoparticles (116, 117), and polymer-based polymersomes (118) or polyplexes (119). Nanobodies are advantageous for the decoration of the surface of nanoparticles due to their small size and the absence of an Fc-domain, as it decreases the chance of immunogenic responses and delay the clearance of these nanobody-targeted nanoparticles (120). *In vitro* experiments employing nanobodies as targeting moieties of nanoparticles have shown improved binding to the target cells (112, 115, 117, 121). *In vivo*, nanoparticles do not effectively cross endothelial barriers. It has been proposed that accumulation of the targeted nanoparticles within tumors is facilitated by the enhanced permeability and retention effect. The abnormal structure of rapidly growing tumor vasculature, combined with the lack of proper lymphatic drainage, leads to the accumulation of nanoparticles (122, 123). In cases where endothelial cells of the tumor vasculature express specific cell surface markers, it is feasible to specifically address nanoparticles to the tumor vasculature.

Release of the drugs from the particles can be achieved by leakage or by mechanical destruction by ultrasound or intracellular degradation. The first liposomes that were decorated with anti-EGFR nanobodies were internalized into the target cell (112). Anti-EGFR nanobody-targeted polymeric micelles containing doxorubicin were significantly more effective at inhibiting tumor growth and prolonging the survival of animals compared with untargeted micelles (114).

VIRAL AND CELLULAR DELIVERY OF ANTITUMOR NANOBODIES AND HEAVY CHAIN ANTIBODIES

While size does matter, it is not the only factor determining the delivery of nanobody-based biologics to tumor cells. Tumor delivery is controlled by a complex interplay of factors, many of

which are still poorly understood: the site of injection (e.g., intravenous, subcutaneous, intratumor), transport *via* the blood and lymphatics, diffusion through the endothelial cell and basement membrane into the interstitial space, hydrodynamic pressure in the blood vs. the tumor tissues, elimination of biologics from the system (e.g., by renal filtration, hepatic excretion, endocytosis by cells), binding to non-tumor cells, and binding to proteins (e.g., albumin, rheumatoid factor, other preformed or induced antibodies). It may, therefore, be of interest to consider and test other options for the delivery of antitumor nanobodies and heavy chain antibodies.

One interesting option is to use circulating cells of the immune system for antibody delivery. Immune cells are not or less effected than proteins by hydrodynamic pressure, endothelial barriers, and renal or hepatic excretion. Cells can effectively migrate through endothelial barriers and into the tumor microenvironment. Cells are eliminated by apoptosis and phagocytosis rather than by renal or hepatic excretion. A potentially powerful technique is to transduce T cells or NK cells to express a CAR on the cell surface (124, 125). CARs typically contain a scFv linked *via* a transmembrane domain to cytosolic activation domains. In nanobody-based CARs, the relatively unstable scFv is simply replaced by a stable nanobody (126–129). CAR-expressing T cells and NK cells can serially bind and kill many tumor cells expressing the target antigen. It is also conceivable that immune cells can be similarly transduced to secrete antitumor nanobodies, Nb-hcAbs, and other nanobody-based biologics.

Adeno-associated viruses (AAV) have been used successfully as gene-therapy vectors, i.e., for the long-term expression of a therapeutic proteins *in vivo* (130, 131). For example, intramuscular injection of AAV encoding HIV-neutralizing antibodies or a CD4-Fc fusion protein led to long-term production of the encoded antibodies and protection of mice from HIV infection (131, 132). In a recent proof of principle study, an AAV-encoded bispecific nanobody was effectively expressed *in vivo* and exhibited therapeutic efficacy in a mouse model of amyloidosis (133). It is conceivable that AAV can similarly be engineered for local and/or long-term expression of antitumor nanobodies *in vivo*.

CONCLUSION AND PERSPECTIVES

Nanobodies, nanobody-based heavy chain antibodies, and nanobody-drug conjugates have a huge potential as antitumor therapeutics. The US Food and Drug Administration recently granted fast track designation for caplacizumab, a bivalent nanobody targeting von Willebrand factor. Ablynx, the leading nanobody biotech company, has submitted an application for European Marketing Authorisation for caplacizumab, which thus may well become the first nanobody approved for therapy. Before nanobody-based antitumor therapeutics follow suite, additional preclinical and clinical studies are warranted. Antitumor nanobodies and antitumor Nb-hcAbs may overcome some of the obstacles that hamper therapies with antitumor mAbs. *In vivo* studies have underscored the favorable

biodistribution of nanobodies, including deep penetration into tumors. Numerous nanobody-based biologics have shown anti-tumor efficacy in preclinical studies *in vivo*. Naked nanobodies can antagonize growth factor receptors and block ion channels and ecto-enzymes in the tumor microenvironment. Fusion of one or more nanobodies to the hinge and Fc-domains of a human immunoglobulin yields highly soluble and versatile heavy chain antibodies. Importantly, because nanobodies do not bind light chains and because they do not show any tendency to aggregate, nanobody-based bispecific hcAbs do not suffer from the VH-VL pairing problem of bispecific conventional antibodies. Heavy chain antibodies are roughly half the size of conventional antibodies and, thus, may show better tissue penetration than conventional mAbs, while retaining the capacity to recruit the complement system, NK cells, and macrophages. Genetic fusion of nanobodies to peptide tags opens a path for marking tumor cells for attack by tag-specific T cells or NK cells transduced with a tag-specific CAR or with universal tag-specific cytotoxic antibodies. Sortagging and introduction of cysteine residues at specific framework positions allow easy conjugation to virtually any chemical moiety, including chelators for radionuclides, NIRFs, polyethylene glycol, liposomes, and nanoparticles. Preliminary studies indicate that it may well be worthwhile to further explore other modes for effective targeting of tumor cells with antitumor nanobodies, including Nb-CAR-expressing T cells and NK-cells and AAV encoding antitumor nanobodies.

V-domains display a much lower intrinsic immunogenicity than Fc domains across species barriers. Moreover, nanobodies show higher sequence identity to human VH domains than do murine VH domains and divergent framework residues routinely are “humanized” in clinical nanobodies. While fusion to the hinge and Fc domains of human IgG does not add any additional immunogenicity, fusion to toxins, peptide tags, and chemical conjugation add potentially immunogenic epitopes. Moreover, as with conventional antibodies, the antibody paratope can induce an anti-idiotypic antibody response in a fraction of patients. As distinct nanobodies to the same target protein become available, switching to a different antitumor nanobody will become a therapeutic option. Assuming that progress will continue at the present pace, it is likely that the future repertoire of clinicians will include an increasing battery of nanobody-based antitumor therapeutics.

AUTHOR CONTRIBUTIONS

FK-N and PB conceived the topic; PB, JH, and FK-N wrote the manuscript.

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Single-Domain Antibodies and the Promise of Modular Targeting in Cancer Imaging and Treatment

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Monoclonal antibodies and their fragments have significantly changed the outcome of cancer in the clinic, effectively inhibiting tumor cell proliferation, triggering antibody-dependent immune effector cell activation and complement mediated cell death. Along with a continued expansion in number, diversity, and complexity of validated tumor targets there is an increasing focus on engineering recombinant antibody fragments for lead development. Single-domain antibodies (sdAbs), in particular those engineered from the variable heavy-chain fragment (VHH gene) found in Camelidae heavy-chain antibodies (or IgG2 and IgG3), are the smallest fragments that retain the full antigen-binding capacity of the antibody with advantageous properties as drugs. For similar reasons, growing attention is being paid to the yet smaller variable heavy chain new antigen receptor (VNAR) fragments found in Squalidae. sdAbs have been selected, mostly from immune VHH libraries, to inhibit or modulate enzyme activity, bind soluble factors, internalize cell membrane receptors, or block cytoplasmic targets. This succinct review is a compilation of recent data documenting the application of engineered, recombinant sdAb in the clinic as epitope recognition “modules” to build monomeric, dimeric and multimeric ligands that target, tag and stall solid tumor growth *in vivo*. Size, affinity, specificity, and the development profile of sdAbs drugs are seemingly consistent with desirable clinical efficacy and safety requirements. But the hepatotoxicity of the tetrameric anti-DR5-VHH drug in patients with pre-existing anti-drug antibodies halted the phase I clinical trial and called for a thorough pre-screening of the immune and poly-specific reactivities of the sdAb leads.

Keywords: camelid heavy-chain antibody, drug-like properties, bioavailability, immunogenicity, broad epitope coverage, poly-specificity

INTRODUCTION

The success of monoclonal antibodies (mAbs) in cancer therapy is driven by the overall efficacy of targeted therapies. The rate of approval of recombinant mAbs continues to outperform that of small molecules in all indications and in particular for the treatment of cancer (1, 2). However, a recent rate of advancement of antitumor candidate leads from preclinical to clinical trial was estimated to be only 20% (3). One approach to improving this success rate is to focus early on a set of characteristics termed “developability” based on high-throughput qualification tests

applicable to mAb hits for a particular target. Two “developability” issues impacting candidate bioavailability are off-target binding and aggregation that can also result in toxicity and immune-reactivity. A candidate with a favorable profile is more likely to emerge from a large set of hits with a broad epitope coverage, by screening out off-target reactive mAbs (4) and guaranteeing “manufacturability,” or stability and solubility, of the lead candidate early in the pipeline (5–8). Camel and shark serum have provided a source of versatile antibody therapeutics with good “developability” and “manufacturability” prospects (6, 9–11). Most recombinant, variable heavy-chain (or VHH) single domains from homodimeric IgG2 and IgG3 found in camelids and VNAR of the so-called Ig new antigen receptor of sharks display higher solubility (above 1 mg/mL) and rapid refolding after temperature or chemical denaturation in comparison with the heterodimeric VL–VH domains in a Fab fragment (**Figure 1A**) (12, 13). VHH expression yield, whether in the periplasm of *Escherichia coli* or the cytoplasm of eukaryotic cells

Abbreviations: A431, epidermoid carcinoma cell line; Abzyme, an antibody with catalytic activity, binding a chemical group and stabilizing the transition state of a given reaction; ADA, anti-drug antibody; ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs; ADM, Adriamycin; BMCD, bone marrow culture-derived macrophages; BMP, bone morphogenetic protein; CA9/CAIX, carbonic anhydrase IX encoded by the CA9 gene; CD47/SIRP α axis, cluster of differentiation 47 and the myeloid inhibitory immunoreceptor signal regulatory protein α signaling axis; CapG, macrophage capping protein oncogene; CD16, cluster of differentiation 16, a low-affinity Fc receptor; CDR, complementarity determining region or antigen-binding domain; CendR, C-end rule motif R/KXXR/K; CEA, carcinoembryonic antigen; cDNA, complementary deoxyribonucleic acid; cMET, tyrosine-protein kinase Met or hepatocyte growth factor receptor (HGFR); CXCR4, fusin or CD184; CXCR7, atypical chemokine receptor 3 or GPCR 159; DAF, decay-accelerating factor; DR5, death receptor 5 of the TNF receptor superfamily (TNFRSF) 5; EGFR, epidermal growth factor receptor, a membrane tyrosine kinase; EpCAM, epithelial cell adhesion molecule or TROP1; EV, extracellular vesicle; Fab, immunoglobulin antigen-binding fragment composed of one constant and one variable domain of each of the heavy and the light chain; Fc, fragment crystallizable region of Ig; FDA, Federal Drug Administration; FR, framework region is a subdivision of the mAb variable region; FTCl, fluorescein isothiocyanate; GFP, green fluorescence protein; GITR, glucocorticoid-induced TNFR-related protein; GPI-DAF, glycosylphosphatidylinositol-anchored decay-accelerating factor; HcAbs, heavy-chain antibodies; HCV, hepatitis C virus; HER2, human epidermal growth factor receptor 2/neu tyrosine kinase, erbB-2; HER3, human epidermal growth factor receptor tyrosine-protein kinase erbB-3; HGF, hepatocyte growth factor; IA, intra auricular; IgG, immunoglobulin G; IgNAR, Ig new antigen receptor; IR, infrared; 131I-SGMIB, iodine-131-labelled N-succinimidyl 4-guanidinomethyl-3-iodobenzoate; iRGF, 9-amino acid cyclic peptide (sequence: CRGDKGPDC) binding tumor cells; i.v., intra venous; mAb, monoclonal antibody; Neae, N-terminal fragment of enterohemorrhagic *E. coli* intimin; NIR, near infrared; NRP-1, neuropilin 1; PBL, peripheral blood lymphocytes; PCR, polymerase chain reaction; PEG2000, poly(ethylene glycol) methyl ether, average Mw 2,000; PD-L1, Programmed death-ligand 1, CD274; p.i., post injection; pl, isoelectric point; PSA, Prostate-specific antigen; PSMA, prostate-specific membrane antigen; p53-HDM2, functional p53 and human double minute 2 interaction; SAS, solvent accessible surface; scFv, Single-chain variable fragment; sdAb, single-domain antibody fragment; SPECT, single-photon emission computed tomography; SPECT/CT, image fusion for anatomic imaging (CT or MRI) and functional imaging (SPECT) computed tomographies; SrtA, sortase A; RANKL, receptor activator of nuclear factor kappa-B ligand; RNA, ribonucleic acid; TNF α , tumor necrosis factor alpha; uPA, urokinase-type plasminogen activator; VCAM1, vascular cell adhesion protein 1; VEGF/Ang2, vascular endothelial factor/angiopoietin-2; VEGFR1, vascular endothelial growth factor receptor 1; VHH, heavy-chain only antibody fragment or nanobody; VH and VL, variable heavy and light chain domains from conventional IgG structures; VNAR, variable new antigen receptor single domain.

is high. Sequence identity of the VNAR domain with canonical human VH falls as low as 25%, while known camelid VHH domains are distinctly close to human VH3 germline sequences and a source of easily humanized single-domain antibody (sdAb) drugs (10, 14–16). In addition, services such as Hybribody, a platform from Hybrigenics for the selection and validation of antibodies derived from a fully synthetic humanized sdAb library displayed on phage, can supply humanized sdAbs to specific targets (**Table 1**, item 3) (17). The immunogenicity of humanized sdAbs may be erroneously overlooked yet it is tested in phase I clinical trials (18). The antigen-specific combining sites may be immunogenic providing sufficient justification for the early use of immunogenicity-screening platforms (19). The detection of anti-drug antibodies (ADA) using highly sensitive ELISAs at Ablynx revealed the benefit of mutating sdAb residues in hydrophobic patches at the C-terminus of VH of single-chain variable fragment (scFv) and VHH fragments, shielded by the CH domains in the original structure (20, 21).

The VHH repertoire is as complex in sequence diversity as is the IgG1 VH camelid counterpart (65–67). Total peripheral blood lymphocytes and lymph node ribonucleic acid (RNA) from alpacas, llamas, dromedaries, and camels are easily extracted to build recombinant VHH libraries. Typically, a VHH phage display library containing 6×10^7 VHHs clones are generated from 200 μ g processed RNA and diverse polymerase chain reaction strategies are available to amplify VHH gene fragments from lymphocyte complementary deoxyribonucleic acid (68, 69). Several reports have confirmed the ease of engineering sdAbs (69, 70) and of selecting specific binders against conformational epitopes in comparison with hit selection of scFv, where library construction shuffles their immune specificity (68, 71, 72).

Two or three VHHs have been combined in a single polypeptide chain to express single, dual, or multimeric specificities without compromising folding or the binding affinities (22, 73). In addition, “self-associating peptide” constructs have been designed to match VHH pairs (69, 74). Concomitantly, the experience gained in site-specific conjugations, in particular those driven by targeted enzymatic reactions, has ensured the preservation of antigen-binding properties of sdAbs (31, 75). The reported affinities of VHH fragments fall in the nanomolar to picomolar range, with binding kinetics comparable to those of conventional antibodies. Selection of stable antigen complexes is often the result of applying selection pressures, such as stringent washing, that enrich a library in VHH with slower off-rates while competitive elution was reported in selecting fragments with novel epitope targeting (70, 76–79). VHH genes are an established source of antibodies, as evidenced by the number of reported co-crystal structures (68, 80–82). **Figure 1A** highlights hallmark VHH residues and, when present, an inter-CDR disulfide bond in the VHH sequence. Around 10% of HcAbs lack these hydrophobic residues mutation but often show longer CDR3 covering putative VL contacts or a hydrophilic substitution of Trp118. Gonzalez-Sapienza et al. suggested a plausible mechanism of selection of HcAb producing B-cells that supports the emergence of independently folding, soluble VH and VHH domains (72).

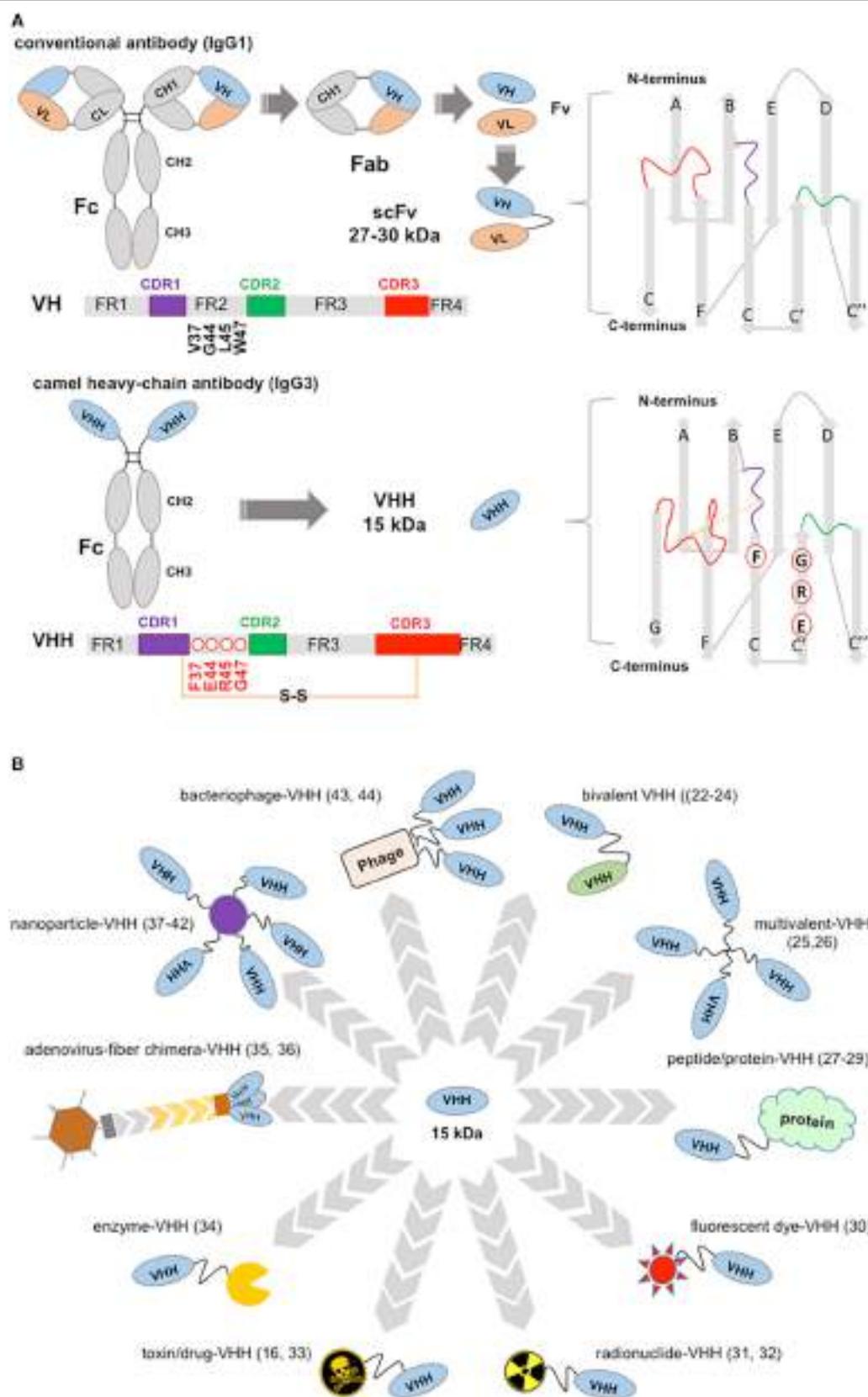
**FIGURE 1 |** Continued

FIGURE 1 | Structure of a “conventional” IgG1 and of a camelid IgG3, showing variable domain differences and illustrations of potential, VHH-based, cancer therapeutics. **(A)** Schematic of an IgG1 showing canonical hypervariable domains (left top diagram) consisting of two light (L) chains, comprising the VL and CL domains, and two heavy (H) chains composed of the VH, CH1, hinge, and CH2 and CH3 domains; and, below a camelid homodimeric heavy-chain IgG3, a heavy-chain antibody (HCAb) (left bottom diagram) which comprises only H chains; each H chain contains a short VHH hinge, CH2, and CH3 domains. The homodimeric heavy-chain IgG2 (not shown) has longer VHH hinge domains compared to IgG3 and comparable CH2, CH3. The smallest intact functional antigen-binding fragment that can be generated from the immunoglobulin G (IgG) canonical variable domains, consists of an oligopeptide linked VH–VL pair known as single-chain variable fragment (top right), while the smallest intact functional antigen-binding fragment of HCAbs is the single-domain VHH (bottom right) known as Nb. VH and VHH bars show framework (FR), complementarity domain regions (CDRs) (color coded), and key residues substitutions. Non-canonical C residues are involved in an inter-CDR disulfide bond in VHH structure. **(B)** VHH-associated strategies in targeting tumors and tumor accessory cells. Top, clockwise: bivalent bi-specific VHH (22–24); multivalent, high-avidity mono-VHH molecules (25, 26); VHH fusions ranging from vascular penetration peptide-VHH to engineered hu-Fab and albumin-binding domains (27–29); fluorescent dye fusions, for example, one spontaneously crossing the blood–brain barrier (30); radionuclide-VHHs (31, 32); toxin-VHH theragnostics (16, 33); chromogenic enzyme fusions: here an alkaline phosphatase-VHH may be applied in ELISA, dot blot, and transferred protein identification in western blot (34); oncolytic virus (35, 36); VHH decorated nanoparticles for therapeutics delivery and in facilitating photothermal therapy (37–42); bacteriophage engineered to display VHH and deliver targeted therapeutics (43) may also be developed for signal amplification in ELISA assays (44).

TABLE 1 | Summarized single-domain antibody (sdAb) research and development in cancer diagnostics and therapy.

Services ^a	Applied technologies	Proposed clinical benefit	Service provider ^b
1. Customizing sdAb engineering	Immune, naïve, and synthetic/humanized libraries phage display, bacterial display, intrabody library services, VHH production (45)	sdAb innovative binder formats, systems biology and target validation tools (46) Pipeline construction (47)	GenScript; Creative BioLabs; Lampire Biological Laboratories; Capralogics, Inc.; ProSci, Inc.; Hybrigenics Corporation, Allele Biotechnology and Pharmaceuticals, Inc.; Qoolabs, Inc.; Abcore Inc.; QVQ Holding BV; Rockland Immunochemicals, Inc.
2. Optimizing sdAb lead candidate selection	Epitope binning and optimum epitope coverage antibodies and sdAb, tested in a pairwise combinatorial manner (8)	Multiple epitope bins reflect functional diversity, support oligoclonal therapy or the simultaneous targeting of biological pathways; watch for off-target binding (48)	Carterra, Inc.; Creative BioLabs
3. Humanizing and screening sequences to diminish sdAb immunogenicity	sdAbs humanization (15, 45) and Identification of potential immunogenic sequences (21)	lower sdAb immunogenicity	GlobalBio, Inc.; Creative BioLabs; Hybrigenics Corporation; EpiVax, Inc.
4. Tailoring the sdAb <i>in vivo</i> half-life	Half-life optimization in circulation (49); Nanobody®-based half-life extension technology	Ozoralizumab, a next-generation bivalent tumor necrosis factor alpha (TNF α) blocker linked to a low-affinity albumin-binding domain	Ablynx; Eddingpharm
Applications ^c	Targeted tumor antigens	Clinical trials	Developer ^b
5. Overcoming monoclonal antibody limitations by targeting inaccessible and intracellular tumor antigens	CapG (50), non-endocytic co-transport and cytoplasmic translocation (51), DR5 (52), dynamic transformation (53), Glioblastoma (54), CA9/CAIX activity (55), p53-HDM2 disruption (56), mesothelin (57)	not initiated or halted	Novartis; ProSci Inc.; Hybrigenics Services; QVQ Holding BV
6. Selecting proficient probes for molecular imaging	^{131}I -SGMIB Anti-HER2 sdAb ^{68}Ga -HER2-sdAb (near infrared) probes in sentinel lymph node detection or residual tumor tissue (58)	Phase I, CAM-VHH1 Study NCT02683083 Phase II PET/CT. Clinical Trial II	Camel-IDS NV, TBM program ^d (social, non-profit organization), QVQ holding BV
7. Targeting known tumor antigens	Epithelial growth factor receptor (59), carcinoembryonic antigen (60), prostate-specific membrane antigen, anti-VEGF/angiopoietin 2 (BI 836880 Nb \circledast), anti-RANKL (ALX-0141 Nb \circledast), TNF α , ADAMTS5	Phase I, Boehringer Ingelheim, anti-VEGF/angiopoietin 2 Nb \circledast , safety in cancer patients Phase I, Ablynx (ALX-0141 Nb \circledast) safety and pharmacokinetic study Anti-ADAMTS5, M6495 Nb \circledast Interventional, Merck KGaG in healthy volunteers. NCT03224702	Ablynx/Merk; Boehringer Ingelheim; Eddingpharm, clinical development, registration and commercialization in Greater China of anti-RANKL Nb \circledast and ozoralizumab; Merck KGaG
8. Targeting immune checkpoints	PD-L1 (61), CD47/SIRP α axis (62, 63), glucocorticoid-induced TNFR-related protein	Early Phase I, $^{99\text{m}}\text{Tc}$ labeled anti-PD-L1 sdAb for diagnostic imaging of non-small cell lung cancer. Pending. NCT02978196	Merck & Co.; Merck KGaG; Ablynx

(Continued)

TABLE 1 | Continued

Applications ^a	Targeted tumor antigens	Clinical trials	Developer ^b
9. Testing molecular mimicry, including anti-idiotypes and abzymes	Ab2 abzymes with alliinase activities (64), self-diversifying antibody library platform (SDALib)	New drug discovery using Abzyme's yeast-based camelid single domain VHH antibody library with self-diversifying ability, to generate VHH antibodies against cancer-related target isoforms	Abzyme Therapeutics, LLC and Ibex BioSciences, LLC partnership

^aServices that support sdAb generation and lead candidates screening.

^bSearch business firm information with preferred online engine.

^cApplications that may broaden the range of tumor targeting lead candidate.

^d<http://www.innovatiennetwerk.be/projects/2275>.

DISTINCTIVE PROPERTIES OF sdAbs

The ease of selecting sdAb under denaturing conditions has assisted in the isolation of “superstable” species with improved resistance to proteases that were proposed as antimicrobial therapeutics of oral intake (83, 84). Li et al. have successfully selected VHH expression products with a high isoelectric point (pI) that spontaneously crossed the blood–brain barrier (transcytosis) (30). High-pI sdAb have been found to penetrate cells and bind to intracellular proteins. For instance, a sdAb that bound specifically to the hepatitis C virus (HCV) protease, selected for its ability to penetrate cells (transbodies), interfered with heterologous HCV replication (15). A sdAb-based anti-β-catenin intrabody was expressed and folded in the cytoplasm retaining its ability to bind to β-catenin (85).

The solvent accessible surface (SAS) area of antigen-VHH and VNAR complexes are comparable to antigen-VH–VL complex SAS indicating that complementarity domain region (CDR) loops involved in antigen binding (**Figure 1A**) contribute similar surface contacts. VHH H1 and H3 loops connecting the β-sheets of the VHH domain are flexible, sometimes longer and packed in a less compact fashion compared to canonical VH of murine and human immunoglobulin G (IgGs) (10, 86). Co-crystal structures of enzyme-VHH and -VNAR complexes showed CDRs that often protruded into the active-site cleft and the derived sdAbs were later shown to inhibit catalysis (65, 66, 87, 88). Alternatively, sdAbs have been selected to stabilize “drugable” targets that display multiple conformations (or conformational plasticity) (79, 82). For example, the urokinase-type plasminogen activator (uPA) from the trypsin-like serine protease family, a target involved in metastasis, is known to adopt high and low activity conformations. Selection of sdAbs against mouse uPA yielded both a catalytic-site inhibitor and an allosteric ligand. Crystal structures of the uPA sdAb complexes revealed high and low activity determinants that provided clues of therapeutic value on the regulatory determinants of uPA and of trypsin-like serine proteases in general (89). **Table 1** documents the pharmaceutical relevance of sdAbs through the number of research and development companies involved in novel sdAb generation, available contract services, lead candidates under clinical trial, and examples of the sdAbs more recently generated against cancer targets.

sdAbs IN IMAGING APPLICATIONS FOR CANCER DIAGNOSTICS

Molecular imaging techniques, of widespread use in the clinic, allow the non-invasive quantitation and visualization of tumors *in vivo* and sdAbs have become promising, small-sized, high-affinity tracers (58, 90–92) (**Figure 1B**). Nuclear imaging probes associated to sdAbs have been evaluated in both single-photon emission computed tomography (SPECT) and positron emission tomography (PET) (90, 93) (**Table 1**, item 6). The most advanced sdAb under clinical evaluation is the ⁶⁸Ga-labeled anti-HER2 sdAb 2Rs15d probe, developed to screen candidates who qualify for treatment with an anti-HER2 therapeutics. A phase I study resulted in high-quality images without adverse reactions and retained 10% of injected activity in blood after 1 h (94). A phase II trial was launched to correlate tumor uptake with HER2 levels in biopsies of 160 metastatic breast carcinoma patients (**Table 1**, item 6). In other studies, 2Rs15d labeled with the prosthetic group *N*-succinimidyl-4-[¹⁸F] fluorobenzoate ([¹⁸F]-SFB) was validated in preclinical models to advance PET imaging (95). The specific uptake of the sdAb 2Rs15d probe in HER2-positive tumor xenografts showed high tumor-to-blood and tumor-to-muscle ratios, high contrast PET imaging and fast renal clearance (4% intra auricular/g at 3 h post injection.). The lead candidate MSB0010853, a biparatopic sdAb labeled with ⁸⁹Zr bound efficiently to HER3 kinase, a potential clinical target associated with resistance to epithelial growth factor receptor (EGFR) and HER2 targeted therapies (96, 97).

Organometallic radiopharmaceuticals are also widely used in diagnosis with SPECT imaging. sdAbs that target either EGFR (98), VCAM1, an 8-kDa fragment of gelsolin or carcinoembryonic antigen (CEA) have been conjugated with ^{99m}Tc (99). Recently, an anti-PD-L1 sdAb labeled with ^{99m}Tc discriminated wild type mice from PD-L1 knock-out mice by SPECT/CT imaging (100). sdAbs used as fluorescence-guided near-infrared wavelength range (NIR) probes are also under preclinical studies addressing sentinel lymph node imaging quality and guiding surgical/endoscopic removal of residual tumor tissue (101). NIR probes, IRDye800CW or IRDye680RD, were conjugated either by lysines or C-terminal cysteine to the 7D12 anti-EGFR sdAb. After IR dye conjugation, comparable specificities and affinities of 7D12 and

the conjugate were measured toward EGFR *in vitro* (58, 102). This study also showed an accumulation of the cysteine-conjugated 7D12 in A431 human tumor xenografts in nude mice or high tumor-to-muscle ratio.

The ultrasound imaging of vessel cell adhesion protein 1 (VCAM1), using specific sdAbs coupled to lipid microbubbles as contrast enhancers, is used to assess potential adhesion sites of melanoma cell extravasation and metastasis (75). Although sdAbs are promising imaging probes renal retention during clearance and toxicity were reported in preclinical studies. Adverse effects were attributed to the polar residue number favoring the interaction with the megalin/cubilin system in the renal tubuli (103). This issue was overcome by mutating positive residues, facilitating filtration at the negatively charged glomerular membrane (104). Toxicity was also controlled by gelofusine or lysine added to the probe (103, 105).

sdAb AGAINST TUMOR TARGETS FOR CLINICAL USE

Single-domain antibodies that bind either hepatocyte growth factor, EGFR, bone morphogenetic protein (TGF β superfamily growth factors), HER2, cMET, or VEGFR1, have been shown to efficiently block tumor cell proliferation (81, 106–109). Zhang et al. (61) have recently shown that KN035, an anti-PD-L1 sdAb, can induce T-cell responses and inhibit tumor growth; the KN035 CDRs structure is remarkably similar to that of the VH of Federal Drug Administration-approved Durvalumab (110). Other sdAbs were developed to target uPA, and chemokine receptors such as CXCR4 and CXCR7 (111). More recently, sdAbs targeting antioxidant enzymes such as membrane catalase and superoxide dismutase were selected for their ability to induce reactive oxygen species-dependent cancer cell apoptosis and found to be synergistic to chemotherapy (112).

Single-domain antibody modules have been engineered into multivalent structures to overcome fast clearance. The anti-DR5 sdAb tetramer showed excellent pharmacokinetics and efficacy in preclinical models, inducing robust antitumor responses and sustained caspase activation *in vivo*. However, in the phase I trial an unexpected hepatotoxicity which triggered hepatocyte apoptosis, later associated to the immune crosslinking of the tetramer in those patients with pre-existing ADA, prompted its discontinuation (113). A bifunctional sdAb, targeting EGFR and TRAIL, inhibits the growth of different tumor cell types that were not responsive to either EGFR-antagonist or death receptor-agonist monotherapies is a clear step forward of the clinical application of sdAb modules (23). To improve the efficacy of a bifunctional therapeutic, the MaAbNA-PEG2000-ADM chimera consisting of an anti-EGFR1 sdAb linked to two anti-HER2 affibodies was conjugated with Adriamycin (114). The bispecific sdAb chimera recognizing CEA and antigen cluster of differentiation 16 (CD16) (NK-cell marker) was linked to a mutated human IgG1 Fc-fragment that equipped the dimer with an effector function (115). The bispecific antibody HER2-S-Fab, an anti-CD16 sdAb that is linked to a anti-trastuzumab Fab, also exhibited a potent tumor growth inhibition in a human tumor xenografts model (29). A multivalent, sdAb-based, in-tandem

trimer was capable of simultaneously binding to CEA, EGFR, and green fluorescence protein with high efficacy for inhibition of human epidermoid carcinoma A431 cell proliferation (26). An interesting approach to increase the half-life of sdAbs without affecting the affinity for its target was the fusion between an anti-TNF α sdAb with an albumin-binding domain derived from *Streptococcus zooepidemicus* (~39-fold half-life increase with respect to the sdAb alone, Table 1, item 4) (28).

Targeting tumors with ionizing radiation is also a promising area for growth for sdAb therapeutics. The most relevant *in vivo* study demonstrated that i.v. administration of the sdAb anti-HER2 labeled with ^{177}Lu , a γ -emission radionuclide, completely prevented tumor growth in mice with small HER2-positive tumors (32). The α -emitting radionuclides ^{213}Bi and ^{211}At coupled to sdAbs are tentatively used to treat minimal residual disease and micro-metastasis and their clinical application is being intensely explored (116).

EMERGING DRUG-DELIVERY STRATEGIES THAT USE sdAbs

To improve solid tumor penetration an EGFR-targeted sdAb was fused to an iRGD, a cyclic domain selective of $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ integrins that carries a CendR motif that binds neuropilin 1 (NRP-1) (117). The efficacy of this construct was measured in BGC-823 multicellular spheroids that overexpress EGFR, NRP-1, and integrins. The anti-EGFRsdAb-iRGD showed better performance in reducing spheroid size than anti-EGFRsdAb or cetuximab. *In vivo*, anti-EGFRsdAb-iRGD-FITC was shown to bind to $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ expressed in the tumor vessels, malignant cells, and cancer-associated stromal cells, penetrating further than the anti-EGFR-FITC (27). Recently, anti-EGFRsdAb-iRGD was conjugated to silk fibroin nanoparticles loaded with paclitaxel, resulting in a significant anti-neoplastic activity in EGFR-expressing cells *in vitro* and *in vivo* (41).

Single-domain antibody has been successfully used to retarget oncolytic adenovirus to a non-cognate receptor following the incorporation of an anti-CEA sdAb into the adenovirus capsid fiber (Figure 1B). This modification was shown to control viral tropism, entry, and gene transfer specifically in CEA-overexpressing cells (36, 118). sdAb displayed on genetically engineering phage combined with target drugs or imaging probes has recently been proposed for preclinical evaluation (43, 119).

Single-domain antibodies have been used to retarget nanoparticles with particular diagnostic or therapeutic properties (120, 121). Branched gold nanoparticles functionalized with an anti-prostate-specific antigen sdAb were shown to destroy cancer cells in response to laser irradiation in a preclinical model of photothermal therapy (37). Pegylated liposomes, schematized in Figure 1B, may be re-directed away from the reticuloendothelial system by coupled sdAbs and are under preclinical evaluation as drug nanocarriers (39, 40). A novel potent delivery system based on extracellular vesicles (EVs) has recently been described where an anti-EGFR sdAb was anchored on the surface of EVs via glycosylphosphatidylinositol signal peptides derived from the decay-accelerating factor significantly improving EV targeting (42).

PLATFORMS FOR THE GENERATION OF NEW sdAbs

Epitope recognition and coverage appear to be dependent on immune-selection pressure of VH and VHH sequences *in vivo* and by the library diversity (122, 123). To amplify antigenic epitope coverage, naïve and semi-synthetic libraries are being promoted to amplify antigen epitope coverage often limited by B-cell IgG amplification *in vivo*. Low affinities may be matured or optimized as required. sdAb discovery may now count on high-throughput, high-resolution broad epitope coverage analysis and poly-specificity and affinity screening tools to increase the likelihood of selecting sdAbs with the desired therapeutic functions (**Table 1**, item 2) as well as to discriminate between functional sdAbs, such as those that can trigger receptor internalization (124) and polyreactive leads (8).

Three novel VHH library presentation and selection platforms have been recently proposed for a high-throughput selection of sdAb to integral membrane tumor antigens, or proteins overexpressed on the surface of whole cells or on virus-like particles (70, 123). Two of the platforms were designed to identify binders to antigen diluted in lysates or in complex mixtures for the discovery of sdAbs that bind critical pathway targets (78, 125). Rosotti et al. reported high throughput, parallel selection and characterization strategies to identify phage-displayed sdAbs against receptors expressed on murine bone marrow-derived dendritic cells (123). As a result of *en masse* cloning and whole-cell screening, the *in vivo* biotinylation of selected VHH facilitated the identification of targets. The isolated VHH were effectively mapped, or binned, by epitope, and target coverage was recorded [also see Ref. (126), **Table 1**, item 2].

Salema and Fernandez optimized the display of VHH on Gram-negative *E. coli*, and the direct expression of selected VHH clones, by anchoring the expression product on the outer membrane by fusing to the N-terminal, intimin β-domain (Neae) (78, 127, 128). High-affinity clone selection was optimized by magnetic cell sorting on immobilized recombinant biotinylated antigen (MACS) or by flow cytometry on whole cells (Cells) (78).

A third sdAb selection platform was presented by Cavallari using a Gram-positive Staphylococcal (*Staphylococcus aureus*) display of sdAb (125). Here, VHH clones were engineered with the signal peptide from staphylococcal enterotoxin B, with the sortase A (SrtA) LPXTG motif, to display folded VHH on the surface. Endogenous SrtA covalently, and irreversibly, coupled expressed sdAb on the outer membrane. A nucleophilic attack of the SrtA sdAb-acyl intermediate by polyglycine nucleophile-biotin was used to release and biotinylate selected VHH clones. The major advantages of bacterial display were the efficiency of selection as reflected by a high “hit” frequency, or high frequency of success, in comparison to hit selection by phage display, and minimum avidity. Also attractive is the choice of evaluating selected sdAbs by flow cytometry or in SPR binding assays

directly enabling screening sdAbs by epitope and a discrimination of poly-specificity in a high-throughput mode (78, 128).

CONCLUDING REMARKS

Single-domain antibodies are soluble, stable, recombinant proteins that fold independently and display an outstanding versatility. The hardware-building concept of “plug and play” appears as an excellent paradigm in which sdAbs are part of a therapeutics generation tool kit that includes engineered recombinant sdAbs, radionuclides, dyes, peptides, proteins, nanostructures, phage, and virus.

Currently, 20–25% of the mAbs in clinical development for cancer and non-cancer indications are recombinant human antibodies derived from phage display libraries or from transgenic mice. Five antibody “fragments” (scFv) were reported in clinical phase 2/3 this past year. These include a human scFv-doxorubicin loaded liposome; two scFv conjugates, a humanized anti-EpCAM scFv-immunotoxin conjugate; and an anti-fibronectin extra-domain B human scFv for cancer indications.

The unexpected toxicity of the anti-DR5 tetramer, TAS266, opened the question of pre-existing immunity against sdAb. This issue has been addressed by developing sensitive immune serum assays and immunogenicity-screening platforms (**Table 1**, item 3, EpiVax) to identify the safer lead candidates, helping reduce the risk of clinical trial failure of sdAb-based drugs. The promise of recombinant, engineered, antibody-based building modules with optimal efficacy and bioavailability may soon translate into tangible cancer drugs.

AUTHOR CONTRIBUTIONS

MI, LP, SW, OP, and GC: conception, design, and writing of the review manuscript.

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Immunomodulatory Monoclonal Antibodies in Combined Immunotherapy Trials for Cutaneous Melanoma

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In the last few years, there has been a twist in cancer treatment toward immunotherapy thanks to the impressive results seen in advanced patients from several tumor pathologies. Cutaneous melanoma is a highly mutated and immunogenic tumor that has been a test field for the development of immunotherapy. However, there is still a way on the road to achieving complete and long-lasting responses in most patients. It is desirable that immunotherapeutic strategies induce diverse immune reactivity specific to tumor antigens, including the so-called neoantigens, as well as the blockade of immunosuppressive mechanisms. In this review, we will go through the role of promising monoclonal antibodies in cancer immunotherapy with immunomodulatory function, especially blocking of the inhibitory immune checkpoints CTLA-4 and PD-1, in combination with different immunotherapeutic strategies such as vaccines. We will discuss the rational basis for these combinatorial approaches as well as different schemes currently under study for cutaneous melanoma in the clinical trials arena. In this way, the combination of “push and release” immunomodulatory therapies can contribute to achieving a more robust and durable antitumor immune response in patients.

Keywords: monoclonal antibodies, immune checkpoint blockade, combined tumor immunotherapy, clinical trials, cutaneous melanoma

INTRODUCTION

An important role for the immune system in cancer biology has been proposed for decades. However, immunotherapy has been very recently recognized as an effective treatment to control tumor growth and dissemination. Cutaneous melanoma (CM) is a highly mutated and immunogenic tumor and has been a rich field for the development of tumor immunology and immunotherapy (1–3). There is strong evidence of common tumor antigens (AgS) as well as patient's own tumor neoantigens

Abbreviations: Ags, antigens; APCs, Ag-presenting cells; CM, cutaneous melanoma; CTLA-4, cytotoxic T-lymphocyte antigen 4; DCs, dendritic cells; DFS, disease-free survival; ICKB, immune checkpoint blockade; ID, intradermally; IV, intravenous; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid-derived suppressor cell; MM, metastatic melanoma; mAb, monoclonal antibodies; neoAgS, neoantigens; OS, overall survival; PAMP, pathogen-associated molecular patterns; PD-1, programmed cell death-1/CD279; SC, subcutaneous; Tregs, regulatory T cells; TLR, toll-like receptors; TAA, tumor-associated Ag; TIL, tumor infiltrating lymphocytes.

(neoAgs) which are recognized by the immune system (4); of tumor infiltration by specific immune populations and their clinical correlation (5); and of tumor immunoediting including immune escape strategies (6). More recently, the discovery of multiple immune checkpoint mechanisms, either stimulatory or inhibitory pathways of the immune system, which can be targeted with monoclonal antibodies (mAbs), has burst into intense clinical research. In particular, immune checkpoint blockade (ICKB) with mAbs immunotherapy has proved for the first time to improve overall survival (OS) of CM metastatic patients (7). Nowadays, immunotherapeutic approaches including ICKB with mAbs is the fourth cancer treatment modality along with surgery, radiotherapy, and chemotherapy/targeted therapy. The use of ICKB has expanded beyond CM and these therapies are now approved for the treatment of several metastatic tumors, including renal cell carcinoma, non-small-cell lung carcinoma, squamous cell carcinoma of the head and neck, urothelial carcinoma, and Hodgkin lymphoma (www.fda.gov/drugs).

The inhibitory immune checkpoint molecules act as physiological brakes that prevent potentially harmful immune responses and autoimmunity. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is constitutively expressed in regulatory T cells (Tregs) but is only upregulated in T-cells after activation. Physiologically, CTLA-4 transmits an inhibitory signal to T cells to shut down immune responses by interaction with CD80 and CD86 molecules, which are expressed at the surface of Ag-presenting cells (APCs) such as dendritic cells (DCs) and macrophages; CTLA-4 also contributes to the inhibitory function of Tregs (8). Ipilimumab (Bristol-Myers Squibb) targets CTLA-4, blocking the inhibitory signal, unleashing cytotoxic T cells to eliminate the cancer cells (7). Another inhibitory immune-checkpoint that has been extensively targeted with mAbs is the axis programmed cell death-1/CD279 (PD-1) and their ligands PD-L1 (B7-H1/CD274) and PD-L2 (B7-DC/CD273) (9). PD-1 is expressed on the membrane of activated T lymphocytes; PD-L1 and PD-L2 are expressed in APCs and also in some tumor cells. PD-1 and PD-L1/PD-L2 binding induce a coinhibitory signal that limits the development of the T-cell response. Several blocking mAbs targeting PD-1 are currently indicated to treat different tumors, such as nivolumab (Bristol-Myers Squibb) (10) and pembrolizumab (Merck Sharp & Dohme) (11). Accordingly, there are mAbs targeting PD-L1, such as atezolizumab (Roche), avelumab (EMD, Serono), and durvalumab (Astra Zeneca). Blocking of CTLA-4 molecule would affect the initial priming phase while targeting the PD-1/PD-L1 axis would interfere more profoundly with the effector phase of the anti-immune response. Although ICKB has shown to potentiate long-lasting antitumor immune response in the metastatic setting, about only 30% of patients achieve durable responses to ICKB with mAbs, thus intense research is ongoing to unravel the mechanisms involved in both primary and acquired ICKB resistance.

Now that ICKB has proven to induce clinical responses for several tumor pathologies, it is currently being investigated in combination with other immunomodulatory strategies in clinical trials, in an attempt to improve antitumor immune responses. ICKB can be combined with antagonists of immunosuppressive molecules or different immunostimulatory treatments, such as active tumor

vaccines, administration of cytokines, tumor-specific mAbs, and adoptive cell therapy (ACT). As shown in **Figure 1**, future immunotherapy can be seen from an integrative point of view, allowing the combination of different approaches that target both the tumor cells and the immune microenvironment, with impact in the systemic immune status. In this review, we have selected several examples of such combinatory strategies that are currently being investigated in clinical studies to discuss the rationale and potential results (www.clinicaltrials.gov) (**Tables 1–3**). Most of these trials are in the initial phases (I-II) and are directed toward advanced metastatic tumor patients including CM, mainly to optimize dose regimens and observe safety, side effects, and initial response in patients, including potential immunogenicity and antitumor immune response.

CLINICAL TRIALS

ICKB Combined with Immunostimulatory Strategies

Vaccines

Therapeutic vaccines are under investigation but still have shown only limited clinical benefit for patients with CM and other tumors. The rationale of therapeutic vaccination is to boost the patient's immune system to induce a pro-inflammatory $T_{H}1$ immune response targeting both shared common tumor Ags and patient-specific neoAgs generated by somatic mutations in a personalized fashion. Vaccines can be prepared as peptides, tumor lysates, or irradiated whole tumor cells, administered with adjuvants to potentiate the immune response or as autologous DCs loaded with the tumor-Ag source.

The Phase III pivotal study that allowed FDA approval of ipilimumab to treat unresectable Stage III–IV melanoma patients was in fact designed to determine the safety and efficacy of ipilimumab in combination with BMS-734019 vaccine, a tumor-associated Ag (TAA) gp100-peptide vaccine, versus vaccine or ipilimumab alone (NCT00094653) (7). The main results of that study were that ipilimumab, with or without a vaccine, in comparison to vaccine alone, improved OS of metastatic CM patients. Severe adverse events were observed but most of them were reversible and manageable. In 2015, a pooled analysis was performed of long-term survival data of 1861 patients from Phase II and III studies (NCT00032045, NCT00058279, NCT00077532, NCT00094653, NCT00135408, NCT00261365, NCT00289627, NCT00289640, NCT00324155, and NCT00623766), some of them including combined vaccination with peptides, the majority of patients receiving the 3 mg/kg regimen. Also, data from additional 2,985 patients from an expanded access program were analyzed. A plateau in the survival curve was observed, beginning at approximately 3 years, which was independent of prior therapy or ipilimumab dose, supporting the impact of ipilimumab in long-term survival for advanced CM patients (12).

Recent combination trials of ICKB with peptide vaccines are shown in **Table 1**. 6MHP, a melanoma vaccine comprised of 6 MHC-II-restricted helper peptides administered subcutaneous (SC) and intradermally (ID) as water-in-oil emulsions with Montanide ISA-51, in combination with pembrolizumab

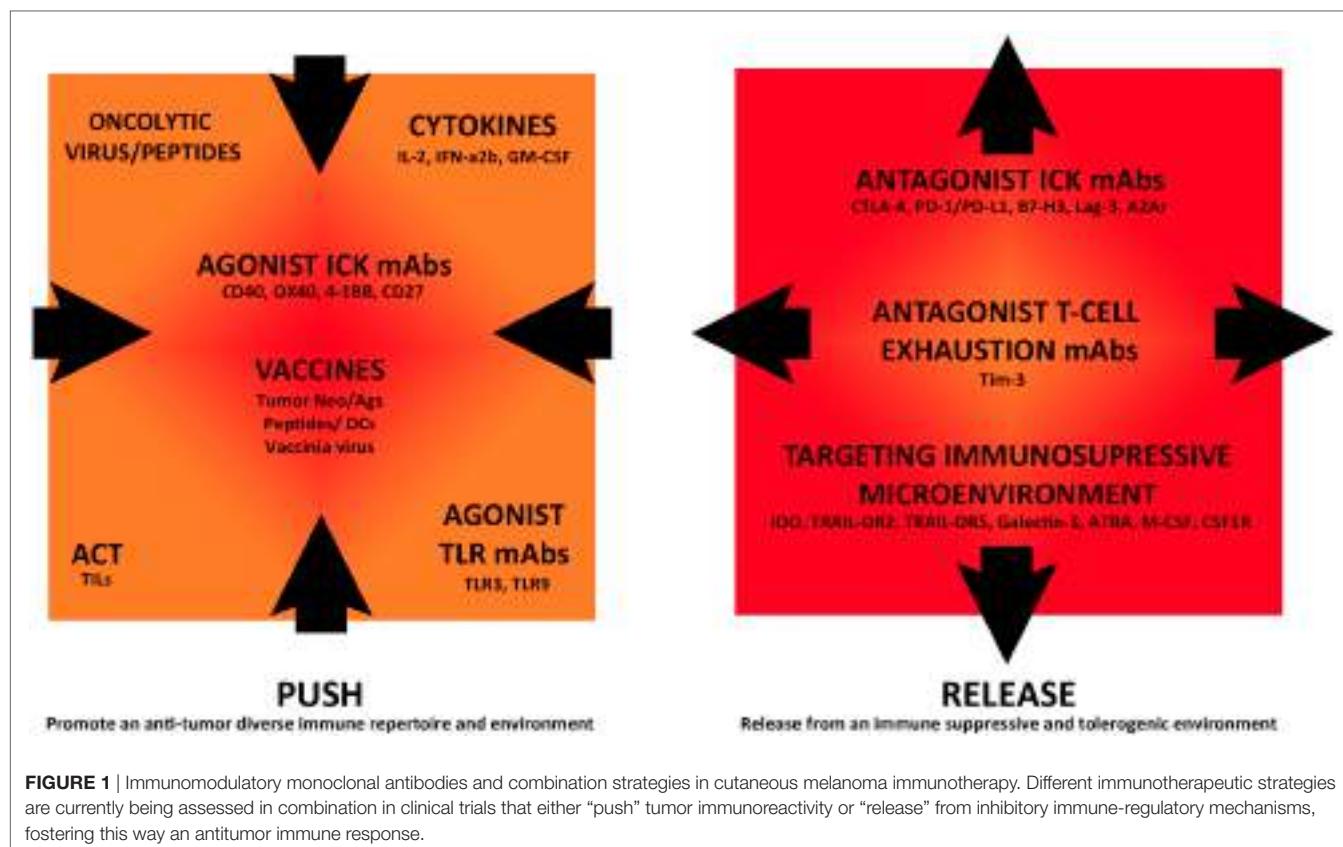


FIGURE 1 | Immunomodulatory monoclonal antibodies and combination strategies in cutaneous melanoma immunotherapy. Different immunotherapeutic strategies are currently being assessed in combination in clinical trials that either “push” tumor immunoreactivity or “release” from inhibitory immune-regulatory mechanisms, fostering this way an antitumor immune response.

(NCT02515227) or ipilimumab (NCT02385669), is under investigation. NCT03047928 trial will combine nivolumab with a peptide vaccine consisting of PD-L1 and indoleamine 2,3-dioxygenase (IDO) peptides. T-cell reactivity against PD-L1 and IDO in the tumor microenvironment and in the peripheral blood of CM patients with cytotoxic activity has been reported (13). Thus boosting specific T cells that recognize immune regulatory proteins such as IDO and PD-L1 may directly modulate immune regulation. In the protocol, patients will be treated with nivolumab every second week as long as there is a clinical benefit. The PD-L1/IDO peptide vaccine is given from the start of nivolumab and every second week for the first six vaccines and thereafter every fourth week up to 1 year. NCT01176461 tested the side effects of an investigational vaccine consisting of gp100280-288 and NY-ESO-1157-165 MHC-I peptides and adjuvant Montanide ISA-51-VG, combined with escalating doses of antagonist PD-1 mAb BMS-936558. A cohort of patients will not receive the vaccine. Phase I trial results indicated that combination was well tolerated and induced responses lasting up to 140 weeks (14).

Another interesting strategy is being tested in NCT02897765, combining nivolumab with NEO-PV-01, a personalized vaccine therapy designed to target mutated proteins which are present uniquely on an individual's tumor neoAg, with poly-ICLC as the adjuvant. Patients will receive 240 mg IV nivolumab every 2 weeks and those patients who have not achieved a complete response to nivolumab alone at week 12 will receive NEO-PV-01+ adjuvant SC, in up to four distinct sites while continuing therapy with

nivolumab. The study will monitor side effects and Ag-specificity in peripheral CD8 $^{+}$ and CD4 $^{+}$ T cell responses and tumor biopsies, which will be assessed after treatment.

NCT01302496 studies ipilimumab combined with TriMix vaccine. The TriMix-DC vaccine is a DC-based vaccine that can induce a T-cell repertoire that recognizes the TAA MAGE-A3, MAGE-C2, tyrosinase, and gp100 in an HLA-restricted way, in unresectable stage III-IV melanoma patients (15, 16). To prepare TriMix-Dc vaccine, autologous DCs are coelectroporated with TriMix mRNA (a combination of CD40L, caTLR4, and CD70 encoding mRNA) in combination with one of four TAA mRNAs linked to an HLA-II targeting signal. After electroporation, the four different TriMixDC-MEL cellular constituents (i.e., DCs expressing one of the four Ags) are mixed at equal ratios and cryopreserved until vaccination. The study is complete but results are still unpublished.

NCT02432963 proposes another combination strategy with ICKB and vaccines, testing a modified Vaccinia Virus Ankara Vaccine expressing tumor protein p53 (p53MVA Vaccine) in combination with pembrolizumab, in patients with tumors with overexpression or mutation of p53. Immune monitoring in peripheral blood samples obtained through the protocol will analyze T-cell reactivity to p53, myeloid-derived suppressor cell (MDSC) and Tregs immunosuppressive populations, and other selected subsets including PD-1 $^{+}$, PDL-1 $^{+}$, and PDL-2 $^{+}$ cells.

Finally, in a Phase I/II study (NCT02275416), ipilimumab (3 mg/kg, every 3 weeks for a total of four doses) in combination

TABLE 1 | Clinical trials combining immune checkpoint blockade with immunostimulatory strategies.

Trial identifier/ study phase/ status	Combination therapy	Patient condition	Sponsor	Official study title	Study design
NCT02515227 Phase I/II (recruiting, 2015)	• Pembrolizumab (antagonist PD-1, mAb) • 6MHP peptide vaccine (6 class II MHC-restricted helper peptides)	Metastatic melanoma (MM)	Craig L Slingluff, Jr	A trial to evaluate the safety, immunogenicity, and clinical activity of a helper peptide vaccine plus PD-1 blockade	6MHP vaccine (200 mg/each six peptides), mixed 1/1 with Montanide ISA-51, will be administered intradermally (ID)/subcutaneous (SC) at days 1, 8, 15, 43, 64, and 85. Pembrolizumab 200 mg intravenous (IV)/3 weeks/2 years
NCT02385669 Phase I/II (recruiting, 2015)	• Ipilimumab [antagonist (CTLA-4) mAb] • 6MHP peptide vaccine (6 class II MHC-restricted helper peptides)	MM	Craig L Slingluff, Jr	A Phase I/II trial to evaluate the safety, immunogenicity, and clinical activity of a helper peptide vaccine plus CTLA-4 blockade in advanced melanoma (Mel62; 6PAC)	6MHP vaccine (200 mg/each six peptides), mixed 1/1 with Montanide ISA-51, will be administered ID/SC at days 1, 8, 15, 43, 64, and 85. Ipilimumab will be administered 3 mg/kg IV/3 weeks/four doses
NCT03047928 Phase I/II (recruiting, 2017)	• Nivolumab (antagonist PD-1 mAb) • PD-L1/indoleamine 2,3-dioxygenase (IDO) peptide vaccine	MM	Inge Marie Svane	Combination therapy with nivolumab and PD-L1/IDO peptide vaccine to patients with MM	• Patients receive nivolumab IV 3 mg/kg biweekly until progression. • Vaccine administration starts concomitantly with nivolumab, biweekly six times, then every fourth week up 1 year. A vaccine consists of 100 µg IDO peptide, 100 µg PD-L1 peptide, and 500 µl Montanide as adjuvant. • Patients who complete all vaccines will continue nivolumab treatment after standard guidelines
NCT01176461 Phase I (ongoing, not recruiting, 2010)	• BMS-936558: antagonist PD-1 mAb • Peptide vaccine: MART-1, NY-ESO-1, gp100 • Adjuvant: Montanide ISA 51 VG	MM	H. Lee Moffitt Cancer Center, National Cancer Institute (NCI), Bristol-Myers Squibb, Medarex	A pilot trial of a vaccine combining multiple class I peptides and Montanide ISA 51 VG with escalating doses of anti-PD-1 antibody BMS-936558 for patients with unresectable Stages III/IV melanoma	• Arm 1: Phase I dose escalation cohort. Six doses of BMS-936558 and six peptide vaccines administered/2 weeks/12 weeks. • Arm comparator: BMS-936558 without peptide vaccine
NCT02897765 Phase I (recruiting, 2016)	• NEO-PV-01 personalized vaccine • Nivolumab • Poly-ICLC	Metastatic tumors including MM	Neon Therapeutic Inc., Bristol-Myers Squibb	An open-label, Phase IB study of NEO-PV-01 + adjuvant with nivolumab in patients with melanoma, non-small-cell lung carcinoma or transitional cell carcinoma of the bladder	• Nivolumab 240 mg IV infusion/2 weeks. Patients who have not achieved a CR to nivolumab alone at week 12 will receive NEO-PV-01 + adjuvant SC (one vial of pooled peptides per injection site) in up to four distinct sites (each extremity or flanks) while continuing therapy with nivolumab
NCT01302496 Phase II (completed, 2017)	• TriMix-DC vaccine • ipilimumab (antagonist CTLA-4 mAb)	MM	Bart Neyns, Vrije Universiteit Brussel	A two-stage Phase II study of autologous TriMix-DC therapeutic vaccine in combination with ipilimumab in patients with previously treated unresectable stage III or IV melanoma	Patients will receive five TriMix-DC doses. All administrations but first will be preceded by ipilimumab 10 mg/kg. NED patients will be offered ipilimumab maintenance (10 mg/kg q12wks)
NCT02432963 Phase I (recruiting, 2015)	• Pembrolizumab (antagonist PD-1 mAb) • modified vaccinia virus ankara vaccine expressing p53	Metastatic tumors including MM	City of Hope Medical Center	A Phase I study of a p53MVA vaccine in combination with pembrolizumab	Patients receive pembrolizumab IV followed by p53MVA Vaccine at least 30 min later once in weeks 1, 4, 7
NCT02275416 Phase I/II (recruiting, 2014)	• Ipilimumab (antagonist CTLA-4 mAbs). • Biological: UV1 vaccine (peptide based-vaccine directed to hTERT) • Biological: GM-CSF	MM	Ultimovacs AS	Safety of UV1 vaccination in combination with ipilimumab in patients with unresectable or metastatic malignant melanoma	• Ipilimumab (3 mg/kg)/3 weeks/four doses. UV1 vaccine 300 µg plus GM-CSF 75 µg ID in the lower abdomen every 4 weeks up to 28 weeks, at weeks 36 and 48
NCT00058279 Phase I/II (completed, 2006)	• Ipilimumab (antagonist CTLA-4 mAb) • Aldesleukin (IL-2)	Intraocular/ skin MM	NCI	MDX-CTLA4 combined with IL-2 for patients with MM	Patients received 3.0 mg/kg ipilimumab every 3 weeks and IL-2 (720,000 IU/kg every 8 h to a maximum of 15 doses)

(Continued)

TABLE 1 | Continued

Trial identifier/ study phase/ status	Combination therapy	Patient condition	Sponsor	Official study title	Study design
NCT02983045 Phase I/II (recruiting, 2016)	• Nivolumab (antagonist PD-1 mAb) • NKTR-214 (IL-2)	Metastatic tumors including MM	Nektar Therapeutics	A Phase 1/2, open-label, multicenter, dose escalation and dose expansion study of NKTR-214 and nivolumab in patients with select locally advanced or metastatic solid tumor malignancies	• Phase 1: patients will receive NKTR-214 every 14/21 days, in combination with 240 mg/360 mg nivolumab every 14/21 days. • Phase 2: additional patient cohorts will be dosed at the recommended Phase 2 dose/schedule of NKTR-214 and nivolumab (as determined by Phase 1 of the trial)
NCT02748564 Phase I/II (recruiting, 2017)	• Pembrolizumab (antagonist PD-1 mAb) • aldeleuskin (IL-2)	Metastatic tumors including MM	Rutgers, The State University of New Jersey	A Phase 1b/II trial of interleukin-2 in combination with pembrolizumab for patients with unresectable or MM	Patients will receive pembrolizumab IV every 3 weeks and aldesleukin IV every 8 h for up to 14 doses at weeks 4, 7, 16, 19, 28, and 31 in the absence of progression/toxicity
NCT01608594 Phase I (ongoing, not-recruiting, 2013)	• Ipilimumab (antagonist CTLA-4 mAb) • HDI (high-dose IFN-a2b)	MM	Ahmad Tarhini	Neoadjuvant combination biotherapy with ipilimumab (3 or 10 mg/kg) and high-dose IFN- α 2B in patients with locally/regionaly advanced/recurrent melanoma: a randomized safety, efficacy and biomarker study	Patients will receive IFN-a2b at 20 MU/m ² /day IV for 5 consecutive days/4 weeks, followed by 10 MU/m ² /day SC thrice a week/2 weeks, followed by definitive surgery. After recovery, IFN-a2b will be resumed at 10 MU/m ² /day SC, thrice a week/46 additional weeks. IFN-a2b will be given concurrently with ipilimumab at 3 or 10 mg/kg
NCT01708941 Phase II (ongoing, not-recruiting, 2013)	• Ipilimumab (antagonist CTLA-4 mAb) • HDI (high-dose IFN-a2b)	MM	NCI	A randomized Phase II study of ipilimumab at 3 or 10 mg/kg alone or in combination with high-dose interferon-alpha in advanced melanoma	There are different cohorts where patients either receive higher dose ipilimumab, higher dose ipilimumab plus HDI, lower dose ipilimumab, and lower dose ipilimumab plus HDI
NCT02112032 Phase I (recruiting, 2014)	• MK-3475 (antagonist PD-1 mAb) • PegIFN-a2b	MM	Hassane M. Zarour, MD	Phase 1 study of anti-PD-1 antibody MK-3475 and PegIFN-a2b for advanced melanoma	2 years treatment with MK-3475: 2 mg/kg every 3 weeks IV and PegIFN-a2b: 1 μ g/kg every week SC
NCT02339324 Phase I (recruiting, 2015)	• Pembrolizumab (antagonist PD-1 mAb) • HDI	MM	University of Pittsburgh	Neoadjuvant combination biotherapy with pembrolizumab and high dose IFN- α 2b in patients with locally/regionaly advanced/recurrent melanoma: safety, efficacy and biomarker study	• Induction phase (first 6 weeks): pembrolizumab IV for two doses/4 weeks concurrently with HDI IV \times 5 consecutive days/week/4 weeks, followed by SC every other day 3x each week/2 weeks. • Surgery phase (week 6–8). • Maintenance phase (following recovery from surgery): pembrolizumab IV infusion/3 weeks given concurrently with HDI SC every week/46 additional weeks
NCT02174172 Phase I (recruiting, 2014)	• Atezolizumab (antagonist PD-L1 mAb) • bevacizumab (antagonist VEGF mAb) • ipilimumab (antagonist CTLA-4 mAb) • obinutuzumab (antagonist CD20 mAb) • IFNa2b • Peg-IFNa2b	Metastatic tumors including MM	Hoffmann-La Roche	A Phase Ib study of the safety and pharmacology of atezolizumab (anti-PD-L1 antibody) administered with ipilimumab, interferon-alpha, or other immune-modulating therapies in patients with locally advanced or metastatic solid tumors	• Arm A: atezolizumab with ipilimumab; • Arm B: atezolizumab with IFN-A2b; • Arm C: Atezolizumab With PEG-IFN-A2b; • Arm D: atezolizumab with PEG-IFN-A2b and bevacizumab; • Arm E: atezolizumab with obinutuzumab
NCT02009397 Phase I/II (recruiting, 2012)	• Ipilimumab (antagonist CTLA-4 mAb) • rhuGM-CSF	MM	J Graham Brown Cancer Center	A Phase I/II open-label study of ipilimumab and GM-CSF administered to unresectable Stage IIIIC and Stage IV melanoma patients	IV ipilimumab followed by SC GM-CSF, for up to four cycles
NCT02652455 Phase I (recruiting, 2016)	• Nivolumab (PD-1 antagonist mAb) • autologous tumor infiltrating lymphocyte (TIL) • CD137 agonist mAb • cyclophosphamide • fludarabine • IL-2	MM	H. Lee Moffitt Cancer Center and Research Institute	A pilot clinical trial combining PD-1 blockade, CD137 agonism and adoptive cell therapy for MM	• Patients will receive treatment with nivolumab prior to tumor removal for TIL growth. • Surgery and TIL growth ex vivo. • Patients lymphodepleting chemotherapy with cyclophosphamide and fludarabine; TIL infusion; interleukin-2 treatment. • The first six participants will not receive nivolumab prior treatment for comparison

(Continued)

TABLE 1 | Continued

Trial identifier/ study phase/ status	Combination therapy	Patient condition	Sponsor	Official study title	Study design
NCT01701674 Phase I (ongoing, not-recruiting, 2012)	• Ipilimumab (CTLA-4 antagonist mAb) • autologous TIL • cyclophosphamide • IL-2	MM	H. Lee Moffitt Cancer Center and Research Institute	Costimulation with ipilimumab to enhance lymphodepletion plus adoptive cell transfer and high dose IL-2 in patients with MM	Combination of ipilimumab followed by lymphodepletion with chemotherapy, TIL infusion, and high dose IL-2
NCT02027935 Phase II (recruiting, 2015)	• Ipilimumab (CTLA-4 antagonist mAb) • autologous CD8+ • cyclophosphamide • IL-2	MM	M.D. Anderson Cancer Center	Phase II study of cellular adoptive immunotherapy using autologous CD8+ antigen-specific T cells and anti-CTLA4 for patients with MM	• Patients' leukapheresis to get and cultivate CD8+ T cells. • Cyclophosphamide 300 mg/m ² for lymphodepletion. • IV Infusion of 10 ¹⁰ T cells/m ² . • IL-2 250,000 U/m ² SC every 12 h for 14 days. • Ipilimumab 3 mg/kg IV 24 h postinfusion and days 22, 43, and 64
NCT03123783 Phase I/II (recruiting, 2017)	• Ipilimumab (antagonist CTLA-4 mAb) • APX005M (agonist CD40 mAb)	MM	Apexigen, Inc.	A study to evaluate the safety and efficacy of the CD40 agonistic antibody APX005M administered in combination with nivolumab in subjects with non-small-cell lung cancer and subjects with MM	Subjects will receive intravenously APX005M in combination with nivolumab until disease progression, unacceptable toxicity or death
NCT02706353 Phase I/II (recruiting, 2017)	• Pembrolizumab (antagonist PD-1 mAb) • APX005M (agonist CD40 mAb)	Metastatic tumors including MM	M.D. Anderson Cancer Center	Phase I/II dose escalation and cohort expansion of safety and tolerability study of intratumoral CD40 agonistic monoclonal antibody APX005M in combination with systemic pembrolizumab in patients with MM	• Dose escalation phase: initial dose APX005M 0.1 mg injected into 1–3 tumors every 3 weeks/four doses up to maximum tolerated dose (MTD). All participants will receive pembrolizumab IV 2 mg/kg/3 weeks. • Dose expansion phase: APX005M MTD, same of pembrolizumab dosage
NCT02554812 Phase II (ongoing, 2015)	• Avelumab (antagonist PD-L1 mAb) • utomilumab (agonist 41BB mAb) • PF-04518600 (agonist OX-40 mAb) • PD 0360324 (neutralizing M-CSF mAb)	Metastatic tumors including MM	Pfizer	A Phase 1b/2 open-label study to evaluate safety, clinical activity, pharmacokinetics and pharmacodynamics of avelumab (MSB0010718c) in combination with other cancer immunotherapies in patients with advanced malignancies	• Arm A: avelumab + utomilumab at three different dose levels. • Arm B: dose escalation PF-04518600 + avelumab. • Arm C: dose escalation PD 0360324 + avelumab. • Arm D: dose escalation utomilumab + PF-04518600 + avelumab. Afterward, dose expansion utomilumab + PF-04518600 + avelumab in selected tumor types
NCT02643303 Phase I/II (recruiting, 2016)	• Durvalumab (antagonist PD-1 mAb) • Tremelimumab (antagonist CTLA-4 mAb) • polyICLC (TLR3 agonist molecule)	Metastatic tumors including MM	Ludwig Institute for Cancer Research	A Phase 1/2 study of <i>in situ</i> vaccination with tremelimumab and IV durvalumab (MEDI4736) plus the toll-like receptor (TLR) agonist PolyICLC in subjects with advanced, measurable, biopsy-accessible cancers	• Phase I, cohort A: IV durvalumab + IT/IM polyICLC; cohort B: tremelimumab + IT/IM polyICLC; cohort C: durvalumab + tremelimumab + IT/IM polyICLC. Phase II: once the recommended combination has been determined, subsequent subjects will follow this dosing scheme
NCT02644967 Phase I/II (recruiting, 2015)	• Ipilimumab (antagonist CTLA-4 mAb) • pembrolizumab (antagonist PD-1 mAb) • IMO-2125 (TLR-9 agonist)	MM	Idera Pharmaceuticals, Inc.	A Phase 1/2 study to assess the safety and efficacy of intratumoral IMO-2125 in combination with ipilimumab or pembrolizumab in patients with MM	• Cohort 1: IMO-2125 IT weekly, then once/3 weeks. Ipilimumab IV at 3 mg/kg • Cohort 2: IMO-2125, IMO-2125 IT weekly, then once/3 weeks. Pembrolizumab, IV at 2 mg/kg/3 weeks
NCT02668770 Phase I (recruiting, 2016)	• Ipilimumab (antagonist CTLA-4 mab) • MGN1703 (TLR-9 Agonist molecule)	Metastatic tumors including MM	M.D. Anderson Cancer Center	A Phase I trial of ipilimumab (immunotherapy) and MGN1703 (TLR agonist) in patients with advanced solid malignancies	Dose escalation and expansion group of MGN1703 doses, SC or ID ipilimumab will be administrated 3 mg/kg/cycle 8 days following MGN1703 administration
NCT02981303 Phase II (recruiting, 2016)	• Pembrolizumab (antagonist PD-1 mAb) • Imprime PGG (PAMP)	Metastatic tumors including MM	Biothera	A multicenter, open-label, Phase 2 study of imprime PGG and pembrolizumab in subjects with advanced melanoma failing front-line treatment with checkpoint inhibitors or triple negative breast cancer failing front-line chemotherapy for metastatic disease	• Imprime PGG IV 4 mg/kg on days 1, 8, 15/3-week treatment cycle. • Pembrolizumab IV 200 mg/kg following Imprime infusion

TABLE 2 | Clinical trials combining immune checkpoint blockade with targeting of immunosuppressive molecules.

Trial identifier/ study phase/ status	Combination therapy	Patient condition	Sponsor	Official study title	Study design
NCT02743819 Phase II (recruiting, 2016)	• Pembrolizumab (antagonist PD-1 mAb) • ipilimumab [antagonist (CTLA-4) mAb]	Metastatic melanoma (MM)	University of Chicago	Phase II study of pembrolizumab and ipilimumab following initial anti-PD1/L1 antibody	Pembrolizumab plus ipilimumab. • Arm A: progression on anti-PD1/L1 antibody • Arm B: stable disease more than 24 weeks or initial response on anti-PD1/L1 antibody
NCT02381314 Phase I (recruiting, 2015)	• Ipilimumab (antagonist CTLA-4 mAb) • enoblituzumab (B7-H3 mAb)	Metastatic tumors including MM	MacroGenics	A Phase 1, open-label, dose escalation study of MGA271 in combination with ipilimumab in patients with melanoma, non-small-cell lung cancer, and other cancers	Enoblituzumab will be administered IV once/week (51 doses) to determine maximum tolerated dose (MTD) in combination with ipilimumab, which is administered IV/3 weeks/four doses
NCT02460224 Phase I/II (recruiting, 2015)	• PDR001 (antagonist PD-1 mAb) • LAG525 (antagonist LAG-3 mAb)	Metastatic tumors including MM	Novartis Pharmaceuticals	A Phase I/II, open label, multicenter study of the safety and efficacy of LAG525 single agent and in combination with PDR001 administered to patients with advanced malignancies	• Arm A: LAG525 single treatment arm. • Arm B: LAG525 plus PDR001 combination arm. • Arm C: LAG525 single treatment arm in Japanese patients
NCT02655822 Phase I (recruiting, 2016)	• CPI-444 (blocking adenosine-A2A receptor inhibitor) • atezolizumab (antagonist PD-L1 mAb)	Metastatic tumors including MM	Corvus Pharmaceuticals, Inc.	A Phase 1/1b, open-label, multicenter, repeat-dose, dose-selection study of CPI-444 as single agent and in combination with atezolizumab in patients with selected incurable cancers	• Cohort I: CPI-444 100 mg orally twice daily for the first 14 days/each 28-day cycle. • Cohort II: CPI-444 100 mg orally twice daily for 28 days/each 28-day cycle. • Cohort III: CPI-444 200 mg orally once daily for the first 14 days/each 28-day cycle. • Cohort IV: CPI-444 MTD + atezolizumab IV
NCT02817633 Phase I (recruiting, 2016)	• Antagonist PD-L1 mAb • TSR-022 (antagonist Tim-3 mAb)	Metastatic tumors including MM	Tesaro, Inc.	A Phase 1 dose escalation and cohort expansion study of TSR-022, an Anti-TIM-3 monoclonal antibody, in patients with advanced solid tumors	• Part 1: Dose Escalation. 1a: dose escalation TSR-022 alone. 1b: dose escalation TSR-022 plus anti-PD-1 antibody. 1c: Phase 2 TSR-022 MTD plus anti-PD-1 antibody. • Part 2: expansion cohorts of Part 1
NCT02608268 Phase I/II (recruiting, 2015)	• PDR001 (antagonist PD-1 mAb) • MBG453 (antagonist Tim-3 mAb)	Metastatic tumors including MM	Novartis Pharmaceuticals	Phase I-1b/II open-label multi-center study of the safety and efficacy of MBG453 as single agent and in combination with PDR001 in adult patients with advanced malignancies	• Cohort 1: MBG453 dose escalation. • Cohort 2: MBG453 dose escalation in combination with PDR001
NCT02983006 Phase I (recruiting, 2016)	• Nivolumab (antagonist PD-L1 mAb) • DS-8273a (TRAIL-DR5 mAb)	MM	New York University School of Medicine	A Phase 1 study of TRAIL-DR5 antibody DS-8273a administered in combination with nivolumab in subjects with unresectable Stage III or Stage IV melanoma	DS-8273a: starting dose 4 mg/kg IV Q 3 weeks. Dose Escalation: 8 mg/kg IV Q 3 weeks, 16 mg/kg IV Q 3 weeks, 24 mg/kg IV Q 3 weeks, 2 mg/kg IV Q 3 weeks, 4 mg/kg IV Q 3 weeks. • nivolumab: 5 mg/kg IV Q 3 weeks
NCT02471846 Phase I (recruiting, 2015)	• Atezolizumab (antagonist PD-1 mAb) • GDC-0919 (IDO inhibitor)	Metastatic tumors including MM	Genentech, Inc.	A Phase Ib, open-label, dose-escalation study of the safety and pharmacology of GDC-0919 administered with atezolizumab in patients with locally advanced or metastatic solid tumors	• Relapsed cohorts to PD1/PD-L1 blockade will receive GDC-0919 at MTD. • Untreated advanced patients will receive escalation doses of atezolizumab and GDC-0919 combinations. • An expansion cohort of atezolizumab and GDC-0919 combination at MTD
NCT02318277 Phase I/II (recruiting, 2014)	• Durvalumab (blocking PD-L1 mAb) • epacadostat (IDO-1 inhibitor molecule)	Metastatic tumors including MM (B7H3+)	Incyte Corporation	A Phase 1/2 study exploring the safety, tolerability, and efficacy of epacadostat (INCB024360) in combination with durvalumab (MEDI4736) in subjects with selected advanced solid tumors (ECHO-203)	Durvalumab IV at selected dose levels every 2 weeks plus epacadostat 25 mg BID as starting dose, followed by dose escalations until MTD
NCT02327078 Phase I/II (recruiting, 2014)	• Nivolumab (PD-1 antagonist mAb) • epacadostat (IDO-1 inhibitor)	Metastatic tumors including MM	Incyte Corporation	A Phase 1/2 study of the safety, tolerability, and efficacy of epacadostat administered in combination with nivolumab in select advanced cancers (ECHO-204)	• Phase 1: nivolumab IV 3 mg/kg/2 weeks plus epacadostat 25 mg BID as starting dose, followed by dose escalations. • Phase 2: nivolumab 240 mg 2 weeks plus epacadostat MTD

(Continued)

TABLE 2 | Continued

Trial identifier/ study phase/ status	Combination therapy	Patient condition	Sponsor	Official study title	Study design
NCT02073123 Phase I/II (recruiting, 2014)	• Ipilimumab (antagonist CTLA-4 mAb) • pembrolizumab (antagonist PD-1 mAb) • nivolumab (antagonist PD-1 mAb) • indoximod (IDO inhibitor)	MM	NewLink Genetics Corporation	A Phase 1/2 study of the concomitant administration of indoximod plus immune checkpoint inhibitors (CPIs) for adult patients with advanced or MM	• Indoximod 1,200 mg BID concurrently with ipilimumab IV 3 mg/kg/3 weeks/four doses. • Indoximod 1,200 mg BID and pembrolizumab IV at 2 mg/kg/3 weeks. • Indoximod 1,200 mg BID and nivolumab IV at 3 mg/kg/4 weeks
NCT02117362 Phase I (recruiting, 2014)	• Ipilimumab (antagonist CTLA-4 mAb) • GR-MD-02 (Galectin-3 Inhibitor)	MM	Providence Health & Services	Phase IB study of a galectin inhibitor (GR-MD-02) and ipilimumab in patients with MM	Cohorts with escalating doses of GR-MD-02 (1, 2, 4, 8 mg/kg) 1 hour before 3 mg/kg of ipilimumab on days 1, 22, 43, and 65
NCT02403778 Phase II (ongoing, not-recruiting, 2015)	• Ipilimumab (CTLA-4 antagonist mab) • All-trans retinoic acid (ATRA)	MM	University of Colorado, Denver	Ipilimumab and ATRA combination treatment of Stage IV melanoma	• Arm A: ipilimumab 10 mg/kg/3 weeks/four doses. • Arm B: ipilimumab 10 mg/kg/3 weeks/four doses plus 150 mg/m ² ATRA orally for 3 days surrounding ipilimumab dosage
NCT02807844 Phase I/II (recruiting, 2016)	• PDR001 (antagonist PD-1 mAb) • MCS110 (blocking MCSF mAb)	Metastatic tumors including MM	Novartis Pharmaceuticals	A Phase Ib/II, open label, multicenter study of MCS110 in combination with PDR001 in patients with advanced malignancies	MCS110 combined with PDR001
NCT02452424 Phase I/II (recruiting, 2015)	• Pembrolizumab (PD-1 mAb) • PLX3397 (CSF1R inhibitor)	Metastatic tumors including MM	Plexxikon	Phase 1/2a study of double-immune suppression blockade by combining a CSF1R inhibitor (PLX3397) with an Anti-PD-1 antibody (pembrolizumab) to treat advanced melanoma and other solid tumors	• Part 1: open-label, sequential PLX3397 dose escalation with a fixed dose of pembrolizumab (200 mg, IV) in approximately 24 patients with advanced solid tumors. • Part 2: extension cohort
NCT02880371 Phase I/II (recruiting, 2016)	• Pembrolizumab (antagonist PD-1 mAb) • ARRY-382 (CSF1R)	Metastatic tumors including MM	Array BioPharma	A Study of ARRY-382 in combination with pembrolizumab, a programmed cell death receptor 1 (PD-1) antibody, for the treatment of patients with advanced solid tumors	• Part A: escalating doses of ARRY-382 with pembrolizumab 2 mg/kg. • Part B: ARRY-382 at MTD with pembrolizumab 2 mg/kg. • Part C: ARRY-382 at MTD with 200 mg pembrolizumab

with GM-CSF (75 µg) and UV1 peptide based-vaccine directed to hTERT is being tested in unresectable Stage III or Stage IV melanoma patients. UV1 vaccine (300 µg) will be administered by injecting ID in the lower abdomen before and between treatments with ipilimumab, thereafter every 4th week up to 28 weeks, and then at weeks 36 and 48. This study will analyze safety and tolerability of the combination and also will measure specific T-cell responses, quality of life, and treatment response by CT scans. A search for potential biomarkers of efficacy and safety studies will be also performed.

Cytokines

There are several trials combining ICKB with typical cytokines first assessed in CM patients as non-specific immunotherapy treatments, such as IL-2, IFN- α 2b, and GM-CSF (Table 1). Aldesleukin is a recombinant analog of the endogenous cytokine IL-2 that has immunoregulatory and antineoplastic activities. It promotes activation of T, B, and NK cells; however, serious related adverse events were seen upon IL-2 administration (17). IL-2 was approved for treatment of metastatic renal cell carcinoma in 1992 and for metastatic melanoma (MM) in 1998 by FDA. Nowadays, IL-2 monotherapy is not the optimal and standard

treatment for both metastatic renal cell carcinoma and MM but efforts to further improve the efficacy of IL-2 therapy are focused on combined therapies. Results from NCT00058279 combining ipilimumab with IL-2 revealed immune-related adverse events. A non-synergistic effect was observed, since the 22% objective response rate observed, results from the additive effect of the expected response rate for each therapy; however, long-term responses were still observed (12). NCT02983045 ongoing study will analyze ICKB in combination with NKTR-214, a prodrug for IL-2, conjugated to six releasable PEG chains. In a preclinical CM mouse model, this molecule showed 20 times preferential activation of CD8⁺ T cells (IL2R β) over Treg cells (IL2R α) in comparison to aldesleukin. In this model, NKTR-214 proved efficacy as a single agent, and long-term immunity when combined with antagonist CTLA-4 mAb, in addition to resistance to tumor rechallenge (18). NCT02748564 Phase Ib/II study will evaluate the safety and tolerability of IL-2 when given in combination with pembrolizumab to patients with advanced CM.

Adjuvant IFN- α 2b increases disease-free survival (DFS) although not OS in CM patients but it is accompanied with considerable toxicity (19, 20); it is not universally considered as a gold standard treatment (21). Besides, the optimal dose and duration

TABLE 3 | Other combinations in clinical trials with immunomodulatory monoclonal antibodies.

Trial identifier/ study phase/ status	Combination therapy	Patient condition	Sponsor	Official study title	Study design
NCT01740297 Phase I/II (completed, 2015)	• Ipilimumab (antagonist CTLA-4 mAb) • talmogene laherparepvec (oncolytic virus)	Metastatic melanoma (MM)	Amgen	Phase 1b/2, multicenter, open-label trial to evaluate the safety and efficacy of talmogene laherparepvec and ipilimumab compared to ipilimumab alone in subjects with unresected, stage IIIB-IV melanoma	• Experimental: Phase 1b and Phase 2 Arm 1. Talmogene laherparepvec plus ipilimumab. • Active Comparator: Phase 2 Arm 2. Ipilimumab
NCT02263508 Phase Ib/ III (recruiting, 2014)	• Pembrolizumab (antagonist PD-1 mAb) • talmogene laherparepvec (oncolytic virus)	MM	Amgen	A Phase 1b/3, multicenter, trial of talmogene laherparepvec in combination with pembrolizumab (MK-3475) for treatment of unresectable stage IIIB to IVM1c melanoma (MASTERKEY-265/ KEYNOTE-034)	• Experimental: Phase 3 Arm 1, talmogene laherparepvec and pembrolizumab (MK-3475). • Experimental: Phase 3 Arm 2: placebo and pembrolizumab (MK-3475)
NCT02272855 phase II (ongoing, not- recruiting, 2014)	• Ipilimumab (agonist CTLA-4 mAb) • HF10 (vaccinia virus)	MM	Takara Bio Inc	A Phase II study of combination treatment with HF10, a Replication-competent HSV-1 oncolytic virus, and ipilimumab in patients with Stage IIIB, Stage IIIC, or Stage IV unresectable or metastatic malignant melanoma	Patients will receive 1.10 ⁷ TCID50/mL HF10 (four injections/once a week; two injections/ once at 3 weeks) and ipilimumab 3 mg/kg IV/3 weeks/four total doses
NCT03003676 Phase I (recruiting, 2016)	• Pembrolizumab (antagonist PD-1 mAb) • ONCOS-102 (oncolytic virus)	MM	Targovax Oy	A pilot study of sequential ONCOS-102, an engineered oncolytic adenovirus expressing GMCSF, and pembrolizumab in patients with advanced or unresectable melanoma progressing after PD1 blockade	Patients will receive three doses of intratumoral (i.t.) injection of ONCOS-102 (days 1, 4, and 8) at 3 × 10 ¹¹ viral particles (VP), preceded by intravenous (i.v.) cyclophosphamide priming 1–3 days prior to day 1. They will then receive pembrolizumab i.v., 2 mg/kg, on day 22 (week 3) and every 3 weeks thereafter until the end of treatment visit on day 169 (week 24)
NCT01986426 Phase I (recruiting, 2013)	• Ipilimumab (antagonist CTLA-4 mAb) • pembrolizumab (antagonist PD-1 mAb) • LTX-315 (lytic peptide)	Metastatic tumors including MM	Lytix Biopharma AS	A Phase I, open-label, multiarm, multicenter, multi-dose, dose escalation study of LTX-315 as monotherapy or in combination with either ipilimumab or pembrolizumab in patients with transdermally accessible tumors	• Arm A: LTX-315 monotherapy at single/ sequential lesions. • Arm B: LTX-315 monotherapy at concurrent multiple lesions. • Arm C: LTX-315 plus ipilimumab in MM patients. • Arm D: LTX-315 plus pembrolizumab in triple-negative breast cancer patients
NCT02302339 Phase II (recruiting, 2016)	• Glembatumumab vedotin (gpNMB conjugate-drug mAb) • varilimumab (CD27 agonist mab) • nivolumab/ pembrolizumab (antagonist PD-1 mAb)	MM	Celldex Therapeutics	A Phase 2 study of glembatumumab vedotin, an anti-gpNMB antibody– drug conjugate, as monotherapy or in combination with immunotherapies in patients with advanced melanoma	• Cohort A: glembatumumab vedotin IV on day 1/21 day cycle. • Cohort B: glembatumumab vedotin IV on day 1/21 day cycle. Varilimumab IV on day 1 of cycles 1, 2, 4, 6, 8 and 10. • Cohort C: glembatumumab vedotin IV on day 1/21 day cycle. Nivolumab/pembrolizumab administered according to institutional standard of care
NCT02076633 Phase II (completed, 2015)	• L19IL2 (HDAC4 mab conjugated with IL-2) • L19TNF (HDAC4 mab conjugated with TNF)	MM	Philogen S.p.A.	A Phase II study of intratumoral application of L19IL2/L19TNF in melanoma patients in clinical Stage III or Stage IV M1a with presence of injectable cutaneous and/or SC lesions	Patients will be treated with intratumoral injections of 10 Mio IU L19IL2 and 312 µg L19TNF once weekly for up to 4 weeks
NCT02315066 Phase I (recruiting, 2015)	• PF-04518600 (agonist OX40 mAb) • PF-05082566 (agonist 41BB mAb)	Metastatic tumors including MM	Pfizer	A Phase 1, open-label, dose escalation study of Pf-04518600 as a single agent and in combination with Pf-05082566 in patients with selected locally advanced or metastatic carcinomas	• Part A1 – PF-04518600 will be administered IV every 14 days starting at a dose of 0.01 mg/ kg, increasing until maximum tolerated dose (MTD) is determined. • Part B1 - PF-04518600 will be administered IV every 2 weeks starting at a dose of 0.1 mg/kg and PF-05082566 will be administered IV 4 weeks starting at a dose of 20 mg. Increases in dose will continue until MTD is determined
NCT02714374 Phase I (recruiting, 2016)	• Eculizumab (C5 neutralizing mab) • GL-ONC1 (vaccinia virus)	Metastatic tumors including MM	Kaitlyn Kelly, MD	An open label, non-randomized Phase 1b study to investigate the safety and effect of the oncolytic virus GL-ONC1 administered intravenously with or without eculizumab prior to surgery to patients with solid organ cancers undergoing surgery for curative- intent or palliative resection	• Arm A: GL-ONC1 escalation dose. • Arm B: GL-ONC1 escalation dose plus eculizumab, single dose on week 1/day 1 at 900 mg 60–90 min prior to GL-ONC1

of IFN- α 2b treatment are still unclear (22, 23). IFN- α 2b would have several mechanisms of action, from induction of apoptosis in tumor cells to activation of monocytes and macrophages favoring Ag processing. There are several ongoing trials combining ICKB with IFN- α 2b or PEG-IFN- α 2b (NCT01608594, NCT01708941, NCT02112032, NCT02339324, and NCT02174172). Low doses of cytokine GM-CSF proved to be a strong monocyte attractant and necessary to differentiate monocytes into DCs promoting a T_H1 response (24); a combination of ipilimumab with this cytokine is also on the way (NCT02009397).

Adoptive Cell Therapy

One approach to restore the functionality of effector immune cells is to cultivate autologous tumor infiltrating lymphocyte (TIL) *ex vivo* after tumor resection and infused them back into the patient (25); this is defined as ACT. Combination of ACT with ICKB may counteract any inhibitory immune checkpoint signal from the tumor microenvironment, provided that T cell effectors have been expanded and activated *in vitro* in the presence of tumor Ags previous to treatment (Table 1). NCT02652455 will compare the effect of nivolumab administration prior to tumor resection and *in vitro* culture of TILs. These will be cultivated *ex vivo* with agonist CD137 mAb to augment T cell proliferation and infused them after chemotherapy-induced lymphodepletion of patients. They will be treated *in vivo* with IL-2 to support T cell proliferation. NCT01701674 will study the effect of ipilimumab before leukapheresis, while NCT02027935 will do it afterward.

Stimulatory Immune Checkpoints

CD40 is a costimulatory receptor that is essential for activating both innate and adaptive immune systems (26). CD40 binds its ligand CD40L, which is transiently expressed on T cells and other non-immune cells under inflammatory conditions. A wide spectrum of molecular and cellular processes is regulated by CD40 engagement including the initiation and progression of cellular and humoral adaptive immunity. Use of agonist CD40 mAbs with high-affinity fosters activation of APCs (DCs, monocytes, and B cells), leading to stimulation of tumor-specific immune responses. Recently, it was reported in a mouse tumor model that use of agonist CD40 mAb reversed resistance to PD-1, downregulating PD-1 levels in T cells via IL-12 production (27). Agonist CD40 mAb APX005M is currently being evaluated in Phase I/II trials in combination with ipilimumab (NCT03123783) or pembrolizumab (NCT02706353) (Table 1). NCT02554812 trial combines avelumab in different cohorts with agonist mAbs toward T cells costimulatory molecules 4-1BB and OX-40 (28) or neutralizing mAb toward M-CSF/CSF1 (macrophage colony-stimulating factor) (29).

Toll-Like Receptors (TLRs)/PAMP

Toll-like receptors can detect a broad range of human pathogens, as well as a variety of molecules such as PAMP (pathogen-associated molecular patterns) that indicate tissue damage. This recognition triggers a cascade of innate and adaptive immune responses that fully activate the immune system. Agonist TLR

mAbs support this response. It was reported that triggering of TLR3 induces T-cell activation and a strong upregulation of HLA-I and PD-L1 in neuroblastoma and melanoma cells (30, 31). Therefore, ICKB will counteract limitation of the T cell response induced by TLR signaling. Ongoing trials include combinations of PD-1 and CTLA-4 ICKB with agonist TLR3 and TLR9 mAbs (NCT02643303, NCT02644967, and NCT02668770). Trial NCT02981303 will assess Imprime PGG, a β -1,3/1,6 glucan PAMP molecule isolated from the cell wall of a proprietary Saccharomyces, in combination with pembrolizumab (Table 1).

ICKB Combined with Targeting of Immunosuppressive Molecules/Pathways

Other ICKB

Immune checkpoint blockade is also being assessed in combinations with the targeting of other molecules/pathways that promote an immunosuppressive environment (Table 2). For instance, there are trials targeting several ICKB. NCT02743819 trial combines pembrolizumab with ipilimumab in advanced patients which, following treatment with PD-1/PD-L1 mAb, either progress or present stable disease/initial response for more than 24 weeks. NCT02381314 studies in B7-H3 expressing tumors such as CM, the combination of ipilimumab with enoblituzumab, a B7-H3 mAb was designed to improve ADCC by increasing FcR affinity. NCT02460224 analyzes the combination of LAG525 and PDR001, antagonist mAbs for LAG-3 and PD-1, respectively. LAG-3 is an immune checkpoint that binds a non-holomorphic region of the MHC-II molecule and has an important role in the tumor microenvironment. It was reported that soluble-LAG-3 binds to immature DCs, promoting their maturation (32). However, LAG-3 is involved in alternative activation of plasmacytoid DCs in melanoma lesions (33). Interaction of MHC-II on APCs with LAG3 downregulates T-cell proliferation and activation (34). In agreement, LAG-3 mediates resistance to apoptosis on MHC-II expressing melanoma cells (35). LAG-3 is substantially expressed on melanoma TILs, including those with potent immunosuppressive activity. LAG3 was shown to have a synergistic action with the PD-1/PD-L1 axis, critical for releasing an antitumor immune response. In a mouse melanoma model, tumor-specific CD4⁺ effector T-cells showed traits of chronic exhaustion, with high expression levels of PD-1, TIM-3, 2B4, TIGIT, and LAG-3 inhibitory molecules. Blockade with a combination of anti-PD-L1 and anti-LAG-3 mAbs overcame the requirement to deplete tumor-specific Tregs in this model (36). The PD-1 expression on CD8⁺ TILs identified a repertoire of clonally expanded tumor-reactive cells, including mutated neoAg-specific CD8⁺ T-cells; these cells expressed LAG-3 and Tim-3 (37). It has recently been described that ipilimumab expanded T cells in patients with higher expression levels of CD27, intracellular CTLA-4, TIM-3, and LAG-3, which can be taken into account for future combination trials (38).

Adenosine-A2A receptor (A2Ar), an ectonucleotidase that catabolizes the hydrolysis of extracellular adenosine monophosphate (AMP) to adenosine, is a novel metabolic target for ICKB. In preclinical models, it was shown that expression of A2Ar on myeloid cells suppressed T and NK cell responses in

the solid tumor microenvironment (39). Also, A2Ar blockade enhanced antitumor activity of PD-1 and CTLA-4 ICKB (40). NCT02655822 is on the way combining PD-1 ICKB with an A2Ar inhibitor molecule.

T-Cell Exhaustion

TIM-3 was first identified as a specific T_H1 receptor. When it binds to galectin-9, it generates an inhibitory signal that results in apoptosis of T_H1 cells (41). TIM-3 was also described as a marker of T-cell exhaustion (38). TIM-3 is also expressed by NK cells and naive DCs, acting in synergy with TLR signaling to induce inflammation. Expression of TIM-3 in monocytes and macrophages promotes phagocytosis of apoptotic cells through interaction with phosphatidylserine, which enhances Ag cross-presentation (42). Also, TIM-3 binds HMGB1, impairing its recruitment of nucleic acids into endosomes, a key step in the sensing of DNA by the innate immune system, promoting tumor escape (43). Notably, it was shown that PD-1 and Tim-3 limited the expansion of tumor Ag-specific CD8⁺ T cells induced by a melanoma peptide vaccine, as dual blockade enhanced the expansion and cytokine production of vaccine-induced CD8⁺ T cells *in vitro* (44). NCT02817633 and NCT02608268 are ongoing combining ICKB with an antagonist Tim-3 mAb (**Table 2**).

Tumor Immune Microenvironment

NCT02983006 trial is testing the combination of nivolumab with DS-8273a (**Table 2**). This agonist mAb showed selective targeting of MDSC through TNF-receptor TRAIL-DR2, without affecting other mature myeloid or lymphoid cells (45). As an agonist of TRAIL-DR5 to induce apoptosis in tumor cells, in a Phase I trial DS-8273 as monotherapy was well tolerated but no objective responses were observed, although decreases in MDSC temporally associated with DS-8273a exposure were observed (46).

Indoleamine 2,3-dioxygenase is the first and rate-limiting enzyme involved in tryptophan catabolism, which can halt T-cells growth. In cancer, IDO is expressed within the tumor itself as well as in the tumor microenvironment, where it promotes the establishment of peripheral immune tolerance to tumor Ags. On the tumor side, lymph node CM cells express IDO, recruiting Treg, which is associated with a poor outcome (47). At the tumor microenvironment, IDO promotes MDSC recruitment by a mechanism dependent on Tregs (48); it also inhibits NK cell function along with PGE-2 (49). Activated T cells *in vitro* induce MDSC function through IL-10; these MDSC secrete ARG-1 and IDO and express PD-L1 and MHC-II, leading to upregulation of PD-1 and LAG-3 on T-cells, promoting an immunosuppressive tumor microenvironment (50). In CM patients, high levels of circulating PD-L1⁺ cytotoxic T-cells were associated with increased expression levels of CTLA-4 in Tregs and IDO in MDSC and plasmacytoid DCs. All these parameters were related to a negative outcome, independent of disease stage (51).

It is interesting to note that IDO is an immunogenic protein, therefore, activation of pro-inflammatory IDO-specific CD4⁺ responses may delay or overcome the immunosuppressive actions of IDO, consequence of early expression in maturing APCs; however, IDO-specific Tregs may enhance IDO-mediated

immune suppression (47). In mouse melanoma models, IDO is an essential mechanism of resistance to ICKB, including CTLA-4 and PD-1. CTLA-4 blockade combined with IDO inhibitors strongly synergizes to mediate tumor rejection (49). It is postulated that following melanoma infiltration by lymphocytes, upregulation of PD-L1, IDO, and Tregs is regulated by an intrinsic immune mechanism (52). And that combination of CTLA-4, PD-1/PD-L1, and IDO blockade restores IL-2 production and proliferation of CD8⁺ T-cells (53). It was recently reported that melanoma expresses high levels of IDO and galectin-3, which upregulate Tregs, suppressing the expansion of tumor-specific T cells cultivated for ACT, which could be reversed by blockade of IDO and galectin-3 (54). Trials combining ICKB with inhibitors of IDO (NCT02471846, NCT02318277, NCT02327078, and NCT02073123) or galectin-3 (NCT02117362) are currently on the way (**Table 2**).

NCT02403778 trial proposes the combination of ipilimumab with all-trans retinoic acid (ATRA), a derivative of vitamin A (**Table 2**). ATRA induces maturation of immunosuppressive MDSCs into myeloid cells (55, 56); this was shown to be of benefit in a lung cancer vaccine and ACT for sarcomas (57, 58). Thus, this combination is designed to decrease MDSCs and differentiate immature monocytes into mature DCs and increase tumor Ag-specific T-cell responses. In this trial, ATRA single-arm versus ATRA with ipilimumab combined-arm will be compared.

Finally, other combinations of ICKB and targeting the tumor microenvironment include antagonist mAbs for M-CSF/CSF1 (NCT02807844) or its receptor MCSFR/CSF1R (NCT02452424 and NCT02880371) (**Table 2**). Interaction of $\alpha 4\beta 1$ integrin from extracellular matrix with MCSF receptor leads to the activation of Rac2 and regulation of macrophage toward a M2 immunosuppressive phenotype (59). In a melanoma mouse model, targeting of CSF1R on MDSCs overcomes resistance to IDO-expressing melanoma cells (60).

Other Combinations with Immunomodulatory mAbs

There are several oncolytic viruses that have shown to promote an immunogenic cell death leading to a T_H1 response; combination with ICKB is aimed to sustain in time this tumor microenvironment (61). Talimogene laherparepvec (T-VEC, Imlytic) is a genetically modified, attenuated, herpes simplex virus type 1 designed to promote an antitumor response through selective viral replication in tumor cells and stimulation of systemic antitumor immunity through GM-CSF (62). This was the first oncolytic viral therapy to be approved by the FDA in 2015 for intralesional treatment of unresectable lesions in patients with melanoma recurrent after the initial surgery. The combination of T-VEC with ipilimumab in a Phase I trial proved to be safe and appeared to have greater efficacy than single agents (NCT01740297) (63). NCT02263508 is a Phase 1b/3 study that will assess the combination of talimogene laherparepvec with pembrolizumab in unresectable CM patients. Combination studies with other oncolytic virus include NCT02272855 trial, which will analyze CTLA-4 ICKB with HF10, an oncolytic virus that has shown to induce angiogenesis and affluence of CD8⁺ T-cells at

the tumor microenvironment (64). Finally, in the NCT03003676 study the combination of pembrolizumab with ONCOS-102, an engineered oncolytic Adenovirus expressing GM-CSF, will be analyzed in CM patients that have progressed to the PD-1 blockade.

The NCT01986426 study is designed to assess the safety, tolerability, and efficacy of different intratumoral dosing regimens of LTX-315; a lytic-peptide that induces immunogenic cell death; it will be assessed as monotherapy or in combination with ipilimumab or pembrolizumab. It was shown in mouse models that this peptide overcomes tumor resistance to CTLA-4 ICKB (65) (**Table 1**).

The NCT02302339 study will examine the effectiveness and safety of glembatumumab vedotin as monotherapy and in combination with either nivolumab or pembrolizumab (**Table 3**). Glembatumumab vedotin mAb is conjugated to the cytotoxic drug monomethyl auristatin E. This mAb targets glycoprotein NMB, expressed on the surface of tumor cells, releasing the drug and inducing tumor cell death. Combinations with immunotherapy include pembrolizumab/nivolumab and varlilumab, an agonist mAb of the T-cell costimulatory receptor CD27. NCT02076633 trial also examines conjugated mAbs for treatment of metastatic CM patients; L19IL2 targets melanoma cells through HDAC4 and it is conjugated to IL-2; instead L19TNF is conjugated to TNF α , exerting its major effects *via* a preferential toxicity for the endothelial cells of the tumor-associated vasculature, therefore, increasing an antitumor immune response. Preclinical data suggest that intratumoral administration of these conjugates can be more effective.

Another approach explores the synergy of two agonist mAbs targeting the T-cells costimulatory molecules OX40 and 41BB (25, 66) (NCT02315066) (**Table 3**). Finally, NCT02714374 ongoing trial is combining GL-ONC1, a genetically modified oncolytic vaccinia virus, with eculizumab, a neutralizing C5 mAb, with the goal that GL-ONC1 remains in the body long before being cleared by the immune system.

DISCUSSION

Combined immunotherapy involving mAbs and other immunomodulatory strategies is an emerging field. There is no still any such combination therapy approved. The immune system should be considered as one interrelated signaling network where targeting different points may act synergistically to promote anticancer effects. Proper immune stimulation and blockade of immunosuppression can be seen as a “push and release” strategy, where both are critical for the efficacy of an anti-cancer immunotherapy (**Figure 1**). ICKB is currently being assessed in combination with immune stimulatory strategies, such as vaccination, cytokines, ACT, stimulatory immune checkpoint agonists, and targeting of TLRs. Other approaches combine ICKB with targeting of several immune suppressive mechanisms, such as blocking other immune checkpoints, T-cell exhaustion inhibition, and promotion of an antitumor microenvironment.

The idea of combining ICKB with immunomodulatory strategies such as vaccines is very attractive, given that in some cases amplification of Ag-specific T cells, as well as the induction of

antibodies recognizing tumor Ags are observed after vaccination. Blocking of immune checkpoints may result in effector T cells that could attain potent tumor destruction more potently; a useful T helper function and modulation of Tregs will result in the expansion of cytotoxic T cell effectors. However, vaccines, in general, have shown a low 10–15% rate of clinical responses, with still a lack of efficacy to eradicate tumor masses and avoid further dissemination in metastatic patients (67). Most of the clinical trials revised in this work are being assessed in advanced CM patients, thus immune suppression, both systemic and local, can be hard to overcome even with ICKB, since there are patients that do not respond at all. Vaccination strategies may be more useful when administered in the adjuvant setting to control tumor relapse in high-risk stage II–III CM patients (68), and thus, their combination with ICKB may hold the promise of durable clinical benefit avoiding metastases to distant organs and achieving prolonged OS.

Assessment of clinical responses in ICKB cancer treatments can be challenging since traditional Response Evaluation Criteria In Solid Tumors, RECIST, may underestimate the actual response that can be delayed and atypical, as evidenced in patients treated with ICKB (69). The immune-related response criteria have been established to allow patients to continue treatment after the first progression until a new progression is presented, given the chance of eventual clinical benefit to more patients (70). It should be taken into account that there are both constitutive and acquired ICKB resistance mechanisms compromising treatment outcome (71–73).

Identification of early predictors of response is desirable to identify patients that would benefit the most and avoid unnecessarily prolonged treatments. Regarding ICKB biomarkers at the local level, an association of PD-L1 expression in pretreatment tumor biopsies with objective response to anti-PD-1/PD-L1 therapy has been observed given the constant finding that PD-L1 expression is enriched in anti-PD-1/PD-L1 therapy responders in several tumors (74). Weighted-average ORR across several studies for patients whose tumors were tested for PD-L1 is 29%; if the tumor expresses PD-L1, these increases to 48%. However, a significant proportion of responding patients with PD-L1 negative-tumors were observed. Also, high-density infiltration of CD8 T-cells in tumor biopsies has been associated with PD-L1 expression in tumor cells; it was associated independently with an improved prognosis, with increased time to development of brain metastases in CM patients (75). Also, it was recently reported that PD-L2 expression in metastatic CM was associated with immune infiltration and a better prognosis independently of therapy of choice (76). Other proposed biomarkers for selecting which patients are likely to benefit from cancer immunotherapies are the tumor mutational load and microsatellite instability in the stability. CM is a tumor with a high rate of mutation (1), and thus with a higher probability of neoAg generation, increasing the number of immunogenic structures that could stimulate a more potent and broad repertoire of antitumor immune effectors. In the same way, microsatellite instability is a frequent event in CM which accounts for tumor immunogenicity, contributing to making of CM a pathology suitable for immunotherapy (77).

At the peripheral level, serum IL-8 concentrations actually reflect tumor burden (78). It was recently reported that measurement of serum IL-8 levels 2–3 weeks following starting therapy can predict response and OS in metastatic CM patients treated with PD-1 ICKB, even before imaging evaluation (79). Also, there are recent publications by two independent groups which reported that assessment of circulating cell-free DNA from tumors in CM patients receiving ICKB treatment is an accurate predictor of tumor response, PFS and OS, as patients with elevated ctDNA on therapy had a poor prognosis (80, 81).

In the combination of several immunotherapy strategies, identifying which patients are likely to respond to therapy will be even more challenging. This is given the complexity of the immune system, and the limited understanding of its regulation and multiple interactions between immune cells, immune-modulating molecules, tumor cells, and other compartments of the tumor microenvironment, such as the lymphatic and blood systems.

CONCLUSION

Monoclonal antibodies have gained evidence of their effectiveness for cancer treatment. This seems to be the tip of an iceberg, as we are learning that not only targeting the tumor but also

modulating the immune response, may be a powerful way to achieve long-term clinical responses. In this way, mAbs can be again considered “magic bullets” targeting molecules with different immunomodulatory effects. We have revisited most of the current clinical trials that explore combined use of immunomodulatory mAbs with different immunotherapeutic approaches, with the aim to improve and/or potentiate clinical responses in CM patients. This is an exciting and expanding research field that is rapidly spreading to other tumor types.

AUTHOR CONTRIBUTIONS

MA and MB: conception and design of the review; manuscript writing. JM: conception and design of the review.

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Glycogen Synthase Kinase 3 Inactivation Compensates for the Lack of CD28 in the Priming of CD8⁺ Cytotoxic T-Cells: Implications for anti-PD-1 Immunotherapy

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The rescue of exhausted CD8⁺ cytolytic T-cells (CTLs) by anti-Programmed Cell Death-1 (anti-PD-1) blockade has been found to require CD28 expression. At the same time, we have shown that the inactivation of the serine/threonine kinase glycogen synthase kinase (GSK)-3 α/β with small-interfering RNAs (siRNAs) and small molecule inhibitors (SMIs) specifically down-regulates PD-1 expression for enhanced CD8⁺ CTL function and clearance of tumors and viral infections. Despite this, it has been unclear whether the GSK-3 α/β pathway accounts for CD28 costimulation of CD8⁺ CTL function. In this article, we show that inactivation of GSK-3 α/β through siRNA or by SMIs during priming can substitute CD28 co-stimulation in the potentiation of cytotoxic CD8⁺ CTL function against the EL-4 lymphoma cells expressing OVA peptide. The effect was seen using several structurally distinct GSK-3 SMIs and was accompanied by an increase in Lamp-1 and GZMB expression. Conversely, CD28 crosslinking obviated the need for GSK-3 α/β inhibition in its enhancement of CTL function. Our findings support a model where GSK-3 is the central cosignal for CD28 priming of CD8⁺ CTLs in anti-PD-1 immunotherapy.

Keywords: T-cells, glycogen synthase kinase-3, programmed cell death 1, Tbet, cancer

INTRODUCTION

Naive T-cells are cells that have not encountered cognate antigen are essential for responses to novel pathogens. In this instance, activation requires a combination of stimulatory signals (1). The first signal is provided by the T-cell receptor (TCR) upon lymphocyte interaction with major histocompatibility class (MHC) antigens on the antigen-presenting cells (APCs) within the immune synapse (2). The second signal for T-cell activation is provided by CD28 and other costimulatory coreceptors on T-cells (3–6). CD28 is a well-defined costimulatory molecule found on lymphocytes, which interacts with B7 (CD80 and CD86) proteins on the APC (7, 8). TCR signaling alone can result in the lymphocyte undergoing cell death, or becoming anergic and thus unable to respond to antigen (9). Simultaneous signaling through CD28 and the TCR gives rise to sustained activation characterized by interleukin (IL)-2 production and cell-cycle entry (8, 10–12). Anti-CD28

crosslinking using monoclonal antibodies (MAbs) that augment CD28 cosignaling, especially on CD4⁺ T-cells, leading to increased interleukin 2-receptor (IL-2R), CD69 expression and proliferation (13). Conversely, Fab fragments of antibodies in mice, inhibit T-cell responses and can induce long-term heart allograft survival (14), and ameliorate experimental autoimmune encephalomyelitis (15).

We and others have shown that CD28 can complement and amplify TCR signaling (8, 12, 16, 17). In addition, CD28 can generate signals independently of TCR engagement (6, 18–20). The Tyr-Met-Asn-Met (YMNM) motif in the cytoplasmic tail of CD28 binds the adaptor growth factor receptor-bound protein 2 (GRB-2) (5, 11, 21–25) and the p85 regulatory subunit of phosphoinositide 3-kinase (PI-3K) resulting in the activation of AKT (21, 26, 27). This, in turn, leads to optimal *IL-2*-gene activation (11, 28), the expression of the anti-apoptotic protein BCL-X_L, and the induction of an antigen response *in vivo* (11, 24, 29, 30). In this context, CD28 is linked to the serine threonine kinase; glycogen synthase kinase-3 (GSK-3). GSK-3 is constitutively active in T-cells, facilitating the exit of nuclear factor of activated T-cells (NFAT-c1) from the nucleus (31). CD28 signaling *via* PI-3K leads to the phosphorylation and inactivation of GSK-3, thus increasing IL-2 production and T-cell proliferation (32, 33).

Programmed cell death 1 (PD-1; PDCD1) is a member of the CD28 supergene family which negatively regulates T-cell function (3, 34, 35). PD-1 is expressed in response to T-cell activation and contributes to the exhaustion of CD8⁺ T-cells during chronic infection (36, 37). The coreceptor binds to ligands, programmed cell death ligand 1 and 2 (PD-L1/L2), on lymphoid and non-lymphoid cells (38–40). Immune checkpoint blockade (ICB) with anti-PD-1 or anti-PD-L1 has also proven highly successful in the treatment of human cancers, alone or in combination with anti-CTLA-4 (41, 42). PD-1 expression on tumor-infiltrating CD8⁺ T-cells correlates with impaired effector cell function (3, 43). We recently showed that GSK-3 is a central regulator of PD-1 expression and that the inactivation of GSK-3 using small molecule inhibitors (SMIs) downregulates PD-1 expression resulting in enhanced clearance of viral infections and cancer (44, 45). Recently, it has also been shown that PD-1 check-point blockade requires CD28 expression (46–48).

Here, we show that inhibition of GSK-3 α/β by either small-interfering RNAs (siRNAs) or SMIs can substitute CD28 stimulation in the potentiation of CD8⁺ cytolytic T-cell (CTL) function. We propose that GSK-3 is the key mediator that is responsible for CD28 priming of CD8⁺ CTLs in T-cell immunity and in response to anti-PD-1 ICB immunotherapy.

RESULTS

Recently, we reported that the inactivation of GSK-3 α/β with siRNAs and drug inhibitors specifically downregulate PD-1 expression for enhanced CD8⁺ CTL function and clearance of tumors and viral infections (44, 45). We also previously reported CD28 costimulation can induce the phosphorylation of GSK-3 and hence its inactivation (33, 49). To assess CD8⁺ CTL function in response to antigen-presentation, we utilized MHC

class I-restricted OVA specific-TCR transgenic (OT-1) mice with a TCR specific for the SIINFEKL peptide of OVA L^{a} presented by H-2k^b. Control samples showed an increase in killing targets concurrent with an increase in effector/target (E/T) ratios. As previously shown (44), inhibition of GSK-3 with the SMI, SB415286, increased killing of EL4 target cells loaded with OVA peptide as measured at day 6 (**Figure 1A**). We next assessed the role of CD28 in this process. To this end, cultures were coincubated with soluble CTLA-4 IgG to block the interaction between CD28 and CD80/86 on presenting cells. EL4 cells express CD80 (50) and were therefore used as target cells. CTLA-4-IgG effectively inhibited the level of CTL killing of target cells (left panels). Intriguingly, the addition of SMI SB415286 completely restored normal levels of high CTL killing of targets at all E/T ratios (right panels). This ability of a GSK-3 SMI to bypass CD28 blockade by CTLA-4-IgG indicated that the inhibition of GSK-3 can substitute for the signal that is normally provided by anti-CD28. Further to this, as expected from our previous work, SB415286 suppressed the expression of PD-1 under all conditions (**Figure 1B**).

Anti-CD28 crosslinking has been found previously to augment CD28 signaling (13, 51). To assess this in the context of CD8⁺ CTLs, cultures were coincubated with anti-CD28 to crosslink the CD28 coreceptor for 7 days followed by an assessment of CTL function. Under these conditions, anti-CD28 greatly potentiated the killing potential of CTLs at all E/T ratios (left panel). Interestingly, this level of enhanced killing was similar to that induced by GSK-3 SMI SB415286 (left panel). Further, the level of increased killing induced by anti-CD28 could not be further enhanced by SB415286 and *vice versa*. In the same vein, anti-CD28 coculture reduced the expression of PD-1 on CD8⁺ T-cells, similar to that seen with SB415286 (**Figure 1B**). Although it was originally assumed that CD28 would provide costimulation needed for the expression of PD-1 as in the case of CTLA-4 (52), we observed the opposite result. This was consistent with the generation of signals *via* GSK-3 whose inhibition also suppressed PD-1 expression. Consistent with this, CTLA-4-IgG blockade of CD28 was seen to increase PD-1 expression (left panel). This suggested that the normal engagement of CD28 by CD80/86 might also act to suppress PD-1 expression. Flow cytometry showed that SB415286 downregulated PD-1 expression on OVA peptide activated cells was accompanied by increased expression of Lamp-1 and GZMB in T-cells (**Figure 1C**).

In a related approach, anti-CD28 or CTLA-4 IgG was added to cells expressing siRNA for GSK-3 α/β (**Figure 2**). In the scrambled control, anti-CD28 acted to increase the level of response. In addition, the knock-down of GSK-3 α/β with siRNA increased the level of response to that of anti-CD28 such that the addition of anti-CD28 has no further effect. While CTLA-4-IgG markedly reduced the response of OT-1 T-cells expressing scrambled siRNA, it had no effect on cells expressing GSK-3 α/β siRNA. Using a different approach, these data confirmed that GSK-3 inhibition could substitute for the signal provided by anti-CD28. In turn, the increased killing was reflected by a decrease in PD-1 expression (**Figure 2B**) and an increase in GZMB and Lamp-1 expression (**Figure 2C**).

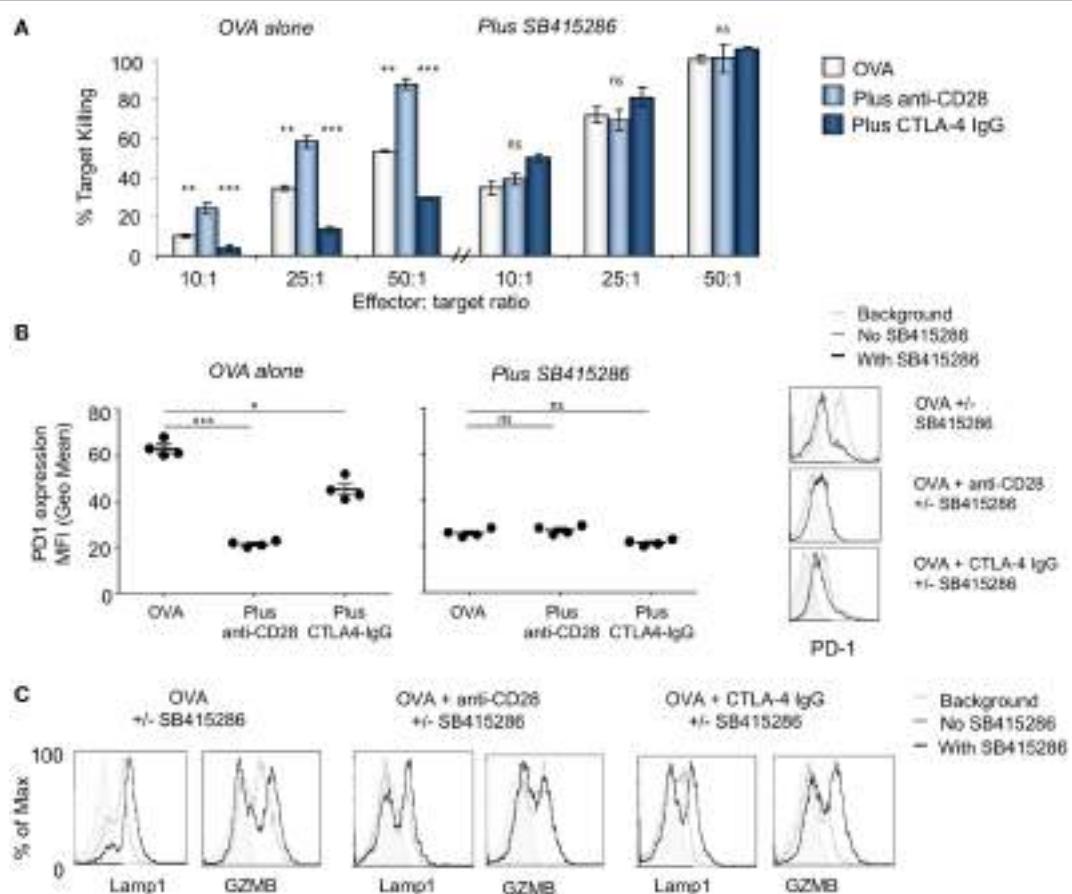


FIGURE 1 | T-cell activation with anti-CD28 enhances cytolytic T-cell (CTL) killing of antigen specific target cells through glycogen synthase kinase 3 (GSK-3). **(A)** OT-1 CD8⁺ CTLs were activated with OVA peptide incubated in the presence (right panel) or absence (left panel) of SB415286 with or without anti-CD28 or blocking CD28 (CTLA-4 IgG fusion protein). After 5 days, CTLs were washed and counted before incubation with target (OVA-EL4) cells at the ratios shown for 4 h. Lactate dehydrogenase release was measured as an indication of target cell killing. Histogram depicts measurements normalized for background non-specific killing. OVA alone: light gray bars; anti-CD28: light blue bars; CTLA-4 IgG: dark blue bars (error bars based on triplicate values in individual experiments, data shown representative of four independent experiments). **(B)** Histogram showing MFI values of programmed cell death 1 (PD-1) expression as measured by flow cytometry. **(C)** Flow cytometry profiles of GZMB and Lamp-1 in the presence and absence of SB415286 alone, combined with anti-CD28 or CTLA-4 IgG. Error bars based on triplicate values in individual experiments; data shown representative of three independent experiments.

Importantly, the ability of GSK-3 inhibition to substitute for anti-CD28 in increasing CD8⁺ CTL function was seen with the use of different GSK-3 inhibitors; SB216763, CHIR99021, and L803-ments (**Figure 3**). Each have distinct structures but share a common target (53, 54). In each case, CD28 blockade by CTLA-4-IgG was reversed by the addition of any one of the four inhibitors used. Together, these data also support a key role for GSK-3 inhibition as a mediator of CD28 regulation of CD8⁺ T-cell killing.

To assess the *in vivo* effect of CTL priming, OVA peptide in the presence or absence of SB415286 was injected intravenously into OT-1 transgenic mice followed by the harvest of spleens at day 7 (**Figure 4**). T-cells from extracted spleens were then subjected to further *ex vivo* stimulation for another 7 days in the presence or absence of SMI SB415286, anti-CD28, or CTLA-4-IgG followed by assessment of *ex vivo* killing of EL4-OVA targets. From this, it was observed that the *in vivo* administration of SMI enhanced cytolytic responses compared to OVA peptide alone (**Figures 4A,B**, left panel). This increase was also

observed with OVA peptide alone primed cells when incubated with the GSK-3 SMI *in vitro* (**Figure 4A**, left panel). This finding showed that the cells were effectively primed *in vivo* with the SMI. In the case of cells primed with OVA peptide alone, the addition of anti-CD28 *in vitro* enhanced killing, whereas no additional effect was seen on cells primed with both OVA peptide and SMI. The addition of CTLA-4-IgG *in vitro* demonstrated the effects of priming with OVA peptide alone to be overcome by CD28 blockade. However, this was overcome by additional SMI *in vitro* (**Figure 4B**, left panel). Flow cytometry showed that priming with SMI, in addition to OVA peptide, slightly increased Lamp-1 and GZMB expression compared to OVA peptide alone. Further, anti-CD28 increased the numbers of CTLs expressing GZMB and Lamp-1, and this effect was reversed by CTLA-4-IgG (right panels). SMI had no further effect on anti-CD28-treated cells, but did overcome the CD28 blockade. Under both priming conditions, PD-1 expression was reduced in the presence of anti-CD28 to the same level as that

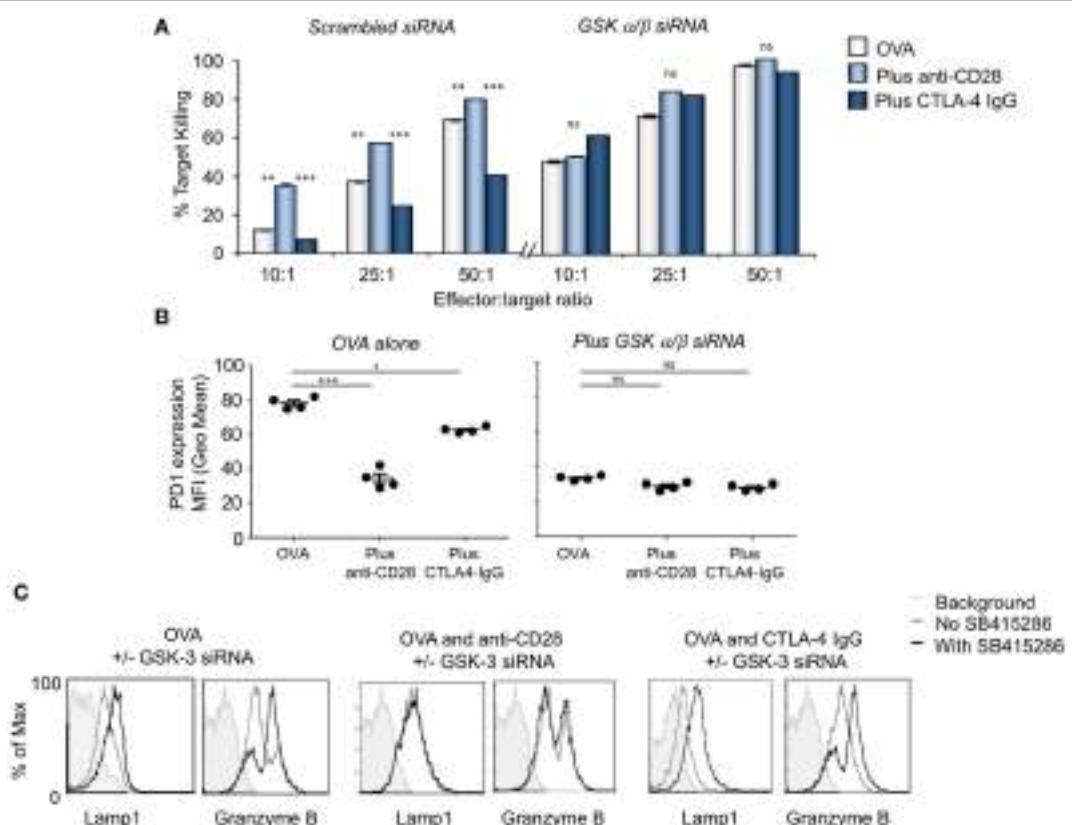


FIGURE 2 | CD28 activation is comparable to glycogen synthase kinase 3 (GSK-3) inactivation enhancing cytolytic function. **(A)** OT-1 CD8⁺ cytotoxic T-cells (CTLs) were transfected with scrambled (left panel) or GSK-3 (right panel) small-interfering RNA (siRNA) prior to activation with OVA peptide and incubated with or without anti-CD28 or blocking CD28 (CTLA-4 IgG fusion protein). After 5 days CTLs were washed and counted before incubation with target (OVA-EL4) cells at the ratios shown for 4 h. Lactate dehydrogenase release was measured as an indication of target cell killing. Histogram depicts measurements normalized for background non-specific killing. OVA alone: light gray bars; anti-CD28: light blue bars; CTLA-4 IgG: dark blue bars (error bars based on triplicate values in individual experiments, data shown representative of four independent experiments). **(B)** Histogram showing MFI values of programmed cell death 1 (PD-1) expression as measured by flow cytometry. **(C)** Flow cytometry profiles of GZMB and Lamp-1 in either scrambled or GSK-3 siRNA transfected cells stimulated with Ova alone, or combined with anti-CD28 or CTLA-4 IgG. Error bars based on triplicate values in individual experiments; data shown representative of three independent experiments.

seen with SMI. These data showed that GSK-3 inhibition *in vivo* augmented CTL function to a similar level as achieved *in vitro* with anti-CD28.

DISCUSSION

Both CD28 and the serine/threonine kinase GSK-3 α/β have been found to play important roles in the activation of T-cells (4, 5, 44). The PI-3K/3-phosphoinositide-dependent protein kinase 1 (PDK1)/AKT signaling axis is central to cellular homeostasis, cell growth and proliferation (55, 56). We previously showed that GSK-3 α/β inactivation with siRNAs and SMIs specifically downregulates PD-1 expression which leads to enhanced CD8⁺ CTL function and clearance of viral infections and cancer (44, 45). Despite this, it has been unclear how the GSK-3 pathway is linked to CD28 costimulation in the generation of CD8⁺ CTL function. We previously showed that CD28 has a cytoplasmic YMNM motif for binding to PI-3K, and that the pathway promotes the phosphorylation and

inactivation of GSK-3 (21, 27, 33). The binding motif for PI-3K is phosphorylated by the src kinases, p56^{lck} and p59^{fyn} (22). Here, we show that GSK-3 inactivation substitutes for CD28 in the priming of cytotoxic CD8⁺ T-cells, while the enhanced cytotoxic function induced by anti-CD28 Mab crosslinking obviates the effects of GSK-3 SMIs.

Our first observation was that GSK-3 inactivation, using either siRNAs or SMIs, could substitute for CD28 in providing cosignals for enhanced cytotoxicity. GSK-3 inactivation reversed the effects of CD28 blockade with CTLA-4-IgG in the cytotoxic response OT-1 CTLs against EL4 cells expressing the OVA peptide. This was seen at all effector to target ratios studied. In each case this enhanced function was accompanied by an increase in Lamp-1 and GZMB expression. The efficacy of SMIs indicated that the inhibition of the catalytic activity of GSK-3, and not its potential role as a molecular scaffold for the binding of other proteins, was primarily responsible for increased function. Further, the effects were seen with four different SMIs with distinct structures whose shared property is the inhibition of

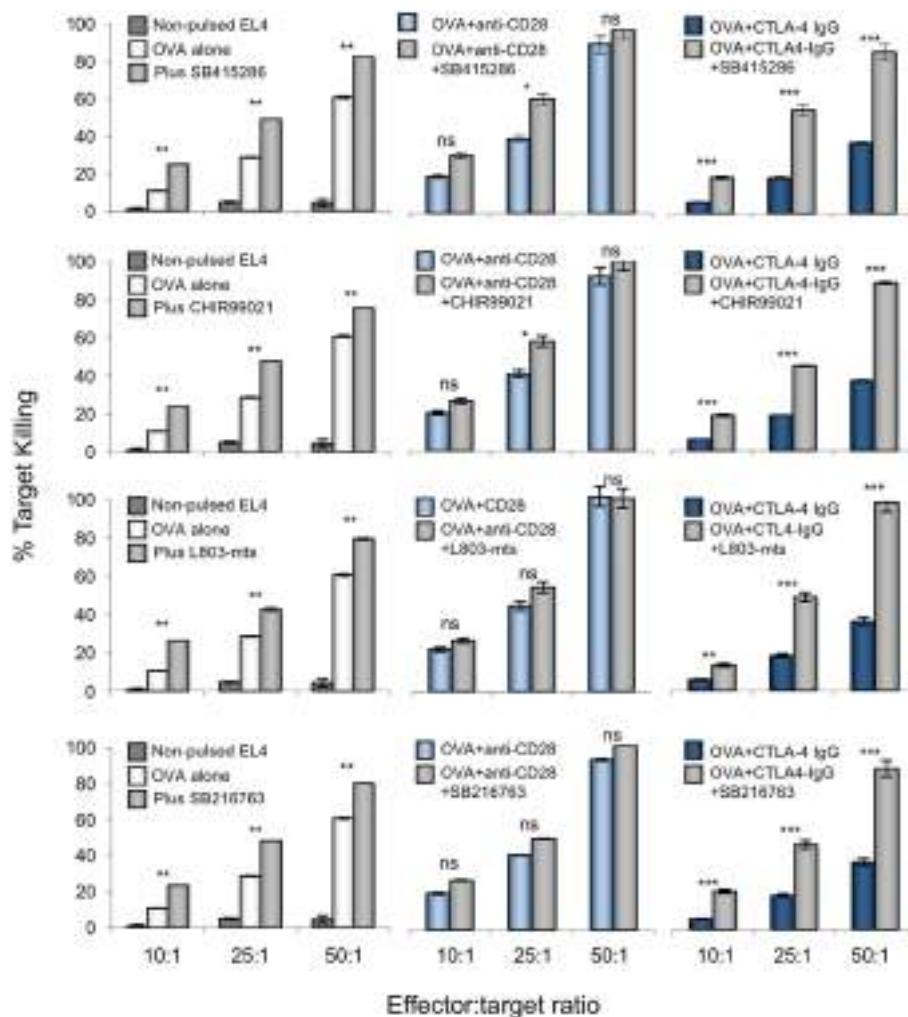


FIGURE 3 | Anti-CD28 enhances cytolytic T-cell (CTL) killing of antigen-specific target cells to similar extent as glycogen synthase kinase 3 (GSK-3) inhibitors. OT-1 CD8⁺ CTLs were activated with OVA peptide incubated in the presence or absence of one of four small molecule inhibitors (from top to bottom; SB415286, CHR99021, L803-mts, SB216763) with or without anti-CD28 or blocking CD28 (CTLA-4 IgG fusion protein). After 5 days, CTLs were washed and counted before incubation with target (OVA-EL4) cells at the ratios shown for 4 h. Lactate dehydrogenase release was measured as an indication of target cell killing. Dark blue bars on left panel depicts background non-specific killing (non-pulsed target cell death). Error bars based on triplicate values in individual experiments; data shown representative of three independent experiments.

GSK-3. These included ATP-competitive inhibitors SB216763, CHR99021, and L803-mts, where SB216763 has a greater preference of inhibition for the GSK-3 α isoform, while CHR99021 and L803-mts preferentially inhibits GSK-3 β (54, 57). Our previous work assessed longevity of the effectiveness of the SMIs by monitoring PD-1 expression in mice coinjected with EL4 tumors and a single injection of SMI. These data indicate that the effects of SB415286 were sustained for over 7–10 days (44).

The close relationship between CD28 and GSK-3 was also observed by the ability of anti-CD28 MAb crosslinking to override or substitute for GSK-3 SMI inhibition in the potentiation of CTL function. While anti-CD28 blocks the interaction between CD28 and CD80/86, it also crosslinks the coreceptor in the generation of cosignals. CD28 crosslinking by CD80/86 is generally thought to be suboptimal, while the higher concentration of

anti-CD28 can be more effective in occupying and crosslinking the coreceptor. Consistent with this, anti-CD28 MAb PVI greatly enhanced the killing function of OT-1 CTLs against OVA-EL4 targets. The level of increased killing was identical to the level observed with the addition of GSK-3 SMIs. The addition of GSK-3 SMI SB415286 to cultures that had been incubated with anti-CD28 provided no further potentiation of the CTL response and *vice versa*. This was confirmed in both *in vitro* and *in vivo* assays. This is reminiscent of the similarity in the effects of GSK-3 SMIs and anti-PD-1 blockade (44). Whether a similar relationship between GSK-3 and CD28 exists in CD4⁺ T-cells and operates in response to activating CD28 superagonists (58) remains to be studied.

Overall, we propose a model where GSK-3 is the center of effects mediated *via* CD28 (Figure 5). Recently, it was reported

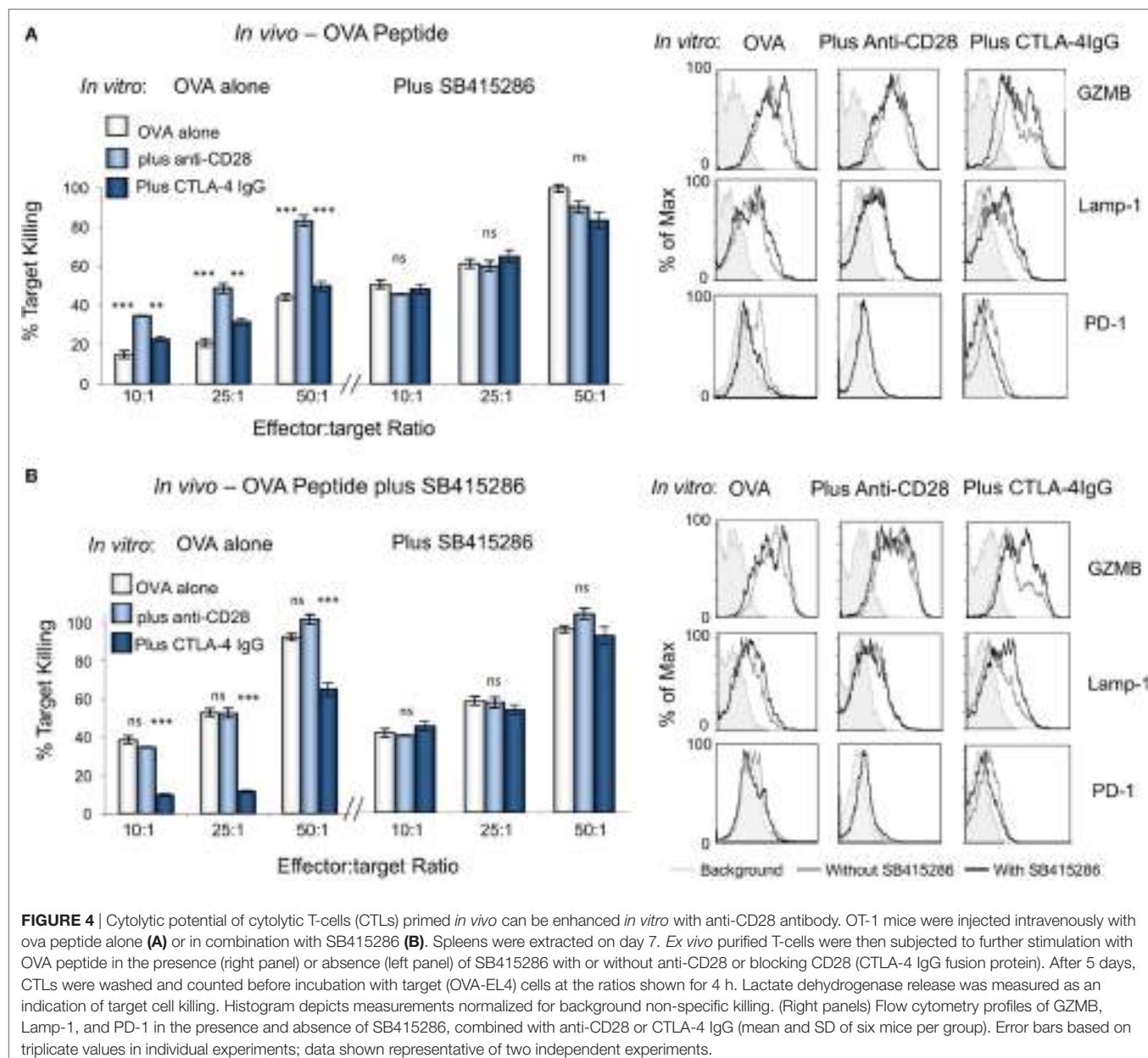


FIGURE 4 | Cytolytic potential of cytolytic T-cells (CTLs) primed *in vivo* can be enhanced *in vitro* with anti-CD28 antibody. OT-1 mice were injected intravenously with OVA peptide alone (**A**) or in combination with SB415286 (**B**). Spleens were extracted on day 7. *Ex vivo* purified T-cells were then subjected to further stimulation with OVA peptide in the presence (right panel) or absence (left panel) of SB415286 with or without anti-CD28 or blocking CD28 (CTLA-4 IgG fusion protein). After 5 days, CTLs were washed and counted before incubation with target (OVA-EL4) cells at the ratios shown for 4 h. Lactate dehydrogenase release was measured as an indication of target cell killing. Histogram depicts measurements normalized for background non-specific killing. (Right panels) Flow cytometry profiles of GZMB, Lamp-1, and PD-1 in the presence and absence of SB415286, combined with anti-CD28 or CTLA-4 IgG (mean and SD of six mice per group). Error bars based on triplicate values in individual experiments; data shown representative of two independent experiments.

that the rescue of exhausted CD8⁺ T-cells by anti-PD-1 blockade requires CD28 expression (46, 47). One proposed mechanism was the de-phosphorylation of CD28 by PD-1-associated Src homology region 2 domain-containing phosphatase (SHP)-2 (48). By connecting these observations to our findings, we propose a new model for the mechanism by which anti-PD-1 ICB operates in immunotherapy (see Figure 5). In the absence of anti-PD-1 ICB, PD-1-associated phosphatases SHP-1 and SHP-2 would dephosphorylate the CD28 YMNM motif for the activation of PI-3K. In the presence of anti-PD-1 ICB, the activation of SHP-1/2 is blocked, allowing for the phosphorylation of the CD28 YMNM motif and the recruitment of PI-3K (4, 5). PI-3K produces phosphatidylinositol (3,4,5) trisphosphates (PIP3)

which serve as plasma membrane docking sites for proteins with pleckstrin-homology (PH) domains. CD28 induced PI-3K would promote PDK1 to the membrane where it would activate serine/threonine kinase AKT (also known as protein kinase B or PKB). AKT would in turn inhibit GSK-3 by phosphorylation of sites of human GSK-3 α (Ser21) and GSK-3 β (Ser9). As we have shown (44, 45), GSK-3 inhibition up-regulates the transcription of the transcription factor Tbx21 (Tbet) that inhibits PD-1 expression. We propose that CD28 regulation of GSK-3 accounts for the requirement for CD28 in the rescue of the response of CD8⁺ T-cells to anti-PD-1 blockade (46, 47). Further studies are needed to assess the full range of targets of the CD28-GSK-3-Tbet-PD-1 axis in T-cell biology.

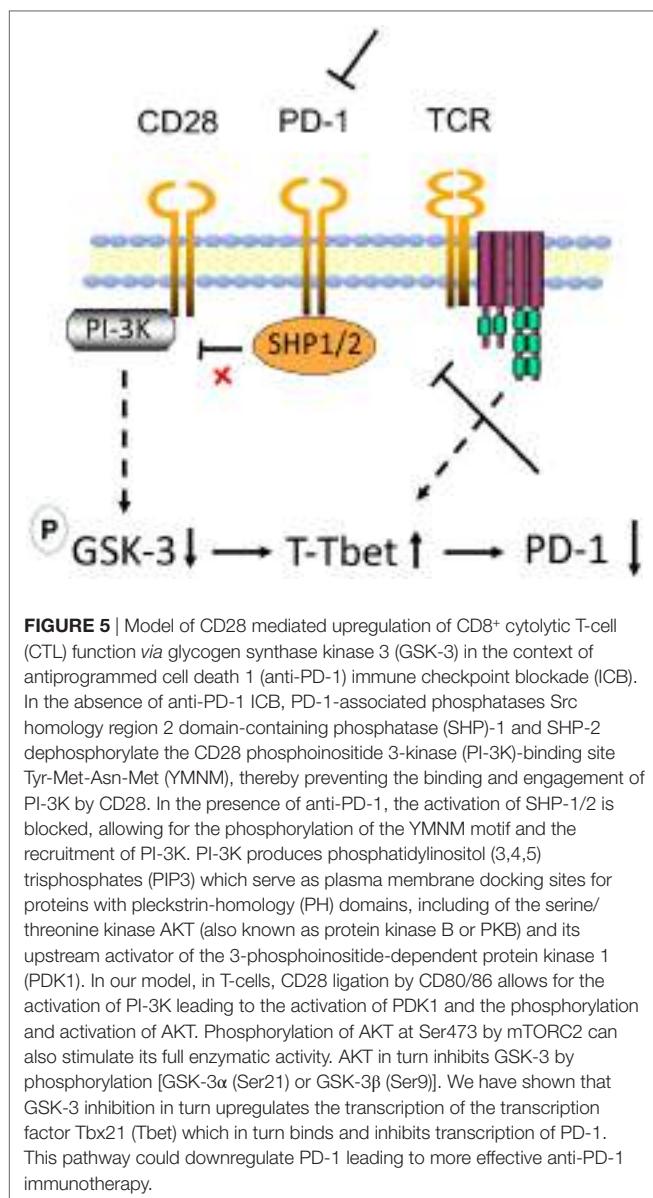


FIGURE 5 | Model of CD28 mediated upregulation of CD8⁺ cytolytic T-cell (CTL) function via glycogen synthase kinase 3 (GSK-3) in the context of anti-programmed cell death 1 (anti-PD-1) immune checkpoint blockade (ICB). In the absence of anti-PD-1 ICB, PD-1-associated phosphatases Src homology region 2 domain-containing phosphatase (SHP)-1 and SHP-2 dephosphorylate the CD28 phosphoinositide 3-kinase (PI-3K)-binding site Tyr-Met-Asn-Met (YMNM), thereby preventing the binding and engagement of PI-3K by CD28. In the presence of anti-PD-1, the activation of SHP-1/2 is blocked, allowing for the phosphorylation of the YMNM motif and the recruitment of PI-3K. PI-3K produces phosphatidylinositol (3,4,5) trisphosphates (PIP3) which serve as plasma membrane docking sites for proteins with pleckstrin-homology (PH) domains, including of the serine/threonine kinase AKT (also known as protein kinase B or PKB) and its upstream activator of the 3-phosphoinositide-dependent protein kinase 1 (PDK1). In our model, in T-cells, CD28 ligation by CD80/86 allows for the activation of PI-3K leading to the activation of PDK1 and the phosphorylation and activation of AKT. Phosphorylation of AKT at Ser473 by mTORC2 can also stimulate its full enzymatic activity. AKT in turn inhibits GSK-3 by phosphorylation [GSK-3 α (Ser21) or GSK-3 β (Ser9)]. We have shown that GSK-3 inhibition in turn upregulates the transcription of the transcription factor Tbx21 (Tbet) which in turn binds and inhibits transcription of PD-1. This pathway could downregulate PD-1 leading to more effective anti-PD-1 immunotherapy.

MATERIALS AND METHODS

Mice

C57BL/6-OT-1Tg and wt mice were used throughout the majority of the study. The research on mice was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body Home Office UK PPL No. 70/7544.

Cells and Cultures

OVA specific CD8⁺ cytolytic T-cells were generated by incubating isolated splenocytes from OT-1 mice with SIINFEKL peptide of OVA (OVA_{257–264}) at 10 ng/mL for 5–7 days. In certain cases, naive OT-1 T-cells were isolated from spleens using T-cell enrichment

columns (R&D) and subjected to nuclear transfection (see method below). In the case of purified naive T-cells, the thymoma EL4 cell line was used to present OVA_{257–264} to primary T-cells. EL4 cells were incubated with 10 nM OVA_{257–264} peptide (Bachem) for 1 h at 37°C and treated with mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) (final concentration of 10 µg/mL) prior to mixing with primary T-cells by coculturing at a ratio of 1:5 of EL4 and T-cells to generate cytotoxic T-cells. In either case, CTLs were generated in the presence or absence of SMI and/or anti-CD28 or CTLA-4-Ig (inhibitors/Abs added simultaneously with OVA-stimulation for 5–7 days) prior to washing and analysis by FACs, PCR, or cytotoxicity assays. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 mM beta-mercaptopethanol, sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and streptomycin (GIBCO).

Antibodies/Reagents

Stimulations were performed using 10 nM OVA_{257–264} peptide (Bachem), anti-CD28 (clone PV1, bioXpress), and CTLA-4 IgG Fusion Protein (BD Pharmingen) where stated. SMI (GSK-3 inhibitor) was obtained from Abcam plc, and suspended in DMSO to give a stock solution of 25 mM and diluted to a concentration of 10 µM *in vitro*. Fluorescently labeled Abs to GZMB, PD-1, and Lamp-1 (CD107a) were obtained from Biolegend.

Cytotoxicity Assays

Cytotoxicity was assayed using a Cytotox 96 nonradioactive kit (Promega) following the instructions provided. In brief, purified T-cells were plated in 96-well plates at the effector/target ratios shown using 10⁴ EL4 (ova peptide-pulsed) target cells per well in a final volume of 200 µL per well using RPMI lacking phenol red. Lactate dehydrogenase release was assayed after 4 h incubation at 37°C by removal of 50 µL supernatant from each well and incubation with substrate provided for 30 min and the absorbance read at 490 nm using the Thermomax plate reader (Molecular Devices). Percentage cytotoxicity = [(experimental effector_{spontaneous} – target spontaneous)/(target_{maximum} – target spontaneous)] × 100. All cytotoxicity assays were reproducible in at least three independent assays (59).

Nuclear Transfection

The 3.0 µg GSK-3 α / β siRNA was added to 1 × 10⁶ PBMC that had been washed in PBS and resuspended in 100 µL of Nucleofector™ solution for T-cells (Amaxa Biosystems, Cologne, Germany). Cells were transferred into a cuvette and electroporated using program X-01 of the Nucleofector™ (Amaxa Biosystems), and then immediately transferred into prewarmed cRPMI medium supplemented as recommended. GSK-3 α / β specific and control siRNA were synthesized by Cell Signaling Technology. Control cells were transfected with 3.0 µg siRNA using the same protocol. Transfected cells were rested 24 h, before assays commenced.

Priming OT-1Tg Cells *In Vivo*

Ova peptide (1 µg) was injected intravenously into OT-1Tg mice with and without SB415286 (100 µg) in 100 µL of PBS.

Spleens were harvested after 7 days and T-cells purified before further stimulation *in vitro* for 5 days with the indicated antibodies.

Statistical Analysis

The mean and SE of each treatment group were calculated for all experiments. The number of samples is indicated in the figure legends. Unpaired Student's *t*-tests or ANOVA tests were performed using the InStat 3.0 software (GraphPad). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

ETHICS STATEMENT

The research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical

review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB) Home Office UK PPL No. 70/7544.

AUTHOR CONTRIBUTIONS

CR supervised, contributed conceptionally, and helped to write the article. AT contributed conceptionally, conducted experiments, and helped to write the article.

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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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PD-1/PD-L1 Blockade: Have We Found the Key to Unleash the Antitumor Immune Response?

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PD-1–PD-L1 interaction is known to drive T cell dysfunction, which can be blocked by anti-PD-1/PD-L1 antibodies. However, studies have also shown that the function of the PD-1–PD-L1 axis is affected by the complex immunologic regulation network, and some CD8+ T cells can enter an irreversible dysfunctional state that cannot be rescued by PD-1/PD-L1 blockade. In most advanced cancers, except Hodgkin lymphoma (which has high PD-L1/L2 expression) and melanoma (which has high tumor mutational burden), the objective response rate with anti-PD-1/PD-L1 monotherapy is only ~20%, and immune-related toxicities and hyperprogression can occur in a small subset of patients during PD-1/PD-L1 blockade therapy. The lack of efficacy in up to 80% of patients was not necessarily associated with negative PD-1 and PD-L1 expression, suggesting that the roles of PD-1/PD-L1 in immune suppression and the mechanisms of action of antibodies remain to be better defined. In addition, important immune regulatory mechanisms within or outside of the PD-1/PD-L1 network need to be discovered and targeted to increase the response rate and to reduce the toxicities of immune checkpoint blockade therapies. This paper reviews the major functional and clinical studies of PD-1/PD-L1, including those with discrepancies in the pathologic and biomarker role of PD-1 and PD-L1 and the effectiveness of PD-1/PD-L1 blockade. The goal is to improve understanding of the efficacy of PD-1/PD-L1 blockade immunotherapy, as well as enhance the development of therapeutic strategies to overcome the resistance mechanisms and unleash the antitumor immune response to combat cancer.

Keywords: PD-1, PD-L1, immune checkpoint blockade, biomarker, MSI, TMB, resistance mechanism, combination immunotherapy

INTRODUCTION

It is widely known that ligation of programmed cell death protein 1 (PD-1, also known as CD279) (1) with PD-1 ligand 1 (PD-L1, also called B7-H1 or CD274) (2, 3) activates a critical immune checkpoint leading to T cell dysfunction, exhaustion, and tolerance; high-affinity anti-PD-1 or anti-PD-L1 monoclonal antibodies (mAbs) (4), which block PD-1–PD-L1 interaction, can reverse the immune checkpoint, releasing the brake on T cell responses. However, neither PD-1 nor PD-L1 expression is specific for the reversible T cell dysfunction state, and the effect of PD-1/PD-L1 blockade can be

context-dependent. In addition, PD-1 signaling and the mechanism of action of anti-PD-1/L1 mAbs are not completely understood.

Despite these discrepancies and unknowns, PD-1/PD-L1 blockade has achieved great clinical success in combating cancers. Durable response could also be achieved in PD-L1⁻ patients (5, 6). Nonetheless, a large proportion of patients, including those with PD-L1⁺/PD-1⁺ expression, do not respond to PD-1/PD-L1 blockade. Some rational combination therapies have shown synergy *in vivo* or in clinical trials (as well as immune-related toxicities, unfortunately). This article summarizes functional and clinical studies of PD-1/PD-L1 and the resistance mechanisms for PD-1/L1 blockade, and discusses several important questions arising from the disparate data, with the goal of increasing understanding of PD-1, PD-L1, and PD-1/PD-L1 blockade.

PD-1 AND PD-1 EXPRESSION: MARKERS OF T CELL EXHAUSTION OR ACTIVATION

Contrary to the common perception that PD-1 and PD-L1 expression is a marker of T cell dysfunction associated with cancer and chronic viral infection, PD-1 and PD-L1 can also be expressed under normal physiologic conditions. PD-1 is expressed on 40–80% of memory T cells but not on naïve T cells in the peripheral blood of healthy human adults, and PD-1 expression levels do not directly affect the cytokine production function of CD8⁺ T cells (7).

PD-1 expression may indicate T cell activation, because PD-1 is expressed only on activated T cells *in vivo*, and not on resting T cells. *PD-1 (PDCD1)* mRNA is mainly expressed in the thymus *in vivo*, with additional possible distribution in the spleen and lung (1). PD-1 protein can be detected in normal murine thymus and spleen T cells at low levels (8), but is strongly induced on thymocytes and T cells in the spleen and lymph nodes after stimulation with an anti-CD3 mAb *in vitro* (9) and increased on T cells in the spleen and liver after tumor cell injection *in vivo* (10). PD-1 is also expressed on activated B cells *in vitro* after stimulation with anti-IgM antibodies, but was undetectable on activated macrophages or dendritic cells (9, 11). In human reactive tonsils, PD-1 is expressed primarily on T cells, as well as a small subset of follicular dendritic cells (12).

The association of PD-1 expression with antigen-specific T cells has also been illustrated in cancer patients. PD-1 expression was significantly higher on antigen-specific CD8⁺ T cells than other CD8⁺ T cells in metastatic melanoma lesions in the same patients (13). In a melanoma mouse model, compared with tumor-ignorant bystander CD8⁺ T cells, tumor-specific CD8⁺ T cells infiltrating the same tumor had significantly higher levels of PD-1, LAG-3, CD69 (activation marker), and 4-1BB (costimulatory molecule) expression and gained 1,414 activation-related (but not exhaustion-related) accessible chromatin regions (14). Adoptive T cell therapy with cells expanded from PD-1⁺CD8⁺ tumor-infiltrating lymphocytes (TILs), but not from PD-1⁻ or bulk CD8⁺ TILs, showed tumor-reactivity and therapeutic benefit *in vivo* (15).

On the other hand, PD-1 expression is associated with suboptimal costimulation and T cell dysfunction when antigen is

presented on non-activated or non-professional antigen-presenting cells (16, 17), and PD-1 expression is often induced by high antigen concentration and prolonged antigen stimulation (18, 19). PD-1 may not be a good T cell activation marker because PD-1 surface expression is not rapidly induced on stimulated CD4⁺/CD8⁺ T cells. PD-1 expression has been shown to be increased 24–48 h after stimulation *in vivo* (20–22), 5–7 days after antigen experience (17), 3–8 days after adoptive transfer of pre-activated antigen-reactive CD8⁺ T cells (14), and 19 days after immunization *in vivo* (19), although *PDCD1* mRNA expression was shown to be increased at an earlier time point, as was the suppression of T-cell function. An *in vivo* kinetics study of T cell response to hepatitis B virus infection also showed that after intrahepatic antigen recognition, CD8⁺ T cells first showed rapid induction and decline of IFN-γ-producing capacity, followed by delayed T cell expansion and an increase in cytolytic activity, and the functional oscillation coincided with strong PD-1 induction on antigen-specific T cells (23).

Furthermore, in a melanoma model, the “exhausted” (showing reduced cytokine production capability) tumor-reactive CD8⁺ T cells, compared with “non-exhausted” bystander CD8⁺ T cells, had *Pdcld1* upregulation but downregulation of genes involved in CD8⁺ T cell survival and function (*Il7r*, *Bcl2*, *Cxcr3*, *Ifngr1*, and *Ifngr2*) (14). In patients with metastatic melanoma, tumor-infiltrating T cells had high PD-1 expression and decreased functional avidity compared with T cells infiltrating normal tissues, whereas circulating peripheral blood T cells had minimal PD-1 expression comparable with that in healthy donors. Smaller fraction of antigen-specific CD8⁺ T cells in metastatic melanoma lesions produced IFN-γ compared with those circulating in blood, which was inversely correlated with PD-1 expression (13). Similarly, PD-1 expression gradually increased in TILs with tumor growth but not on spleen T cells in a melanoma tumor model; although a higher percentage of TILs produced IFN-γ after stimulation *ex vivo* compared with spleen T cells, the amount of IFN-γ produced by TILs was lower, and smaller percentage of TILs produced TNF-α (19). In a colon cancer model, the cellular expression levels of PD-1 on intratumoral T cells inversely correlated with the function of CD8⁺ T cells (24).

During chronic infection with lymphocytic choriomeningitis virus (LCMV), *PDCD1* mRNA levels were upregulated in “exhausted” CD8⁺ T cells with impaired cytokine production and proliferation, but *PDCD1* was not upregulated in functional LCMV-specific memory CD8⁺ T cells during acute viral infection (25). Paradoxically, PD-1 protein expression was not limited to chronic LCMV infection, and PD-1 protein was also transiently expressed on CD8⁺ T cells in acute viral infection and downregulated along with LCMV clearance, suggesting that PD-1 protein expression is not a specific marker of exhaustion (25). In fact, during acute infection with rapid control of the viral infection, PD-1^{lo} cells mainly produced antiviral cytokines and PD-1^{hi} cells were the main mediators of cytotoxicity activity (26). Similarly, during chronic mycobacterial infection *in vivo*, PD-1⁺ T cells were not functionally exhausted (highly proliferative and could differentiate into cytokine-secreting T cells), and probably critical for antigen-specific T cell responses (27). Moreover, during tumor growth in a mouse model, although increased PD-1 and

LAG-3 expression was accompanied by decreased T-cell effector function, enhancing fatty acid catabolism increased PD-1 expression and improved T-cell effector function; conversely, inhibiting fatty acid catabolism decreased PD-1 expression and impaired T-cell function (28).

PD-1^{hi} expression also does not mark T cell exhaustion in patients with autoimmune disease or cancer. In patients with rheumatoid arthritis, PD-1^{hi}CXCR5⁺CD4⁺ cells are expanded in pathologically inflamed non-lymphoid tissues and are functionally active (promoting B cell responses) (29). In follicular lymphoma patients, PD-1⁺ T cells include both functionally “exhausted” (unable to produce cytokines) PD-1^{lo}T cells and PD-1^{hi} “non-exhausted” follicular helper T cells (CXCR5⁺BCL6⁺CD4⁺, supporting the growth and survival of B cells, and secreting IL-21 and IL-4) (30). Increased PD-1⁺ cells in tumor biopsies have been associated with either favorable prognosis in patients with follicular lymphoma (31, 32), lung cancer (33), ovarian cancer (34), or poor survival in cancer patients (35, 36). Furthermore, in melanoma patients, PD-1⁺ T cell clones are antigen-specific T cell clonotypes with higher functional avidity and reactivity (IFN- γ and TNF- α production after activation) than PD-1⁻ T cell clones (37), and PD-1 expression can be used as a biomarker for neoantigen-specific T cells in TILs and in the peripheral blood (38–40). The discrepancies in association of PD-1 expression with T-cell function (exhaustion or avidity) may reflect the complex interplay between various driving forces and effectors of the PD-1 pathway, suggesting that factors other than PD-1 are also important for T-cell functionality.

Similar to PD-1, PD-L1 expression can also be a marker of immune activation. PD-L1 is often not expressed in cell lines *in vitro* but is induced on tumors and in the tumor microenvironment (exceptions include some lymphoma and myeloma cell lines) (10, 41). IFN- γ produced by effector T cells soon after but not before activation of immune response (23), is the major inducer of PD-L1 expression at the transcription level (42). Supporting this, in metastatic melanoma samples, PD-L1⁺ cell densities were shown to significantly correlate with CD8⁺ T cell densities in the tumor and at the invasive tumor margin (43). IFN- γ and TLR ligands induce PD-L1 through the JAK/STAT/IRF-1, MEK/ERK, and MyD88/TRAF6 pathways (44–47). JAK2 (46), MEK/ERK, and p38 MAPK (48) signaling pathways were critical for PD-L1 expression in Hodgkin lymphoma cells. Furthermore, PD-L1 expression is also induced on immune cells after immune activation, including dendritic cells, macrophages, B cells (8, 11), T cells (49), and natural killer cells (50), and this is mediated through the cytokine/chemokine and STAT3 pathways (50–52).

Immune responses are not the only processes that can induce PD-L1 expression; tumor-intrinsic oncogenic pathways can also upregulate PD-L1 expression. For example, oncogenic c-Jun (AP-1) and STAT3 signaling (53), and hypoxia-inducible factor HIF-1 α (54) upregulate PD-L1 expression transcriptionally; the oncogenic epigenetic writer EZH2 (55) and epigenetic reader BET4 upregulate PD-L1 (56), whereas the epigenetic eraser histone deacetylase downregulates PD-L1 expression (57). In addition, loss of PTEN function and oncogenic activation of the PI3K/AKT/mTOR pathway increase PD-L1 expression post-transcriptionally (58, 59) [however, *in vivo* PTEN loss did not

always affect PD-L1 expression significantly (60)]. Moreover, CSN5, induced by NF- κ B p65 (61), and novel CMTM6/4 transmembrane proteins (62, 63) decrease ubiquitination and stabilize PD-L1. EGF signaling induces PD-L1 glycosylation and antagonizes GSK3 β -mediated PD-L1 phosphorylation and degradation (64). Enhanced glycolysis and lactate production activate transcriptional coactivator TAZ and induce PD-L1 expression on tumor cells (65). The glycolytic intermediate pyruvate can also metabolically control PD-L1 expression on macrophages through the BMP4/p-SMAD1/5/IRF-1 signaling pathway (66).

Furthermore, PD-L1 is also expressed under normal conditions in both lymphoid and non-lymphoid tissues on human placental trophoblasts, myocardial endothelia cells, and cortical thymic epithelial cells (8, 11, 42), which is involved in peripheral tolerance and immune privilege (67–69). PD-L1 expression has been correlated with either poorer or better survival of cancer patients (70, 71). Taking together, these findings show that, similar to PD-1, PD-L1 expression is not a specific marker for T cell activation or exhaustion.

PD-1 AND PD-L1 EXPRESSION AS DRIVER OR BIOMARKER OF IMMUNE SUPPRESSION: TUMOR-DRIVEN OR HOST-DRIVEN EVOLUTION

As mentioned above, PD-L1 expression can be either immunogenic (tumor-extrinsic, driven by the immune system) (72) or oncogenic (tumor cell-intrinsic, driven by intrinsic mechanisms in cancer cells). It has been controversial whether the immunogenic and oncogenic PD-L1 expression on tumor cells or PD-L1 expression on activated host immune cells is essential for immune evasion. Recently, four studies addressed this question *in vivo* and showed that although all forms of PD-L1 expression contribute to immune suppression in a non-redundant fashion, the relative roles (i.e., predominant or minor) of immunogenic tumor-derived PD-L1 and host-derived PD-L1 expression in suppressing T cell cytotoxicity and infiltration varied depending on the mouse models used, which had different levels of tumor immunogenicity (73–76). *PD-L1* gene deletion in highly immunogenic MC38 colorectal adenocarcinoma tumors resulted in loss of protection from T cell cytotoxicity, whereas the growth of MC38 tumors in PD-L1/PD-L2-knockout (PD-L1^{-/-}/L2^{-/-}) mice was as robust as in wild-type mice, which elegantly demonstrated that induced tumor PD-L1 expression directly and sufficiently inhibits antitumor immunity, serving as far more than a marker of an ineffective immune response (74).

Similarly designed experiments demonstrated that oncogenic PD-L1 expression in BRAF/PTEN melanoma tumors only slightly inhibited antitumor immunity (74), whereas immunogenic PD-L1 expression on non-tumor cells was critical for immune evasion. Similarly, in a mouse model of melanoma tumors with low immunogenicity, host PD-L1 and PD-1 expression on non-tumor cells is essential for suppressing antitumor immunity. Therefore, although the prevailing notion is that tumors exploit

the PD-1 pathway and evade immune response by actively over-expressing PD-L1, this “adaptive immune resistance mechanism” is largely limited to immunogenic PD-L1 expression (74), which is ultimately driven by the host immune response (72).

Although tumor PD-L1 expression in the MC38 model has a driver role, tumor PD-L1-mediated immune suppression has local limitations, which one study proposed as the “molecular shield” functional model. In this model, PD-L1 forms only a temporal molecular shield to protect PD-L1⁺ tumor cells, and the cytolytic function of T cells against other PD-L1⁻ tumor cells with the same antigen is not impaired (77), likely because a close proximity between PD-1–PD-L1 and immunologic synapses is required for PD-L1 function to disturb the T-cell receptor (TCR)–major histocompatibility complex (MHC) interaction. This functional mode is somewhat like another mechanistic model, in which PD-1–PD-L1 interaction increases T cell motility through inhibition of TCR-driven “stop signals” (78). Consistent with this functional model, two (73, 74) of the four recent studies mentioned above showed that tumor PD-L1 expression can protect only PD-L1⁺ tumor cells from cytolytic T cell killing *in situ*, and not PD-L1⁻ cells *in trans*, conferring a selective growth advantage on PD-L1⁺ tumor cells.

However, as shown in mouse models and in cancer patients, immunogenic tumor PD-L1 expression is heterogeneous (76) and transient (75), which does not support the idea that tumor-derived PD-L1 expression is required for tolerance induction and maintenance or that PD-L1⁺ tumor clones are preferably selected during tumorigenesis. It is postulated that PD-L1⁻ tumor cells escape immune surveillance through alternative mechanisms such as decreased MHC expression, increased PD-L2 expression on PD-L1⁻ tumor cells, stromal remodeling, and epithelial–mesenchymal transition (73), as well as compensatory PD-L1 expression on host cells, including T cells (79–81), antigen-presenting cells, monocytic myeloid-derived suppressor cells (MDSCs), and host tissues (81, 82). The compensatory PD-L1 expression can be both IFN-γ-dependent and IFN-γ-independent (75), and may be able to trigger a vicious cycle of immune suppression in the tumor microenvironment (83). Moreover, PD-1 signaling was recently proposed to affect antigen-presenting cells more than tumor cells owing to the increased CD80/CD86 expression on antigen-presenting cells, given that the CD28 receptor is the primary target for PD-1/SHP2-mediated dephosphorylation, as was newly discovered in that study (84). Therefore, host-derived PD-L1 appears to be indispensable for the inhibitory function of the PD-L1/PD-1 axis. However, whether the minor role of the oncogenic PD-L1 expression in the BRAF/PTEN melanoma model applies to tumor PD-L1 expression upregulated by other tumor-intrinsic mechanisms in different types of cancer is unclear.

Furthermore, the driver role of PD-1 on host T cells in immune suppression is demonstrated by the fact that MC38 tumors were completely cleared in PD-1-knockout (PD-1^{-/-}) mice. TILs from PD-1^{-/-} mice had an increased ratio of CD8⁺ cells to regulatory T cells (Tregs) and granzyme expression compared with TILs from wild-type mice. In contrast, MC38 tumors (with immunogenic PD-L1 expression) grew similarly robust in PD-L1^{-/-}/L2^{-/-} mice as in wild-type mice; PD-L1^{-/-}/L2^{-/-} mice and wild-type

mice had similar CD8/Treg ratios and PD-1, granzyme, and Ki-67 expression levels in TILs (74). In addition, earlier studies also showed that blockade of PD-1, but not PD-L1, by genetic deletion or mAbs cleared the tumor growth in tumor models (10, 74, 85), and PD-L1 knockout *in vivo* had no effect on PD-1 expression in TILs (74).

Together, these studies may suggest that immune responses are ultimately regulated by the host rather than the tumor. However, another study showed that continuous antigen encounters and TCR stimulation, rather than factors associated with the tumor microenvironment, induce PD-1 expression and T cell dysfunction (17), which is “imprinted” at the premalignant and early malignant phase and later evolves into a therapeutically irreversible state. In line with the idea of antigen dictation of immune response, increased PD-1 expression in expanded blood CD8⁺ cells from patients following viral immunotherapy was not necessarily a target for improving the efficacy of viral immunotherapy (86); immunogenic personalized mutanome vaccines have induced durable clinical response in melanoma patients (87, 88). However, resistance to personalized neoantigen vaccines can still be developed through β2M deficiency and other unclear mechanisms in some patients in these personal neoantigen vaccine trials, and patients receiving PD-1 blockade combination therapy achieved complete regression (87, 88). Moreover, in a tumor model, although tumor vaccines increased antigen-specific TILs, they did not decrease PD-1 expression, which impaired the effector function of TILs, nor did they decrease the percentage of MDSCs in the tumor lesions (which accumulated since early-stage and accentuated after immunization) (19). In a clinical trial of immunization in patients with metastatic melanoma, the expansion and function (tested *in vivo* and *in vitro*) of stimulated antigen-specific CD8⁺ T cells by cancer vaccines were also regulated by increased PD-1 expression (89).

The critical role of antigen was also shown in a mouse model with LCMV infection: T cells functioned normally during acute (Armstrong strain) infection with transient PD-1 expression but were exhausted during chronic (clone 13) infection with stable PD-1 expression (25). Although exhausted CD8⁺ T cells could be reinvigorated by anti-PD-L1 therapy *in vivo*, T cells became re-exhausted with persistent PD-1 expression if antigen concentration remained high (90). Therefore, persistent tumor antigens appeared to be the dictator for PD-1 expression and T cell re-exhaustion. However, this was not supported by antigen withdrawal *in vivo* experiment. After antigen clearance, exhausted T cells and anti-PD-L1-treated exhausted T cells failed to down-regulate PD-1 expression (or T-bet and Eomes expression) and had poor recall response upon antigen re-challenge (90).

A study assessing changes in chromatin accessibility during viral infection revealed that acute LCMV infection resulted in stable (5–10%) and dynamic ($\geq 25\%$) changes in accessible chromatin regions in antigen-specific effector and memory CD8⁺ T cells. In contrast, chronic infection uniquely enriched accessible chromatin regions for NFAT and Nr4a family transcription factors (including enhancers of the *PDCD1* locus) but partially lost the accessibility to some regions (such as *Satb1* and *Il7r* loci) in exhausted CD8⁺ T cells, although exhausted CD8⁺ T cells and effector CD8⁺ T cells shared chromatin accessibility at promoter

regions of key effector-related genes, including *Ifng*, *Gzma*, *Gzmk*, *Fasl*, and *Prf1*, as well as inhibitory receptor genes, including *Tim3*, *Lag3*, and *Ctla4* (91). Anti-PD-L1 therapy *in vivo* caused only minimal epigenetic profile changes in exhausted T cells; instead, the T cell reinvigoration by PD-L1 blockade resulted from transcriptional rewiring with different transcription factors (NF-κB, Jun/AP-1, IRFs, and CTCF, instead of “partnerless” NFATc1, NFAT:AP-1, Nr4a1, Nur77, Eomes, and Egr2) in the epigenetic landscape (90). The epigenetic inflexibility is thought to contribute to re-exhaustion with antigen stimulation without memory-like recall response after anti-PD-L1 treatment (90), suggesting the importance of host T cell-intrinsic regulatory factors including PD-1.

Similar to this unsustained therapeutic effect in viral infection models, an anti-PD-L1 mAb was shown to have only transient antitumor effects in a mouse model, in contrast to the complete suppression of myeloma growth by gene knockout of PD-1 (85). Anti-PD-L1 therapy *in vivo* led to tumor regression with increased antigen-reactive T cell infiltrate and increased IFN-γ and TNF-α production upon antigen stimulation *ex vivo*. However, PD-L1 blockade had only a moderate effect on gene activation and chromatin accessibility in tumor-infiltrating T cells, including upregulation of a few functionally important genes (including granzyme and serpin genes) and dampened accessibility in limited motifs binding NFAT, NFAT:AP-1, TCF, and bZIP:IRF transcription factors. In contrast, 450 accessible regions (including those accessible for Nr4a and NFAT) were gained in “exhausted” T cells compared with “non-exhausted” T cells before the treatment (14).

Furthermore, in an inducible liver cancer model, dysfunction of antigen-specific T cells lasting for more than 30 days was not rescued either after antigen withdrawal or after a decrease in PD-1 levels in TILs by anti-PD-1/PD-L1 therapy (17), suggesting that the dysfunction state was maintained by multiple factors rather than PD-1 alone. Irreversibility of these TILs, which will be discussed more in later sections, somewhat resembled the unresponsiveness of tolerant/anergic T cells to PD-L1 blockade (92). In these settings, PD-1 appeared to be a biomarker rather than the central driver of immune suppression.

PD-1 AND PD-L1: FUNCTIONALLY DEPENDENT OR INDEPENDENT IN DRIVING IMMUNE SUPPRESSION

The receptor and ligand relationship between PD-1 and PD-L1 was discovered by Freeman et al. in 2000 (2), and the relationship between PD-1 and PD-1 ligand 2 (PD-L2, also called B7-DC or CD273) was discovered by Latchman et al. in 2001 (93). PD-1 ligation leads to T cell exhaustion (decreased proliferation and effector function) (25), apoptosis (94, 95), or anergy/tolerance (a hyporesponsive state of T cells to a specific antigen that can be induced by lack of costimulation) (96–99). Functional studies have demonstrated that PD-1 receptor ligation is required for PD-1 to prevent T cell activation, and the inhibitory effect of PD-1 ligation depends on TCR strength (21, 22, 42) and co-localization of PD-1 with CD3 and/or CD28 (20, 100).

Molecularly, PD-1 ligation inhibits CD28-mediated costimulation (2, 20, 93); prevents TCR-driven stop signals (78); inhibits TCR signaling in both CD8⁺ and CD4⁺ T cells; blocks cell cycle progression in CD4⁺ T cells; downregulates expression of antiapoptotic molecules and proinflammatory cytokines; and upregulates expression of Cbl-b ubiquitin ligase in CD8⁺ T cells (20, 93, 100–104). For B cell-derived PD-1 expression, coligation of the PD-1 cytoplasmic region with the B cell receptor (BCR) inhibited BCR signaling *in vitro* (105). Inhibition of TCR/BCR signaling is mediated by the protein tyrosine phosphatase SHP2, which is recruited to the PD-1 immunoreceptor tyrosine-based switch motif upon PD-1 ligation and dephosphorylates ZAP70 (in T cells), Syk, Igβ, PLCγ2, and ERK (in B/T cells) and other downstream kinases, including PI3K/AKT (20, 93, 102, 105, 106). Although SHP2 can be associated with PD-1 immunoreceptor tyrosine-based switch motif with TCR stimulation in the absence of PD-1 engagement, PD-1 engagement is required to block T cell activation (20).

However, in contrast to these earlier studies, a recent study showed that CD28 and Lck (a kinase associated with CD4/CD8 that phosphorylates CD3/TCR, CD28, and PD-1), but not TCR, were the preferred targets of dephosphorylation by PD-1-bound SHP2 in a biochemical reconstitution system (84). PD-1 co-clustered with CD28 in plasma membrane microclusters in a PD-L1-dependent manner but only partially segregated with TCR in stimulated CD8⁺ T cells. Furthermore, intact cell assays using Jurkat T cells and Raji B cells confirmed that CD28, but not TCR, was dephosphorylated after PD-1 ligation with PD-L1; however, the dephosphorylation was only transient (84).

The downregulated PI3K/AKT pathway in T cells upon PD-1 ligation is important for the cell cycle, proliferation, survival, apoptosis, and metabolism. PD-1 also inhibits the PI3K/AKT pathway by inhibiting phosphorylation of PTEN in the C-terminal tail, which decreases PTEN stability but increases PTEN phosphatase activity (107). Because the PI3K/AKT/mTOR pathway is critical for metabolic reprogramming, PD-1 expression and ligation has been linked to metabolic dysfunction in T cells. As shown *in vitro*, ligation of PD-1 on CD4⁺ T cells inhibited glycolysis (106) and glucose transporter Glut1 as well as transportation and catabolism of glutamine, but augmented lipolysis and fatty acid oxidation (108), which promotes Treg development over that of effector T cells (109, 110). In multiple graft-vs.-host disease (GVHD) models, PD-1 expression was shown to increase levels of reactive oxygen species, which was dependent on oxidative metabolism of fat in both CD4⁺ and CD8⁺ T cells, facilitating CD8⁺ T cell apoptosis (95). Conversely, PD-1/PD-L1 blockade partially decreased the generation of reactive oxygen species and cell death of alloreactive PD-1^{hi}, but not PD-1^{lo}, T cells and increased the severity of GVHD (95). However, in patients with viral infection, exhausted virus-specific CD8⁺ T cells were dependent on glycolysis with high Glut1 and PD-1 expression and depolarized mitochondria which could be rescued by a signal 3 (111) cytokine IL-12, compared with the non-exhausted CD8⁺ T cells within the same patients with metabolic flexibility of utilizing mitochondrial oxidative phosphorylation to fuel the effector function (112). A recent study showed that *in vivo* hypoglycemia and hypoxia metabolic stress caused CD8⁺ T cell exhaustion

(which was independent of the PD-1 pathway however); fatty acid catabolism enhanced in CD8⁺ T cells (which was also observed in melanoma patients) partially preserved antitumor effector functions of CD8⁺ TILs but upregulated (possibly indirectly) PD-1 expression; PD-1 blockade synergizes (but did not change) this metabolic reprogramming in inhibiting tumor growth (28). In a B cell leukemia model with increased PD-1 and PD-L1 expression over time in the leukemic microenvironment, impaired T cell metabolism directly contributed to T cell dysfunction, whereas *in vivo* and *in vitro* PD-1 blockade was not sufficient to improve T-cell function (113).

Opposite to the PD-1 function in suppressing glycolysis, enhanced glycolysis induces PD-L1 expression (65), which in turn promotes glycolysis in tumor cells and restricts T-cell function by metabolically competing for glucose (114). Of note, PD-1 signaling inhibits the PI3K/AKT/mTOR and MAPK/ERK pathways in T cells but PI3K/AKT and MEK/ERK signaling pathways activate PD-L1 expression in tumor cells. Tumor PD-L1 promotes MTORC1 signaling but inhibits MTORC2 and autophagy (115). Metabolic competition or adaptation between tumor cells and T cells (114) may contribute to these contrasting pathways, and the paradoxical results in transplantation models: allogeneic donor T-cells in PD-L1-deficient GVHD mice had increased aerobic glycolysis and oxidative phosphorylation (116), whereas donor PD-L1-deficient T cells in wild-type mice had reduced aerobic glycolysis, oxidative phosphorylation, fatty acid metabolism, and cytokine production (117).

In line with the requirement of PD-1 ligation for its suppressive function, in follicular lymphoma, which has very low PD-L1 expression, only subsets of PD-1⁺ T cells have exhausted phenotypes and function (30, 118). However, exhaustion of terminally differentiated PD-1^{hi}CD44^{int}CD8⁺ T cells during chronic viral infection appeared not to depend on PD-L1 expression, because anti-PD-L1 mAbs could not rescue these PD-1^{hi} T cells from apoptosis or restore the effector function (119). Moreover, PD-1 and PD-L1 expression may be temporally non-overlapping; a kinetics study observed a rapid but transient burst of IFN- γ production at 4 h after adoptive T cell transfer, whereas loss of IFN- γ expression coincided with delayed strong PD-1 induction (23).

PD-L2, the second PD-1 natural ligand, has higher affinity than PD-L1 for PD-1 (120, 121). However, PD-1–PD-L2 interaction is much less functionally significant than the PD-1–PD-L1 interaction owing to the low expression of PD-L2, and PD-1–PD-L1 interaction is sensitive to PD-L2 competition only when PD-L2 levels are very high (120). In sharp contrast to PD-L1, PD-L2 is rarely expressed in lymphohematopoietic and non-hematopoietic tissues (8, 122), except human placental endothelium and medullary thymic epithelial cells (42). PD-L2 can be induced on dendritic cells, macrophages, activated T cells (8, 11, 21, 42), B cells (123–125), and cancer cells by IL-4 through IL-4R/STAT6 in inflammatory macrophages (126), the NF- κ B pathway in dendritic cells (8), and IFN- β /IFN- γ in melanoma cells (47). Furthermore, several studies showed that PD-1 and PD-L1, but not PD-L2, induce T cell tolerance and apoptosis, preventing auto/alloimmune responses (16, 67, 97, 116, 127, 128). These data may suggest that PD-1's suppressive function is largely dependent on PD-L1 but not PD-L2 expression.

In contrast, PD-L1 and PD-L2 can exert inhibitory function independent of PD-1 by binding to B7-1 (CD80) (129) and RGMb (130), respectively. The binding affinity of PD-L1–CD80 is less than that of PD-1–PD-L1 (49). Studies showed that PD-L1–CD80 interaction, but not PD-L1–PD-1 interaction, is responsible for the induction and maintenance of T cell tolerance (131, 132), and that interaction between PD-L1 and PD-1 does not lead to T cell anergy *in vitro* (77). In contrast, in nonobese diabetic (NOD) mouse models, loss of PD-1, but not PD-L1, on antigen-specific CD4⁺ T cells resulted in increased proliferation of CD4⁺ T cells and infiltration of the pancreas during type 1 diabetes (133).

However, early studies showed that similar to the dependence of PD-1 function on receptor ligation (20), the inhibitory activity of PD-L1 and PD-L2 requires the expression of PD-1 (2, 93); in fact, PD-L1 expression in T cells, natural killer cells, and peripheral tissues can have a costimulatory effect with unknown receptors (3, 50, 117, 134–142). PD-L1 expressed on activated CD8⁺ T cells was shown to promote survival and effector function of CD8⁺ T cells during the contraction phase following immunization/antigen stimulation (134). PD-L1 expression in pancreatic islet beta cells was shown to accelerate allograft rejection, increase CD8⁺ T cell proliferation, and promote autoimmune diabetes (135). Likewise, PD-L1 expression induced on donor T cells augmented GVHD lethality (117). A recent study showed that after CD4⁺ T depletion in hematopoietic cell transplantation *in vivo*, PD-L1–CD80 interaction augmented survival and expansion of donor CD8⁺ T cells, resulting in strong graft-vs.-leukemia effects. In contrast, interaction of PD-L1 in recipient tissues with PD-1 on donor CD8⁺ T cells prevented GVHD (139), suggesting that PD-L1's inhibitory function depends on PD-1. These contradictory results suggest that PD-L1 interactions with PD-1, CD80, and other unknown receptors have context-dependent functions. Unidentified receptors of PD-L2 with stimulatory function have also been reported (143–145).

PD-1 BLOCKADE AND PD-L1 BLOCKADE BY GENE KNOCKOUT OR ANTIBODIES: EFFICACIES AND LIMITATIONS

Blocking of the PD-1/PD-L1 pathway by genetic deletion or using anti-PD-1/PD-L1 antibodies has been studied in various preclinical models and the results are quite variable, likely owing to the different roles of PD-1 and PD-L1 in different genetic and immunologic settings. Unlike CTLA-4 germline knockout CTLA-4^{-/-} mice, which spontaneously and rapidly developed fatal lymphoproliferative disease with massive expansion of activated T cells (146, 147), PD-1^{-/-} mice with different genetic backgrounds slowly developed lupus-like proliferative arthritis, glomerulonephritis, splenomegaly, or dilated cardiomyopathy with high-titer autoantibodies in early PD-1 studies (148–150), suggesting that PD-1 can inhibit B cell proliferation and differentiation. In a later study, PD-1 knockout in NOD mice specifically accelerated the onset and frequency of type I diabetes, with strong T helper 1 (Th1) polarization of T cells infiltrating into islets (151). Loss of PD-1, but not PD-L1, was further confirmed to be responsible for the proliferation and infiltration of reactive CD4⁺

T cells during type 1 diabetes in an adoptive T cell transfer model (133). PD-1 also plays a role in positive and negative selection of T cells, as indicated by the altered thymocyte repertoire in PD-1^{-/-} TCR-transgenic mice (152) and *in vitro* (104).

In contrast, PD-L1^{-/-} mice appeared normal but were susceptible to experimental autoimmune hepatitis (induced by accumulation of antigen-activated CD8⁺ T cells in the liver) (153) and experimental autoimmune encephalomyelitis (induced by myelin-reactive CD4⁺ Th1 cells) (81). PD-L1^{-/-} lupus-susceptible (MRL^{+/+}) mice developed autoimmune myocarditis and pneumonitis with increased PD-1⁺ macrophage and T cell infiltrates in the heart and lung (154). PD-L2^{-/-} mice exhibited enhanced antigen-specific T cell response and breakdown of oral tolerance compared with wild-type controls (155).

In tumor-formation models, PD-1^{-/-} mice completely suppressed the tumorigenesis of PD-L1⁺ myeloma cells (85); PD-1 deficiency also inhibited the hematogenous dissemination of poorly immunogenic tumors (which were PD-L1⁻ *in vitro*) in PD-1^{-/-} mice (10, 85). In viral infection models, both PD-L1^{-/-} (25) and PD-1^{-/-} mice (156) died from immunopathologic damage within a week after being infected with the LCMV clone 13 strain, which causes chronic infections in wild-type mice. However, both PD-L1^{-/-} and PD-1^{-/-} mice exhibited normal T cell responses to acute LCMV infection and controlled the infection as the wild-type mice did (25, 156). The lethal consequence of chronic infection was a result of systemic vascular leakage due to severe perforin-mediated cytolysis with enhanced CD8⁺ T cell activity (156). These results may suggest that the effectiveness of antiviral immune response is determined by the strain of virus or antigen but not the PD-1/PD-L1 axis, whereas high cytolytic activity due to PD-1/PD-L1 absence results in immunopathologic tissue damage over a prolonged period (23). These results may also suggest that PD-1/PD-L1 interaction has a positive role in generating effective antiviral responses. Indeed, further studies showed that *PD-1* deletion in virus-specific CD8⁺ T cells enhanced T cell proliferation in the acute phase, but overstimulation and robust proliferation lead to increased apoptosis during the contraction phase, as well as accumulation of more cytotoxic but terminally differentiated (Eomes^{hi} cells evolved from T-bet^{hi} progenitor cells), “deeply exhausted” CD8⁺ T cells during chronic LCMV infection (157).

Interestingly, PD-L1 blockade with anti-PD-L1 antibodies during the early-phase (on days 4–6) of systemic LCMV clone 13 infection also caused vascular permeability and ultimately fatal circulatory collapse (156), but anti-PD-L1 therapy on days 23–40 after infection restored the function of exhausted CD8⁺ T cells (proliferation, cytokine production, degranulation, and viral control) with or without CD4⁺ T cell depletion (25). Although CD4⁺ T cell help is critical for sustained CD8⁺ T cell cytotoxic function during chronic LCMV infection (158), other studies showed that combining PD-L1 blockade with CD4⁺ T cell depletion (159) or Treg cell depletion (160) could rescue deeply exhausted CD8⁺ T cells during the late stage of infection and may result in a significant reduction in viral load.

Although the autoimmune diseases against self-antigens were much milder and at later onset in PD-1/PD-L1/L2 deficient mice than in CTLA-4^{-/-} mice, anti-PD-1 mAbs exhibited stronger

antitumor effects than anti-CTLA-4 mAbs in tumor models (10, 25). The enhanced antitumor immunity is believed to result from the occupancy of the PD-1 receptor by anti-PD-1 mAbs which prevents PD-1 from interacting with its natural ligands PD-L1/L2. It has been demonstrated that PD-1 blockade with anti-PD-1 mAbs can increase proliferation and cytokine production of antigen-specific T cells (4, 10, 161), expand intratumoral frequencies of CD8⁺ effector memory T cells (162), enhance the cytotoxicity activity of effector T cells (preferably PD-1⁺ memory T cells with higher functional avidity) (37), augment recruitment of effector cells into the tumor site (4, 10, 161), decrease T cell mobility and enhance stable T-dendritic cell interaction (78), and promote CD8⁺ T cell priming (97, 163) [however, some studies showed that PD-1 blockade alone did not affect CD8⁺ T cell priming and costimulation from CD27 or CD28 may also be required for T cell priming (164, 165)].

In addition, PD-1 expression was found on 64% of freshly isolated natural killer cells from patients with multiple myeloma (166). Anti-PD-1 treatment *in vitro* with a CT-011 antibody (however, its specificity for PD-1 has been questioned) enhanced natural killer cell trafficking, immune complex formation, and cytotoxicity against PD-L1-bearing multiple myeloma cells (166). In multiple tumor models, IL-18 upregulated PD-1 expression on mature natural killer cells only in lymphoid organs but not in tumors; anti-PD-1 therapy *in vivo* abrogated IL-18-mediated metastases (167). PD-1 expression was also found on tumor-associated macrophages in patients with colorectal cancer (168) and on tumor-infiltrating myeloid dendritic cells in ovarian cancer patients (169). PD-1/PD-L1 blockade alone or combined with anti-CD47 therapy *in vivo* increased macrophage phagocytosis but decreased tumor growth and increased survival of mice (168). PD-1 blockade *in vitro* or *in vivo* enhanced dendritic cell function, including cytokine (TNF- α and IL-6) release, antigen presentation, and costimulation owing to NF- κ B activation. PD-L1 blockade also increased cytokine release although to less extent (169, 170).

Similar to PD-1 blockade, PD-L1 blockade with anti-PD-L1 mAbs was also shown to increase cytokine production of T helper cells, enhance the cytolytic activity of cytotoxic T cells, and lengthen the duration of antigen-driven T cell migration arrest *in vitro* (78, 100, 171, 172). PD-L1 blockade strongly enhanced proliferation and cytokine production of memory or recently activated T cells from peripheral blood of healthy donors *ex vivo*, but only slightly enhanced naive T cell activation during a primary response (173). In contrast, a study showed that anti-PD-1/PD-L1 mAbs *in vivo* enhanced IFN- γ production but inhibited naïve CD4⁺ T cell proliferation, mediated by IFN- γ from CD4⁺ T cells and nitric oxide from macrophages (174). In a murine model of chronic colitis induced by adoptive transfer of CD4⁺CD45RB^{hi} T cells, PD-L1 blockade treatment before (but not after) the onset of severe colitis suppressed T cell expansion and Th1 cytokine production and prevented the development of colitis (141).

However, the effects of PD-1/PD-L1 blockade were contextual in viral infection models. PD-L1 blockade and PD-1 blockade were effective only for exhausted T cells during chronic LCMV infection, and they did not increase virus-specific CD8⁺

T cells during acute infection (25, 26). Moreover, in a chronic LCMV infection model, PD-L1 blockade rescued only the rescueable subset of exhausted CD8⁺ T cells and not the more terminally differentiated (PD-1^{hi}CD44^{int}) subset of CD8⁺ T cells (119). Similarly, adoptive transfer of CXCR5⁺CD44^{hi} but not CXCR5⁻CD44^{lo}CD8⁺ T cells (the former had PD-1^{lo}TIM-3^{lo} expression and higher effector function) reduced the viral load in mice chronically infected with LCMV; the therapeutic effect was further enhanced with anti-PD-L1 combination (175). T cell terminal differentiation during chronic viral infection was also shown to be associated with the Eomes^{hi}PD-1^{hi}BLIMP-1⁺T-bet^{lo} phenotype (converted from T-bet^{hi}PD-1^{int} cells) and increased cytotoxicity but decreased co-production of IFN- γ and TNF- α (176); the therapeutic reversibility of Eomes^{hi}PD-1^{hi}T-bet^{lo} cells compared with T-bet^{hi}PD-1^{int} cells was not examined in that study (176). Another study demonstrated opposite results, showing that anti-PD-L1 or anti-PD-1 therapy during chronic viral infection *in vivo* expanded only the TCF1⁺ memory-like CD8⁺ T cells with PD-1^{hi}T-bet^{lo}Eomes⁺ expression but not terminally differentiated TCF1⁻CD8⁺ T cells (177). However, whether the effector functions of expanded TCF1⁺CD8⁺ T cells were restored was not shown.

PD-1/PD-L1 blockade also had no effect on established T cell anergy in autoimmune models (92) nor on “non-reversible” dysfunction of T cells in tumor models. In a breast cancer mouse model, the PD-1^{hi}-expressing CD8⁺ T cell population failed to be rescued by anti-PD-1 therapy, showing increases in the Treg/CD8⁺ T ratio, in contrast to CD8⁺ T cells with PD-1^{lo} surface expression, which were sensitive to anti-PD-1 mAb in a colon cancer mouse model (24). Several studies demonstrated that the therapeutic reversibility correlated to the duration of dysfunction. In a tamoxifen-inducible autochthonous liver cancer model, dysfunctional tumor-specific CD8⁺ T cells could be rescued by PD-1/PD-L1 blockade in the early-phase, but after 30 or more days the dysfunction was irreversible (17). Notably, this timing effect is opposite to that for PD-L1 blockade during systemic LCMV infection [fatal during the early-phase (156) but effective on days 23–40 (25)]. Also, PD-1 blockade at early time points following viral immunotherapy did not improve durable control of metastatic disease *in vivo* despite the high frequency of PD-1⁺TIM-3⁺CD8⁺ T cells (86).

Transcriptional factors (17) and epigenetic programs may define the function of tumor-specific T cells in TILs and therapeutic reprogrammability (178). Dysfunctional TILs were found to lose access to some intergenic/intragenic regions (probably enhancers), including those in *Ifng*, *Cd5*, and *Tcf7*, but gain access to some NFATC1-binding sites, including those in *Pdcld1*, *Ctla4*, *Cd38*, and *Egr1/2*. The reprogrammability of dysfunction, as assessed by whether the ability to produce IFN- γ and TNF- α was regained after anti-PD-1/PD-L1 therapy, is associated with the discrete chromatin state of T cells—i.e., the “plastic dysfunctional state” at early tumorigenesis and the “fixed dysfunctional state” after day 14–35—and the differential expression of TCF and NFAT family transcription factors. The chromatin changes associated with the fixed dysfunction state included closed TCF/FOS motifs and opened E2F/ETS/KLF motifs. Antigen exposure in tumors has a pivotal role in determining the chromatin state in

T cells, whereas PD-1^{hi}-expressing CD8⁺ T cells can be in either a plastic or fixed dysfunctional state (178).

However, PD-L1 and B7/CD28 expression in these viral infection models and tumor models, which may be relevant for the therapeutic efficacy, were unclear. For example, terminal differentiated TILs with reduced IFN- γ production may induce very low PD-L1 expression, contributing to the hyporesponsiveness to anti-PD-1/L1 therapy, if pre-existing PD-1–PD-L1 interaction is required for the anti-PD-1/L1 therapy to have a positive effect. It has been shown *in vitro* that PD-1 engagement with anti-PD-1 mAbs inhibited rather than enhanced CD4⁺ T cell expansion and cytokine production with optimal ICOS or suboptimal CD28 costimulation (20, 21, 101) and inhibited glycolysis and glutamine catabolism in T cells (106, 108). However, it is unclear why anti-PD-1 mAbs do not activate similar inhibitory signaling in T cells after blocking the PD-1–PD-L1 interaction in PD-L1⁺ tumors. Also unknown are whether after anti-PD-1 mAbs occupy PD-1, blocked PD-L1 will bind to the alternative CD80 receptor and whether the PD-L1–CD80 interaction in tumors is inhibitory or stimulatory.

In contrast, anti-PD-L1 mAbs, which do not bind to PD-1, should not induce *de novo* inhibitory signaling in T cells in PD-L1⁻ tumors. In addition, anti-PD-L1 mAbs block both PD-1 and CD80 interaction with PD-L1, suggesting that anti-PD-L1 mAbs may have higher efficacy than anti-PD-1 antibodies in PD-L1⁺ tumors. However, treatment with anti-PD-L1 mAbs will not block PD-1–PD-L2 interaction or decrease PD-1 expression, and PD-L1 is broadly expressed in normal tissues, which may suggest that anti-PD-L1 mAbs are less efficacious in PD-1⁺ PD-L2⁺ scenarios but have more immune-related toxicities than anti-PD-1 mAbs.

In preclinical models, comparison between PD-1 blockade and PD-L1 blockade showed inconsistent or contradictory results. Several studies demonstrated that PD-1 and PD-L1 blockade had similar efficacy in preclinical models with PD-L1⁺ tumors (19, 77). In tumor-formation mouse models, PD-1 blockade showed striking efficacy in inhibiting hematogenous dissemination of tumor cells with poor immunogenicity, but PD-L1 blockade had no effect (10). However, PD-L1 blockade was more effective than PD-1 blockade in restoring the function of exhausted T cells in PD-L1-expressing mice with chronic viral infection (25). Moreover, an antibody against PD-L1 on myeloid dendritic cells improved T cell antitumor immunity, although it did not block PD-1–PD-L1 interaction (179). PD-L1 blockade had a stronger effect than PD-1 blockade in breaking T cell anergy *in vivo* in an OT-1 T-cell anergy model. Anergy prevention required early treatment with PD-1 or PD-L1 antibodies after tolerogen exposure, whereas delayed treatment had no effect in preventing T cell anergy (127). The ineffectiveness of PD-1/PD-L1 antibodies in breaking established T cell tolerance, in sharp contrast to the effectiveness in preventing tolerance induction, was also observed in other mouse models (92, 97, 159). In contrast, an anti-PD-L1 mAb, which specifically blocks PD-L1/CD80 but not PD-L1/PD-1 interaction (131), was able to break the pre-established T-cell anergy. However, another study showed that in NOD mice, both PD-L1 and PD-1 blockade enhanced the interactions of tolerized T cells with antigen-bearing dendritic

cells, abrogated tolerance, and induced rapid development of autoimmune diabetes, whereas CTLA-4 blockade or anti-CD80 had no such effects (78).

In addition to anti-PD-1 antibodies, small-molecule compounds and peptide antagonists have been reported to inhibit the interaction between PD-1 and PD-L1 (180–183), but their clinical efficacies and dependence on PD-1/PD-L1 expression are currently unknown.

CLINICAL PD-1 BLOCKADE AND PD-L1 BLOCKADE IN CANCER PATIENTS: SUCCESSES AND FAILURES

Immune checkpoint blockade with anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies has changed the paradigm of cancer treatment. Compared with the CTLA-4 antibodies, anti-PD-1/L1 antibodies have the advantage of lower toxicities (184–186). Currently, the US Food and Drug Administration (FDA) has approved two anti-PD-1 mAbs (PD-1 blockade), nivolumab (Opdivo; Bristol-Myers Squibb Co.) and pembrolizumab (KEYTRUDA; Merck and Co., Inc.), and three anti-PD-L1 mAbs (PD-L1 blockade), atezolizumab (TECENTRIQ; Genentech Oncology), avelumab (BAVENCIO; EMD Serono, Inc.), and durvalumab (IMFINZI; AstraZeneca UK Limited), for the treatment of cancer. The approvals were based on a high objective response rate (ORR), durability of response, or improved survival rate as demonstrated in successful clinical trials (**Tables 1** and **2**).

Anti-PD-1 mAbs as single agents or combined with chemotherapy or ipilimumab (anti-CTLA-4 mAb) have been approved for the treatment of the following cancers as first-line, second-line, third-line, or later-line therapies: melanoma (6, 184, 187–195), non-small cell lung cancer (NSCLC) (5, 196–200, 219), classical Hodgkin lymphoma (202, 203, 220), renal cell carcinoma (201), head and neck squamous cell carcinoma (HNSCC) (204), urothelial carcinoma (205–207), microsatellite instability-high (MSI-H) cancers (including colorectal cancer and other solid cancers) (208–210), hepatocellular carcinoma (211), and gastric or gastroesophageal junction adenocarcinoma [approval to pembrolizumab (**Table 1**); however, only nivolumab phase 3 results are available (221)]. Anti-PD-L1 mAbs as single agents in first-line, second-line, or salvage therapies have been approved in urothelial carcinomas (212–215, 222), NSCLC (216, 217), and Merkel cell carcinoma (218). Many clinical trials in different cancer types or settings are still ongoing and some have shown good results, such as the phase 3 PACIFIC clinical trial for durvalumab as consolidation therapy in patients with stage III NSCLC (223). The ORRs with PD-1/PD-L1 blockade as monotherapy in relapse/recurrence settings largely differ by disease entities; the ORR is close to 70% in classical Hodgkin lymphoma which frequently has 9p24 copy number alterations (202), ~40% in skin cancers, ~20% in lung cancers, ~25% in renal cancer, 13–23% in bladder cancer, and 13–16% in HNSCC. PD-1 blockade and PD-L1 blockade largely showed similar efficacy, although the ORRs were ~5% higher with PD-1 blockade than with PD-L1 blockade in NSCLC, and results of PD-L1 blockade need to be validated in phase 3 studies.

However, anti-PD-1/PD-L1 therapies did not work in all cancers [e.g., chronic lymphocytic leukemia (224)]. Although most of responses were more durable than traditional therapies, some patients who initially responded to checkpoint blockade experienced relapse [acquired resistance; however, a small subset of relapsed patients could still respond to continuing blockade therapy; the rate was 3.6% in urothelial carcinoma patients treated with atezolizumab (225)]. Moreover, recently five phase 3 studies have failed to meet the endpoints [first-line nivolumab alone or durvalumab plus tremelimumab compared with chemotherapy; nivolumab, pembrolizumab, or atezolizumab as a later-line therapy compared with chemotherapy or standard treatment (226, 227), **Table 3**], even though blockade has shown clinical activity in phase 1/2 trials (212, 228–231). Two phase 3 clinical trials of pembrolizumab in multiple myeloma have been placed on full clinical hold owing to increased risk of death.

In addition, hyperprogression, a new pattern of disease progression after anti-PD-1/PD-L1 monotherapy, that is associated with elderly age and worse overall survival but not specific tumor types, has been identified in ~9% of cancer patients (232, 233). A higher rate of hyperprogression (regional recurrence in most cases without any cases of pseudoprogression), 29%, was retrospectively identified in patients with HNSCC (234). The different rates may result from differences in hyperprogression definition and size of the cohorts, since in the HNSCC cohort, hyperprogression was significantly associated with shorter progression-free survival but not with overall survival. These unexpected clinical observations may reflect our incomplete understanding of the PD-1/PD-L1 pathway and immune regulation mechanisms.

MOLECULAR DETERMINANTS AND PREDICTIVE BIOMARKERS FOR PD-1/PD-L1 BLOCKADE IMMUNOTHERAPY: PD-L1⁺, TUMOR MUTATIONAL LOAD, T CELL FUNCTIONAL STATE, OR OTHER HOST FACTORS

Given the high cost and potential toxicities of the treatment, efforts have been made to identify predictive biomarkers for selecting patients who are most likely to benefit from anti-PD-1 immunotherapy. PD-L1 is the first and most studied biomarker for PD-1 blockade (188, 235). Theoretically, PD-1 blockade should work only in PD-1⁺ PD-L1⁺ patients and not in PD-1⁻ patients (4) or PD-L1⁻ patients (most PD-L1⁻ cases are PD-L1⁻) (**Figure 1**), because PD-1 ligation is indispensable for PD-1-mediated suppression, and in the absence of PD-1 natural ligand, anti-PD-1 mAbs can act as PD-1 agonists to inhibit rather than enhance PD-1⁺ CD4⁺ T-cell function (20, 21, 101). However, in multiple clinical trials, PD-L1 negativity was not found as an excluding factor for patient selection (**Table 1**). Durable clinical response to PD-1 blockade was also observed in some PD-L1⁻ patients with unknown PD-L2 status (although with a lower response rate in most studies). Furthermore, in some studies of squamous NSCLC and renal cell carcinoma, the efficacy of PD-1 blockade (response rate or survival outcome) in PD-L1⁻ patients was similar to or even better than that in PD-L1⁺ patients (5, 201). The predictive

TABLE 1 | Brief summary of the results of anti-PD-1 therapy clinical trials leading to US food and drug administration approval.

Antibody; reference	Clinical trial	Efficacy	PD-L1 biomarker
Melanoma			
Pembrolizumab; Robert et al. (187)	Phase 1b KEYNOTE-001 trial in 173 patients with advanced melanoma progressed following ipilimumab and if <i>BRAF</i> ^{V600} mutation positive, a BRAF and/or MEK inhibitor	ORR: 26%; 88% of responses were durable	Pooled analysis (<i>n</i> = 451) by Daud et al. (188): membranous PD-L1 expression (22C3 mAb) in tumor and immune cells was scored 0–5; higher scores were associated with better ORRs, PFS, and OS; with a ≥1% cutoff for PD-L1+, HR: 0.51 for PFS and 0.50 for OS; ORR: 8–12% in PD-L1+ patients (durable response), 22–53% in PD-L1+ patients with a PD-L1 score 2–5
Pembrolizumab; Ribas et al. (189)	Phase 2 KEYNOTE-002 trial in 540 patients with unresectable or metastatic melanoma who were refractory to prior ipilimumab and if <i>BRAF</i> ^{V600} mutation positive, a BRAF inhibitor	For 2–10 mg/kg pembrolizumab vs. chemotherapy, 6-month PFS: 34–38 vs. 16% (HR: 0.57/0.50, <i>p</i> < 0.0001); ORR: 21–25 vs. 4%; see final update according to Hamid et al. (190) on the right	For 2–10 mg/kg pembrolizumab vs. chemotherapy, 24-month PFS: 16–22 vs. <1%; 24-month OS: 36–38 vs. 30% (HR: 0.86/0.74, <i>p</i> = 0.117/0.011, non-significant); ORR: 22–28 vs. 4%; DOR: 73–74 vs. 13% of responders had no progression; OS was consistent across PD-L1 groups but pembrolizumab is favored over chemotherapy in PD-L1+ patients
Pembrolizumab, first- or second-line alone; Robert et al. (191)	Phase 3 KEYNOTE-006 trial in 834 patients with advanced melanoma previously untreated or received no more than one line of prior systemic therapy	6-month PFS: 47.3 or 46.4%; 12-month OS: 74.1 or 68.4%; ORR: 33.7 or 32.9%	PFS and OS were better in PD-L1+ patients compared with PD-L1− patients. Pembrolizumab vs. ipilimumab: better PFS in both PD-L1+ and PD-L1− groups (HR: 0.53/0.52 and 0.67/0.76), better OS only in PD-L1+ patients (HR: 0.55/0.58)
Nivolumab; Weber et al. (192)	Phase 3 CheckMate 037 trial in 405 patients with advanced melanoma who progressed after ipilimumab or ipilimumab and a BRAF inhibitor if <i>BRAF</i> ^{V600} mutation positive	ORR 31.7 vs. 10.6% for chemotherapy	ORR with nivolumab vs. with chemo: in PD-L1+ patients (surface expression, cutoff: ≥5% tumor cells, Dako; prevalence: 49%), 43.6 vs. 9.1%; in PD-L1− patients, 20.3 vs. 13.0%
Nivolumab; first-line alone; Robert et al. (6)	Phase 3 CheckMate 066 trial in 418 previously untreated patients who had metastatic melanoma without a BRAF mutation	Improved ORR and survival rates compared with dacarbazine: ORR: 40 vs. 13.9%; 1-year OS: 72.9 vs. 42.1%; median PFS: 5.1 vs. 2.2 months (all <i>p</i> < 0.001)	ORR improvement in PD-L1+ (≥5% tumor cells) patients (prevalence: 35.4%): 52.7 vs. 10.8%; in PD-L1− patients: 33.1 vs. 15.7%. OS improvement: HR for death, 0.30 in PD-L1+ patients and 0.48 in PD-L1− patients
Nivolumab alone or combined with ipilimumab, first-line; Larkin et al. (193)	Phase 3 CheckMate 067 trial in 945 previously untreated patients with metastatic melanoma	Median PFS: 11.5 months with nivolumab plus ipilimumab vs. 2.9 months with ipilimumab (<i>p</i> < 0.001), or 6.9 months with nivolumab alone (<i>p</i> < 0.001)	With nivolumab alone, in PD-L1+ patients, median PFS: 14.0 months, ORR: 57.5%; in PD-L1− patients, median PFS: 5.3 months, ORR: 41.3%. Combination benefit showed in PD-L1− patients: with combination, ORR: 54.8%, median PFS: 11.2 months; with nivolumab alone, ORR: 41.3%, median PFS: 5.3 months; PD-L1+ cutoff: ≥5% tumor surface expression, Dako 28–8; PD-L1+ prevalence: 23.6%
Combined nivolumab and ipilimumab, first-line; Hodi et al. (194)	Phase 2 CheckMate 069 trial in 142 patients with previously untreated advanced melanoma	For combination vs. ipilimumab alone, ORR: 60 vs. 11%; median PFS: 8.9 vs. 4.7 months; 2-year OS: 63.8 vs. 53.6%	PD-L1 positivity (cutoff: ≥5% tumor cells, Dako 28–8; prevalence: 30%) did not correlate with ORR or PFS
Nivolumab and ipilimumab for adjuvant therapy; Weber et al. (195)	Phase 3 CheckMate 238 trial in 906 patients with resected advanced melanoma	12-month PFS with nivolumab vs. with ipilimumab: 70.5 vs. 60.8% (<i>p</i> < 0.001)	12-month PFS in PD-L1+ (cutoff: ≥5% tumor cells, Dako 28–8) patients (prevalence: ~34%), 81.9 vs. 73.8%; in PD-L1− patients, 64.3 vs. 53.7%
NSCLC			
Nivolumab; Brahmer et al. (5)	Phase 3 CheckMate 017 trial in 272 patients with advanced, refractory squamous NSCLC	For nivolumab vs. docetaxel, ORR: 20 vs. 9% (<i>p</i> = 0.008); 1-year OS: 42 vs. 24% (<i>p</i> < 0.001); median PFS: 3.5 vs. 2.8 months (<i>p</i> < 0.001)	Tumor PD-L1 membranous expression (Dako 28–8) was neither prognostic nor correlated with response; PD-L1+ prevalence: 52–54, 36, and 31% using cutoffs of ≥1, ≥5, and ≥10%, respectively
Nivolumab; Borghaei et al. (196)	Phase 3 CheckMate 057 trial in 582 patients with advanced, refractory, or relapsed non-squamous NSCLC	For nivolumab vs. docetaxel, ORR: 19 vs. 12% (<i>p</i> = 0.02); median OS: 12.2 vs. 9.4 months (<i>p</i> = 0.002); 1-year OS: 51 vs. 39%; 1-year PFS: 19 vs. 8%	Tumor PD-L1 membrane expression (Dako 28–8) correlated with greater efficacy; only in PD-L1+ patients, nivolumab was superior; PD-L1+ prevalence: 53–55, 38–41, and 35–37% using cutoffs of ≥1%, ≥5%, and ≥10%, respectively

(Continued)

TABLE 1 | Continued

Antibody; reference	Clinical trial	Efficacy	PD-L1 biomarker
Pembrolizumab; Garon et al. (197)	Phase 1 KEYNOTE-001 trial in 495 patients with advanced NSCLC	ORR: 19.4%; median DOR: 12.5 months; median PFS: 3.7 months; median OS: 12.0 months	In PD-L1 ^{hi} ($\geq 50\%$ tumor cells with membranous expression; anti-PD-L1 clone 22C3, Merck) patients (prevalence: 23.2%), ORR: 45.2%; median PFS: 6.3 months; median OS: not reached
Pembrolizumab; Herbst et al. (198)	Phase 2/3 KEYNOTE-010 trial in 1,034 patients with previously treated PD-L1 ⁺ ($\geq 1\%$ tumor) advanced NSCLC	For 2 or 10 mg/kg pembrolizumab vs. docetaxel, median OS: 10.4 ($p = 0.0008$) or 12.7 ($p < 0.0001$) vs. 8.5 months; no difference in PFS	In PD-L1 ^{hi} ($\geq 50\%$, Dako 22C3) patients (prevalence: 40–44%), median OS: 14.9 months ($p = 0.0002$) or 17.3 ($p < 0.0001$) vs. 8.2 months; median PFS: 5.0 ($p = 0.0001$) or 5.2 ($p < 0.0001$) vs. 4.1 months
Pembrolizumab, first-line alone; Reck et al. (199)	Phase 3 KEYNOTE-024 in 305 patients with PD-L1 ^{hi} ($\geq 50\%$) advanced NSCLC	For pembrolizumab vs. chemotherapy, ORR: 44.8 vs. 27.8%; median PFS: 10.3 vs. 6.0 months ($p < 0.001$); 6-month OS: 80.2 vs. 72.4% ($p = 0.005$)	PD-L1 ^{hi} ($\geq 50\%$; Dako PD-L1 IHC 22C3 pharmDx assay) prevalence: 30.2%
Pembrolizumab, first-line combination; Langer et al. (200)	Phase 2 KEYNOTE-021 trial in 123 patients with previously untreated advanced, non-squamous NSCLC	For pembrolizumab plus chemo vs. chemotherapy alone, ORR: 55 vs. 29% ($p = 0.0032$); improved PFS (HR: 0.53, $p = 0.01$) no OS improvement; median DOR: 8 vs. 4.9 months	Combination benefit was shown in PD-L1 ^{hi} ($\geq 50\%$; prevalence: 27–33%) and PD-L1 ⁻ ($< 1\%$; prevalence: 35–37%) groups but not in the PD-L1 ^{inter} (1–49%; prevalence: 32–37%) group. ORR: 80, 57, and 26%, respectively; membranous PD-L1 expression, Dako IHC 22C3 pharmDx assay
Renal cell carcinoma			
Nivolumab; Motzer et al. (201)	Phase 3 CheckMate 025 trial in 821 patients with advanced clear cell renal cell carcinoma	For nivolumab vs. everolimus, ORR: 25 vs. 5% ($p < 0.001$); median OS: 25.0 vs. 19.6 months ($p = 0.002$); no PFS improvement	Median OS with nivolumab vs. with everolimus: in PD-L1 ⁺ patients, 21.8 vs. 18.8 months; in PD-L1 ⁻ patients, 27.4 vs. 21.2 months; PD-L1 ⁺ cutoff: $\geq 1\%$ tumor cells, membranous expression, Dako assay; prevalence: 24%
Classical Hodgkin lymphoma			
Nivolumab; Younes et al. (202)	Phase 2 CheckMate 205 trial in 80 patients with classical Hodgkin lymphoma that failed to respond to autologous hematopoietic stem cell transplantation and brentuximab vedotin	ORR: 66.3%; 6-month PFS: 76.9%; 6-month OS: 98.7%	High and low tumor PD-L1 H score (prevalence: both 26%) showed correlation with complete response and progression, respectively; H score was calculated by multiplying the% of PD-L1 ⁺ malignant cells [by double staining with anti-PD-L1 (405.9A1) and anti-PAX5 mAbs] by the average intensity of positive staining (1, 2, or 3+)
Pembrolizumab; Chen et al. (203)	Phase 2 KEYNOTE-087 trial in 210 patients with classical Hodgkin lymphoma that progressed after autologous hematopoietic stem cell transplantation and/or brentuximab vedotin	ORR: 69%; 6-month PFS: 72.4%; 6-month OS: 99.5%; 75.6% of patients had a response for ≥ 6 months	Clinical activity was seen across all PD-L1 groups defined by PD-L1 intensity score, tumor-membrane staining score, and histiocyte score (QualTek IHC assay); 90.4% of patients had an intensity score of 3; 88.1% had 100% PD-L1 ⁺ membrane staining; 71.8% had a histiocyte score of 3
HNSCC			
Pembrolizumab; Larkins et al. (204)	Phase 1b KEYNOTE-012 trial in 174 patients with recurrent or metastatic HNSCC	ORR: 16%; DOR: 2.4+ to 27.7+ months; 82% had response durations of ≥ 6 months	PD-L1 ⁺ (cutoff: $\geq 1\%$ tumor cells, membranous expression) prevalence: 65%
Nivolumab; Ferris et al. (205)	Phase 3 CheckMate 141 in 361 patients with recurrent HNSCC	For nivolumab vs. standard therapy, ORR: 13.3 vs. 5.8%; median OS: 7.5 vs. 5.1 months (HR: 0.70, $p = 0.01$); 1-year OS: 36.0 vs. 16.6%; no PFS improvement	Nivolumab vs. standard therapy: in PD-L1 ⁺ patients, median OS: 8.7 vs. 4.6 months, HR: 0.55; in PD-L1 ⁻ patients, median OS: 5.7 vs. 5.8 months, HR: 0.89; PD-L1 ⁺ (cutoff: $\geq 1\%$ tumor cells, membranous expression, Dako 28-8) prevalence: 57.3%

(Continued)

TABLE 1 | Continued

Antibody; reference	Clinical trial	Efficacy	PD-L1 biomarker
Urothelial carcinoma			
Nivolumab; Sharma et al. (206)	Phase 2 CheckMate 275 trial in 270 patients with metastatic urothelial carcinoma	ORR: 19.6%; median OS: 11.30 months for PD-L1 ⁺ patients, 5.95 months for PD-L1 ⁻ (<1%) patients	ORR: 28.4 or 23.8% in PD-L1 ⁺ patients using ≥5% or ≥1% PD-L1 ⁺ cutoff (prevalence: 31 and 46%, respectively); 16.1% in PD-L1 ⁻ patients; tumor-membrane PD-L1 expression was evaluated by the Dako PD-L1 IHC 28-8 pharmDx kit
Pembrolizumab; Bellmunt et al. (207)	Phase 3 KEYNOTE-045 trial in 542 patients with advanced urothelial cancer	For pembrolizumab vs. chemotherapy, ORR: 21.1 vs. 11.4% (HR: 0.73, $p = 0.001$); median OS: 10.3 vs. 7.4 months ($p = 0.002$); no PFS improvement	Pembrolizumab was more superior to chemotherapy in patients with ≥10% PD-L1 combined positive score (prevalence: 30.3%): median OS, 8.6 vs. 4.2 months (HR: 0.57, $p = 0.005$); PD-L1 combined positive score was the % of PD-L1 ⁺ tumor and immune cells relative to tumor cells, Dako PD-L1 IHC 22C3 pharmDx assay
MSI-H/dMMR solid tumors			
Pembrolizumab; Le et al. (208)	Phase 2 NCT01876511 trial in 41 patients with progressive metastatic carcinoma	For dMMR vs. mismatch-repair-proficient colorectal cancer, ORR: 40 vs. 0%; immune-related PFS: 78 vs. 11%	
Pembrolizumab; Le et al. (209)	Phase 2 NCT01876511 trial in 86 patients with advanced dMMR cancers (12 types)	ORR: 53%; median PFS/OS: not reached	
Nivolumab; Overman et al. (210)	Phase 2 CheckMate 142 trial in 74 patients with recurrent or metastatic dMMR/MSI-H colorectal cancer	ORR: 31.1%; median DOR: not reached; estimated 1-year OS: 86%	
Hepatocellular carcinoma			
Nivolumab; El-Khoueiry et al. (211)	Phase 1/2 CheckMate 040 trial in 154 patients with advanced hepatocellular carcinoma	ORR: 14.3%; DOR: 3.2 to 38.2+ months; 91% of responses lasted 6+ months; 55% of responses lasted 12+ months	Responses were observed regardless of PD-L1 levels (tumor-membrane expression, Dako PD-L1 IHC 28-8 pharmDx assay)
Gastric cancer			
Pembrolizumab; Ref ^a below	Phase 2 KEYNOTE-059 trial in 259 patients with recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma	In 7 MSI-H patients (prevalence: 3%): ORR: 57%; DOR: 5.3+ to 14.1+ months	In 143 PD-L1 ⁺ (≥ 1% PD-L1 combined positive score) patients: ORR: 13.3%; DOR: 2.8 to 19.4+ months; 58% of responses lasted 6+ months; 26% of responses lasted 12+ months; PD-L1 combined positive score was the % of PD-L1 ⁺ tumor and immune cells relative to tumor cells, Dako PD-L1 IHC 22C3 pharmDx kit

ORR, objective response rate; PFS, progression-free survival (rate); OS, overall survival (rate); DOR, duration of response; HR, hazard ratio; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; MSI-H, microsatellite instability-high; dMMR, mismatch-repair deficient.

^a<https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm577093.htm>.

values of the percentage and cellular levels of PD-1 expression in correlation with PD-L1 expression were unclear in these clinical studies.

Unlike tumor PD-L1 expression, which has shown predictive value for the efficacy of anti-PD-1 therapy in most studies (Table 1), PD-L1 expression on immune cells in the tumor microenvironment was more correlated with treatment response to anti-PD-L1 therapy (212, 236) (Table 2). However, the correlation of PD-L1 expression with response was absent in other studies, with similar ORRs or improved survival rates occurring across all PD-L1 subgroups (213, 216). Even in studies showing correlations, some patients who lacked both tumor and immune cell expression of PD-L1 still responded to anti-PD-L1 therapy (212, 218). The mechanisms of response to anti-PD-1/L1 therapy

in these PD-L1⁻ patients are unknown, posing intriguing questions. One plausible explanation would be failure to detect PD-L1 expression owing to technical reasons or temporal and spatial expression (for example, clustered PD-L1 expression in the early time course of T cell activation and the dynamic PD-L1 expression on circulating T cells).

Efforts have been made to improve the prediction accuracy, by standardizing the detection antibodies and immunohistochemistry assays (Tables 1 and 2), separately assessing PD-L1 expression on tumor cells and PD-L1 expression on non-tumor cells, and optimizing PD-L1 cutoffs. Because most studies have used low PD-L1 cutoffs (≥1% or ≥5%), and PD-L1 function is limited to local inhibition, acting as the “molecular shield” of PD-L1⁺ cells, we would postulate that the association of PD-L1 expression

TABLE 2 | Brief summary of the results of anti-PD-L1 therapy clinical trials leading to US food and drug administration approval.

Antibody	Clinical trial	Efficacy	PD-L1 biomarker	Reference
Urothelial carcinoma (bladder cancer)				
Atezolizumab	Phase 2 IMvigor210 trial in 310 patients with previously treated inoperable locally advanced or metastatic urothelial carcinoma	ORR: 15%; 84% of responses were ongoing; ORR in patients with $\geq 5\%$ PD-L1 immune cells (IC) score vs. in patients with $<1\%$ IC score: 27 vs. 8% or 26 vs. 13% ($p < 0.0001$)	Percentage of PD-L1+ immune cells in the tumor microenvironment correlated with response; prevalence of $\geq 5\%$ PD-L1 IC score: 32%; prevalence for $<1\%$ IC score: 33%; Ventana SP142 PD-L1 assay	Rosenberg et al. (212)
Atezolizumab, first-line alone	Phase 2 IMvigor210 trial in 119 patients with cisplatin-ineligible locally advanced or metastatic urothelial cancer	ORR: 23%; 70% of responses were ongoing; median PFS: 2.7 months; median OS: 15.9 months	Responses occurred across all PD-L1 subgroups according to the % of PD-L1+ immune cells in the tumor microenvironment; prevalence for $\geq 5\%$ PD-L1 IC score: 27%; Ventana SP142 PD-L1 assay	Balar et al. (213)
Durvalumab	Phase 1/2 trial (NCT01693562) in 191 patients with locally advanced or metastatic urothelial carcinoma	ORR: 17.8%; median PFS: 1.5 months; median OS: 18.2 months; 1-year OS rate: 55%	ORR in patients with high PD-L1 scores ($\geq 25\%$ tumor cells, Ventana SP263 PD-L1 Assay) vs. in patients with low/0 PD-L1 scores: 26.3 vs. 4.1%	Powles et al. (214)
Avelumab	Phase 1b JAVELIN Solid Tumor trial in 242 patients with refractory metastatic urothelial carcinoma	ORR: 13.3–16.1%; median response duration had not been reached		(215)
NSCLC (lung cancer)				
Atezolizumab	Phase 3 OAK trial in 850 patients with previously treated NSCLC	For atezolizumab vs. docetaxel, median OS: 13.8 vs. 9.6 months ($p = 0.0003$); ORR: 14 vs. 13%; DOR: 16.3 vs. 6.2 months	In PD-L1+ patients (prevalence: 54%), median OS: 15.7 months with atezolizumab vs. 10.3 months with docetaxel ($p = 0.0102$); in PD-L1- patients, median OS: 12.6 vs. 8.9 months; PD-L1+ cutoff: $\geq 1\%$ tumor or immune cells; Ventana SP142 PD-L1 assay	Rittmeyer et al. (216)
Atezolizumab	Phase 2 POLAR trial in 277 patients with previously treated advanced or metastatic NSCLC	For atezolizumab vs. docetaxel, median OS: 12.6 vs. 9.7 months ($p = 0.04$); ORR: 14.6 vs. 14.7%	PD-L1 on both tumor and immune cells were evaluated, Ventana SP142 PD-L1 assay; compared with docetaxel, OS with atezolizumab was improved in patients with $\geq 1\%$ score (prevalence: 68%) but not in patients with $<1\%$ score (HR 0.59 and 1.04; $p = 0.005$ and 0.87, respectively); ORR with atezolizumab was improved in patients with $\geq 50\%$ scores (prevalence: 16%), 37.5 vs. 13.0%, but decreased in patients with 5–49% scores (prevalence: 37%), 7.7 vs. 15.6%	Fehrenbacher et al. (217)
Merkel cell carcinoma (skin cancer)				
Avelumab	Phase 2 JAVELIN Merkel 200 trial in 88 patients with refractory metastatic Merkel cell carcinoma	ORR 31.8%; 82% of responses were ongoing	ORR: 34.5% in PD-L1+ patients (prevalence: ~78%); 18.8% in PD-L1- patients; PD-L1+ cutoff: $\geq 1\%$ tumor cells, detected by Merck anti-PD-L1 clone 78-10	Kaufman et al. (218)

ORR, objective response rate; PFS, progression-free survival; OS, overall survival; DOR, duration of response; HR, hazard ratio; NSCLC, non-small cell lung cancer.

with immune activation status, rather than the correlation with immune suppression strength, may underlie the predictive value of PD-L1 expression for anti-PD-L1 therapy. Interestingly, in NSCLC, anti-PD-1 therapy (pembrolizumab) demonstrated superiority over chemotherapy in patients with $\geq 50\%$ or $<1\%$ tumor PD-L1 scores, but this benefit was absent in patients with 1–49% tumor PD-L1 scores (200). This non-linear correlation reappeared in an anti-PD-L1 study in NSCLC, in which atezolizumab compared with docetaxel was associated with improved ORR in the $\geq 50\%$ PD-L1+ group but decreased ORR in the 1–49% group (217). The predictive 50% cutoff of PD-L1 expression has been included in the FDA indication for pembrolizumab in metastatic NSCLC tumors as frontline therapy (199).

A recent biomarker study using longitudinal tumor samples from patients with metastatic melanoma showed that expression of PD-1, LAG-3, and PD-L1 in early on-treatment (median: 1.4 months after initiation of treatment), but not in pre-treatment (median: 3 months prior to treatment), biopsies was highly predictive for response to PD-1 blockade, suggesting the inability to accurately predict the clinical response before anti-PD-1 therapy (237). In this study, some responders had no PD-1/PD-L1 expression in pre-treatment samples but had high immune marker expression in on-treatment samples; conversely, many non-responders had high PD-L1 expression in pre-treatment samples but had low PD-L1 expression in on-treatment samples. The observation that PD-L1- patients turned into PD-L1+ patients

TABLE 3 | Examples of anti-PD-1/L1 clinical trials that missed the endpoint or were discontinued owing to increased risk of death.

Regimen	Clinical trial	Efficacy	Toxicities	Reference
OPDIVO				
Nivolumab as first-line monotherapy compared with chemotherapy	Phase 3 CheckMate 026 trial in 423 patients with previously untreated stage IV or recurrent NSCLC with PD-L1 scores $\geq 5\%$	For nivolumab vs. chemotherapy, median PFS: 4.2 vs. 5.9 months (HR: 1.15; $p = 0.25$; missed the endpoint); median OS: 14.4 vs. 13.2 months (HR: 1.02)		Carbone et al. (226)
Nivolumab compared with investigator's choice chemotherapy	Phase 3 CheckMate 037 trial in 405 patients with ipilimumab-refractory advanced melanoma	For nivolumab vs. chemotherapy, higher and more durable responses but no survival improvement: median OS: 16 vs. 14 months; median PFS: 3.1 vs. 3.7 months		Larkin et al. (227)
KEYTRUDA				
Pomalidomide and low-dose dexamethasone with or without pembrolizumab	Phase 3 KEYNOTE-183 trial in 249 patients with refractory or relapsed multiple myeloma	ORR: 34% in the pembrolizumab arm vs. 40% in the control arm; time-to-progression: 8.1 vs. 8.7 months (HR: 1.14)	At median follow-up of 8.1 months, 29 deaths in the pembrolizumab arm vs. 21 deaths in the control arm (HR: 1.61)	http://www.onclive.com/web-exclusives/fda-discloses-data-on-halted-pembrolizumab-myeloma-trials
Lenalidomide and low-dose dexamethasone with or without pembrolizumab	Phase 3 KEYNOTE-185 trial in 301 patients with newly diagnosed and treatment-naïve multiple myeloma	ORR: 64% in the pembrolizumab arm vs. 62% in the control arm; HR for time-to-progression: 0.55	At a median follow-up of 6.6 months, 19 deaths in the pembrolizumab arm compared to 9 deaths in the control arm (HR: 2.06)	
Pembrolizumab compared with standard treatment	Phase 3 KEYNOTE-040 trial in 495 patients with previously treated recurrent or metastatic HNSCC	Missed the primary endpoint of OS [HR: 0.82 (95% CI: 0.67–1.01); $p = 0.03$ (one-sided)]		Larkins et al. (204) and Ref ^a below
TECENTRIQ				
Atezolizumab compared with chemotherapy	Phase 3 IMvigor211 trial in 931 patients with previously treated locally advanced or metastatic urothelial cancer	Failed to meet the primary endpoint of improving OS		http://www.roche.com/media/store/releases/med-cor-2017-05-10.htm
IMFINZI				
First-line durvalumab alone or combined with tremelimumab compared with chemotherapy	Phase 3 MYSTIC trial in previously untreated metastatic NSCLC	Did not improve PFS of patients with PD-L1 scores $\geq 25\%$ compared with chemotherapy		Peters et al. (231) and Ref ^b below

NSCLC, non-small cell lung cancer; PFS, progression-free survival; HR, hazard ratio; OS, overall survival; ORR, objective response rate; HNSCC, head and neck squamous cell carcinoma; CI, confidence interval.

^a<http://www.onclive.com/web-exclusives/pembrolizumab-falls-short-in-phase-iii-head-and-neck-cancer-trial>.

^b<https://www.astazeneca.com/media-centre/press-releases/2017/astazeneca-reports-initial-results-from-the-ongoing-mystic-trial-in-stage-iv-lung-cancer-27072017.html>.

appeared to suggest that immunogenic PD-L1 expression was induced after anti-PD-1 therapy. However, results from *in vitro* experiments (20, 21, 101) suggest that in PD-L1⁻ patients, binding of anti-PD-1 mAbs to PD-1 will inhibit IFN- γ production, and therefore, the baseline PD-L1⁻ status should not be changed after anti-PD-1 therapy. In contrast to these discrepancies, hyperprogression after anti-PD-1/L1 therapy tended to be associated with PD-L1 negativity (232, 233).

Because PD-L1 expression in on-treatment tumors predicted response to anti-PD-1 treatment (237), one would postulate that inducibility of PD-L1 expression can predict effectiveness of PD-1 blockade. Consistently, JAK2/STAT1 signaling is increased in classical Hodgkin lymphoma (238) which showed high ORRs [(220) and **Table 1**] to PD-1 blockade. Conversely, JAK1/2 and APLNR loss-of-function mutations, which result in non-inducibility of tumor PD-L1 expression by IFN- γ , have been associated with

primary or acquired resistance to PD-1 blockade in solid tumors; PD-1 blockade was ineffective for these patients even if their mutational load was high (239–241). However, PD-L1 should still be inducible on nonmalignant immune cells, which did not harbor JAK1/2 and APLNR mutations as tumors did, suggesting that other immune escape/suppressive mechanisms may also contribute to the treatment resistance in these patients. Indeed, JAK1/2 or IFN- γ pathway gene mutations were not always found to be associated with clinical response (242, 243).

Microsatellite instability arising from mismatch-repair deficiency is the second predictive biomarker (208, 244) approved by FDA (245). MSI-H tumors have high levels of neoantigens associated with a strong local and systemic immune response (246). In addition, MSI-H tumors were shown to display upregulation of multiple immune checkpoints, including PD-1, which may limit the vigorous immune microenvironment (247), making PD-1

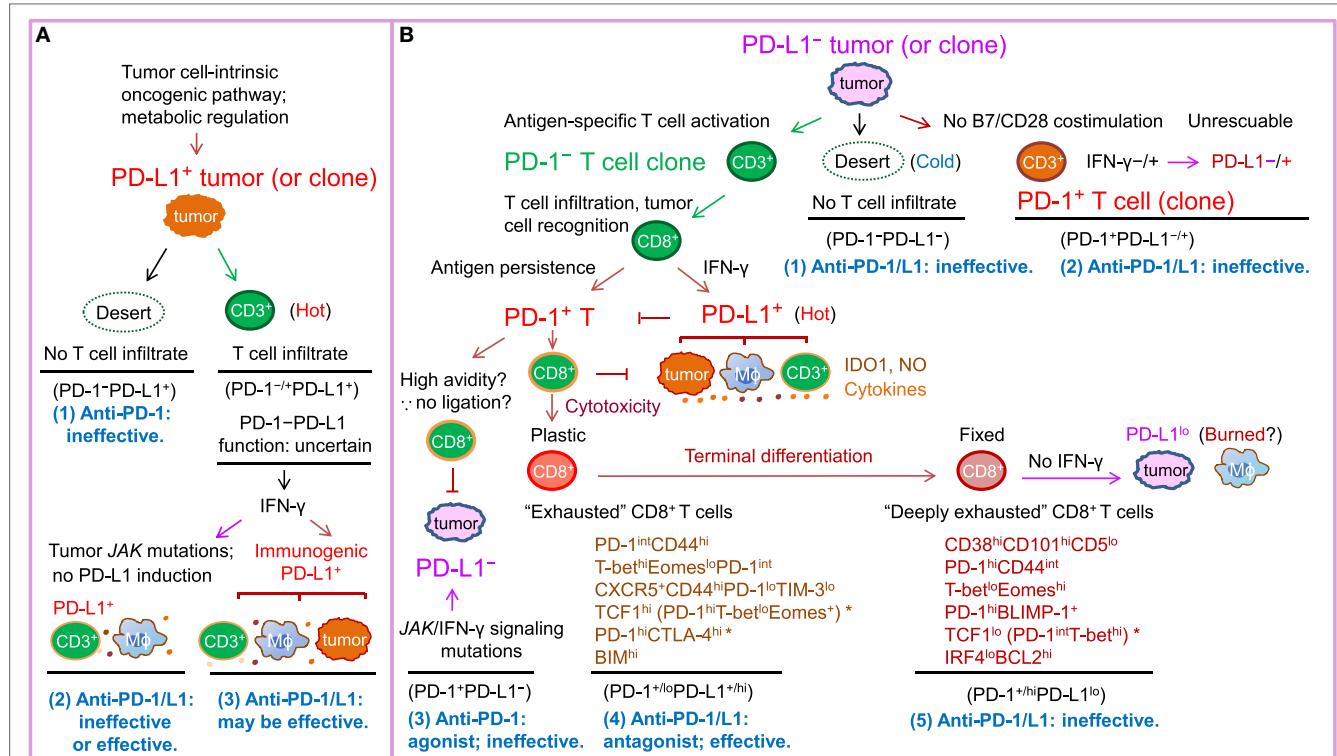


FIGURE 1 | Schematic illustration of PD-1/PD-L1 expression in the tumor setting as a marker of T cell activation and driver of T cell dysfunction, as well as a predictive biomarker for response to PD-1/PD-L1 blockade in PD-L1⁻ and PD-L1⁺ tumors according to the prevailing notion. PD-L2, which is infrequently expressed and potentially has PD-1-independent positive function, is not depicted in the figure for clarity. The PD-L1-CD80 axis is also not illustrated because its role and significance in the cancer setting is unclear. **(A)** In tumors (or tumor clones) with cell-intrinsic PD-L1 expression driven by the oncogenic pathways, whether anti-PD-1/PD-L1 is effective may depend on the activity of the PD-1-PD-L1 axis. If T-cell infiltration is lacking (a “desert”-like immune landscape, or “cold” tumors), or PD-1 is not expressed on T cells, anti-PD-1 therapy will not elicit a *de novo* T cell response. If the tumor is infiltrated with immune cells (“hot” tumor) and the oncogenic or immunogenic PD-L1 expression suppresses T cell activation by binding to PD-1 within the T-cell receptor microclusters, anti-PD-1/PD-L1 therapy can be effective. IDO1, NO (nitric oxide), and suppressive cytokines in the tumor microenvironment may contribute to resistance to PD-1/PD-L1 blockade therapy. **(B)** In tumors without cell-intrinsic PD-L1 expression, tumors (or tumor clones) with low immunogenicity (“cold” tumors) or costimulation may not respond to anti-PD-1/PD-L1 therapy, whereas tumors (or tumor clones) with a high neoantigen load elicit antitumor T cell responses (“hot”) but their response to anti-PD-1/PD-L1 therapy varies. Antigen-specific CD8⁺ T cells secrete IFN-γ, which may turn PD-L1⁻ tumors into PD-L1⁺ tumors infiltrated with PD-L1⁺ macrophages, dendritic cells, and T cells. However, if tumors do not have IFN-γ receptors or have JAK2 mutations, tumors may remain PD-L1⁻ and not respond to anti-PD-1/PD-L1 treatment or respond if PD-L1 is induced on non-tumor immune cells. In PD-L1⁺ tumors, prolonged antigen stimulation gradually induces PD-1 expression on antigen-specific T cells. PD-1 ligation with PD-L1 induced on tumors, antigen-presenting cells, and T cells in hot tumors in turn suppresses antitumor function of effector T cells, leading to T cell “exhaustion” (a term initially used for T cell dysfunction during chronic viral infection). Early-phase T cell “exhaustion” is plastic and can be reversed by PD-1/PD-L1 blockade; in contrast, if T cell dysfunction is fixed after terminal differentiation, “deeply exhausted” T cells cannot be rescued by PD-1/L1 blockade. Inflexibility in transcriptional and epigenetic programs may contribute to the therapeutic irreversibility of deeply exhausted T cells. Potential markers suggested by studies in tumor models, viral infection models, and cancer patients are summarized below the labels for these two different dysfunctional stages of PD-1⁺CD8⁺ T cells. * indicates disparities in PD-1 levels in the literature (please refer to the text for details).

blockade a rational treatment approach. In metastatic colorectal cancer, the ORR with pembrolizumab was 40% in MSI-H patients compared with 0% in mismatch-repair-proficient patients (208). In an expanded study of advanced mismatch-repair-deficient cancers across 12 different tumor types, the objective radiographic response rate was 53% and the complete response rate was 21% (209).

High tumor mutational burden and neoantigen load, which are fairly common across cancer types compared with the uncommon MSI-H (248), have also been correlated with sensitivity to PD-1 blockade (higher ORR and/or prolonged survival) in melanoma, NSCLC, glioma (243, 249–252), and likely across types of solid cancers (252). In addition, high numbers of indel

mutations were found in renal cell carcinomas, and frameshift indel count was associated with response to PD-1 blockade in melanoma patients (253). Conversely, high copy number loss burden was associated with resistance to checkpoint blockade (242). However, classical Hodgkin lymphoma has a high ORR but not a high mutational burden. Some gene mutations may correlate with treatment resistance (such as *JAK2* and *B2M*). Although a study showed that neoantigen load correlated with T-cell infiltration in colorectal cancers (254), another study showed that the density of immunogenic antigens did not correlate with T-cell infiltration and local immunity in melanoma (255). To reduce whole-exome sequencing and enhance the clinical applicability of tumor mutational burden, targeted comprehensive genomic

profiling (248) and small next generation sequencing panels (256) have been developed. Progress has been made in understanding the association of response with particular gene (such as DNA repair genes *BRCA2* and *POLE*; potentially also *PMS2*, *MSH2/6*, and *MLH1*) mutations and clonal neoantigens, as well as T cell clones responding to PD-1/L1 blockade (243, 248, 250, 256–259). *POLE* mutations have been shown to be associated with not only higher mutational burden (248) but also immune signatures and lymphocytic infiltration independent of MSI-H status in endometrial cancer (260). However, particular gene mutations and alterations (such as loss of *PTEN* and *CDKN2A*) and mutational burden showed inconsistent significance in studies (60, 242, 243, 258). Tumor mutation load and clonal mutation load (less heterogeneity) were associated with overall survival and response to nivolumab in ipilimumab-naïve patients but not in patients who had previously progressed on ipilimumab (243). In the latter group of patients, response to PD-1 blockade was inconsistently associated with T cell clonality (242, 243).

Some T cell-derived biomarkers have also been found to be predictive of response to PD-1 blockade in patients with advanced melanoma; these biomarkers include high baseline CD8⁺ and PD-1⁺ density at the invasive tumor margin and inside the tumor, proximity between PD-1⁺ and PD-L1⁺ cells, clonal TCR repertoire (43, 242), BIM expression in tumor-reactive PD-1⁺CD8⁺ T cells (261, 262), and higher proportion of PD-1^{hi}CTLA-4^{hi} cells with a partially exhausted T cell phenotype (capable of producing IFN-γ but lost the ability to produce TNF-α and IL-2) within CD8⁺ TILs (263). Baseline *PDCD1* mRNA expression was also associated with progression-free survival after anti-PD-1 therapy in a pooled cohort of cancer patients (264). However, the findings that PD-1^{hi}CTLA-4^{hi} TILs that were preferably expanded after anti-PD-1 therapy in melanoma patients (263) counters the findings in preclinical models [PD-1^{hi} T cells were irreversible (178) and anti-PD-1 therapy was effective only in tumors with low frequencies of PD-1⁺ T cells (24)].

In addition, in preclinical models, low levels of CD38, CD101, and CD30L whereas high levels of CD5 surface expression (178), low to intermediate levels of PD-1 expression on CD8⁺ T cells (24), as well as high TCF1 (177) and IRF4 nuclear expression were associated with T cell plastic dysfunctional state whereas high BCL2 expression in CD8⁺ T cells was associated with fixed dysfunctional state (178). The potential of these biomarkers may be clarified in future anti-PD-1/L1 clinical trials.

Moreover, several non-T host factors, including absolute lymphocyte count, relative eosinophil count, ≤ 2.5 -fold elevation of serum lactate dehydrogenase, and the absence of metastasis other than soft-tissue/lung metastasis, have also been associated with improved overall survival in melanoma patients treated with pembrolizumab (265). However, efficacy comparison with controlled arms (anti-PD-1 therapy compared with traditional therapy) will be more informative (266). Also notably, a retrospective analysis found a five-factor {serum lactate dehydrogenase elevation, age < 65 years, female sex, previous ipilimumab treatment [however, this factor was non-significant in the earliest pembrolizumab trial (184)], and liver metastasis} prediction scale was associated with lower ORRs to anti-PD-1 therapy (267). Although studies have shown that response to anti-PD-L1 therapy was associated with a

Th1 gene signature in on-treatment samples (236), a recent study found that early decrease of IL-8 (a Th1-associated cytokine) levels in the serum 2–3 weeks after anti-PD-1 therapy was predictive of response in melanoma and NSCLC patients, including rare cases [0.6–4% (268, 269)] with pseudoprogression (270). A prospective trial in melanoma patients found that response to anti-PD-1 therapy induced genomic contraction, which was associated with pronounced pre-existing immune signatures in pre-treatment samples, including TCR/PD-1/IFN-γ/IL-2/PI3K signaling signatures as well as MHC class II and other genes resembling a macrophage signature (243).

The gut microbiome in cancer patients has been shown to influence PD-1 blockade efficacy. Clinical responses to anti-PD-1 immunotherapy were associated with high diversity and relative abundance of Ruminococcaceae bacteria in prospectively collected microbiome samples from patients with metastatic melanoma (271) and relative abundance of *A. muciniphila* in patients with NSCLC, renal cell carcinoma, or urothelial carcinoma (272). In addition, commensal *Bifidobacterium* was shown to confer improved anti-PD-L1 efficacy *in vivo* (273). Mechanisms accounting for the favorable prognosis may include increased tumor infiltration of CD8⁺ T cells, more effector T cells than Tregs in systemic circulation, dendritic cell function, IL-12 secretion, anabolic metabolism, and systemic inflammation (271–273), but the mechanistic links for these immunomodulatory effects remain unknown. PD-1 also regulates the gut microbiota and the function and survival of IgA-producing plasma B cells, but this effect can be abrogated by PD-1 blockade, as was shown *in vivo* (274).

OVERCOMING RESISTANCE TO PD-1/PD-L1 BLOCKADE: VARIOUS COMBINATION STRATEGIES

Like a tug-of-war, the actions of immune response and tumor development resist each other. PD-1 blockade may have anti-tumor effects in cancer patients (275) but this is not always sufficient for a clinical response. Resistance mechanisms may come from either the immune system or the tumor. The ratio of immunologic reinvigoration to tumor burden, but not the magnitude of reinvigoration alone, was found to be predictive of response to pembrolizumab and overall survival in patients with advanced melanoma (276). Maximized innate and adaptive responses, achieved through combination therapies, were capable to eliminate large, advanced, poorly immunogenic tumors in mice (277).

Multiple tumor- or immune-driven resistance mechanisms have been identified and targeted in combination with PD-1 blockade. First, absence of “signal 1” and T cell activation leads to ineffectiveness of anti-PD-1/L1 monotherapy (278). Studies have shown that *B2M* mutations, deletions, or loss of heterozygosity, which leads to loss of MHC class I expression and failure of antigen recognition, is a potential mechanism for immune escape and resistance to PD-1 blockade in patients with melanoma (239, 279). Clinical outcome of anti-PD-1/PD-L1 therapy was shown to correlate with MHC class II positivity in a unique subset of

melanoma cells (typically MHC class II is expressed only on immune cells in solid tumors), as well as increased CD4⁺ and CD8⁺ TILs in melanoma patients (280).

However, a surprisingly high frequencies of decreased or absent expression of β 2M/MHC class I (79% overall; 92% in *PD-L1/L2* amplified cases) and MHC class II (67%) were found in 108 patients newly diagnosed with classical Hodgkin lymphoma (88% of patients had nodular sclerosis Hodgkin lymphoma; 82.5% were negative for Epstein-Barr virus) (281). High frequencies of abnormal MHC expression were also observed in another 233 patients with Epstein-Barr virus-negative classical Hodgkin lymphoma (83.2% for MHC class I and 46.8% for MHC class II) (282). Because classical Hodgkin lymphoma has a high ORR to PD-1 blockade, these data may suggest that non-T responses also play important roles in the effect of PD-1 blockade, which is supported by a study showing that after PD-1 blockade, genes implicated in cytotoxicity and natural killer cell function were upregulated in patients (283). In addition to natural killer cells whose antitumor function is MHC-independent, invariant natural killer T cells can be activated by signals from a lipid-CD1d complex (284), and alloreactive CD8 T cells demonstrated cytotoxicity effector function against MHC class I-deficient allogeneic cells in a TCR-independent manner (285). To enhance antigen recognition and T cell response, chimeric antigen receptor T cell therapies, bispecific T-cell engagers, oncolytic viruses, vaccination, and intratumoral IL-12 plasmid electroporation have been combined with PD-1/PD-L1 blockade (86, 286–290) but the clinical results are currently unavailable.

Second, because the absence of costimulation (“signal 2”) can result in T cell anergy (278), impaired costimulation could lead to ineffectiveness of PD-1/PD-L1 blockade. This is supported by recent studies showing that rescue of exhausted CD8⁺ T cells with PD-1 blockade requires CD28/B7 costimulation in a mouse model with chronic viral infection (291) and that response to PD-1 blockade requires the presence of both CD4⁺ and CD8⁺ T cells as well as CD28 and CD80/CD86 costimulation in a murine melanoma tumor model with low mutational load (165). However, an earlier study showed that PD-1 blockade *in vivo* leads to accelerated rejection of heart allografts only in the absence of CD28 costimulation, accompanied by expansion of alloreactive T cells and enhanced generation of effector T cells (292).

Although PD-1 is expressed only after T cell activation, which requires costimulation (9), it has been shown that PD-1 can be induced without CD28 costimulation (11); in fact, lack of costimulation leads to upregulation of PD-1 (16). In one study of patients with early-stage lung cancer, 10–80% of tumor-infiltrating CD8⁺ T cells were CD28⁻ (291). CD28 could be lost during aging, with repeated antigenic stimulation, and after exposure to some cytokines (293). Therefore, insufficient CD28 costimulation could be an important resistance mechanism for PD-1 blockade. Consistent with the high efficacy of PD-1 blockade in Hodgkin lymphoma, CD28 is strongly or moderately expressed on T cells surrounding CD80/CD86hi-expressing Reed-Sternberg cells (294–296). In contrast, chronic lymphocytic leukemia has no or low levels of CD80/CD86 expression on leukemia cells (297–299) with immunologic synapse formation defects (300) and is resistant to pembrolizumab in a clinical trial (224).

In addition to the CD28 pathway, the CD40–CD40L costimulatory pathway has been shown to be required for the ameliorative effects of anti-PD-L1 therapy and plays a critical role in rescue of exhausted CD8 T cells (301). Anti-CD40 agonists, which alone could effectively reverse cytotoxic T cell exhaustion by activating the mTORC1 pathway *in vivo*, significantly enhanced action of PD-1 antagonists in chronic infection *in vivo* (302). In addition, combining PD-1/PD-L1 blockade with costimulatory agonist antibodies to CD27 (164), CD137 (4-1BB) (303, 304), TLR3/7/9 (305–307) [TLR3 is also a safe vaccine adjuvant (308)], GITR (309), STING (310), or OX40 [the synergy to restore function of exhausted CD8⁺ T cells was only observed under helpless (no CD4⁺ T cell) condition (311)] have demonstrated enhanced antitumor effects in preclinical models. However, sequential (delayed anti-PD-1) but not concurrent anti-OX40 and anti-PD-1 treatment (combination) *in vivo* resulted in increased efficacy which required both CD4⁺ and CD8⁺ T cells (312).

Third, although anti-PD-1/PD-L1 antibodies block PD-1–PD-L1 interaction, they do not affect PD-1/L1 expression. Studies have demonstrated that expanded exhausted CD8⁺ T cells reactive to anti-PD-1/PD-L1 therapy *in vivo* retain high PD-1 expression (25); PD-1/PD-L1 blockade was shown to enhance IFN- γ and PD-L1 expression (42, 72) and increase tumor-infiltrating PD-1⁺ T cell frequencies (14). One preclinical study showed that the antitumor effect of anti-PD-1 therapy required the presence of PD-1^{lo}CD8⁺ T cells before treatment and decreased frequencies of tumor-infiltrating PD-1^{hi}CD8⁺ T cells below a threshold after the anti-PD-1 therapy (24). However, clinical studies showed that PD-1^{hi} expression before treatment (263) or on treatment correlated with response to PD-1 blockade in melanoma patients (237).

High PD-1 expression as resistance mechanism is probably more relevant for anti-PD-L1 therapy, which only blocks PD-1–PD-L1 interaction by modulating cytosolic signaling pathways and does not reduce PD-1 expression. In a chronic LCMV infection model and a melanoma tumor model, anti-PD-L1 therapy did neither downregulate the *PDCD1* gene in treated T cells nor did reprogram the epigenetic landscape, including chromatin accessibility to Nr4a and NFAT transcription factors (14, 90).

Strategies to modulate the transcriptional (including epigenetic) and posttranscriptional regulation of PD-1/PD-L1 expression may lead to a more durable response in patients. The transcription factors and pathways positively regulating PD-1 expression include BLIMP-1 (although conflicting results were also reported) (313, 314), IFN- α -IRF9 (315), TGF β -SMAD3 (316), NFATc1 (317), STAT3/4/NFATc1/CTCF (318), the Notch signaling pathway (319), FOXP1 (320), c-FOS (321), STAT1/2 (322), and NF- κ B (323). In contrast, T-bet (324), trimethylation (37, 325, 326), and a chromatin organizer SATB1 (327) negatively regulate *PDCD1* expression. Chromatin accessibility to *PDCD1* enhancers (including the –23.8 kb enhancer) is important for PD-1 expression in exhausted T cells (328).

Fourth, insufficient antitumor activity may result from multiple T cell subtypes and subclones (including those with “fixed” T cell dysfunction) that are not responsive to PD-1/L1 blockade. Dysfunction of these T cell subclones may lead to tumor evolution of subclonal neoantigens, which were associated with primary and acquired resistance to checkpoint blockade in

patients (250, 258). In a cancer model, “fixed” dysfunction of driver-antigen-specific T cells was associated with PD-1, TIM-3, LAG-3, and 2B4 expression (17). Although PD-1 has a uniquely critical role in immune suppression, co-expression of multiple immune checkpoint receptors on T cells resulted in greater T cell exhaustion (329).

Multiple blockade combinations have shown synergistic effects in releasing adaptive immune resistance in preclinical models (330), as well as combination strategies targeting the transcriptional program (17). Histone deacetylase inhibitors have been shown to increase expression of multiple T cell chemokine (paradoxically also PD-L1 expression) and enhance the response to PD-1 blockade *in vivo* (57, 331). EZH2 and DNMT1 inhibitors increased Th1-type chemokines and T-cell infiltration, and augmented the efficacy of PD-L1 blockade therapy *in vivo* (332). Simultaneous blockade of PD-1 and LAG-3 synergistically improved viral control and tumor eradication (329, 333, 334). Combined TIGIT and PD-1 blockade (335), or combined PD-1, TIM-3 (336), and BLTA blockade (337), increased the expansion and effector function of antigen-specific CD8⁺ T cells from melanoma patients *ex vivo*.

The combination of PD-1 blockade and CTLA-4 blockade, which has distinct immunologic effect and activates different T cell populations *in vivo* (283, 338), demonstrated greater antitumor effects than the use of either antibody alone (339, 340). Furthermore, clinical trials have demonstrated remarkable efficacy of combined nivolumab and ipilimumab therapy in melanoma (ORR: ~60%) (194, 341), although combined durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) in NSCLC was not successful in a recent phase 3 study (**Table 3**). Sequential use of nivolumab followed by ipilimumab or in reverse sequence did not reduce the toxicities resulting from concurrent (combination) therapy with nivolumab and ipilimumab, as found in a phase 2 study; nivolumab followed by ipilimumab showed higher response and survival rates but also higher toxicities compared with sequential use of ipilimumab followed by nivolumab, in which the synergistic effect was lost (342).

Fifth, the immunosuppressive tumor microenvironment may contribute to the ineffectiveness of anti-PD-1/L1 treatment. Tregs, MDSCs, M2 macrophages, and their associated cytokines, chemokines, and other soluble factors are well-recognized inhibitory mechanisms orchestrated to suppress antitumor immunity (72). Depletion of tumor-infiltrating Tregs was shown to synergize with PD-1 blockade to eradicate established tumors *in vivo* (343). However, the clinical significance of Tregs was inconsistent in different studies, likely due to the differential function of Treg subsets (344). Moreover, as shown *in vivo*, the suppressive function of NRP1^{+/+} Tregs could be lost and converted to antitumor immunity in the presence of IFN-γ produced by HIF-1α^{hi} NRP1^{-/-} Tregs. This functional fragility signaled through the IFN-γ receptor was required for the effectiveness of PD-1 blockade *in vivo* (345).

Increased MDSCs have been shown to be associated with poor prognosis (346), whereas decrease in macrophages after anti-PD-1 therapy was associated with clinical response in melanoma patients (243). Combination of PD-1/PD-L1 blockade with tumor vaccines only partially restored the effector function

of TILs stimulated by immunization and decreased Treg infiltration, but had little effect on the frequencies of MDSCs in the tumor lesions *in vivo* (19). Anti-PD-L1 blocking mAb augmented IFN-γ-mediated nitric oxide production by macrophages which inhibited CD4⁺ T cell proliferation; nitric oxide synthase inhibitor L-NMMA abrogated the inhibition and increased cytokine production (174). Indoleamine 2,3-dioxygenase (IDO) expression in tumor-associated macrophages and MDSCs induced by IFN-γ during CD8⁺ T cell response, can cause tryptophan deficiency and “metabolic checkpoint” in T cells (347, 348). Combining IDO inhibitors with anti-PD-1 therapy was shown to increase effector T-cell infiltration *in vivo* (349), and this combination has shown promising results in ongoing clinical trials (350). In addition, upregulation of IL10 and macrophage/monocyte chemotactic genes was associated with resistance to anti-PD-1 therapy (259). Combination of PD-1 blockade with IL-10 neutralization *in vivo* resulted in reduced tumor burden and improved murine survival, accompanied by augmented antitumor function of T cells and decreased infiltration of MDSCs (351). However, recent clinical trials demonstrated that pegylated recombinant IL-10 combined with PD-1 blockade therapy enhanced the antitumor effect (352).

Moreover, a study showed that *in vivo* PD-1⁻ tumor-associated macrophages removed anti-PD-1 mAbs from the surface of PD-1⁺CD8⁺ T cells, mediated by the interaction between FcγII/III receptors and the anti-PD-1 Fc domain glycan (353). Therapeutic inhibition of FcγR interaction enhanced anti-PD-1 efficacy *in vivo*. Also, nivolumab was transferred from human CD8⁺ T cells to macrophages in an *in vitro* coculture system (353), although the IgG4 constant region sequences of nivolumab are designed to contain an S228P mutation to prevent antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (4). It is unknown whether pembrolizumab, which binds to PD-1 at a completely different region than does nivolumab (354), can also be transferred by this FcγR-mediated mechanism. Unlike anti-PD-1 mAbs, selective depletion of Tregs, dependent on activating Fcγ receptors expressed by macrophages, is essential for the activity of anti-CTLA-4 therapy *in vivo* (355, 356).

Sixth, systemic immunity is critical for tumor eradication and protection against new tumors; in the immune network, dendritic cell function and T cell infiltration play an important role (357). Gut dysbiosis (loss of microbial diversity) and antibiotic treatment were associated with shorter progression-free and/or overall survival in cancer patients receiving anti-PD-1 immunotherapy (271, 272). Conversely, improving the gut microbiome may lower the cancer-immune set point and circumvent resistance to PD-1 blockade (272). Peritumoral injection of LCMV alone or combined with PD-1 blockade has also been shown to induce immune surveillance and tumor regression *in vivo* (358).

CONCLUDING REMARKS

Although the complexity of the PD-1/PD-L1 pathway has been revealed, our current understanding of the rejuvenation potential of T cells is only the tip of the iceberg. Accumulating evidence has demonstrated that PD-1 ligation suppresses the effector function of activated T cells; PD-L1 can directly cause tumor immune evasion; and anti-PD-1/PD-L1 mAbs that prevent PD-1-PD-L1

interaction can restore T-cell effector function. However, tumor PD-L1 expression through cell-intrinsic mechanisms may not have a significant role in driving immune suppression; PD-L1 and PD-L2 may also have costimulatory functions; and PD-1/PD-L1 blockade did not always elicit an effective antitumor response in preclinical studies. Moreover, although many anti-PD-1/PD-L1 clinical trials were remarkably successful which have revolutionized the treatment of cancer, some failed to reach the endpoint or resulted in an increased risk of death. In the setting of advanced cancers except Hodgkin lymphoma (likely also MSI-H tumors), only the minority of cancer patients had durable response to PD-1/PD-L1 blockade monotherapy, and some patients even had disease hyperprogression. Classical Hodgkin lymphoma, which does not have a high mutational burden or MHC class I expression, demonstrated a high response rate to PD-1 blockade therapy.

In addition to summarizing these paradoxical results in studies of PD-1/PD-L1 and PD-1/PD-L1 blockade, this review discussed a few open questions from mechanistic and clinical perspectives. As discussed, both PD-1 and PD-L1 are often (but not always) associated with T cell dysfunction; PD-1⁺ and PD-L1⁺ expression can also indicate T cell activation although PD-L1 and PD-1 may be expressed in different stages of immune response; markers to distinguish PD-1⁺ T cells with high functional avidity from exhausted PD-1⁺ T cells are unclear. Both PD-1 and PD-L1 can either independently or independently drive immune suppression. Whether tumor or host factors dictate immunity remains to be determined. Mechanisms that are not completely understood also include those governing PD-1 expression, molecular pathways underlying PD-1/PD-L1 blockade, the difference in PD-1 signaling upon PD-L1 binding and upon anti-PD-1 mAb binding, metabolic crosstalk between tumor cells and T cells, and functional relationship (causal, consequential, or independent) between PD-1/PD-L1 expression and cell metabolism. Molecular delineation and critical node identification may also help clarify the inconsistent preclinical results of blocking PD-1 compared with blocking PD-L1.

It is unclear whether PD-1 blockade has different action (antagonist or agonist) in PD-L1⁺ and PD-L1⁻ patients. Also uncertain is whether this and other differences between PD-L1 and PD-1 (for example, the association of PD-L1 expression with earlier stage of immune activation, the more dynamic PD-1 expression, or other factors which are critical for immune response but differentially associated with PD-1 and PD-L1

expression) underlie the better predictive value of PD-L1 over PD-1 expression as a biomarker for clinical response. Tumor mutational burden has also emerged as a promising biomarker; however, our understanding of clonal mutations, T cell clonality, and neoantigen-reactive TIL clones responsive to PD-1/PD-L1 blockade may be still in its infancy. In addition, infiltration of immune cells, tumor immunogenicity, strength of TCR signaling and costimulation/co-inhibition, T cell differentiation stage and chromatin flexibility, immune cells and soluble factors in the tumor microenvironment, pharmacologic kinetics of antibodies, and systemic immunity may all affect the efficacy of PD-1 blockade. Future studies in the fast advancing field of immunotherapy may shed light on these intriguing questions, develop algorithms to accurately predict the blockade efficacy, and pave the way for a new era of combination immunotherapy.

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ZX-M conceptualized and wrote the manuscript and created the figure. KY contributed to the conception and writing. MZ and JL revised the manuscript. All authors read and approved the final manuscript. The authors thank Erica A. Goodoff from the Department of Scientific Publications, MD Anderson Cancer Center, for her edition of the manuscript.

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Therapeutic Antibodies: What Have We Learnt from Targeting CD20 and Where Are We Going?

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Therapeutic monoclonal antibodies (mAbs) have become one of the fastest growing classes of drugs in recent years and are approved for the treatment of a wide range of indications, from cancer to autoimmune disease. Perhaps the best studied target is the pan B-cell marker CD20. Indeed, the first mAb to receive approval by the Food and Drug Administration for use in cancer treatment was the CD20-targeting mAb rituximab (Rituxan®). Since its approval for relapsed/refractory non-Hodgkin's lymphoma in 1997, rituximab has been licensed for use in the treatment of numerous other B-cell malignancies, as well as autoimmune conditions, including rheumatoid arthritis. Despite having a significant impact on the treatment of these patients, the exact mechanisms of action of rituximab remain incompletely understood. Nevertheless, numerous second- and third-generation anti-CD20 mAbs have since been developed using various strategies to enhance specific effector functions thought to be key for efficacy. A plethora of knowledge has been gained during the development and testing of these mAbs, and this knowledge can now be applied to the design of novel mAbs directed to targets beyond CD20. As we enter the "post-rituximab" era, this review will focus on the lessons learned thus far through investigation of anti-CD20 mAb. Also discussed are current and future developments relating to enhanced effector function, such as the ability to form multimers on the target cell surface. These strategies have potential applications not only in oncology but also in the improved treatment of autoimmune disorders and infectious diseases. Finally, potential approaches to overcoming mechanisms of resistance to anti-CD20 therapy are discussed, chiefly involving the combination of anti-CD20 mAbs with various other agents to resensitize patients to treatment.

Keywords: anti-CD20, monoclonal antibody, Fc gamma receptors, Fc engineering, isotype, resistance, combination therapies

INTRODUCTION

Over the last two decades, monoclonal antibodies (mAbs) have become a key part of treatment regimens for many diseases, including cancer. In 1997, rituximab became the first mAb to receive Food and Drug Administration (FDA) approval in oncology for relapsed/refractory non-Hodgkin's lymphoma (NHL), and has since significantly impacted on a vast number of patients with various B-cell malignancies and, more recently, autoimmune disorders (1, 2). For example, addition of rituximab to conventional [CHOP; cyclophosphamide, hydroxydaunorubicin, vincristine (Oncovin),

[prednisolone] chemotherapy in diffuse large B-cell lymphoma (DLBCL) has resulted in significantly increased progression-free and overall survival at 10-year follow-up (3, 4). By contrast, treatment success is more modest in conditions such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), where response rates are lower and many patients relapse and/or become refractory to treatment (5). Both the success and failure of rituximab has driven the development of further mAb reagents; leading to an increase in our knowledge of how mAb work and how resistance arises (Figure 1).

Interestingly, although much of the current focus in immunotherapy is on checkpoint blockers and other immunomodulatory mAb; in fact, the majority of mAbs approved for use in oncology are the so-called direct-targeting mAb, such as rituximab (6), which are designed to target tumor cells directly. Indeed, mAbs targeting CD20 represent over a quarter of such tumor-targeting mAbs with more in clinical development for conditions outside of cancer (Table 1). Moreover, as many immunomodulatory mAb such as anti-CTLA-4, GITR and OX40 may function as direct-targeting mAb, by deleting regulatory T cells (Tregs) (7–9), the lessons we have learnt from CD20 likely have further relevance in these settings.

In this article, we review developments arising from targeting CD20 and then discuss a range of approaches that are now being applied to improve efficacy, including new antibodies and combination strategies.

CD20 AS A MODEL TARGET

The pan B-cell marker CD20 remains one of the best studied antibody targets to date. Originally named B1, CD20 was discovered in 1980 as the first specific B-cell marker (47). It is a non-glycosylated tetraspanin of the membrane spanning 4-A family, with two extracellular loops (48–50) containing the epitopes for anti-CD20 antibodies (51).

Early studies showed that CD20 forms homotetramers in the cell membrane, suggesting that it may function as an ion channel, and that it disassociates from the B-cell receptor (BCR) upon mAb binding (52). CD20 is now thought to modulate calcium release arising from the BCR: CD20-deficient mouse cells exhibit decreased calcium signaling downstream of BCR engagement, and human B-cells (Ramos) are unable to initiate calcium signaling in the absence of the BCR despite CD20 crosslinking (53, 54). In mice and humans, loss of CD20 results in defects in the ability to generate antibody responses to certain antigens (55, 56).

Importantly, as well as being expressed on normal B-cells, CD20 was also found to be expressed on the surface of malignant B-cells (57). Furthermore, CD20 is expressed on pre-B-cells from an early stage in their development but is not present on the precursor hematopoietic stem cells from which they are derived, and expression is lost during differentiation into antibody secreting plasma cells (58–60). This expression

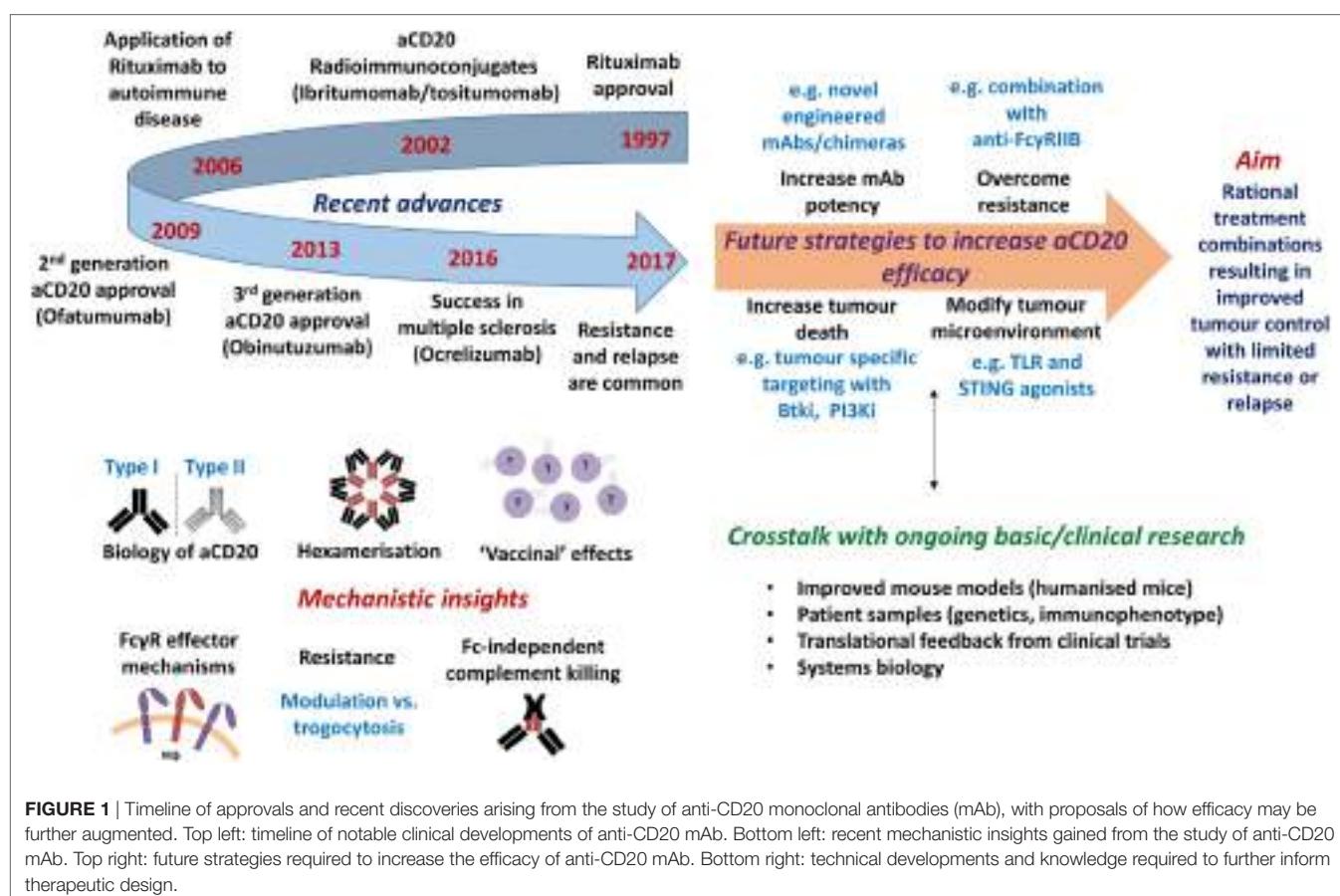


TABLE 1 | Direct-targeting monoclonal antibodies (mAbs) currently approved for use in oncology settings.

Generic name	Brand name	Target	Format	Comments (anti-CD20)	Indication	Food and Drug Administration (FDA) (EMA) approval date/ status	Reference
Rituximab	MabThera; Rituxan	CD20	Chimeric IgG1	Type I	NHL	1998 (1997)	(1–5)
Ibritumomab tiuxetan	Zevalin	CD20	Mouse IgG1	Type II, ⁹⁰ Y radiolabeled	NHL	2002 (2004)	(10, 11)
Ofatumumab	Arzerra	CD20	Human IgG1	Type I, binds small CD20 loop	CLL	2009 (2009)	(12, 13)
Obinutuzumab	Gazyva; Gazyvaro	CD20	Humanized IgG1	Type II, glycomodified	CLL	2013 (2014)	(14–17)
Ocrelizumab ^a	Ocrevus	CD20	Humanized IgG1	Type I	MS	2017 FDA (under review by EMA)	(18, 19)
Veltuzumab ^a	N/A	CD20	Humanized IgG1	Type I, rituximab backbone	Various (i.e., NHL; CLL; ITP)	Clinical trials and/or FDA orphan drug status	(20, 21)
Ocaratuzumab ^a	N/A	CD20	Humanized IgG1	Type I, Fc-modified	FL; CLL	As above	(22, 23)
Ublituximab ^a	N/A	CD20	Chimeric IgG1	Type I, glycoengineered	Various (i.e., CLL; MS; other)	As above	(24, 25)
Cetuximab	Erbitux	EGFR	Chimeric IgG1		Colorectal cancer	2004 (2004)	(26)
Panitumumab	Vectibix	EGFR	Human IgG2		Colorectal cancer	2006 (2007)	(27, 28)
Necitumumab	Portrazza	EGFR	Human IgG1		NSCLC	2015 (2015)	(29)
Trastuzumab	Herceptin	HER2	Humanized IgG1		Breast cancer	1998 (2000)	(30, 31)
Pertuzumab	Perjeta	HER2	Humanized IgG1		Breast cancer	2012 (2013)	(32, 33)
Ado-trastuzumab emtansine	Kadcyla	HER2	Humanized IgG1	Drug conjugate	Breast cancer	2013 (2013)	(34, 35)
Brentuximab vedotin	Adcetris	CD30	Chimeric IgG1	Drug conjugate	NHL; large cell lymphoma	2011 (2012)	(36, 37)
Daratumumab	Darzalex	CD38	Human IgG1		Multiple myeloma	2015 (2016)	(38, 39)
Dinutuximab	Unituxin	GD2	Chimeric IgG1		Neuroblastoma	2015 (2015)	(40, 41)
Alemtuzumab	Lemtrada, MabCampath	CD52	Humanized IgG1		CLL; MS	As Campath—2001 (2001) As Lemtrada—2014 (2013)	(42, 43)
Olaratumab	Lartruvo	PDGFR α	Human IgG1		Soft tissue sarcoma	2016 (2016)	(44, 45)

Table modified from (46).

^aAdditional anti-CD20 mAbs in clinical development and/or for clinical indications outside of cancer are also shown. Withdrawn mAbs are excluded.

NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; MS, multiple sclerosis; ITP, idiopathic thrombocytopenic purpura; FL, follicular lymphoma; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; GD2, disialoganglioside 2; PDGFR α , platelet-derived growth factor receptor alpha.

pattern is close to ideal for a target antigen: it minimizes the potential for off-target toxicity and retains humoral protection against previously encountered pathogens (61), while allowing for repopulation of the B-cell compartment after cessation of anti-CD20 treatment.

Another property that affords CD20 ideal target antigen status is its expression level: it is highly expressed, with approximately 100,000 CD20 molecules expressed on the surface of normal B-cells (with similarly high levels on most malignant cells) (62), which facilitates efficient target opsonization and deletion (63). Moreover, given the extracellular structure of the molecule, the available mAb binding epitopes are located close to the plasma membrane, a feature that has been reported to facilitate efficient binding and recruitment of effector mechanisms for deletion (64, 65). Perhaps less important but also worthy of consideration are that CD20 has no known ligand to interfere with mAb binding and does not exhibit extracellular post-translational modifications, reducing the variation in, and potential loss of, binding epitopes (49).

TYPE I AND TYPE II ANTI-CD20 ANTIBODIES

Anti-CD20 mAbs also have the capacity to redistribute CD20 within the plasma membrane into lipid rafts (66). Functionally, this redistribution may be important for the role of CD20 in BCR signaling (67). However, it also has significant implications for anti-CD20 antibodies themselves. The ability (or lack thereof) of mAbs to redistribute CD20 into lipid rafts has served as a useful classification system for anti-CD20 antibodies (68, 69). mAbs such as rituximab and ofatumumab that bind CD20 and cause compartmentalization into lipid rafts are classified as type I antibodies, whereas those that bind CD20 but cause no redistribution, such as obinutuzumab, are known as type II antibodies (51). In addition to a convenient basis for antibody nomenclature, the type I/II distinction describes key differences in antibody characteristics: first, opsonization of CD20 $^{+}$ target cells with type I mAb results in binding twice as many antibody molecules per cell as a type II antibody (63). This is thought to

be due to differences in the modes of binding between the two antibody types, as suggested by X-ray crystallography structures and tomography analysis of type I and II mAbs in complex with CD20 (70). Type I antibodies are proposed to bind CD20 tetramers in a manner that does not block binding of subsequent antibodies, whereas type II antibodies are thought to bind across the tetramer, blocking the binding of further mAbs (51).

The redistribution of CD20 and the associated mAb into lipid rafts is also functionally important with regard to the antibody effector functions induced. Due to the enhanced clustering of antibody Fc regions, type I antibodies are able to potently induce complement-dependent cytotoxicity (CDC), whereas type II antibodies do not induce CDC to a similar extent (51). However, type II antibodies have been reported to induce a greater degree of directly induced, non-apoptotic cell death upon binding to target cells (71). This mechanism has been shown in both B-cell lines as well as primary B-CLL cells (72). The enhanced clustering of type I antibodies renders them more susceptible to internalization, resulting in lysosomal degradation and a reduction in surface CD20 expression (73). Known as antigenic modulation, this is thought to be an important mechanism of resistance to type I anti-CD20 treatment.

Importantly, since the very first studies on CD20 mAb carried out with B1 and 1F5 (74), it has been clear that targeting the same surface marker with different mAb can have profound differences in response. Among many other lessons, this has been an important one that study of CD20 has revealed. In fact, subsequent work by Niederfellner et al. revealed that type I and II mAbs bind an extremely similar epitope on the same loop of CD20 and it is likely that only the orientation of binding differs between these mAbs but that this results in profound differences in activity (75).

MECHANISMS OF DIRECT-TARGETING MAB FUNCTION

As alluded to above, therapeutic mAbs are able to elicit multiple effector functions after binding to their target antigen. The study of anti-CD20 mAbs has contributed to the understanding of almost all of these, including signaling through the target molecule, triggering cell death, initiating the complement cascade, and engagement of Fc gamma receptors (FcγRs) triggering FcγR-dependent responses such as target cell lysis or engulfment (76).

Direct Binding Effects

Monoclonal antibody binding can have multiple direct effects on the target cell. For example, binding to a receptor can block binding of the relevant ligand, such as is the case with cetuximab binding the epidermal growth factor receptor (EGFR), inhibiting soluble EGF binding; thereby reducing proliferation and survival signaling to the tumor (26). With CD20, direct effects are again dependent on the mAb type; type I mAb triggering a limited degree of apoptosis, which is likely reflective of BCR signaling and type II mAb provoking a non-apoptotic lysosomal form of cell death (69). How this is triggered is still

the subject of much debate, but is likely related to reactive oxygen species production (77).

Complement-Dependent Cytotoxicity

All anti-CD20 mAb used in the clinic to date have been of the IgG1 subclass and so are able to activate the complement cascade once bound to target-expressing cells, triggering complement-dependent cytotoxicity (CDC). This process begins with the binding of C1q and follows the sequential activation of several proteases that cleave serum complement proteins in a specific order, generating enzymatic complexes that trigger further protein recruitment and processing (78). The end result of the cascade is threefold: the liberation of soluble molecules that act as anaphylatoxins to recruit immune effector cells; the deposition of cell bound cleavage fragments, largely C3b, acting as opsonins promoting target cell phagocytosis; and, finally, formation of a membrane attack complex (MAC) in the target cell membrane (79).

It has recently been shown how the proximity of binding to the membrane affects the effector functions engaged by an antibody, as had been previously suggested by the enhanced complement activating ability of ofatumumab (65, 80). Ofatumumab (2F2) is a type I anti-CD20 mAb (Table 1) that recognizes an epitope comprising both extracellular loops, binding closer to the cell membrane than rituximab (12). This membrane proximity is linked to the increased CDC seen with this antibody compared to rituximab (13). Ofatumumab has shown activity against rituximab-resistant CLL cells *in vitro*, despite their low CD20 expression, and has been approved for CLL treatment (13, 80).

Although CDC has been studied for many years, it was only recently revealed, using mAbs to CD20 and other targets, that IgG adopts a hexameric conformation in order to interact efficiently with the six head domains of C1q (81). The formation of hexamers on the target cell surface results from non-covalent interactions between adjacent Fc regions, increasing C1q binding avidity and subsequent CDC efficacy (81). This observation prompted a series of new developments in mAb engineering. Specific mutations capable of enhancing hexamerization of IgG and hence CDC were identified, namely E345R, E430G, and S440Y (81). Introducing the E345R mutation into anti-CD20 (IgG1-7D8) significantly increased Daudi cell lysis in comparison to wild-type IgG1 (81). In a further study, de Jong et al. showed the applicability of these findings to mAbs targeting different target antigens (i.e., CD52), target cell lines with differing levels of CD20 and complement regulatory proteins, and also confirmed improved efficacy in comparison to wild-type mAb in a tumor model (82).

Despite the obvious potential of such Fc region engineering for enhanced CDC, introducing multiple hexamer-enhancing mutations is likely to be detrimental, as double (E345R/E430G; RG) (82) and triple (E345R/E430G/S440Y; RGY) (81, 82) mutants formed hexamers in solution (RG—7.7%, RGY—73%) (82). RGY also activated complement in the absence of target cells, as measured by C4d generation (81). Although to a lesser degree than double and triple mutants, some single mutants also resulted in the formation of a small percentage of hexamers in solution (1.2% for

E345R), target-independent complement activation, and accelerated clearance of antibody from the circulation (82). However, an important finding was that amino acid substitutions at positions E345 and E430 (resulting in enhanced hexamer formation on the target cell) was not restricted to R and G, respectively. Moreover, when the preferred mutations (E435K or E430G) were introduced into the type I anti-CD20 mAbs 7D8 and rituximab, an increase in CDC in 5/6 CLL samples in comparison to wild-type mAbs was observed (with one of the CLL samples being refractory to CDC due to having a very low CD20 expression).

Intriguingly, it was also shown that the inefficient CDC induced by type II anti-CD20 mAbs (11B8) (82), or an anti-CD38 mAb containing IgG2 and IgG4 Fc regions (81) could be partially overcome by introduction of hexamer-enhancing mutations. Alternatively, the poor CDC mediated by anti-EGFR (2F8) was overcome by forcing monovalent binding of antibody to the target (81), indicating that the orientation of mAbs on the target cell is important for hexamer formation. However, CDC mediated by the type I anti-CD20 mAb 7D8 was not enhanced when only capable of monovalent binding (81). Although rituximab is able to adopt a monovalent binding to target antigens due to a relatively high off-rate (83), this explanation for enhanced CDC in the case of 7D8 is unlikely as 7D8 has a lower off-rate (83) and also induces more CDC in comparison to rituximab in the presence or absence of hexamer-enhancing mutations (82). Nevertheless, these results suggest that the CDC capability of a mAb may be increased by forcing hexamerization at the level of the target, and that a single hexamer-enhancing mutation is probably sufficient. However, what remains to be seen is whether these mutations also augment Fc γ R-mediated mechanisms and elicit greater efficacy *in vivo*.

Fc γ R-Mediated Mechanisms

Unique to IgG antibodies are the effects mediated through the Fc γ R family. These receptors are expressed on many different cell types and are essential for several IgG functions (84). Conventionally, Fc γ R-expressing effector cell functions have been ascribed to either natural killer (NK) cells or myeloid effectors (85). NK cells are able to mediate a direct lytic attack on opsonized target-expressing cells through Fc γ RIIIA [and, if present, Fc γ RIIC (86)] through a process termed antibody-dependent cell-mediated cytotoxicity (ADCC) (87).

Another Fc γ R-dependent mechanism is mediated by phagocytic cells, such as macrophages, monocytes, and neutrophils. Similar to ADCC, opsonized target cells trigger signaling through Fc γ Rs expressed on the phagocyte, resulting in actin rearrangement and extension of the phagocytic cell membrane (88). The membrane eventually engulfs the opsonized cell in a phagocytic vesicle, or phagosome, which then fuses with lysosomes within the phagocyte, resulting in degradation of the phagocytosed cell by lysosomal enzymes (85). This mechanism has been termed antibody-dependent cell-mediated phagocytosis (ADCP). In fact, myeloid cells can elicit both phagocytosis and killing of targets (89).

IN VIVO MECHANISMS OF ACTION

The above described effector functions of IgG can all be readily demonstrated through *in vitro* assays (14, 65). However,

knowledge of the relative importance of these effector functions to *in vivo* efficacy is essential to design optimal treatments.

One method applied to shed light on *in vivo* antibody function has been the retrospective analysis of the impact of Fc γ R polymorphisms in human clinical trials. In some trials, this analysis has revealed a significant correlation between the Fc γ RIIIA V158 polymorphism that encodes for higher affinity binding to IgG1 and clinical response (90, 91). This finding supported the paradigm that Fc γ R-mediated effector functions and particularly ADCC through NK cells, which predominantly express only Fc γ RIIIA, were the dominant effector mechanisms for anti-CD20 mAb. These findings also reinforced the bias that NK cells are the principle effectors for anti-CD20 mAb which derives from studies of human peripheral blood mononuclear cells (PBMCs) and blood (in which key effectors such as macrophages and/or neutrophils are lacking). However, it is important to note that several myeloid cells, including macrophages, also express Fc γ RIIIA and that, more recent, larger oncology trials have failed to show strong evidence for this receptor polymorphism as being central to antibody efficacy (92, 93).

With regard to other effector functions studied in humans, data from samples collected from patients treated with rituximab convincingly show that components of the complement system are depleted after mAb administration, and that supplementation of blood from these patients with additional complement components restores complement-mediated lysis *ex vivo* (94). Furthermore, early studies with rituximab suggested that the expression of complement defense molecules, including CD55 and CD59, on target cells was a predictor of poor response to anti-CD20 treatment (95). However, these studies have not been confirmed (96) and, moreover, several negative associations of complement engagement and mAb effector function have been provided (97, 98). Moreover, a polymorphism in the gene encoding C1qA (A276G), known to influence C1q levels, has been linked to responses to anti-CD20, with FL patients having an AG or AA genotype (lower C1q expression) experiencing a significantly longer time to progression following an initial response to rituximab (99), and patients with DLBCL harboring the AA genotype displaying significantly longer overall survival following R-CHOP (100). This seemingly suggests a detrimental role for complement.

Perhaps the best current models for elucidating *in vivo* effector function are mouse models, which facilitate the manipulation of various effector components to establish their relative contribution to antibody efficacy. Initial studies using mice that are defective in the Fc γ chain, and therefore do not express any activatory Fc γ R, showed no response to anti-CD20 therapy, indicating that activatory Fc γ Rs are absolutely required for anti-CD20 therapy (101, 102). Similar studies in mice lacking the key complement mediators C1 or C3 have argued against a major *in vivo* role for complement as an effector mechanism of anti-CD20 antibodies (73, 103, 104). Thus, it would appear that Fc γ R-dependent mechanisms predominate in mediating anti-CD20 therapy in mice.

Studies in mice trying to identify the key cell type(s) for mAb-mediated anti-CD20 depletion have indicated that NK cells are not essential for antibody therapy, as anti-CD20 therapy was

effective in mouse strains with defective NK cells or after NK cell depletion (103, 105). Intriguingly, in the study by Uchida et al., mice deficient in perforin, one of the main NK cell effector molecules, were still capable of depleting the majority of circulating/splenic B-cells (103) further supporting the absence of a role for NK cells and ADCC as an effector function in anti-CD20-mediated depletion. However, macrophage depletion using clodronate liposomes resulted in impaired deletion of normal and malignant B-cells during anti-CD20 therapy (73, 103, 104). This finding argues that myeloid cells, and particularly macrophages, are the most important cell type for anti-CD20 therapy, at least in mice. Other evidence for this comes from intravital imaging, in which macrophages within the liver (Kupffer cells) were imaged engulfing opsonized B-cells after anti-CD20 therapy (106). As above, clodronate liposomes completely abrogated anti-CD20 mediated B-cell depletion.

Finally, although the evidence for a role of Fc γ Rs and macrophages in the setting of anti-CD20 is unequivocal, a recent study by Lee et al. (107) indicates that next-generation mAb formats may be able to elicit alternative means of activity. Those authors used a library screening approach to select variants of rituximab with enhanced C1q binding but no Fc γ R binding, and provided evidence that these mAbs can elicit complement-dependent cellular cytotoxicity (CDCC) and complement-dependent cellular phagocytosis (CDCP) in the presence of serum. In comparison to wild-type rituximab, the aglycosylated variant (RA801) with two complement-enhancing mutations (K320E and Q386R) displayed some activity in Fc γ R-null mice (107) and is, therefore, worthy of consideration as a novel therapeutic; although it should be noted that the models chosen for study represent cell line tumors which may display little complement defense. As such, further experiments are required in fully syngeneic models targeting normal or malignant B-cells in a more physiological setting to confirm these findings, but nonetheless it represents an interesting approach in settings where Fc γ R-mediated effector functions may be limited.

NEUTROPHILS AS ALTERNATIVE EFFECTORS

As described above, macrophages are now widely recognized as key mediators of ADCC/ADCP of IgG-opsonized tumor cells *in vivo*, particularly with regard to anti-CD20 mAb. However, there have also been recent reports that neutrophils may also be involved or at least capable of effector activity with these reagents. Neutrophils are characterized by expression of the glycosylphosphatidyl inositol (GPI)-linked Fc γ R, Fc γ RIIB (CD16B), and to a lesser extent Fc γ RIIA (108) and, therefore, may be expected to be activated by IgG-opsonized tumor cells. Given their abundance in the circulation, it is reasonable to suggest that they can elicit robust effector function.

It has long been known that IgG mAbs are capable of inducing neutrophil-mediated cytotoxicity against B-cell targets. For example, although dependent on the target cell line, anti-human leukocyte antigen (HLA) class II IgG mAbs were shown to mediate ADCC by neutrophil effectors with a clear hierarchy

of isotype (IgG1 > 2 > 3 > 4) albeit less than IgA mAbs (109) (see below). Moreover, in the setting of anti-CD20 mAbs, Golay et al. more recently showed that anti-CD20 IgG mAbs are capable of activating neutrophils and inducing tumor cell phagocytosis, at least *in vitro* (15). Consistent with the neutrophil Fc γ R expression profile, phagocytosis mediated by a glycoengineered variant of rituximab was blocked with F(ab) fragments of either anti-Fc γ RIII or Fc γ RII, and to a greater extent with a combination of both. Intriguingly, as for Fc γ RIIA, the highly homologous Fc γ RIIB was shown to bind with a higher affinity to afucosylated mAbs in comparison to non-glycomodified mAbs (15). In line with this, neutrophil activation (CD11b upregulation, CD62L downregulation, and cytokine secretion) was greater with the glycoengineered (afucosylated) type II anti-CD20 obinutuzumab in comparison to wild-type rituximab. However, comparisons with a non-glycomodified obinutuzumab were not performed in this setting and so the enhanced activation could not be ascribed solely to tighter binding to Fc γ RIIB due to afucosylation. Neutrophils were also clearly capable of mediating cytotoxicity of rituximab-opsonized Raji and Ramos cells in a recent study, with an EC₅₀ only slightly higher than with PBMC effectors (107). This was shown to be Fc γ R dependent, as complement-enhanced, Fc-deficient variants of rituximab (RA801 and RA802) were inefficient in neutrophil-mediated lysis (107). However, these rituximab mutants had restored activity in the presence of neutrophils and serum lacking C9 (so as not to activate MAC formation and classical CDC), with lower EC₅₀'s in comparison to wild-type rituximab, which was blocked by mAbs to the complement receptors (CR) 3 and 4 (107). This shows that in addition to ADCC *via* Fc γ Rs, neutrophils can also participate in CDCC of anti-CD20-opsonized targets *via* CRs.

An alternative effector mechanism of neutrophils was recently proposed by Nakagawa et al., whereby target cell apoptosis is triggered through neutrophil-mediated crosslinking of surface bound rituximab (110). Blocking studies and use of afucosylated rituximab variants suggested that Fc γ RIIB was responsible for such crosslinking. Intriguingly, this phenomenon mirrors the Fc γ R-mediated crosslinking reported for pro-apoptotic anti-TNF-related apoptosis-inducing ligand (TRAIL) mAbs (111). Although neutrophil-mediated ADCC mediated by IgG mAbs, such as in the context of anti-EGFR IgG1 and IgG2 (112), anti-HLA class II (109), or indeed anti-CD20 (107) has been reported, neutrophil-mediated ADCC was not observed in this study (110). This possibly reflects a difference between methods of neutrophil isolation or target cells used. Similarly, no neutrophil activation was observed (as measured by upregulation of CD63 and Fc γ RI), which is possibly related to the fact that Fc γ RIIB is GPI-anchored (without an intrinsic cytoplasmic domain) and, thus, is not expected to signal when crosslinked alone (unless through the crosslinking of associated lipid raft-resident kinases). Nevertheless, this mirrors previous findings whereby the crosslinking of pro-apoptotic anti-Fas (113) or agonistic anti-CD40 mAbs (114, 115) did not require intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing signaling domains of Fc γ RIIB. Similarly, although effector functions such as ADCC are clearly dependent on the immunoreceptor tyrosine-based activation motif (ITAM) signaling domains of activatory Fc γ R (116), Fc γ R-mediated antigen internalization and presentation to

T cells is seemingly ITAM-independent (117). This indicates that both activatory and inhibitory Fc γ R may function independently of their respective ITAM or ITIM domains.

In addition to this *in vitro* work, *in vivo* evidence for a role of neutrophils in the killing of IgG mAb-opsonized tumor cells has also been provided. Although not in the setting of anti-CD20, neutrophils protected against tumor growth following IgG mAb therapy in subcutaneous solid tumor models (melanoma and breast cancer), in an Fc γ R-dependent fashion (118). However, the model used (solid tumor versus hematological) is important to consider, and utilizing the same conditional neutrophil-depletion strategy in B-cell models involving anti-CD20 treatment would be worthwhile. Indeed, in our own studies, depletion studies showed that anti-CD20 mAb-mediated B-cell depletion was independent of neutrophils (119).

Despite the above findings, neutrophil-mediated phagocytosis following mAb engagement is contentious, as a recent study indicated that neutrophils instead mediate the removal of mAb/CD20 complexes from the target cell, in the absence of phagocytosis or target cell death, in a mechanism known as trogocytosis (120). This activity would be expected to be of detriment to the success of mAb therapy. Surprisingly, this trogocytosis was greater for rituximab in comparison to obinutuzumab. In addition to our work on CD20 modulation (73, 121), this may provide a further/alternative explanation for the improved efficacy of obinutuzumab over rituximab observed in CLL patients (16). Similarly, neutrophils have abundant pro-tumor properties (122), suggesting that recruiting neutrophils by direct-targeting mAbs may be undesirable for clinical outcomes.

In summary, IgG mAbs are clearly capable of activating neutrophils. However, potential detrimental functions (i.e., trogocytosis; pro-tumoural functions) should be considered, and the precise role of neutrophils downstream of IgG mAb therapy requires clarification in further studies. Finally, as discussed below, IgG may not be the optimal isotype for recruitment of the favorable attributes of neutrophils, such as ADCC and cytokine/chemokine release (123).

VACCINAL RESPONSES TO MAB THERAPY

The principle success of anti-CD20 mAb has been the direct deletion of the target cells by the effector mechanisms detailed above. However, deletion of tumor cells and their engulfment by myeloid effectors raises the possibility of the induction of a T-cell-mediated immune response to the foreign (mutated) components of the tumor. Although this concept has existed for several years, strong evidence in humans has not been forthcoming with the possible exception of data showing the *ex vivo* re-stimulation of T cells from a small number of patients post-rituximab therapy (124). Regardless, ascribing this activity to mAb-mediated killing of the tumor following Fc γ R-mediated uptake has not been possible. For this reason, more mechanistic proof of concept has been attempted in mouse models.

Dendritic cells (DCs), *via* their surface Fc γ Rs, are adept at internalizing, processing, and presenting or cross-presenting

antigen (Ag) to CD4 $^{+}$ and CD8 $^{+}$ T cells *in vivo*, as highlighted in recent experiments whereby Ag was targeted to specific Fc γ Rs (117). In relation to tumors, however, early work showed that DCs, when loaded with immune complex (IC) and transferred into mice, are capable of presenting Ag to T cells and inducing immune responses that lead to tumor elimination in an antigen-specific manner (125). It was also indicated that Fc γ RIIB regulates DC maturation in response to IC and, therefore, the magnitude of anti-tumor T cell responses *in vivo* (126). This was expected based on previous studies showing that Fc γ RIIB regulates the activity of ICs in *in vivo* alveolitis models (127).

An advance came from studies indicating that such T cell responses will develop *in vivo* following anti-CD20 mAb therapy, rather than *via* artificially generated ICs. First, in a series of tumor challenge and rechallenge experiments, Abes et al. showed that when treated with an anti-CD20 mAb, mice were resistant to tumor growth on rechallenge, and this was dependent on the mAb Fc region (128). Recently, the Fc γ R and cellular requirements for such adaptive, vaccinal effects of mAb therapy using the same model were identified. Using a series of experiments involving conditional DC knockouts, Fc-modified mAbs, and humanized mice, DiLillo and Ravetch provided indirect evidence that macrophage ADCC (*via* Fc γ RIIA), DC uptake of ICs (*via* Fc γ RIIA), and Ag presentation were responsible for the induction of anti-tumor adaptive responses (129).

Intriguingly, both these studies indicated the generation of an adaptive response specific for the CD20 antigen itself, as evidenced by poor survival of mice rechallenged with tumors lacking CD20 (128, 129). Although there are various limitations with these models, such as the utilization of a xenoantigen (human CD20) in mouse (EL4) cell lines, a more recent study also showed that T cells were required for tumor regression of murine A20 tumors following anti-CD20 therapy, as no tumor regression was observed in nude (T cell deficient) mice (130). Notably, Ren et al. also showed a similar requirement for both macrophages [*via* production of type I interferon (IFN)] and DCs in the induction of anti-tumor T cell responses following anti-CD20 therapy, and that CTLA-4 $^{\text{hi}}$ Treg cells, within larger (more established) tumors, may be responsible for “adaptive resistance.” This lends support for an anti-CD20/anti-CTLA-4 combination regimen. However, the particular tumor model employed is likely important, as the anti-CD20/CTLA-4 combination is not effective in all models (unpublished data).

Despite being slightly different in their T cell subset requirement, with CD4 $^{+}$ (128) versus CD8 $^{+}$ T cells (130) being more important for primary tumor clearance following anti-CD20 mAb therapy, the mechanisms involved in the various models are not necessarily mutually exclusive. Specifically, IC formation following initial ADCC, which are then internalized/endocytosed and presented/cross-presented by DCs, likely remains the common link. Similarly, the indicated requirement for macrophage type I IFN may help to explain the efficacy of stimulator of interferon genes (STING) agonist/anti-CD20 combination in our own experiments (119). Furthermore, considering the regulatory role of Fc γ RIIB at the level of the DC, it can be hypothesized that anti-Fc γ RIIB mAbs in combination with anti-CD20 mAbs (131) (clinical trial NCT02933320, see below)

may favor enhanced activation of DCs by ICs following ADCC, migration to lymph nodes and stimulation of anti-tumor T cells.

Finally, this phenomenon is likely not limited to anti-CD20 mAbs, as similar observations were made using an anti-human EGFR2 (HER2) mouse model (132). In summary, in addition to the principle 4 mechanisms (direct effects, CDC, ADCC, and ADCP), the vaccinal effect of mAb therapy is emerging as an additional potential mechanism of action for direct-targeting mAbs. The above studies did not measure IC production *per se*. It is, therefore, of interest to determine how changes in the nature of ICs (size/valency) influence the vaccinal response (i.e., between different patients, cancer types and treatments, etc.). Recent studies have attempted to define the relationship between various IC parameters and Fc γ R binding and activation (133), and novel assays for the detection of ICs in serum may also assist this endeavor.

ENHANCING ANTI-CD20 MAB FUNCTION THROUGH Fc ENGINEERING

With the progress outlined above in identifying *in vivo* mechanisms of anti-CD20 antibody therapy and the importance of activatory Fc γ Rs, second- and third-generation anti-CD20 antibodies have been developed which utilize several strategies to try and achieve greater efficacy (**Figure 1; Table 1**).

Glycoengineering

Removal of the Fc glycans results in a dramatic decrease in binding to Fc γ Rs and complement activation without affecting antigen binding (134–136). This is thought to be due to changes in the constant heavy (CH) 2 domain structure, possibly through the two CH2 domains collapsing to block the Fc γ R/C1q binding site (137). However, the importance of Fc glycosylation extends beyond simply holding the Fc structure in place (138). Shields et al. found that removal of the core fucose residue, present on most recombinant and serum IgG molecules, resulted in increased Fc γ RIIIA binding up to 50 times, translating into increased NK-mediated ADCC (138). Shinkawa et al. confirmed this and reported increased ADCC using low fucose anti-CD20 mAb (139).

In 2006, the structural basis for this increased binding was reported, with Ferrara et al., showing *via* X-ray crystallography that the fucose residue was sterically blocking a stacking interaction between the Fc glycans and those present on the Asn162-linked glycan of Fc γ RIIIA (140). Absence of the fucose resulted in a closer interaction, explaining the increased affinity. As a result of these findings, several afucosylated antibodies have been developed which exhibit the expected increase in Fc γ RIIIA affinity and ADCC. Currently, afucosylated mAbs targeting CD20 (obinutuzumab) or CC chemokine receptor 4 (CCR4) (mogamulizumab) produced *via* cell line engineering have been brought to the clinic and more may follow (141). While other glycoforms have been linked to specific functions, none have been carried forward to the clinic.

Additional glycomodified anti-CD20 mAbs have been developed, further to obinutuzumab. EMAB-6, an afucosylated

anti-CD20 mAb was generated with a view that it may allow lower doses of chemotherapy used in the treatment of CLL (142). This mAb was able to both bind Fc γ RIIIA more tightly and mediate greater NK-mediated ADCC of CLL cells at lower mAb concentrations in comparison to rituximab (142). A later version of this mAb (LFB-R603, now known as ublituximab) was able to elicit maximal ADCC of target Raji cells at a concentration of 1 ng/ml, in comparison to 100 ng/ml for rituximab (143). Moreover, ublituximab recently showed promising efficacy when combined with the Bruton's tyrosine kinase (Btk) inhibitor ibrutinib in a phase II study of relapsed/refractory CLL patients, with ~90% of patients responding, and two complete responses (24). This combination is currently being assessed in a phase III trial of CLL patients (NCT02301156). Another phase III trial for this indication (NCT02612311) has been initiated involving a distinct combination regimen (see below) and ublituximab was placed on Reichart's "Antibodies to watch in 2017" list (18).

On a final note, although the enhancement of ADCC with afucosylated mAbs cannot be disputed, a recent study utilizing mAbs to Rhesus D antigen (RhD) on erythrocytes indicated that afucosylated mAbs do not elicit greater ADCP, in comparison to a clear enhancement in ADCC (144). This led authors to conclude that the benefit of fucose removal may be restricted to cases where NK cells are known to be involved. How this relates to anti-CD20 mAbs is, therefore, of key interest, especially considering the predominant role of macrophages in this setting (see above).

Fc Engineering

While glycosylation is a post-translational modification and, thus, difficult to precisely control, the IgG Fc backbone is readily amenable for mutation to create more efficacious molecules. Mutagenesis libraries have enabled the identification of IgG Fc variants that are aglycosylated but retain Fc γ R binding and effector functions similar to, or even exceeding that of, glycosylated IgG (145, 146). Extensive Fc backbone mutagenesis and an improved understanding of Fc–Fc γ R interactions has enabled the generation of mAbs with increased affinities for Fc γ Rs and effector function (147). Multiple IgG mutations that increase binding for specific Fc γ R, both activatory or inhibitory, have been reported (148). 200-fold increased binding to Fc γ RIIB (but not Fc γ RIIA) was achieved through a Pro:Asp conversion at position 238 and generated IgG with increased agonistic capacity when applied to anti-CD137 mAb (149). Increased binding to Fc γ RIIA alone, without impacting binding to Fc γ RI or the neonatal Fc receptor (FcRn) has also been reported using an anti-CD20 antibody (150). Increasing binding to activatory Fc γ Rs but not Fc γ RIIB serves to increase the activatory:inhibitory (A:I) ratio (151), enabling greater effector cell activation. A 100-fold increase in ADCC was achieved using Fc mutation to increase Fc γ RIIIA binding (both high- and low-affinity alleles) and applied to several antibodies including rituximab (152). Fc mutations that improve binding to Fc γ RIIA selectively over Fc γ RIIB have also been reported, such as the G236A mutant, which resulted in improved macrophage phagocytosis (153). Furthermore, combination of this mutation with others can result in additive increases in ADCC and ADCP over the wild-type antibody (153).

AME-133v (now known as ocaratuzumab) is an example of an Fc-modified anti-CD20 mAb that is in clinical development for the treatment of B-cell malignancies (**Table 1**). AME-133v contains two mutations in its Fc region and elicits more efficient ADCC than rituximab with PBMCs from both Fc γ RIIIA VV158 and VF/FF158 patients (22). Moreover, 5/23 previously treated FL patients responded in a phase I/II clinical trial (22), suggesting potential efficacy. In separate *in vitro* studies, it was also indicated that ocaratuzumab is capable of mediating ADCC of CLL target cells at a greater level than rituximab and ofatumumab, and at a similar level to obinutuzumab (23).

As discussed above, several mutations are also able to promote hexamerization of IgG and elicit potent C1q binding leading to powerful CDC. Although (to the best of the authors' knowledge) the effect of these mutations on Fc γ R binding has not been reported, there have been some reports that hexamer-enhanced mAb variants also have enhanced Fc γ R effector functions. To this end, de Jong et al. showed that variants (E345K and E430G) of the type II anti-CD20 mAb 11B8 mediated greater ADCC of Raji cells (82), and improvements in ADCC and ADCP were indicated in the setting of a modified immunomodulatory anti-OX40 mAb (154).

Notably, two situations whereby complement-optimized rather than Fc-optimized mAbs may be beneficial were highlighted in the aforementioned study by Lee et al. (107); reducing potential Fc γ R-mediated toxicity and Fc γ RIIB-mediated anti-CD20 mAb modulation, which has been suggested by us to be a rituximab resistance mechanism (73, 121). Finally, the authors speculated that complement-optimized mAb that work independently of Fc γ Rs may be beneficial in the setting of unfavorable Fc γ R polymorphisms (107).

In addition to optimizing affinity of IgG for C1q and Fc γ R interaction, mutation strategies optimizing FcRn binding to improve serum IgG half-life has also been attempted to augment efficacy and reduce dosing frequency. Due to the pH-dependent binding of IgG to FcRn, improving the serum half-life of an IgG requires increased binding to FcRn at pH6 (allowing for greater FcRn binding in acidic endosomes) but unaltered FcRn binding at pH7.4 (thereby allowing release at the cell surface) (155). Numerous mutations have been reported to alter FcRn binding at pH6 (156). As an example, the M428L N434S double mutant on the IgG1 background of bevacizumab and cetuximab yielded increased FcRn binding (~10× fold for bevacizumab) and increased half-life in both human FcRn transgenic mice and cynomolgus monkeys (157). As far as we are aware, this technology has not been tested on anti-CD20 mAb. Given the shorter half-life of rituximab due to internalization, such an approach may be beneficial (73). A mAb targeting respiratory syncytial virus carrying the YTE triple mutant (M252Y/S254T/T256E) to increase FcRn binding at pH6.0 has been tested in humans and been reported to increase mAb half-life up to 100 days (158). Further optimization of Fc structure for optimal IgG half-life could enable the tailoring of IgG molecules to suit specific functions, including both therapeutic and also short-term uses such as labeling for imaging (159). Interestingly, enhanced FcRn binding through various Fc mutations has been combined with glycoengineering to generate low fucose

anti-CD20 mAbs with increased serum half-life, Fc γ RIIIA binding, and ADCC (160).

ISOTYPE SELECTION AND ENGINEERING

All direct-targeting mAbs approved for use in oncology, including anti-CD20 mAbs, are of the IgG class (**Table 1**). However, it has been questioned whether IgG is the optimal therapeutic Ig class and whether efficacy could be improved by adopting other Ig classes. As expected, many of these proposals have used CD20 as their target of choice.

IgA As an Alternative Ig Class

IgA is important in mucosal immunity (123) and, in contrast to IgG, it has only two isotypes (IgA1 and IgA2) (161). Much of the recent interest in using IgA as a therapeutic isotype has been in its potential to recruit the anti-tumor properties of neutrophils, which express the predominant (although not the only) receptor for IgA (Fc α RI, CD89) (123). Crosslinking studies showed that CD89 signaling in neutrophils is efficient, and the use of bispecific mAb constructs (i.e., anti-CD20 × CD89) highlighted that stimulating the interaction between target antigen expressing tumor cells and CD89 on neutrophils efficiently induces cytotoxicity (162). A recent study also indicated that IgA mAbs targeting the melanoma antigen gp75, but not IgG1 or 3, mediated neutrophil ADCC *in vitro* (163). CD89 is also expressed by other myeloid cells including monocytes (and macrophages) (123). Therefore, considering the intricate involvement of macrophages in IgG mAb-mediated target cell depletion (see above), therapeutic IgA mAbs may be able to similarly engage and activate these cells when in sufficient number. However, when compared with IgG, IgA mAbs were limited in their ability to induce mononuclear cell ADCC, which is presumably due to the low percentage (10%) of monocyte effector cells within this cell population, and/or the presence of NK cells (20%) (109) that are not expected to engage IgA mAbs.

Anti-CD20 mAbs of the IgA class have been compared with IgG mAbs in various models. Surprisingly, anti-CD20 IgA2 was capable of mediating CD20 target cell depletion similar to IgG1 in an adoptive transfer model utilizing mice lacking CD89 (164). Pascal et al. also reported activity of IgA2 anti-CD20 in similar adoptive transfer models, although in this setting IgA2 was less effective than IgG1 anti-CD20 (165). Moreover, a different strategy was also employed, whereby DNA constructs encoding anti-CD20 IgG1 and IgA2 were vaccinated following tumor challenge to allow *in vivo* mAb synthesis and, thus, avoid difficulties in IgA purification (165). The survival of mice vaccinated with IgA2 and IgG1 constructs was similar, which is intriguing considering the absence of CD89 expression [as in Lohse et al. (164)]. However, a significantly increased activity of anti-CD20 IgA2 was reported in CD89 transgenic mice in comparison to wild-type mice (165), highlighting the potential for tumor cytotoxicity downstream of IgA interaction with cognate receptor-expressing effector cells *in vivo*.

In these anti-CD20 studies, it was shown that, as expected, IgA mAbs induced neutrophil-mediated cytotoxicity of both cell line and CLL targets to a greater extent than IgG, although

(as expected) the converse was true for mononuclear cells (164). The same trend was observed with anti-HLA class II mAbs (109). Notably, however, IgA was able to recruit more immune cells than IgG in an *in vitro* imaging assay, in a CD89-dependent manner (165). Interestingly, these studies also showed that hIgA anti-CD20 mAbs were capable of inducing CDC of varying CD20⁺ target cells *in vitro* (164, 165). Although of interest, the relevance of this finding *in vivo* is unclear due to retained activity of anti-CD20 hIgA in C1q and C3 knockout mice (164). Despite differences in the kinetics of CDC mediated by IgG1 and IgA2 anti-CD20 being identified, as well as sensitivity to factors such as mAb (165) or serum concentration (164), the unexpected ability of IgA mAbs to induce CDC is nevertheless intriguing from a biological perspective, as IgA antibodies are not expected to engage C1q. Pascal et al. proposed an indirect mechanism for C1q binding downstream of anti-CD20 IgA (165) and recent studies have provided further evidence for a mechanism, now referred to as “accessory CDC,” which occurs in an Fc-independent, BCR-dependent fashion (166). Strikingly, mAbs with no expected CDC functions, namely anti-CD20 F(ab')₂ fragments or IgG4 mAbs with a complement-silencing mutation (K322A), were capable of inducing CDC of BCR⁺ cell lines. The emerging mechanism of such Fc-independent CDC is, therefore, reliant on clustering of the BCR by anti-CD20 mAbs, which favors indirect binding of C1q to surface IgM and subsequent CDC (166). The phenomenon may be limited to anti-CD20 mAbs, as no CDC was observed with IgA1 or IgA2 anti-HLA class II mAbs (109).

IgGA Chimeras

Although IgA mAbs are clearly functional *in vivo*, it is not yet clear how IgA would replace IgG in clinical practice (164). Moreover, IgA molecules have disadvantageous attributes, such as a difficulty of purification and a shorter half-life in comparison to IgG (165). As described, IgA molecules are also not expected to stimulate NK cells, as evidenced by the absence of cytotoxicity observed with mononuclear cells in comparison to IgG (109, 164). For these reasons, there have been efforts to engineer novel mAbs containing the Fc regions of both IgG and IgA, with a view that the resulting molecule will harness the beneficial properties of both Ig classes. Kelton et al. grafted relevant regions of IgA into the Fc region of an anti-HER2 mAb to form a so-called “cross-isotype” IgGA mAb (167). The resulting IgGA mAbs were capable of binding to both Fc_αRI and Fc_γR, and induced neutrophil ADCC and macrophage ADCP of HER2⁺ targets similar to IgA molecules, and to a greater extent than parental IgG mAb. Next, as anti-HER2 mAbs did not elicit CDC, presumably due to the biology of the target, and similar to unmodified anti-EGFR (81, 82), anti-CD20 IgGA was generated. This was capable of inducing greater CDC of CD20⁺ targets in comparison to IgA, and greater CDC at lower concentrations than an IgG variant of the same mAb. However, anti-CD20 IgA did induce some CDC, although in contrast to Lohse et al. (164) this was to a lesser extent than anti-CD20 IgG. This is likely related to the “accessory CDC” mechanism (166) mentioned above.

Notably, the IgGA construct did not bind to Fc_γRIIIA or FcRn (167). As this would be predicted to negatively impact

ADCC/ADCP and IgG recycling, respectively, the functionality of IgGA molecules *in vivo* would be interesting to assess. To this end, a recent study assessed the efficacy of a similar anti-CD20 IgGA molecule which had equivalent pharmacokinetics to anti-CD20 IgG1 (168). Anti-CD20 IgGA treatment of tumor bearing mice (transgenic for CD89 on CD14⁺ myeloid cells) led to an improved regression of tumors in comparison to IgG or IgA, in a CD89-dependent manner. Similarly, a peritoneal model was used to show that the activity of IgA or IgGA *in vivo* requires interaction with CD89 on monocytes/macrophages. However, a limitation of this model is that CD89 was restricted to CD14⁺ cells, with no neutrophil CD89 expression. It is also unclear whether the expression level of the CD89 is comparable to that seen in humans.

Alternatively, in contrast to the grafting used to produce the “cross-isotype” IgGA, Borrok et al. fused the entire CH2/hinge of IgA2 onto the C terminus of an anti-HER2 IgG1 to form a tandem IgG/IgA molecule (169). Similar to the IgGA, this molecule mediated enhanced neutrophil ADCC in comparison to both IgG and IgA2. However, by contrast, it was also capable of inducing NK-mediated ADCC due to retained Fc_γRIIIA binding (169), albeit lower than compared to afucosylated IgG1. Also in contrast to IgGA, tandem IgG/IgA also bound FcRn with a similar affinity to hIgG1 and had a correspondingly similar half-life to IgG1 *in vivo*, therefore overcoming one of the main limitations of IgA. This can be expected as the CH2-CH3 interface contains the IgG binding site for FcRn (170), and is maintained in this molecule. Finally, considering that this study focused on HER2 as a target, comparing anti-CD20 mAbs with a tandem IgG/IgA backbone with cross-isotype IgGA *in vivo* would be worthwhile to identify the most effective molecule.

In summary, IgA mAbs clearly engage various effector mechanisms and can exploit additional killing pathways (i.e., via CD89) compared to IgG. Although IgA in itself may not be able to replace IgG due to reasons of half-life and manufacturability, various chimeric fusions or combination regimens have been designed or suggested that combine the beneficial aspects of both IgG and IgA. It would be interesting to assess how these novel agents influence resistance mechanisms following anti-CD20 mAb therapy. For example, is trogocytosis (120) still induced by chimeric IgG/A molecules and how does this compare to wild-type IgA and G? As highlighted previously (109), an advantage of utilizing IgA mAbs is that interaction with the inhibitory Fc_γRIIB, known to limit effector cell activity (102), would not be expected. Similarly, IgA mAbs would not be expected to interact with Fc_γRIIB on the surface of malignant B-cells, thus limiting Fc_γRIIB-mediated modulation and removal of CD20/antibody complexes from the cell surface (73, 121). It would be interesting to assess how modulation compares with IgG/A chimeras, and whether further modifying these chimeras can reduce Fc_γRIIB binding to improve efficacy/limit resistance mechanisms.

IgE As an Alternative Immunoglobulin Class for mAb Therapies

Further to IgA, the anti-tumor potential of IgE has recently been identified, leading to suggestions that IgE may be an alternative

Ig class for mAb therapeutics. Although IgE is widely recognized as an Ig class implicated in allergy and responses to parasites, Nigro et al. have recently shown that IgE has a role in immune surveillance following tumor challenge (171). Various models were utilized to show that control of tumor growth was mediated in an IgE- and Fc epsilon receptor (Fc ϵ RI)-dependent manner, with an additional role for CD8 $^{+}$ T cells. Further to showing that tumors induce effective IgE responses that can limit tumor growth in a tumor challenge setting, this highlights that the Fc ϵ RI–IgE axis is worth considering in the setting of mAb therapy.

In the setting of anti-CD20, Teo et al. showed that an IgE mAb was capable of activating and inducing cytotoxicity, in an antigen-specific manner, through cells typically involved in allergic responses, namely mast cells or eosinophils derived from cord blood (172). The authors also highlighted the limitation of studies involving PBMCs as effectors (173), where the poor responses observed with IgE mAb are not considered in the absence or paucity of IgE effector cells. Moreover, a crucial concern was highlighted, in that there is a risk of anaphylaxis in the setting of a large circulating tumor burden following anti-CD20 IgE therapy (172). This prevented *in vivo* assessment of IgE anti-CD20 in this setting. It, therefore, needs to be considered how anti-CD20 IgE mAb therapies can be optimized to limit toxicity in patients. Nevertheless, an anti-MUC-1 mAb in a solid tumor model (4T1) was assessed (172). Although the efficacy of the mAb alone was limited, when utilizing a slightly different strategy to aid IgE and chemoattractant synthesis at the tumor site, tumor regression was observed. This highlights the importance of effector cell chemotaxis to the tumor site in the efficacy of anti-IgE mAb therapy.

Alternative IgG Isotypes

In addition to belonging to the IgG class, all but two (Panitumumab, hIgG2 anti-EGFR; ibritumomab, mouse IgG1 anti-CD20) of the direct-targeting mAbs approved for cancer treatment also have a hIgG1 Fc region (Table 1). Therefore, further to altering the class of Ig, changing the isotype has been considered as an alternative to anti-CD20 hIgG1 therapy.

IgG3 As an Alternative Isotype for mAb Therapies

Similar to IgG1, IgG3 is capable of effective Fc-dependent effector functions, such as CDC and ADCC (173). Indeed, IgG3 binds favorably to C1q (173) and broadly binds to Fc γ Rs similar to IgG1 (174). There are numerous differences between IgG1 and 3, however. The latter bears an extremely long hinge region (IgG3—62 amino acids; IgG1—15) and is subject to extensive polymorphism (IgG3—13 allotypes; IgG1—4) (175). IgG3 also has a shorter half-life in comparison to other isotypes (176), an inability to bind protein A (173), and suffers from aggregation issues (177). In many ways, these mirror the disadvantages of IgA (see above). Despite this, some studies have suggested that IgG3 may be a more effective isotype for anti-CD20 mAbs, and have provided strategies to overcome the aforementioned limitations.

Rosner et al. showed that an IgG3 variant of rituximab (C2B8-IgG3) induces greater CDC than the corresponding IgG1 variant, with indications of superior sensitivity to low CD20 densities, such as in the case of CLL cells (177). However, ADCC and ADCP mediated by anti-CD20 IgG1 versus IgG3 were not compared in this study. This greater CDC capability of anti-CD20 IgG3 in comparison to IgG1 was also observed by Natsume et al., although they reported the converse for ADCC, with IgG1 being more effective (178). Similarly, although not in the context of anti-CD20, IgG1 was more capable of inducing ADCP of melanoma cells than IgG3 in a recent study (163) further suggesting that Fc γ R effector functions may not be improved in the setting of IgG3. A molecule comprising the advantageous regions of both IgG1 and IgG3 may, therefore, be beneficial. To this end, similar to the “cross-isotype” IgGA mAb described above, a domain switch variant of rituximab was generated by replacing the CH2/CH3 (Fc) of hIgG1 with same regions of IgG3. One particular mAb (1133) was identified that mediated superior CDC in comparison to hIgG1 and 3 and maintained a similar level of ADCC to hIgG1. Despite a potential benefit of the long hinge of IgG3 in introducing flexibility into the molecule (179), this finding suggests that the long hinge region of IgG3 is not responsible for the enhanced CDC (as 1133 contains the CH1 and hinge region of IgG1). Indeed, it has previously been suggested that a disulfide bond connecting the heavy chains, and not a hinge region *per se*, is required for CDC (179).

However, due to a loss in protein A binding, a known feature of IgG3 mAbs (173), and, therefore, concern about purification of the molecule on an industrial scale, the CH3 domain of mAb 1133 was further modified with increasing amounts of IgG1 sequence. This resulted in a molecule (113F) that was capable of binding to protein A and, importantly, maintained its superior CDC-inducing capabilities. Intriguingly, protein A and Fc γ n both bind to the CH2–CH3 interface of IgG (170), and the shorter half-life of IgG3 in comparison to hIgG1 has been shown to be caused by a single amino acid in this region (R435 in IgG3, H435 in other isotypes) that reduces the ability of IgG3 to compete with other isotypes of IgG for Fc γ n binding at pH 6 and, consequently, increases degradation (180). This is important to consider in the design of mAb therapeutics, but as 113F (in addition to binding to protein A) also contains the H435 site (178), poor pharmacokinetics should not be a limiting factor in this case. The polymorphic nature of IgG3 should nevertheless be considered if designing an IgG3 mAb therapy, as the IgG3 G3m(s,t) allotype contains H435 and has a correspondingly longer half-life (180).

Finally, it was shown that afucosylation improved the ADCC capacity of 113F but did not affect CDC, and that 113F resulted in more effective and prolonged B-cell depletion in a cynomolgus monkey model in comparison to IgG1 (178). This suggests that 113F may also be more effective than anti-CD20 hIgG1 in human patients.

In summary, studies with anti-CD20 mAbs have suggested that IgG3 mAbs may mediate more CDC in comparison to IgG1. However, this finding is inconsistent with distinct target antigens, indicating context-dependent rules. Fc γ R effector mechanisms of IgG3 may also be limited in comparison to

IgG1 *in vivo*, despite having a half-life enhancing mutation (see above), as highlighted in a recent study (163), although whether this translates to CD20 mAbs is unknown. Nevertheless, chimeric IgG1/3 molecules have been developed to combine the effector mechanisms of both IgG1 and 3.

OVERCOMING RESISTANCE AND THE IMMUNOSUPPRESSIVE MICROENVIRONMENT

The two decades of study of CD20 and its mAbs have provided us with a wealth of knowledge for how these reagents work and might be augmented. However, it has become increasingly clear that in addition to tumor intrinsic factors, such as expression level (181, 182), internalization (73), and trogocytosis (183), that tumor extrinsic factors associated with the tumor infiltrate are critical for determining mAb efficacy. A well-recognized hallmark of tumors is their ability to subvert and suppress the host immune system to facilitate their growth (184). Hematologic malignancies exhibit this trend and this may contribute to the tumor resistance often seen with anti-CD20 therapies. For example, CLL cells have been reported to produce the anti-inflammatory cytokine IL-10, which is able to reduce macrophage cytokine production (185), and also to impact upon the gene expression of both CD4⁺ and CD8⁺ T cells and viability of CD4⁺ T cells through surface expression of Fas ligand (186, 187). In addition, certain B-cell subsets have also been reported to produce IL-10, which may contribute to an anti-inflammatory environment within lymphoid organs (188). Tumor-associated macrophages frequently display a pro-tumor phenotype characterized by reduced phagocytosis and production of angiogenic factors (189).

Anti-CD20 therapy has been shown to be highly effective at rapidly depleting CD20 expressing cells from the circulation (190–192). However, circulating B-cells constitute only approximately 2% of the total B-cell population, and thus the penetration and efficacy of anti-CD20 mAbs into lymphoid tissues is crucial to their effectiveness (193). Mouse and primate studies have indicated that increasingly large doses are needed to deplete B-cells from bone marrow, spleen, and lymph nodes (191, 194, 195). As many malignant B-cells reside in lymphoid organs, if they are not eradicated by anti-CD20 therapy, they can act as disease reservoirs enabling re-emergence of the tumor leading to relapse and progression (196). Although next-generation mAb such as obinutuzumab that have followed rituximab have improved depletion efficacy, it is clear that further improvements in treatment regimens are still required (16).

OVERCOMING RESISTANCE TO ANTI-CD20 THERAPY THROUGH COMBINATION

As described above, an immunosuppressive microenvironment is one mechanism known to reduce the efficacy of mAb treatment. As such, attempts to alter the tumor microenvironment to a more favorable, inflammatory state have been made. Agonists

for toll-like receptors (TLRs), known to be important transducers of inflammatory signals in response to pathogen-associated molecular patterns such as LPS, are one group of molecules that have been tested. The synthetic oligodeoxynucleotide TLR agonist CpG, which activates TLR9, in combination with low dose radiotherapy has been reported to have a beneficial impact on B-cell lymphoma patients, inducing a T cell memory response in certain patients (197). Another TLR-9 agonist, 1018 ISS, has been combined with rituximab in follicular lymphoma and reported clinical response and tumor infiltration of CD8⁺ T cells and macrophages (198).

Another class of immunomodulatory molecules recently developed is STING agonists. These cyclic dinucleotides are sensed by cytosolic STING receptors (199). Normally involved in detection of DNA viruses, these agents can induce expression of IFN genes contributing to increased inflammation (199). *In vitro* and *in vivo* experiments using STING agonists have reported a phenotypic change of macrophages to a more inflammatory phenotype, increasing expression of activatory FcγRs crucial for antibody-mediated therapy (119). Accordingly, *in vivo* combination of STING ligands with anti-CD20 mAbs in a model of B-cell lymphoma overcame tumor-mediated immune suppression and resulted in curative treatments for 90% of mice (119).

An alternative immunomodulatory compound being assessed in combination with anti-CD20 mAb is lenalidomide. Lenalidomide is thought to act both through inducing tumor cell death and altering the tumor microenvironment and is approved for use in multiple myeloma (200). Lenalidomide combined with anti-CD20 mAb resulted in a significantly greater overall and complete response rates versus lenalidomide alone in a meta-analysis of refractory/relapsed CLL patients (201). Interestingly, lenalidomide plus anti-CD20 mAb achieved similar complete response rates to those seen with ibrutinib plus rituximab (see below) (202). Lenalidomide plus rituximab has also reported high response rates in untreated indolent NHL (203). The mechanistic basis for these effects is not yet fully resolved.

An alternative means of achieving immune conversion is by combining anti-CD20 mAbs with the so-called immunomodulatory antibodies. These antibodies differ from direct-targeting mAb in that they bind to cells of the immune system (rather than the tumor target) with the aim of activating or de-repressing them to elicit T cell responses. These mAb have achieved remarkable success in the last few years in treating certain patients with melanoma and lung cancer (6). The possibility of combining these agents with direct-targeting anti-CD20 mAbs has been proposed and tested in clinical trials. One such study combined the anti-programmed cell death-1 (PD-1) antibody pidilizumab with rituximab in the treatment of relapsed/refractory follicular lymphoma (204). Albeit for a small sample group, this study reported an increased complete response rate of 52% as compared to only 11% in patients receiving rituximab monotherapy. Nivolumab, another anti-PD-1 antibody, has already been approved for use in refractory Hodgkin's lymphoma after stem-cell transplant (205).

Following a phase I trial finding, ipilimumab was well tolerated in NHL and increased T cell proliferation. A combination trial

involving rituximab and the anti-CTLA-4 antibody ipilimumab is ongoing (206).

Other strategies for improving anti-CD20 therapy aim to address the results of tumor-mediated immune suppression, rather than reverse them *per se*. In our own work, we have attempted to counter the above described Fc γ RIIB-mediated internalization and inhibitory signaling which decreases CD20 therapy efficacy. This has been achieved through the use of an antagonistic anti-Fc γ RIIB antibody that prevents the *cis* binding of anti-CD20 antibody to Fc γ RIIB on the same cell, preventing internalization (131). Furthermore, this effect was also shown for combination of obinutuzumab and alemtuzumab with anti-Fc γ RIIB, suggesting a more general mechanism for reducing antibody internalization and increasing therapeutic efficacy. This has led to the initiation of a clinical trial for combining rituximab with anti-Fc γ RIIB in Fc γ RIIB $^+$ cell malignancies (NCT02933320).

In addition to these immune-related interventions detailed above, recent years have also seen a rapid increase in drugs targeted at specific molecules thought to be involved in malignancy. In many cases, these have been combined with anti-CD20 mAbs for the treatment of B-cell malignancies. One such drug, ibrutinib (Ibruvica), an irreversible inhibitor of Btk has been approved for the treatment of relapsed/refractory CLL and several NHLs owing to high response rates and increased survival (207). Ibrutinib has been combined with anti-CD20 chemoimmunotherapy and yielded increased response rates in relapsed/refractory CLL over chemoimmunotherapy alone (202, 208). Ibrutinib has also been combined with anti-CD20 mAb in, among others, DLBCL and MCL and has achieved high response rates (209, 210). Further trials are ongoing combining ibrutinib with chemoimmunotherapy in various disease settings (211). Despite the apparent efficacy of this combination, ibrutinib has been reported to decrease antibody-induced effector mechanisms both *in vitro* and in cells from patients taking ibrutinib (212). This highlights the importance of considering drug combination mechanisms of action and appropriate dosing schedules to get the maximum benefit for patients.

Another small molecule inhibitor, idelalisib (Zydelig), approved for relapsed/refractory CLL and FL therapy is targeted at the delta isoform of the lipid kinase phosphoinositide-3-kinase (PI3K δ) (213, 214). This isoform is preferentially expressed in leukocytes and expressed in malignant B-cells (215, 216). Targeting of PI3K δ has shown to be effective in the treatment of B-cell malignancies, although toxicity issues have prevented idelalisib from becoming a front line therapy (217, 218). Combination of idelalisib and rituximab was found to be superior to idelalisib alone in relapsed/refractory CLL, and addition of idelalisib to bendamustine–rituximab therapy for CLL patients with a poor prognosis has shown to improve progression-free survival (219, 220). Idelalisib has also shown efficacy in several NHLs as monotherapy and in combination with rituximab and bendamustine (221, 222). Recent work from our group has revealed the pro-apoptotic BH3-only protein Bim to be key to the *in vivo* therapeutic mechanism of PI3K δ inhibition. Addition of a PI3K δ inhibitor to anti-CD20 mAb therapy reduced the accumulation of leukemia cells in the E μ -Tcl1 transgenic mouse model, and also improved survival compared to anti-CD20 mAb or PI3K δ inhibitor alone, in a Bim-dependent

manner (223). Furthermore, combination of a PI3K δ inhibitor with a BCL-2 inhibitor was more effective than either agent alone, reducing leukemic burden by 95% (223).

Venetoclax (Venclexta) is another small molecule inhibitor that targets BCL-2 and is approved for the treatment of relapsed/refractory CLL with 17p chromosomal deletions, based on high response rates in heavily pretreated patients (224, 225). This molecule has also been trialed in combination with rituximab in relapsed/refractory CLL, with high response levels reported (86% overall response rate) (226). Trials combining venetoclax with obinutuzumab are also underway, with preliminary data suggesting that it is highly efficacious in relapsed/refractory and untreated CLL in elderly patients (227, 228). Importantly, venetoclax has been reported to be efficacious in CLL patients who have failed previous kinase inhibitor therapy, such as ibrutinib or idelalisib (229). Another anti-BCL-2 drug, the antisense oligonucleotide Oblimersen sodium, has been tested in combination with rituximab and found to be beneficial in patients with relapsed/refractory NHL (230).

Although segregated in this review by mechanism, combinations of multiple drugs with differing mechanisms of action are being examined alongside anti-CD20 therapy. For example, TG Therapeutics are currently recruiting patients with relapsed/refractory CLL to a trial combining ublituximab (a glycoengineered anti-CD20 antibody) with TGR-1202 (a PI3K δ inhibitor) and pembrolizumab (anti-PD-1 antibody). Whether such an approach is efficacious or indeed viable in terms of health economics remains to be seen.

BISPECIFIC ANTIBODIES (bsAbs)

A further therapeutic approach that is currently being trialed in the clinic is the use of bsAbs. Multiple technologies have been developed for producing bsAbs, incorporating additional Fab domains in various positions and with altered Fc backbone engineering to ensure appropriate heavy chain pairing (231). A bsAb targeting CD19 and CD3 has already achieved approval for relapsed/refractory acute lymphoblastic leukemia (232). An anti-CD20/CD22 bsAb has shown enhanced preclinical activity over the combination of the two parental antibodies, inducing greater apoptosis *in vitro* and improved overall survival and tumor shrinkage *in vivo* (233). Combination of two anti-CD20 mAbs (a type I and a type II) into a tetravalent bsAb produced a molecule that induced enhanced direct cell death over the combination of parental Abs and retained equivalent CDC (234). Furthermore, this molecule had a more potent anti-tumor activity than the combined parental antibodies *in vivo*.

Attempts to increase engagement of the target cell with effector cells using bsAbs have also been made. One example is a CD20(2) × Fc γ RIIIA tribody that binds target CD20 and effector Fc γ RIIIA, irrespective of the V/F158 polymorphism. This construct was superior to rituximab in terms of cell line and patient lymphoma cell lysis, NK-mediated tumor cell killing, and also B-cell depletion in whole blood, and functioned to deplete human B-cells in a mouse model reconstituted with a humanized hematopoietic system (235). A CD20/CD3 bsAb tested in multiple *in vivo* models appeared to act primarily through CD3 expressing

cells, rather than the antibody Fc region of this bispecific humanized IgG (236). Some of these bsAbs, such as the CD20/CD3 molecules REGN1979 (237) and FBTA05 (238), have entered clinical trials for B-cell lymphoma. Despite the termination of the clinical trial for FBTA05, this antibody has been used on compassionate grounds in children with B-cell malignancies refractory to conventional therapy, with some positive results (239).

CD20 MAB IN AUTOIMMUNE SETTINGS

In addition to the treatment of B-cell malignancies, many of the same therapeutic principles learnt from the study of anti-CD20 mAb can be applied to other disease settings, namely autoimmune disease. The rationale for B-cell depletion in autoimmune diseases such as rheumatoid arthritis (RA) is based on the (albeit incompletely understood) role of these cells in disease pathogenesis, namely differentiation into autoantibody-secreting plasma cells and antigen presentation to T cells, and the consequent expectation that their depletion will restore self-tolerance, as discussed in depth elsewhere (240). Nevertheless, it was shown in a double-blind randomized control trial that treating RA patients with rituximab resulted in both prolonged B-cell depletion and significant improvements in symptoms in comparison to methotrexate-treated patients (241). Moreover, a combination of rituximab and methotrexate increased the percentage of patients with improvements in symptoms at 48 weeks post-treatment (241). As a consequence of this (and other studies), rituximab is now FDA-approved for the treatment of RA, as well as the anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV), Wegener's Granulomatosis and Microscopic Polyangiitis (<https://www.fda.gov/Drugs/DrugSafety/ucm109106.htm>). However, contrary to indications of efficacy (242), rituximab showed no significant clinical benefit over control arms in randomized clinical trials of both extrarenal (243) and renal (lupus nephritis) (244) systemic lupus erythematosus (SLE) patients. Nevertheless, it has been estimated that rituximab is used off-label in approximately 0.5–1.5% of SLE patients in Europe, seemingly as a last resort in patients with worse disease (245).

As may be expected, a requirement for Fc γ Rs in the mechanism of action of rituximab in autoimmune disease (as for B-cell malignancies) has been indicated in studies such as by Quartuccio et al., whereby clinical responses of RA patients were significantly greater at 6 months post-rituximab in Fc γ RIIIA V/V patients (246). It is noteworthy that the depletion of B-cells by rituximab may be variable (between patients) and incomplete in autoimmune disease. In the setting of RA, for example, a sensitive flow cytometry technique was used to detect remaining B-cells, and patients with complete depletion of B-cells after a single rituximab infusion had favorable clinical responses in comparison to patients with partial depletion (247). Similarly, when the same methodology was applied to SLE, all patients with complete B-cell depletion had a clinical response to rituximab, which contrasts to patients with incomplete B-cell depletion (248). Intriguingly, a significantly lower depletion of B-cells from SLE patients was observed in comparison to B-cells from RA patients or healthy donors when treated with anti-CD20 mAb in whole blood assays (249).

Several mechanisms may help to explain the variable and/or incomplete B-cell depletion observed with rituximab in autoimmune disease. This may be linked to levels of B-cell-activating factor (BAFF), which is known to increase in RA patients treated with rituximab in periods of B-cell depletion (240). Indeed, a recent retrospective study analyzed two cohorts of AAV patients and showed that a single nucleotide polymorphism in BAFF (TNFSF13B) was associated with responses to rituximab treatment (250). Although the authors of this study conceded that further mechanistic studies are required, this indicates that responses to B-cell depletion may be predicted in advance of rituximab treatment in the future (similar to Fc γ R polymorphisms and degree of B-cell depletion mentioned above), and patients given alternative therapies instead. Modulation of Fc γ RIIB/rituximab complexes may, as for malignant B-cells (73, 121), also be a relevant resistance mechanism in the setting of autoimmune B-cells, as indicated in *in vitro* studies (249) (see below). Finally, results from animal models of SLE have suggested that inefficient depletion in this disease may be due to the presence of autoantibody ICs (251). Recent studies employing chronic viral infection models, also characterized by excessive ICs, have lent support to the hypothesis that high concentrations of ICs may inhibit antibody effector mechanisms (252, 253). Both of these studies utilized anti-CD20 mAb and showed that chronically infected mice were incapable of depleting CD20 $^{+}$ cells (252, 253). This suggests that high levels of circulating ICs should be a concern in setting of anti-CD20 therapy and may result in inefficient B-cell depletion in patients.

Nevertheless, considering such indications of incomplete B-cell depletion using rituximab in autoimmune disease, one fundamental question is how the depletion of B-cells can be improved in the setting of autoimmune disease. Employing next-generation mAbs is an option. To this end, although a non-glyco-engineered type II anti-CD20 mAb induced greater depletion of B-cells in comparison to rituximab in a whole blood assay (249), suggesting a role for the type II nature of the mAb rather than a change in glycosylation, depletion was further increased with the glycomodified (afucosylated) type II mAb obinutuzumab (17). The greater depletion mediated by type II anti-CD20 corresponded to less internalization from the surface of B-cells from healthy donors and RA/SLE patients (249). In the setting of SLE, B-cell depletion by rituximab correlated with the level of surface accessible CD20, and the difference between B-cell cytotoxicity mediated by type I versus type II anti-CD20 mAb correlated with degree of internalization (249). Internalization mediated by type I anti-CD20 could be partially inhibited by use of blocking anti-Fc γ RIIB mAb (249). It can, therefore, be hypothesized that a combination of rituximab with an anti-Fc γ RIIB mAb will increase the efficiency of autoimmune B-cell depletion, for reasons including blockade of such Fc γ RIIB-mediated modulation, or Fc γ RIIB-mediated inhibition of activatory signaling on effector cells (102). Further still, alternative anti-CD20 mAbs have also been/are being developed for the treatment of other autoimmune diseases, namely the humanized mAb veltuzumab for the treatment of ITP (in addition to CLL/NHL) (20), which has a single amino acid change in the complementary determining region (CDR) 3 V $_{\text{H}}$ in comparison to rituximab, and framework regions/Fc domains from the anti-CD22 mAb epratuzumab (21);

and ocrelizumab for the treatment of multiple sclerosis (MS) (**Table 1**). Notably, ocrelizumab was recently shown to significantly decrease disease progression in a phase III trial of primary progressive MS when compared with placebo (19) and was successful in two other trials (18), leading to its FDA approval. Alternatively, the glycoengineered anti-CD20 mAb ublituximab (**Table 1**) is also in clinical trials for the treatment of MS (25) for reasons of increased ADCC/potency (see above). Nevertheless, as with RA (241), the clinical benefit observed following B-cell depletion with anti-CD20 in MS further emphasizes a role of these cells in autoimmune disease pathogenesis (19).

A final factor to consider is the existence of serological evidence of autoimmunity that can precede the development of overt disease by years, as reviewed elsewhere (254, 255). It has, therefore, been questioned whether the development of autoimmune disease can be prevented/delayed. Studies such as PRAIRI (256) have, therefore, tested this, by infusing autoantibody-positive patients that do not yet have overt RA with a single infusion of rituximab (1,000 mg) and prospectively monitoring for disease onset versus placebo controls. The early results indicate that this strategy is able to delay disease onset (256).

CONCLUSION AND SUMMARY

Anti-CD20 mAbs have now been with us as approved clinical reagents for 20 years. As highlighted in **Figure 1**, their

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development and study has fostered a large amount of our current knowledge of therapeutic mAb mechanisms of action and what makes an effective therapeutic target and mAb. In the next 5 years, an increasing number of combination strategies will be investigated in order to improve on the current levels of success. Coupled to this will be an increasing number of new mAb formats, aiming to take advantage of the knowledge gained to date. One important aspect of this development will be an in depth understanding of the disease microenvironment in each case. For example, to improve responses in CLL may not require the same developments as required for NHL and similarly the specific pathologies relating to RA, SLE, and MS may not involve similar solutions.

More widely, we can expect the learnings gleaned from the study of CD20 antibodies will flow into developments for other mAb specificities; particularly where target cell deletion is required. So, in answer to the question “What have we learnt from targeting CD20 and where are we going?” the response should be “a huge amount” and “to an era of combination and advanced antibody engineering leading to improved responses for patients.”

AUTHOR CONTRIBUTIONS

MM and RS wrote the manuscript and both contributed equally. MC edited and wrote the manuscript.

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Monoclonal Antibody Therapies for Hematological Malignancies: Not Just Lineage-Specific Targets

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Today, monoclonal antibodies (mAbs) are a widespread and necessary tool for biomedical science. In the hematological cancer field, since rituximab became the first mAb approved by the Food and Drug Administration for the treatment of B-cell malignancies, a number of effective mAbs targeting lineage-specific antigens (LSAs) have been successfully developed. Non-LSAs (NLSAs) are molecules that are not restricted to specific leukocyte subsets or tissues but play relevant pathogenic roles in blood cancers including the development, proliferation, survival, and refractoriness to therapy of tumor cells. In consequence, efforts to target NLSAs have resulted in a plethora of mAbs—marketed or in development—to achieve different goals like neutralizing oncogenic pathways, blocking tumor-related chemotactic pathways, mobilizing malignant cells from tumor microenvironment to peripheral blood, modulating immune-checkpoints, or delivering cytotoxic drugs into tumor cells. Here, we extensively review several novel mAbs directed against NLSAs undergoing clinical evaluation for treating hematological malignancies. The review focuses on the structure of these antibodies, proposed mechanisms of action, efficacy and safety profile in clinical studies, and their potential applications in the treatment of hematological malignancies.

Keywords: monoclonal antibody, immunotherapy, hematological malignancies, non-lineage antigens, mechanism of action

INTRODUCTION

Cancer treatment is expanding from non-specific cytotoxic chemotherapies to targeted therapies as a consequence of increased knowledge of the pathogenesis of cancer that leads to a better design of treatments to inhibit tumor growth and spread. Most of these therapies consist in monoclonal antibodies (mAbs) that bind to specific antigens (Ags) expressed on the surface of cancer and normal cells, mediating different mechanisms of action (MOA).

IgG antibodies, which are the most commonly used in cancer immunotherapy, show two regions that determine their biologic properties: the variable fragment (Fv), responsible for interaction with Ag and the constant fragment (Fc), responsible for interaction with immune cells or molecules bringing together cells bearing the Ag (or the Ag itself) to components of innate or acquired immunity. The Fc of an antibody is responsible for half-life, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent phagocytosis (ADCP), or complement-dependent cytotoxicity (CDC) (1, 2). Both Fv and Fc determine the different and characteristics of MOA displayed by a single mAb and

its utility as immunotherapeutic agent in cancer. These MOA may work alone or combined. Briefly, a particular mAb may inhibit ligand–receptor interactions, and/or induce proapoptotic signaling, and/or activate innate immune cells or molecules triggering ADCC, ADCP, or CDC, and/or may induce tumor cell killing by targeting regulatory molecules on host immune cells (1, 2). In addition, mAbs can be used to deliver payloads such as cytotoxic agents, toxins, or radioisotopes, which are coupled to the mAb targeting tumor cells (3). One explanation to the rapid growth of mAbs as therapeutic drugs is their plasticity. Antibodies can be engineered at several levels leading to customized modulations in the Fv/Fc properties. Altering the glycosylation status is the most extended modification among all the novel mAbs under development and is used to regulate anti- and proinflammatory properties and to control the binding to Fc receptors (FcRs) to modulate ADCC (4, 5).

In the hematological malignancies field, therapeutic mAbs are especially relevant owing to accessibility to tumor cells, facilitating *in vitro* studies of targets and MOA. In addition, the historical knowledge of the hematopoietic differentiation Ags, usually grouped as cluster of differentiation (CD) Ags, has provided a large number of potential targets in hematological malignancies. Similar to other cancers, tumor-associated Ags recognized by therapeutic mAbs in blood cancers fall into different categories. Many of them are present at the different normal maturation steps of a given lineage and this is why they are called lineage-specific

antigens (LSAs). For example, B-cell differentiation is associated with the expression of CD19, CD20, CD22, and surface Ig (6). Similarly, myeloid differentiation is associated with CD33 expression (7), whereas CD3 is the hallmark of the T-cell lineage (8). These LSAs show significant overlapping expression patterns between leukemia or lymphoma subtypes within the same lineage.

It could be said that most of the LSAs are clinically validated targets in antibody-based therapy. CD20 is a LSA exclusively expressed on B-cells membrane and on the majority of malignant B-cells (6, 9). The “blockbuster” antibody rituximab is the first-in-class anti-CD20 mAb approved for the treatment of B-cell non-Hodgkin lymphoma (B-NHL) and chronic lymphocytic leukemia (CLL); it is by far the most important mAb used in hematological malignancies (10–12). Since its approval in 1997, four additional mAbs targeting different CD20 epitopes and displaying several MOA have been approved by the US Food and Drug Administration (US-FDA) (13–15). These CD20-targeting therapeutic mAbs account for >30% of all current therapeutic mAbs for cancer (3) and reflect the previous tendency to develop improved antibodies against the same LSAs. The MOA of antibodies directed to CD20 are given in **Table 1**.

The success of anti-CD20 mAbs has encouraged drug developers to propose novel LSAs, such as CD19, CD22, or CD79b (**Table 2**) (24–26). Despite these LSAs representing potential candidates for the treatment of B-cell cancers, antibodies directed to CD19 (MOR00208, inebilizumab, or MDX-1342)

TABLE 1 | Characteristics of antibodies directed to CD20.

Target	mAb (commercial name/originator)	IgG class	MOA (compared to RTX)	Type—generation	Active indications in HMs (highest phase)	Reference
CD20	Rituximab (<i>Rituxan</i> /Biogen Idec)	Ch IgG1	CDC ADCC PCD	Type I First	Approved (B-NHL, CLL)	(11, 12)
	Ofatumumab (<i>Arzerra</i> /Genmab)	Fh IgG1	↑ CDC ~ ADCC ↓ PCD	Type I Second	Approved (CLL) I-II (HL) I-II-III (B-NHL)	(13, 16)
	Veltuzumab, IMMU-106 (Immunomedics)	Hz IgG1	↑ CDC ~ ADCC ~ PCD	Type I Second	I-II (CLL, B-NHL) Granted (ITP)	(17, 18)
	Ocrelizumab (<i>Ocrevus</i> /Biogen Idec; Genentech)	Hz IgG1	↓ CDC ↑ ADCC ~ PCD	Type I Second	I-II (FL) disc. Approved (MS)	(19)
	Obinutuzumab (<i>Gazyva</i> /Roche Glycart Biotech)	Hz IgG1 Glyco-Fc	↓ CDC ↑ ADCC ↑ PCD*	Type II Third	Approved (CLL) I-II-III (B-NHL)	(20, 21)
Ocaratuzumab, LY2469298 (Applied Molecular Evolution)	Hz IgG1 Glyco-Fc	~ CDC ↑ ADCC ~ PCD	Type I Third	I-II (FL)	(22)	
Ublituximab, LFB-R603 (LFB Biotech.; rEVO Biologics)	Ch IgG1 Glyco-Fc	~ CDC ↑ ADCC ~ PCD	Type I Third	III (CLL) I-II (B-NHL)	(23)	

Antibodies that reached clinical studies. Biosimilars and immunoconjugates are excluded.

RTX, rituximab; mAb, monoclonal antibody; MOA, mechanisms of action; HMs, hematological malignancies; Ch, human–mouse chimeric; Fh, fully human; Hz, humanized; Glyco-Fc, glycoengineered Fc fragment; ↑, higher; ↓, lower; ~ comparable; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cell-mediated cytotoxicity; PCD, classical programmed cell death; PCD*, non-classical PCD; type I antibodies draw CD20 into lipid rafts, induce CDC, ADCC, and PCD; type II antibodies induce ADCC, PCD* but not CDC; B-NHL, B-cell non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; MS, multiple sclerosis; ITP, immune thrombocytopenic purpura; disc., discontinued in hematological malignancies.

TABLE 2 | Characteristics of antibodies directed to LSAs.

Target	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)	Reference
CD19	Inebilizumab, MED1 551 (Collective Therapeutics)	Hz IgG1 Glyco-Fc	ADCC CDC ADCP	II (CLL/NCT01466153 °C) II (DLBCL/NCT01453205 °C) disc. I-II (B-NHL/NCT02271945 °C) I (MM/NCT01861340 °C)	(31)
	MOR00208, XmAb5574 (Xencor)	Hz IgG1	ADCC ADCP PCD	III (DLBCL/NCT02763319 °R) II (BALL/NCT02763319 °T) II (CLL/NCT02639910 °R) II (B-NHL/NCT01685008 °ANR)	(24, 32)
	MDX-1342 (Medarex)	Fh IgG1 Glyco-Fc	ADCC	I (CLL/NCT00593944 °C)	(33)
CD22	Epratuzumab, AMG-412, IMMU-103 (Immunomedics)	Hz IgG1	ADCC PCD Alterations in CD22 and BCR signaling its action	II (B-ALL/NCT01802814 °R) III (B-NHL/NCT00022685 °C) II (FL/NCT00553501 °C)	(25)

Antibodies that reached clinical studies. Biosimilars and immunoconjugates are excluded.

^aCombined therapy.

^bMonotherapy.

mAb, monoclonal antibody; MOA, mechanisms of action; HMs, hematological malignancies; Ch, human–mouse chimeric; Fh, fully human; Hz, humanized; Glyco-Fc, glycoengineered Fc fragment; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; PCD, programmed cell death; B-NHL, B-cell non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; MM, multiple myeloma; FL, follicular lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; BCR, B-cell receptor; DISC., discontinued; NCT, number of clinical trial (clinicaltrials.gov); C, completed; R, recruiting; T, terminated; ANR, active non-recruiting.

or CD22 (epratuzumab) have yielded only modest responses in clinical studies (9). This low efficacy has been attributed to high Ag internalization rates on mAb ligation (3). Consequently, CD19, CD22, and CD79 have been widely investigated for immunoconjugate therapy with promising clinical results as a single agent with no unexpected safety concerns. Finally, but beyond the scope of this review, it should be mentioned that other antibody formats, such as the bispecific T-cell engager (BiTe) blinatumomab, show promising results when targeting CD19 (27, 28). Thanks to a dual specificity for CD19 and CD3, this BiTe efficiently redirects host T-cells to CD19 expressed in tumor B-cells, although it shows neurological toxicity as treatment-related adverse event (29, 30).

A different group of tumor Ags are the non-lineage-specific antigens (NLSAs), which comprise many molecules that are preferentially expressed by tumor cells but not restricted to specific leukocyte subsets or tissues and include, among others, oncogenic receptors, chemokine receptors (CKRs), and molecules involved in the formation and preservation of the tumor microenvironment (TME). The ubiquitous expression of many NLSAs potentially enables antibodies directed to these molecules to be used in different hematological malignancies, or even universally in cancer.

Limited clinical efficacy of some mAbs targeting LSAs and the advent of patients with refractory diseases to therapies directed to LSAs boosted the research on many NLSAs with a relevant role in the pathogenesis of cancer, especially in B-cell malignancies (9, 34). Moreover, in some disorders the lack or loss of LSA expression in cell membrane may preclude the use of antibodies, thus prompting research of other potential therapeutic targets. This is the case of multiple myeloma (MM), a B-cell disorder where tumor cells do not express CD20 (35) and where novel antibodies directly targeting several NLSAs are a profound

change compared with earlier treatment approaches based on anti-CD20 antibodies.

Efforts to target NLSAs have resulted in an ever-increasing number of new murine, chimeric and human antibodies with proven efficacy in preclinical models. Here, we extensively review the results of several novel mAbs directed against NLSAs undergoing clinical evaluation (Table 3). The review focuses on the structure of these antibodies, proposed MOA, efficacy, and safety profile in clinical studies, and their potential applications in the treatment of hematological cancers.

ANTIBODIES TARGETING GLYCOPROTEINS AND ONCOGENIC RECEPTORS

Pathologic clonal populations express or overexpress different NLSAs which are involved in different oncogenic pathways and may confer an evolutionary advantage to the tumor. In some cases, high expression of the NLSAs is the rationale behind targeting a single receptor. In other cases, this targeting represents an optimal strategy to avoid cancer cell proliferation and survival.

CAMPATH-1 (CD52)

CD52 is a glycoprotein anchored to glycosylphosphatidylinositol (GPI) present on the surface of mature lymphocytes, monocytes and dendritic cells (DCs) (36). CD52 expression is particularly high on T-cell prolymphocytic leukemia (T-PLL), Sézary syndrome (SS), acute lymphoblastic leukemia (ALL), CLL, and acute myeloid leukemia (AML) (36–39), which is the reason why it was selected as therapeutic target despite not having a clear role in the pathogenesis of these conditions. Nonetheless, efficacy as single agent in patients with high-risk CLL (40–42)

TABLE 3 | Characteristics of antibodies directed to NLSAs.

Target	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)	
Glycoproteins and oncogenic receptors	CD52	Hz IgG1	ADCC CDC ADCP?	Approved (CLL) II (T-PLL/NCT01186640 ^a C) II (PTCL/NCT01806337 ^a C)	
	CD38	Hz IgG1	ADCC CDC ADCP Blocks CD38	Approved (MM) II (MCL, DLBCL, FL/NCT02413489 ^a T)	
	Isatuximab, SAR650984 (ImmunoGen)	Hz IgG1	ADCC CDC ADCP Blocks CD38	III (MM/NCT02990338 ^b R) II (T-ALL, T-NHL/NCT02999633 ^b R)	
	MOR202, MOR03087 (MorphoSys)	Hz IgG1	ADCC ADCP Blocks CD38	I-II (MM/NCT01421186 ^b R)	
	SLAMF7 (CS1, CD319)	Elotuzumab, HuLuc63, BMS-901608 (<i>Empliciti</i> /PDL BioPharma)	Hz IgG1	ADCC	Approved (MM)
	CD37	Bl836826 (Boehringer Ingelheim)	Ch IgG1 Glyco-Fc	ADCC PCD	II (DLBCL/NCT02624492 ^b R) I (CLL/NCT01296932 ^a C, NCT02538614 ^b T)
		Otltuzumab, TRU-016 (Trubion Pharmaceuticals)	Fv-Fc	ADCC PCD	I-II (CLL/NCT01188681 ^b C) I (B-NHL/NCT00614042 ^a C)
	CD98 (4F2, FRP-1)	IGN523 (Igenica)	Hz IgG1	ADCC CDC PCD	I (AML/NCT02040506 ^a C)
	DKK-1	BHQ880 (MorphoSys; Novartis)	Fh IgG1 GlycoFc	Blocks DKK-1 ADCC	II (MM/NCT01302886 ^a C, NCT01337752 ^a C)
		DKN-01, LY-2812176 (Eli Lilly)	Hz IgG4	Blocks DKK-1	I (MM/NCT01711671 ^b C, NCT01457417 ^b C)
CD157 (BST-1)	OBT357, MEN1112 (Menarini; Oxford BioTherapeutics)	Hz IgG1 GlycoFc	ADCC	I (AML/NCT02353143 ^a R)	
GRP78 (BiP)	PAT-SM6 (OncoMab GmbH)	Fh IgM	CDC PCD	I (MM/NCT01727778 ^a C)	
TRAIL-R1 (DR4)	Mapatumumab, TRM1, HGS-1012 (Cambridge Antibody Technology)	Fh IgG1	PCD	I (NHL, HL/NCT00094848 ^a C) disc. II (MM/NCT00315757 ^b C) disc.	
ROR-1	Cirmtuzumab, UC-961 (University of California, San Diego)	Hz IgG1	PCD Blocks ROR-1	I-II (CLL, MCL/NCT03088878 ^b NYR) I (CLL/NCT02860676 ^a E, NCT02222688 ^a R)	
Notch-1	Brontictuzumab, OMP-52M51 (OncoMed Pharmaceuticals)	Hz IgG2	Blocks Notch-1	I (HM/NCT01703572 ^a C) disc.	
TfR1 (CD71)	E2.3/A27.15 (University of Arizona)	mlgG1	Blocks TfR1	I (HM/NCT00003082 ^a C)	
EPHA3	Ifabotuzumab, KB004 (Ludwig Institute for Cancer Research)	Hz IgG1	ADCC PCD	I-II (AML/NCT01211691 ^a S)	
HLA-DR	IMMU114, hL243 (Immunomedics)	Hz IgG4	PCD	I (B-NHL/NCT01728207 ^a R)	
G(M2)	BIW-8962 (Kyowa Hakko Kirin Co.)	Hz IgG1	ADCC	I (MM/NCT00775502 ^a T)	

(Continued)

TABLE 3 | Continued

	Target	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)
Chemokine receptors	CCR4	Mogamulizumab, KW-0761 (<i>Poteligeo/Kyowa Hakko Kirin Co.</i>)	Hz IgG1	ADCC	Approved (ATL, CTCL, PTCL) II (NK-lymphoma/NCT01192984 ^a C)
	CXCR4	Ulocuplumab, BMS-936564, MDX-1338 (Medarex)	Hz IgG4	Blocks CXCR4	I-II (AML/NCT02305563 ^b R) I (MM/NCT01359657 ^b C) I (CLL, DLBCL, FL/NCT01120457 ^a C)
		PF-06747143 (Pfizer)	Hz IgG1	ADCC CDC Blocks CXCR4	I (AML/NCT02954653 ^b R)
Soluble factors and associated receptors	BAFF	Tabalumab, LY2127399 (Eli Lilly)	Fh IgG4	Blocks BAFF	II (MM/NCT01602224 ^a C) disc.
	BAFF-R	VAY736 (MorphoSys; Novartis)	Fh IgG1 GlycoFc	Blocks BAFF-R ADCC	I (CLL/NCT02137889 ^a T)
	RANKL	Denosumab, AMG-162 (<i>Prolia; Ranmark; Xgeva/Amgen</i>)	Fh IgG2	Blocks RANKL	III (MM/NCT01345019 ^a ANR) II (MM/NCT00259740 ^a C, NCT02833610 ^b R) II (NHL with hypercalcemia/NCT00896454 ^a C)
IL-6	IL-6	Siltuximab, CNTO-328 (<i>Sylvant/Centocor</i>)	Ch IgG1	Blocks IL-6	II (MM/NCT00911859 ^b C, NCT00402181 ^a C)
	IL-6R	Tocilizumab, R-1569 (<i>Actemra/Chugai Pharmaceutical; Osaka University</i>)	Hz IgG1	Blocks IL-6R	I (CLL/NCT02336048 ^b R) I (MM/NCT02447055 ^b W) disc.
	IL-3R α (CD123)	CSL360 (CSL)	Ch IgG1	ADCC CDC Blocks IL-3R α	I (AML/NCT00401739 ^a C)
		Talacotuzumab, JNJ-56022473, CSL362 (CSL)	Hz IgG1 GlycoFc	ADCC Blocks IL-3R α	III (AML/NCT02472145 ^b ANR) II (MDS/NCT03011034 ^b R)
		XmAb14045 (Xencor)	Fh IgG1	ADCC	I (AML, B-ALL, DC Neoplasm, CML/NCT02730312 ^a R)
IL-2R α (CD25)		KHK2823 (Kyowa Hakko Kirin Co.)	Fh IgG1	ADCC	I (AML, MDS/NCT02181699 ^a ANR)
		Basiliximab, SDZ-CHI-621 (<i>Simulect/Novartis</i>)	Ch IgG1	Blocks IL-2R α	II (AML, CML, ALL, CLL, HL, MM/NCT00975975 ^a C)
		Daclizumab	Hz IgG1	Blocks IL-2R α	II (ATL/NCT00001941 ^a C) II (MM, NHL/NCT00006350 ^b C)
IGF-1R (CD221)	Ganitumab, AMG-479 (Amgen)	Fh IgG1	Blocks IGF-1R	I (NHL/NCT00562380 ^a C)	
	Figitumumab, CP-751871 (Pfizer)	Fh IgG2	Blocks IGF-1R	I (MM/NCT01536145 ^a C) disc.	
	Dalotuzumab, MK-0646 (Pierre Fabre)	Hz IgG1	Blocks IGF-1R	I (MM/NCT00701103 ^a C) disc.	
	AVE1642 (ImmunoGen)	Hz IgG1	Blocks IGF-1R	I (MM/NCT01233895 ^a C) disc.	
GM-CSF (CSF2)	Lenzilumab, KB003 (KaloBios Pharmaceuticals)	Hz IgG1	Blocks GM-CSF	I-II (CMML/NCT02546284 ^a R)	
	HGF	Ficlatuzumab, AV-299 (Aveo Pharmaceuticals)	Hz IgG1	Blocks HGF	I (NHL, HL, MM/NCT00725634 ^b C) I (AML/NCT02109627 ^b R)

(Continued)

TABLE 3 | Continued

Target	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)
Adhesion molecules	CD44	Hz IgG1	Blocks CD44	I (AML/NCT01641250 °C)
	VLA-4 (CD49d)	RG7556, RO5429083 (Chugai Biopharmaceuticals; Roche)		I-II (MM/NCT00675428 °T) disc.
	ICAM-1 (CD54)	Natalizumab, BG-0002-E (Tysabri/Elan Corporation)	Hz IgG4	I (MM/NCT01025296 °C) II (SMM/NCT01838369 °C)
Angiogenesis	VEGF-A	Fn IgG1	ADCC ADCP PCD	I (MM/NCT00486759 °T) disc. II (FL/NCT00193492 °C)
Endosialin (CD248, TEM1)	Bevacizumab (Avastin/Genentech; Hackensack University Medical Center)	Hz IgG1	Blocks VEGF-A	II (MM/NCT00482495 °C, NCT00473590 °C) II (CLL/NCT00290810 °C, NCT00448019 °C) disc. III (DLBCL/NCT00486759 °T) disc.
	Ontecizumab, MOFAB-004 (Ludwig Institute for Cancer Research; Morphotek)	Hz IgG1	Blocks endosialin	I (HM/NCT01748721 °C)

Antibodies that reached clinical studies. Biosimilars and immunon conjugates are excluded.

^aMonotherapy.

^bCombined therapy.

mAb, monoclonal antibody; MOA, mechanisms of action; HMs, hematological malignancies; Ch, human–mouse chimeric; Fn, fully human; Hz, humanized; m, mouse; Glyco-Fc, glycoengineered Fc fragment; CDC, complement-dependent cytotoxicity; ADC, antibody-dependent cell-mediated phagocytosis; PCD, programmed cell death; B-NHL, B-cell non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; B-ALL, B-cell acute lymphoblastic leukemia; T-PLL, T-cell prolymphocytic leukemia; PTCL, peripheral T-cell lymphoma; CTCL, cutaneous T-cell lymphoma; ATL, adult T-cell leukemia and lymphoma; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; DC, dendritic cells; disc., discontinued in hematological malignancies; NCIT, number of clinical trial [clinicaltrials.gov]; C, completed; R, recruiting; T, terminated; ANR, active non-recruiting; NVR, not yet recruiting; E, enrolling by invitation; S, suspended; W, withdrawn.

prompted the approval of the anti-CD52 mAb alemtuzumab as front-line therapy in CLL. The main MOA of alemtuzumab are CDC and ADCC (36) which are likely to be involved in its efficacy in SS and T-PLL (39). Curiously, side effects associated with immune-suppression and infections were more frequent in B-cell than in T-cell malignancies, probably due to off-target activities (43). Despite being one of the few working weapons in T-cell malignancies, alemtuzumab was withdrawn in 2012, due to a strategic decision, and now is only available through an international compassionate use program.

CD38

In some leukocytes, this type II transmembrane glycoprotein behaves both as an ectoenzyme (NADase/ADPR cyclase) and as a receptor involved in cell adhesion, calcium flux and signal transduction (44, 45). While its expression was low to moderate on lymphoid and myeloid cells, both normal and tumor plasma cells exhibited high levels of CD38, making it an attractive target for MM (44, 45). In 2015, daratumumab, a humanized anti-CD38 IgG1 mAb, became the first mAb approved for MM (46). In preclinical models, daratumumab caused cell death through ADCC, CDC, ADCP, and blocking of CD38 that inhibits its enzymatic activity and induces apoptosis in a caspase-dependent manner (47–50). In addition, it has been recently suggested that depletion of CD38⁺ immunosuppressive regulatory T (Tregs) and B-cells and myeloid-derived suppressor cells (MDSCs) increase antitumor effector T-cell responses (51). Altogether, these MOA are responsible for daratumumab single-agent efficacy as demonstrated by two phase I–II trials in pretreated MM patients (NCT00574288; NCT01985126) that prompted FDA approval of daratumumab (52, 53). Moreover, daratumumab shows promising results both in the relapsing/refractory setting (rrMM) and in the upfront setting when combined with other potent MM therapeutics, including lenalidomide, dexamethasone and bortezomib (54–56). As a result, the FDA granted “Breakthrough Therapy” designation to daratumumab in combination with lenalidomide–dexamethasone or bortezomib–dexamethasone for the treatment of previously treated MM.

In the light of the aforementioned results, it is not difficult to find several anti-CD38 mAbs under clinical development. Isatuximab, with similar MOA to daratumumab, has shown promising results in ongoing phase I–II studies in rrMM both in monotherapy (NCT01084252) (57) or combined with immunomodulatory drugs (IMIDs) or dexamethasone (NCT01749969) (58). Another mAb is MOR202, which lacks CDC activity, but still shows promising results in ongoing trials both in monotherapy or in combination (NCT04121186) (59, 60). Last, but not least, anti-CD38 mAbs are attracting the interest in many other B-cell malignancies expressing surface CD38 including CLL, mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), and transformed follicular lymphoma (FL) (NCT02413489) (44, 52, 61).

Signaling Lymphocytic Activation Molecule Family Member F7 (SLAMF7; CS1)

This glycoprotein is moderately expressed by normal plasma cells and by cytolytic lymphocyte subsets such as natural killer (NK) cells,

NKT cells or CD8⁺ T-cells (62, 63). As their normal counterpart, MM plasma cells express SLAMF7, but at higher levels (62, 63) as a consequence of an amplification of chromosome 1q23 region, where SLAMF7 gene is located, which is very frequent in aggressive MM (62, 64). SLAMF7 expression in MM does not correlate with other high-risk cytogenetic abnormalities or the degree of disease progression (62, 63), thus validating SLAMF7 as a potential target.

The humanized IgG1 mAb elotuzumab was the first-in-class anti-SLAMF7 to be approved by the FDA in 2015, and the second antibody marketed for the treatment of MM (65). Similar to daratumumab, elotuzumab has several MOA *in vitro*, although it seems to predominantly act through ADCC *in vivo* (63, 66–68) since homozygosity for the high-affinity Fc γ RIIIa Val significantly prolonged median period free survival in clinical settings (69). In addition, elotuzumab is an agonistic mAb, which activates NK cells, further enhancing their cytotoxicity through a unique SLAM-associated pathway. Conversely, MM cells lack the SLAM-associated adaptor EAT-2 thus preventing proliferation upon elotuzumab binding (70, 71).

In contrast to daratumumab, elotuzumab has demonstrated limited activity as a single agent in both preclinical and clinical studies (63). The effects on NK cell activity observed in MM patients may be explained by elotuzumab activity relying on ADCC. Also, the paradox of NK cells becoming targets may also contribute to the lack of objective responses in rrMM patients treated with elotuzumab as single-agent (72). Therefore, to reach its maximum efficacy, elotuzumab needs to be combined with other antimyeloma agents such as lenalidomide-dexamethasone (NCT00742560, NCT01239797) (66, 73, 74) or bortezomib-dexamethasone (69, 75). Currently, several studies are examining different combinations either in the upfront or the relapsed/refractory settings.

CD37

This heavily glycosylated tetraspanin is highly expressed by mature B-cells and B-cell malignancies, including CLL and NHL (76–78). The exact function of CD37 has not yet been elucidated, although it seems to be important for T-cell-dependent B-cell responses, and may be involved in both pro- and antiapoptotic signaling (78). In addition, recent evidence confirms CD37 expression on the surface of CD34⁺/CD38⁻ AML stem cells (LSCs), which are considered the root of tumor drug resistance and recurrence (79). For this reason, despite initially conceived as a lineage-specific therapy for B-cell malignancies, anti-CD37 mAbs are also being tested as therapeutics in AML.

CD37 has unique properties for generating therapies as low internalization rates allows the preservation of its ADCC activity (76). For this reason, different kinds of IgG formats targeting CD37 are currently in clinical development. BI836826 is an Fc-engineered, chimeric IgG1 that mediates potent ADCC and induces apoptosis on CD37-overexpressing cells (80). This mAb is undergoing phase I-II studies for the treatment of CLL and B-NHL, either as a single agent or in combination with ibrutinib, idelalisib or rituximab. A number of anti-CD37 immunoconjugates are also in advanced clinical phases (79, 81, 82) (**Table 6**).

Of special interest is the modular homodimer called otlertuzumab (TRU-016) formed by a single-chain Fv linked to the

hinge region and Fc domain of hIgG1 (148, 149). Otlertuzumab induces apoptosis directly *via* binding to the CD37 protein, which results in up-regulation of the proapoptotic protein BIM (also termed BCL2L11) (150). In addition, otlertuzumab triggers Fc-dependent cytotoxicity (ADCC) but does not induce complement activation. In B-cell malignancies, otlertuzumab has shown efficacy as a single agent or combined with other therapeutic drugs in preclinical models (151, 152) as well as in phase I (NCT00614042) and phase II (NCT01188681) studies (149, 153). Other studies in B-NHL patients (NCT01317901) further confirm that combination regimens are well tolerated and lead to higher response rates (154). As a consequence, novel clinical trials are recruiting patients to evaluate combinations with standards of care in B-NHL such as rituximab, obinutuzumab, idelalisib, and ibrutinib.

CD98

The CD98 heterodimer consists of a type II single-pass transmembrane glycoprotein (also known as 4F2 Ag heavy chain or FRP-1) with two biochemical functions depending on the coupled light chain (155). Upon binding to the cytoplasmic tail of the integrin beta-chain it mediates adhesive signals thereby controlling cell proliferation, survival, migration, epithelial adhesion and polarity. In addition, CD98 contributes to the amino acid transport processes through the binding to one of the six permease-type amino acid transporters including L-type amino acid transporter 1 and 2 (LAT-1 and LAT-2) (155, 156), whose localization and proper function rely on the CD98 heavy chain (157). Both CD98-mediated activities take place on fast-cycling cells undergoing clonal expansion, such as AML cells, where CD98 supports elevated growth rates and contributes to proliferation, survival, and metastasis (158). Few approaches target metabolic cancer, and most of them are based on small molecules against CD98-associated light chains (158). In this context, targeting CD98 heavy chain with antibodies provides an alternative approach as demonstrated by IGN523, a novel humanized anti-CD98 mAb with robust preclinical activity against established lymphoma tumor xenografts (158). IGN523 elicits strong ADCC, mild CDC, and induces lysosomal membrane permeabilization that elicits caspase-3- and caspase-7-mediated apoptosis in the presence of crosslinking antibody. But the most differential feature of IGN523 is the inhibition of essential amino acid (phenylalanine) uptake by rapidly proliferating tumor cells that ultimately results in caspase-3- and -7-mediated apoptosis (158). IGN523 has been evaluated in a completed Phase I study for rrAML (NCT02040506), although results are not published yet (158, 159).

Dickkopf-1 Protein (DKK1)

This NLSA is related to the canonical Wnt/beta-catenin signaling pathway. DKK1 is a soluble inhibitor that binds simultaneously the transmembrane receptors Kremen-1 or 2 and the Wnt/beta catenin coreceptor LRP5/6 (160). This extracellular binding leads to endocytosis of the DKK1-associated complex that impairs a subsequent activation of Wnt/beta-catenin signaling. The first association between DKK-1 and cancer was described in MM patients suffering osteolytic lesions MM (160). Later, on

preclinical studies have demonstrated that a neutralizing DKK mAb reduces osteolytic bone resorption, increases bone formation, and controls MM growth (161–163). BHQ880 and DKN-1 are neutralizing humanized IgG1 mAbs, which are being tested in phase I–II studies in MM. Most of the studies (NCT00741377, NCT01457417) are using anti-DKK-1 mAbs in combination with antimyeloma therapy, except the phase II study that evaluated the efficacy of BHQ880 in monotherapy in previously untreated patients with high risk smoldering MM (phase II, NCT01302886). Overall, BHQ880 was well tolerated but the clinical benefits were limited (164). For this reason, other studies were designed to test the efficacy of anti-DKK-1 antibodies in the setting of MM with bone alterations combined with specific agents such zoledronic acid (NCT00741377).

CD157 (BST-1)

This GPI-linked membrane protein has a close resemblance to CD38 and a significant role in myeloid cells trafficking and pre-B-cell growth (165–167). It is, therefore, not surprising that high levels of CD157 can be found in B-ALL cells and in most primary AML patient samples, including the LSCs compartment (168). Based on this rationale, a novel defucosylated IgG1 termed OBT357/MEN1112 validated CD157 as a therapeutic target in AML *in vitro* and *ex vivo* models (168). Now, the potent ADCC observed in preclinical phases is under evaluation in a phase I study in AML patients (NCT02353143).

Glucose-Regulated Protein 78 (GRP78; BiP; HSP5a)

Members of the heat shock protein-70 family, if expressed on the cell membrane, are NLSAs of interest in mAb-based cancer therapy. GRP78 is a protein with multiple functions related to its different cellular locations. It may control the unfolded protein response, the macroautophagy or prosurvival pathways activated by PI3K/AKT. In some circumstances like glucose starvation, hypoxia or protein mal folding, GRP78 is translocated to the membrane, where it mediates, in general, cytoprotective responses (169). Many tumor cells, including MM, overexpress GRP78 on the outer plasma membrane to promote tumor survival, proliferation, and motility and this overexpression correlates with an adverse prognosis and drug resistance (170). Interestingly, normal plasma cells do not express the molecule on their membrane (171). Based on this, GRP78 is an ideal candidate for immunotherapeutic intervention of MM. Recently, the natural fully human IgM PAT-SM6 (initially isolated from a patient with gastric cancer) was evaluated as monotherapy in a phase I study in rrMM (NCT01727778). Results show that PAT-SM6 is well tolerated but has modest clinical activity (169). PAT-SM6 lacks ADCC activity thus its MOA mainly relies on apoptosis and to a lesser extent CDC (171, 172). Interestingly, patients who received prior treatment with proteasome inhibitors responded much better to PAT-SM6 than patients who had been previously treated with IMIDs or other chemotherapeutics. Hence, future clinical studies will focus on synergistic combinations with proteasome inhibitors to induce better clinical responses (press release by Patrys).

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor-1 (TRAIL-R1; DR4)

This protein, also known as death receptor 4, is a cell surface receptor that binds TRAIL (ApoL2) and activates the extrinsic apoptotic pathway (173). After binding of either ligand or agonist antibodies to TRAIL-R1, a death-inducing signaling complex (DISC) starts the recruitment, cleavage and activation of caspases-3, -6, -7, resulting in the characteristic programmed cell death (PCD) (173). The expression of TRAIL-R1 is minimal or absent in healthy tissues. In contrast, this receptor is frequently detected in cancer, including B-cell malignancies (174–176). This rationale boosted the development of the agonist anti-TRAIL-R1 IgG1 antibody mapatumumab (HGS-ETR1). Unlike native TRAIL, mapatumumab has longer half-life and binds specifically to TRAIL-R1 and not to the other TRAIL receptors (177). Like TRAIL, mapatumumab mediates caspase-dependent apoptosis by binding TRAIL-R1. In preclinical models of hematological malignancies, mapatumumab induced apoptosis in a wide spectrum of human cancers and promoted tumor regressions in xenograft models (175–179). Interestingly, in a recent study, the combination of mapatumumab with low dose bortezomib potentiated the uptake of myeloma cell apoptotic bodies by DC and induced antimyeloma cytotoxicity by both CD8⁺ T-cells and NK cells (180). Based on this, it has been suggested that mapatumumab may also promote endogenous antitumor immune responses.

Results from a phase II study (NCT00094848) demonstrated that mapatumumab was capable of producing clinical responses when used as single agent in patients with B-NHL (181), particularly FL. Of interest, immunohistochemistry analysis suggested that strong TRAIL-R1 staining in tumor specimens was not a requirement for mapatumumab activity in FL (181). In another phase II study in MM (NCT00315757), no differences in efficacy were observed between patients receiving mapatumumab plus bortezomib and the control group. What remains unclear is whether immunosuppressive effects of bortezomib could affect the ability of mapatumumab to promote immune responses (180).

Receptor Tyrosine Kinase-Like Orphan Receptor 1 (ROR-1)

This type I membrane glycoprotein lacks catalytic activity but is essential for ligand binding and signal transduction in the non-canonical Wnt pathway. It is considered an oncofetal Ag since it is highly expressed during early embryonic development, where it modulates neurite growth, but absent in most adult tissues (182). Ubiquitously found in human cancers, ROR-1 protein is highly expressed on the surface of CLL cells and several other B-cell malignancies where it favors invasion, metastasis and therapeutic resistance (183).

Cirmtuzumab (UC-961) is the first IgG1 directed against a functional epitope of the extracellular domain of ROR-1. It blocks ROR-dependent non canonical Wnt5a signaling through ROR-1 dephosphorylation, thus blocking tumor cell proliferation, migration and survival, leading to tumor cell death by apoptosis (184, 185). Preclinical and phase I studies have shown good tolerability

and moderate activity of cirmtuzumab when used as a single agent but with apparent synergistic activity with other agents like ibrutinib (184, 185). Interestingly, cirmtuzumab acts like other kinase inhibitors mobilizing ROR1-expressing CLL cells, thereby preventing progression in protective niches and providing an additional MOA (185). Currently, cirmtuzumab is facing phase I-II studies as single agent in rrCLL (NCT02222688) or in combination with ibrutinib in rrCLL or rrMCL (NCT03088878). Finally, the interest in ROR-1 as target is supported by two bi-specific antibodies (ROR1-CD3-DART and APVO425) that aim to redirect cytotoxic T-cells to ROR-expressing cells (182) and by a novel anti-ROR1 single-chain (sc) antibody able to induce apoptotic death of CLL lines and primary CLL cells (186).

Notch-1

Aberrant signaling of this Notch family transmembrane receptor has been implicated in cancer, cancer stem cells, and tumor vasculature (187). Indeed, Notch1 is a well-characterized oncoprotein in T-ALL and lymphomas where activating Notch1 mutations are responsible for approximately 60% of T-ALL cases (188, 189). In preclinical models, blocking the extracellular region of Notch1 with antibodies decreased T-ALL tumor growth by inhibiting cancer cell growth and by disrupting angiogenesis (190, 191). Brontictuzumab (OMP-52M51) is the only anti-Notch-1 antibody tested in clinical settings (NCT01703572). Although completed, the results from this study have not been published and development in hematological cancers was discontinued. Severe grade adverse events could explain this, as Notch-1 inhibition causes gastrointestinal side effects (190, 191). Finally, antibodies against DLL4, a ligand of Notch-1, are alternatives to target this pathway, including OMP-21M18 or MEDI0639. Already tested *in vitro* and *in vivo* in solid tumors, they are currently under evaluation in several ongoing clinical trials. Nonetheless, no development has been reported for blood cancers (192).

EphrinA3 (EPHA3)

This member of the ephrin subfamily of receptor protein-tyrosine kinases can be considered an oncofetal Ag since it is not expressed in normal healthy adult tissues but is overexpressed by a variety of tumor types instead, including most hematological malignancies (193), where it plays an important role in tumor cell proliferation. Ifabotuzumab (KB004) is a humanized, non-fucosylated IgG1 mAb targeting EphrinA3 which induces apoptosis and stimulates ADCC (193). One phase I-II study (NCT01211691) tested for the utility of KB004 as a single agent in patients with heavily pretreated AML. KB004 was well tolerated but the efficacy was very limited with responses observed in patients with fibrotic myeloid diseases (194). In this study, it was postulated that low expression of EPHA3 in various myeloid leukemic cell subsets or the ability of KB004 to be internalized upon Ag binding are likely explanations for KB004 ineffectiveness (194). Based on this ability to be internalized, an alternative approach targeting EPHA3 with an immunoconjugate was proposed (195).

HLA-DR

Ligation of HLA-DR by antibodies is one of the oldest approaches to eliminate hematological tumors, since most of them express

high levels of this MHC class II molecule (196). Anti-HLA-DR antibodies with different MOA such as apolizumab, Lym-1, and 1D09C3 eventually had no convincing clinical response in several clinical trials and were discontinued (197). In addition, anti-HLA-DR mAbs are potent inducers of complement activation, which plays a pivotal role in the pathogenesis of mAb infusion side effects (197). To our knowledge, there is only one ongoing phase I study recruiting patients to test the safety and efficacy of an anti-HLA-DR antibody called IMMU-114 in B-cell disorders (NCT01728207). This drug is a humanized IgG4 form of murine anti-human HLA-DR mAb, L243, which recognizes a conformational epitope in the alpha chain of HLA-DR. Due to safety concerns related to the expression of HLA-DR in non-tumor cells, IMMU-114 was specifically generated to kill tumor cells avoiding CDC or ADCC (198). IMMU-114 binding to tumor B-cells results in antiproliferative effects and apoptosis and has demonstrated efficacy in preclinical models (198). Although the exact mechanism has yet to be fully elucidated, it appears to induce hyperactivation of ERK- and JNK-dependent mitogen activated protein kinase signaling pathways that may lead to mitochondrial membrane depolarization and reactive oxygen species generation. This eventually leads to an induction of tumor cell apoptosis and a reduction in tumor cell proliferation (198).

ANTIBODIES TARGETING CHEMOKINE RECEPTORS

Chemokines are small chemotactic cytokines that bind to specific surface seven transmembrane domain G protein-coupled receptors, or CKRs. Upon binding of their ligands, CKRs promote cell survival, proliferation, and adhesion, contributing to mammalian development and organogenesis, thus playing a central role in homeostasis and the maintenance of innate and acquired immunity (199). In cancer, CKRs may associate with tumor cells facilitating their survival, proliferation, and metastasis (200, 201). Moreover, they may also promote an immunotolerant milieu by recruiting Treg, tumor-associated macrophages (TAMs) or MDSCs that opens the way to tumor growth, angiogenesis, and immune evasion (202–205). For all these reasons, tumor-associated CKRs are considered suitable targets for cancer therapy (206). Nevertheless, generating antibodies against these Ags is particularly challenging due to, among other reasons, a complex and unstable native conformation (206, 207). So far, few anti-CKRs are under study in preclinical or early clinical phases and only one has been approved for clinical use (208).

C-C-Motif-Chemokine Receptor 4 (CCR4)

Under homeostasis, this receptor and its ligands, the chemokines CCL17 and CCL22, predominantly contribute to the biology of Th2, Th17, Treg, and skin-homing T-cells positive for cutaneous lymphocyte antigen (CLA) (209–211). In addition, CCR4 has been implicated in the pathogenesis of inflammatory diseases and cancer, being overexpressed in several T-cell disorders including adult T-cell leukemia-lymphoma (ATL), peripheral T-cell lymphoma (PTCL), and cutaneous T-cell lymphoma (CTCL) (212–214).

In cancer therapy, mogamulizumab (KW-0761) is the first approved and clinically tested antibody against a CKR and, in addition, the first glycoengineered antibody to be marketed. This IgG1 antibody is directed to the N-terminal region of human CCR4. Despite this, it does not block the interaction between CCR4 and its ligands, thereby not interfering with CCR4-mediated protumor functions or migration (215, 216). Moreover, it does not bind complement molecules either. Nevertheless, its Fc was selectively defucosylated to reach a potent ADCC *via* high-affinity binding to the FcγRIIIa on effector cells (215, 217). As a result, phase I and II clinical trials investigating mogamulizumab in T-cell malignancies demonstrated its effectiveness and led to the approval for use in Japan for rrATL in 2012 and rrCTCL in 2014 (208, 218). Given the safety and efficacy of mogamulizumab, different clinical studies are underway for T-cell disorders (208, 219). In addition, based on preclinical evidence, studies are being conducted to establish whether other diseases could be targeted by mogamulizumab therapy, including certain NK-cell lymphoproliferative disorders (220) and Hodgkin's lymphoma (HL) (221). Interestingly, in HL, the majority of the cells in TME are TAMs, Tregs, and CD4⁺ Th2 cells recruited by chemokines secreted by tumor cells such as CCL17 (222). This infiltrate probably enables tumors to escape from immune surveillance. Therefore, it is conceivable that targeting CCR4-positive cells in HL niche might revert this immunosuppressive environment enhancing the antitumor immunity. Indeed, in CTCL patients, a single dose of mogamulizumab decreased the fraction of CCR4-positive malignant T-cells, with a concomitant reduction of CCR4⁺ Tregs (223). Notably, similar effects on Treg subsets were observed in melanoma patients (224). All together, these results prompted phase I-II clinical studies in solid tumors not expressing CCR4 in order to evaluate the potential of mogamulizumab as immunomodulatory drug. Finally, the lack of neutralization of CCR4-ligands interaction by mogamulizumab leaves room to novel mAbs able to target this interaction. In preclinical phases, mAb1567 and its high-affinity variant (mAb2-3) were able to abolish CCR4-mediated chemotaxis of malignant cells and Tregs (225, 226). Moreover, *in vitro* studies confirmed that both antibodies mediated CDC and ADCC (225, 227), whereas the derivative mAb2-3 affected Treg functions and survival by means of CD25 shedding (226).

C-X-C-Motif-Chemokine Receptor 4 (CXCR4)

This CKR and its chemokine CXCL12 (or stromal cell-derived factor 1α) regulate hematopoietic development, lymphoid tissue architecture, and hematopoietic cell trafficking. Additionally, this couple controls organogenesis and development in several tissues (228, 229), hence CXCR4 is not surprisingly overexpressed in more than 23 different human cancers and has been demonstrated to be particularly relevant in B-cell malignancies like B-ALL, CLL, or MM (230, 231), T-cell malignancies such as T-ALL (232), and myeloid malignancies like AML (233). In these conditions, CXCR4 causes tumor cell trafficking and homing into lymphoid and non-lymphoid tissues where CXCL12 is produced. Here, the couple CXCR4/CXCL12 keeps leukemic cells

in close contact with stromal cells and extracellular matrix that together provide growth-promoting and antiapoptotic signals which facilitate resistance to chemotherapy and disease relapse (234–239). All together, these data strongly indicate that therapeutic strategies targeting the CXCL12–CXCR4 axis represent an attractive investigative approach to disrupt the leukemia–stromal interaction.

The first anti-CXCR4 clinically tested was ulocuplumab (BMS-936564), an IgG4 that blocks CXCL12 binding to its receptor thereby inhibiting CXCL12-induced migration and calcium flux (240). In this context, ulocuplumab is comparable to AMD3100 (Plerixafor-Mozobil), a small molecule CXCR4 inhibitor. However, ulocuplumab, but not AMD3100, induces caspase-independent apoptosis on a panel of cell lines and primary samples from AML, CLL, and MM patients (240–242). Both mechanisms contribute to the efficacy of ulocuplumab as monotherapy observed in xenograft models of the aforementioned diseases (240). The first clinical report on ulocuplumab suggests safe and significant antileukemia activity in AML patients, achieving fairly respectable complete remissions (CR/CRI) of 51%, and, notably in four patients, CR/CRI was documented after a single dose of ulocuplumab monotherapy (NCT01120457) (243). Results on other conditions are not available yet. Another IgG4 targeting CXCR4 is LY2624587, a humanized antibody deeply modified to eliminate half-antibody exchange associated with human IgG4 isotypes (244). Similar to ulocuplumab, LY2624587 inhibits CXCL12 binding to CXCR4 thus abrogating CXCR4-mediated survival and migration. The first clinical trial of LY2624587 (NCT01139788) was completed on 2011; however, results have not yet been published. Besides IgG4 isotypes, novel anti-CXCR4 antibodies with IgG1 isotype are demonstrating to be effective in preclinical phases. This is the case of hz515H7 (245) or PF-06747143. The latter was the first anti-CXCR4 mAb with an IgG1 scaffold to be evaluated in humans (NCT02954653), specifically in AML patients (246, 247). Like IgG4 formats, IgG1 antibodies are antagonist that block tumor cell chemotaxis toward CXCL12 and induce tumor cell apoptosis in either presence or absence of stromal cells (245, 246). In contrast, IgG1 isotypes trigger potent ADCC and CDC, which are involved in the antitumor effect observed in AML and CLL models as monotherapy or in combination with standard therapy (245–247). Currently, there is evidence suggesting that anti-CXCR4-IgG4 antibodies are generally safe although they induce short-term toxicity affecting the process of normal hematopoiesis with the result of myelosuppression, or a deleterious effect on immune cells where CXCR4 is widely expressed (243). In addition, the off-target adverse event of hyperleukocytosis was reported in a number of patients. Finally, owing to the ubiquitous expression of CXCR4, long-term effects should be carefully evaluated, even more with the upcoming IgG1 molecules as they may trigger off-target ADCC or CDC.

CCR2 and Others

CCR2 is another CKR targeted by an antibody under clinical development. Plozalizumab (MLN-1202) is a neutralizing antibody that showed a positive effect in phase II for the treatment of bone metastases (NCT01015560) (206). Interestingly, recent

preclinical evidence suggests that targeting CCR2 may be effective in the setting of AML (248) and MM (249). MM cells from patients with bone lesions overexpress CCR2, while osteoclasts secrete chemokines that act as growth factors for tumor cells. In this scenario, targeting CCR2 could reduce MM cells survival and prevent drug resistance similar to CXCR4 antagonism (249). Many other CKRs with pathogenic role in hematological malignancies were preclinically validated as good targets for mAb-based therapy. This includes antibodies against CCR7 (34, 250) and CCR9 (251).

Recent evidence on a CXCL12-neutralizing RNA oligonucleotide reveals that targeting the chemokine instead of the CKR may interfere with CXCR4-mediated drug resistance in CLL and MM (252). These data support a rationale for clinical development of mAbs targeting chemokines instead of their corresponding receptors. However, to date no mAb targeting chemokines has been included in a clinical trial for cancer therapy. There are two plausible explanations. First, targeting chemokines does not activate the host immune response against tumor cells. Second, a cell surface-restricted receptor is more efficiently targeted than delocalized secreted chemokines (206, 207). Moreover, a recent study in cynomolgus monkeys demonstrated that targeting the chemokine CCL21 with a novel mAb (QBP359) requires impractical large doses and frequent administration to maintain suppression of CCL21 in the clinical setting. In other words, it is difficult to target soluble proteins with high synthesis rates, a common characteristic to many chemokines (253).

ANTIBODIES TARGETING THE TUMOR NICHE

Malignant cells are surrounded by different types of leukocytes and stromal cells that compose an extremely relevant source of soluble factors and adhesion molecules that promote tumor progression and escape from conventional treatments (254, 255). We have already referred in a separate section how clinical antibodies neutralize CKRs and their protumorigenic activities in the TME. Below we review other approaches to disrupt the tumor niche, including: (i) neutralizing soluble survival/growth factors (mainly cytokines) or their associated receptors, all of them validated and valuable targets of antibody-based therapies of immunological disorders (256); (ii) blocking adhesion molecules that lodge tumor cells to their protective niche (257, 258); and (iii) blocking angiogenesis, an important process during development and vascular remodeling (259) that feeds tumor growth and progression (259, 260).

Soluble Factors and Associated Receptors

B-Cell Activating Factor (BAFF) and A Proliferation Inducing Ligand (APRIL)

These TNF-family members are produced as type II transmembrane proteins that are then proteolytically cleaved and released in soluble form (261). BAFF and APRIL are produced by a variety of hematopoietic and non hematopoietic cells including stromal microvascular endothelial cells and osteoclasts. Both factors share two receptors: transmembrane activator and cyclophilin

ligand interactor (TACI) and B-cell maturation antigen (BCMA; CD269). Additionally, BAFF binds strongly to BAFF receptor (BAFF-R) (261). These receptors have distinct expression patterns and mediate separate functions. BAFF-R is absent on B-cell precursors but is gained on immature B-cells upon acquiring a functional BCR, which is critical for survival and maturation of immature B-cells. TACI is expressed on memory B-cells and is necessary for T-independent responses and promotion of class switch recombination in B-cells. Last, BCMA expression is restricted to plasmablasts and plasma cells and promotes their long-lived survival (261–263).

BAFF and APRIL are particularly relevant in MM, where BCMA and both soluble factors are augmented in samples from patients compared to healthy donors, and ligand–receptor interactions lead to increased survival of malignant cells (264–267). Moreover, higher concentrations of APRIL may promote resistance to lenalidomide, bortezomib and other standard-of-care drugs, and also may drive expression of programmed cell death ligand 1 PD-L1, interleukin (IL)-10, and TGF β on BCMA $^+$ tumor cells creating an immunosuppressive niche that favors tumor cells (266). In recent years, compelling evidence has suggested that neutralizing APRIL or BAFF could diminish MM cell survival, revert the immunosuppressive phenotype on BCMA $^+$ cells and reduce resistance of malignant cells to treatment, regardless of the presence of protective stromal cells (266, 268–270). Tabalumab (LY2127399), a human IgG4 mAb that neutralizes membrane-bound and soluble BAFF, was entered in clinical trials after demonstrating both antitumor activity and osteoclastogenesis inhibition in xenograft models of MM (270). Results in two different studies showed limited efficacy of tabalumab in combination with standard-of-care drugs (NCT00689507, NCT01602224) (271, 272). In the near future, new molecules will burst into the field such as BION-1301, an anti-APRIL neutralizing mAb able to fully suppress *in vitro* APRIL-induced B-cell IgA and IgG class switching (273).

Other antibodies block BAFF-R, including VAY736 and belimumab, or are aimed to deliver payloads to tumor cells expressing BCMA as exemplified by two novel immunoconjugates in clinical studies: AMG 224 and GSK2857916 (Table 6). Notably, GSK2857916 acts through multiple mechanisms. It specifically blocks cell growth via G2/M arrest, induces caspase 3-dependent apoptosis in MM cells, and strongly inhibits colony formation by MM cells. Furthermore, GSK2857916 recruits macrophages and mediates ADCP of MM cells (274). Finally, BI836909 and JNJ-6400795 are the first MM cell-specific BiTEs in development, and both target BCMA/CD3 (275). Clinical studies to evaluate safety and efficacy of both BiTEs are still recruiting MM patients (NCT02514239 and NCT03145181).

Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)

This soluble member of the TNF family is a key mediator in the pathogenesis of a broad range of skeletal diseases since it binds to RANK on preosteoclasts and mature osteoclasts which are involved in bone resorption (276). In particular, malignant plasma cells produce RANKL leading to an imbalance between

bone formation and resorption, local bone lysis and the development of osteolytic lesions in MM patients (277, 278). Denosumab is a human IgG2 antibody that binds to the soluble and cell membrane-bound forms of RANKL thus preventing RANK-mediated differentiation, activation, and survival of osteoclasts. As a consequence, bone resorption and bone destruction are reduced (279). As expected, and based on its MOA, denosumab is not effective reducing tumor burden neither improving responses in MM nor B-NHL. But denosumab does inhibit RANKL regardless of previous exposure to bisphosphonates, standard-of-care drug in bone lesions (280) and delays the time to the first on-study skeletal-related event with similar results to zoledronic acid, another standard-of-care treatment (NCT00330759) (281). Similar to IgG1, denosumab is a big molecule that, contrary to bisphosphonates, is not cleared by the kidneys. Therefore, current investigation (NCT02833610) is evaluating whether denosumab could fill the unmet need for bone-targeted therapies in MM patients with renal insufficiency (approximately 25–50% of all patients) (282). Finally, denosumab has demonstrated efficacy at solving bisphosphonate-refractory hypercalcemia in hematological cancers (NCT00896454) (283). Despite the current US FDA-approved label for denosumab it does not include MM nor NHL, this situation may be reverted depending on the forthcoming results from these studies.

Interleukin-6

IL-6 is a pleiotropic cytokine with a critical role in the pathogenesis of MM and B-NHL by promoting tumor cell growth and interfering with chemotherapy drugs (284). Different mAbs against IL-6 or its soluble receptor IL-6R have been developed, with the two most promising being the chimeric siltuximab (CNTO 328) that neutralizes the cytokine, and tocilizumab that blocks the receptor (285). Siltuximab, was recently registered for multicentric Castleman's disease and evaluated as a single agent or in combination with other agents in advanced MM and B-NHL (particularly CLL). Again, mAb-based strategies targeting soluble factors produce discouraging results in hematological malignancies. In addition, results are modest probably due to the complex interaction between malignant clones, inflammatory background and host response (NCT00412321, NCT00911859, NCT00401843) (286, 287). However, new investigations aim to uncover the application of siltuximab in the treatment of Waldenström macroglobulinemia and the early phase of smoldering MM (285). Moreover, one trial is exploring the utility of blocking the IL-6R in CLL (NCT02336048).

IL-3 Receptor Alpha Chain (IL-3R α ; CD123)

Interleukin-3 stimulates cell cycle progression in early hematopoietic progenitors and enhances the differentiation of various hematopoietic cells while inhibiting apoptosis (288). IL-3R α is a novel molecular target that has emerged as a highly specific entity for CML, AML blasts, and LSCs (289–291). Notably, normal hematopoietic stem cells have limited expression of CD123 (289, 292). One of the first humanized anti-IL-3R α antibodies tested in clinical trials was CSL360, a chimeric IgG1 molecule that achieved an improvement in blasts and LSCs percentage in bone marrow, but no clinical responses in high-risk rrAML

(NCT00401739) (293). These results showed that the blockade of IL-3R α alone was ineffective, leading to the development of second-generation molecules able to kill IL-3R α -positive tumor cells by means of immune effector mechanisms. The one in most advanced stages is talacotuzumab (JNJ-56022473/CSL362), an Fc-engineered derivative from CSL360 which is undergoing phase II–III studies for rrAML (NCT02992860, NCT02472145). Talacotuzumab induces potent *in vitro* ADCC against IL-3R α -expressing AML blasts/LSC and reduces leukemic cell growth in murine xenograft models of human AML (294). In addition, talacotuzumab inhibits IL-3-stimulated rescue of tyrosine kinases inhibitors (TKIs)-induced cell death, demonstrating that resistance to previous standard-of-care could be reverted (295). Actually, the combination of TKI therapy and talacotuzumab may eliminate leukemic cells *in vivo* more effectively than TKI treatment alone (296). Another second-generation mAb with similar MOA is XmAb14045 that will be tested early in a phase I study (NCT02730312). With different MOA, KHK2823 is a novel non-fucosylated fully human IgG1 mAb which mediates ADCC without inducing CDC. Its safety and efficacy is under evaluation in a phase I study (NCT02181699).

Other approaches targeting CD123 are based on bi-specific platforms. Examples are JNJ-63709178 (NCT02715011), a humanized CD123xCD3 DuoBody and flotetuzumab (NCT02152956), a CD123xCD3 bi-specific antibody-based molecular construct named dual affinity retargeting (DART) molecule. Both bispecific antibodies are effective *in vitro* and *in vivo* in preclinical settings (297). Nevertheless, recent evidence on human CD123-directed T-cells (CAR-T123) shows some concerns regarding toxicity related to off-target events (298), suggesting the possibility that the same effects could be found with the redirection of T-cells by bi-specific antibodies. Finally, owing to CD123 internalization upon mAb binding, novel immunoconjugates aim to target CD123 (**Table 6**).

IL-2 Receptor Alpha Chain (IL-2R α ; CD25)

Commonly expressed by activated T- and B-cells, some thymocytes and myeloid precursors, IL2-R α is also found in most of the malignancies corresponding to such lineages, particularly in ATL where IL2-R α functions as the receptor for human T-cell leukemia virus 1 (HTLV-1) (299). Few mAbs have been developed for T-cell neoplasia. One of them, the chimeric IgG1 basiliximab selectively blocks IL-2R α , thereby preventing IL-2-mediated activation of lymphocytes. Nevertheless, it lacked of activity in patients (299). Another anti-IL-2R α is daclizumab, a humanized antibody which shows potential in T-cell disorders and HL, although its activity needs to be confirmed in a big cohort of patients (300, 301). A likely explanation for the modest results of anti-IL-2R therapy is related to the pharmacokinetics/pharmacodynamics of daclizumab. Indeed, a phase I–II study suggested that higher doses than previously used may be required to achieve clinical responses since high doses were needed to saturate targets in extravascular sites (301). Low activity in phase I–II was also documented for LMB-2, an immunotoxin comprised of the Fv of an anti-CD25 mAb connected to an exotoxin (302). The limited efficacy of naked or toxin-conjugated antibodies has led to the conjugation of basiliximab and daclizumab with radionuclides.

Currently, both molecules are under evaluation. Interestingly, in HL daclizumab linked to radionuclides shows efficacy in patients with tumor cells expressing IL-2R, and in patients whose tumor cells lacking the receptor, suggesting off-target effects on accessory cells (303). Based on the same rationale, both basiliximab and daclizumab are being explored as adjuvant therapy to eliminate IL-2-R α -positive Tregs in MM or to eliminate IL-2-R α -positive naive T-cells to prevent the development of graft-versus host disease.

Type I Insulin-Like Growth Factor Receptor (IGF-1R; CD221)

This ubiquitously expressed tetramer binds insulin growth factor 1 (IGF-1) to activate multiple signaling pathways involved in cell growth, differentiation, migration, and cell survival (304). IGF-1R also mediates anchorage-independent growth and survival, and migration, thus facilitating tumor establishment and progression (305–307). IGF-1R has been widely studied in hematological tumors where a pathogenic role has been found, among others, for myeloid leukemias (308), and several B-cell malignancies (304, 309) but an exceptional role has been uncovered for MM (310). Therefore, the therapeutic potential of IGF-1R has been explored in MM with three different mAbs that directly block IGF-1R: dalotuzumab, AVE1642, and fitigatumab. All of them prevent the binding of IGF-1 and the subsequent activation of PI3K/AKT signal transduction, nonetheless results derived from clinical studies were disappointing (310). In phase I studies, only dalotuzumab showed an evaluable antimyeloma activity (NCT00701103) (311). In contrast, AVE1642 (NCT01233895) and fitigatumab (NCT01536145) did not result in significant improvement as single agents or in combination with standard-of-care drugs (312, 313). Hence, no further evaluation of these mAbs in MM patients is currently ongoing and the development of dalotuzumab in MM was consequently also discontinued. These discouraging results could be explained by the emergence of tumor cell independence from their microenvironment due to intraclonal heterogeneity, the involvement of other growth factors and the existence of hybrid receptors composed of IGF-1R and 2R that can be activated by all IGF ligands (314). Anti-IGF-1R mAbs are unable to neutralize these hybrid receptors. Moreover, it is thought that circulating IGF-1R can interact with the IGF-1R targeting antibodies and prevent their interaction with the IGF-1R on cancer cells (310).

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF; CSF2)

This cytokine is a monomeric glycoprotein secreted by immune cells, endothelial cells and fibroblasts. Overall, GM-CSF participates in the development of innate immune cells since it stimulates stem cells to produce granulocytes and monocytes (315). GM-CSF is also involved in the pathogenesis of chronic myelomonocytic leukemia (CMML), where progenitor expansion through STAT5 signaling is mediated (316). Based on this, lenzilumab (KB003), a humanized antibody formerly developed for inflammation, is being investigated as single agent in CMML (NCT02546284) (317). In primary samples from CMML patients, lentizumab bound the cytokine and directly interrupted binding

to its cognate receptor inducing apoptosis *in vitro* (318). However, the curative application in humans is uncertain as some CD34-positive subsets, including the LSCs, seem to be insensitive to GM-CSF signaling (318). Results from the ongoing clinical study will shed some light on the subject.

Hepatocyte Growth Factor (HGF)

This soluble factor is the only known ligand for the c-Met receptor tyrosine kinase that when bound to HGF activates key oncogenic signaling pathways that increase cell proliferation, survival, migration and invasion in several human cancers, including MM where HGF expression predicts poor prognosis (319). Ficlatuzumab (AV-299) is a potent HGF-neutralizing mAb able to interrupt HGF/c-Met interaction thus inhibiting c-Met-induced phosphorylation, cell proliferation, cell invasion and cell migration. With proven efficacy in solid tumors (320), a phase I study aimed to examine its efficacy in MM and NHL (NCT00725634), although preliminary results indicate that clinical effects are only seen in MM (321).

Adhesion Molecules

CD44

This cell-surface glycoprotein is a receptor for hyaluronic acid (HA), osteopontin, collagen, and matrix metalloproteases, which are typically found in the microenvironment of BM and lymphoid tissues (257). CD44 is particularly expressed by AML-LSCs and CLL cells which take advantage of HA-CD44 signaling to promote leukemic survival *via* PI3K/AKT and MAPK/ERK pathways (257, 322). Since AML-LSCs are more dependent on CD44 for their anchoring in the BM niche than their normal counterparts, CD44 is an exciting target to mobilize leukemic cells out of their protective niche (257). A novel humanized neutralizing mAb, RG7356 (RO5429083), has recently been evaluated alone or in combination with cytarabine in a phase I trial in rrAML patients (NCT01641250). RG7356 does not activate effector cells or complement. Very limited activity was observed in this study although the mAb was well tolerated (323). The ability of CD44 to complex with different partners, overcoming the neutralization mediated by the antibody, may explain this outcome. In CLL, the expression of CD44, in cooperation with VLA-4 and MMP9, helps in creating a protective TME within the lymphoid organs that circumvents spontaneous and drug-induced apoptosis in CLL cells (324). In preclinical models of CLL, RG7356 provoked apoptosis of CLL cells in a caspase-dependent manner and regardless of the presence of protective co-cultured stromal cells or HA, or even regardless of BCR signaling (325). These results indicate that RG7356 might have therapeutic activity in CLL patients.

Very Late Antigen 4 (VLA4; CD49d)

This molecule is the α -chain of the $\alpha 4\beta 1$ integrin heterodimer which is normally expressed on monocytes and lymphocytes cell surface (326, 327). VLA-4 is involved in the firm adhesion step during the extravasation process, mediating the binding to fibronectin or to vascular cell adhesion molecule-1 (VCAM-1) located on the surface of endothelial cells (326, 327). In several hematological malignancies CD49d is considered as one of the

main players at the TME as it mediates both cell–cell and cell–matrix interactions delivering prosurvival signals and protecting tumor cells from drug-induced damage (258, 328–330). Despite this, no anti-VLA4 antibodies are under development for blood cancers. In this context, the recombinant IgG4 anti-CD49d antibody natalizumab, which is an FDA-approved drug for relapsing multiple sclerosis, has demonstrated the potential to revert chemo-sensitivity and to inhibit both *in vitro* and *in vivo* adhesion of MM cells to non-cellular and cellular components of the TME. It also has the potential to arrest tumor growth in a xenograft model of MM (329). Unfortunately, a trial evaluating natalizumab as a single agent in MM patients was terminated due to low enrollment (NCT00675428). Natalizumab is also able to restore drug sensitivity in B-cell lymphomas (328) and primary ALL (330) providing the rationale for the clinical evaluation of natalizumab in many hematological tumors, preferably in combination with novel agents to enhance tumor cell cytotoxicity and improve patient outcome (329). Nevertheless, it should be taken into account that natalizumab treatment is associated with appearance of progressive multifocal leukoencephalopathy which may, in turn, dissuade this approach in hematological malignancies (331).

Angiogenesis

Vascular and Endothelial Growth Factor (VEGF)

This molecule is one of the most important mediators of neoangiogenesis and tumor growth (332). Out of the five members of the VEGF family described to date, VEGF-A and its receptor VEGFR-2 are the main targets of current antiangiogenic agents (332). Bevacizumab is an IgG1 antibody that binds to all isoforms of VEGF-A preventing the interaction with its receptors and their subsequent activation (333). In solid tumor, this antibody promotes a regression of immature tumor vasculature, normalization of remaining tumor vasculature and inhibition of further tumor angiogenesis (334). In hematological malignancies, bevacizumab has been tested as a tool to solve resistances to previous chemotherapies. In myeloid malignancies, bevacizumab has not worked as monotherapy or combined with standard therapies (335). In CLL, preclinical studies demonstrated that bevacizumab was a proapoptotic and antiangiogenic drug (336, 337). Despite having no significant clinical activity as monotherapy (NCT00290810) (338), in combination with chemotherapy regimens the results had a better outcome (339). In FL, a phase II study (NCT00193492) revealed that a combination of rituximab with bevacizumab significantly extended progression free survival, although bevacizumab increased the toxicity as well (340). Encouraged by these works, bevacizumab has been used as adjuvant therapy in many other B-cell disorders, where addition of the anti-VEGF did not show improvement of the therapeutic responses (341, 342). It is tempting to assume that anti-VEGF in combination with other chemoimmunotherapies is a promising therapy for CLL and FL patients, but a close follow-up is recommended to ascertain the potential toxicities, including left ventricular dysfunction and heart failure, observed in many of the cited studies.

Endosialin (CD248; TEM1)

This glycoprotein is selectively expressed in vascular endothelial cells of malignant tumors (343). Targeting endosialin showed

antitumor activity in different preclinical models where endosialin function was suppressed with the antiangiogenic antibody MORAB-004 (ontuzumab) (343). Although the MOA of this drug is not completely understood, it was suggested that endosialin is removed from the cell surface by means of MORAB-004-mediated internalization. MORAB-004 could affect cellular signaling as well as protein–protein interactions that serve to communicate signals in the TME between tumor and stromal cells. Additional work is under way to further establish the exact mechanism of action of MORAB-004 (343). MORAB-004 antitumor activity has been observed in several phase I studies on solid tumors and phase II studies have recently started. Despite the role of endosialin in blood cancers is not fully understood, patients with different blood disorders have been enrolled in a phase I study (NCT01748721), although no results have been published (344).

ANTIBODIES TARGETING IMMUNE CHECKPOINTS

Antitumor innate and adaptive immune responses rely on cell-activation and cell-exhaustion balances which are regulated by stimulatory or inhibitory molecules most belonging to the B7-CD28 and the TNF-TNFR superfamilies (345). Due to their function modulating immune responses, these NLSAs are commonly known as “immune checkpoints.” Although checkpoint targeting with specific antibodies is a relatively new area (346), their accessibility to cell membranes and significance in regulating immune responses made them a very attractive therapy option, as exemplified by the plethora of novel agents that have been already approved or are under intensive studies in solid tumors. In Table 4, an account of the landscape of immune-checkpoint regulators in hematological malignancies is provided. Commonly, immune-checkpoints do not target tumor cells directly, but instead act on lymphocytes to boost their endogenous antitumor activity reversing tumor immune escape (347, 348). It is not our intention to review these types of molecules. Instead, in the next section, we will analyze a second-generation of immunomodulatory antibodies targeting receptors expressed in both tumor and immune cells. These antibodies are armed with a dual MOA combining direct tumoricidal properties with the ability to restore host antitumor immunity.

PD-L1 (B7-H1; CD274)

Programmed death-1 (PD-1) is an inhibitory receptor member of the B7 receptor family with a significant role in immune regulation (349). PD-1 is upregulated on activated T-cells, NK cells, NKT cells, and B-cells among other leukocytes (349–351). In many types of cancers, PD-1 engagement may represent one means by which tumors evade immune surveillance and clearance (349). Cancer cells express PD-L1, a PD-1 ligand that upon binding to PD-1 on tumor-infiltrating lymphocytes (TILs) leads to impairment of antitumor responses through multiple mechanisms including inhibition of T-cell activation and proliferation (352, 353) and increase in T-cell apoptosis

TABLE 4 | Characteristics of antibodies directed to immune checkpoint receptors.

Target/expression	mAb (commercial name/orIGINator)	IgG class	MOA	Active indications in HMs (highest phase)	References
Inhibitory receptors					
Programmed death-1 (PD-1)/T-cells, NK cells, NKT cells, Treg and B-cells	Nivolumab, BMS-936558, MDX-1106 (<i>Opdivo/Medarex; Ono Pharmaceutical</i>)	Fh IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	Approved (HL) III (MM/NCT02726581 ^a R) II-III (AML/NCT02275533 ^b R) II (CLL/NCT 02420912 ^a R) II (DLBCL/NCT02038933 ^a ANR) II (FL/NCT02038946 ^a ANR) I-II (B-NHL, T-NHL/NCT02985554 ^b R) I (CML/NCT02011945 ^a ANR) I (FL/NCT03245021 ^a NYR) I (B-ALL/NCT02819804 ^a R)	(83-88)
Pembrolizumab, MK-3475 (<i>Keytruda/ Merck & Co; The Leukemia & Lymphoma Society</i>)	Hz IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	III (HL/NCT02684292 ^b R) II-III (MM/NCT02906332 ^a ANR) II (DLBCL/NCT02362997 ^a R) II (FL/NCT02446457 ^a R) II (T-NHL, NK-L/NCT03021057 ^b ANR) II (ALL/NCT02767934 ^b R) II (AML/NCT02768792 ^b R) II (MF/SS/NCT02243579 ^b ANR) II (CLL/NCT02332980 ^a R) I-II (MCL/NCT03153202 ^a R)		
Pidilizumab; CT-011, MDV9300 (CureTech)	Hz IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	II (MM/NCT01067287 ^a ANR) II (FL/NCT00904722 ^a C) II (DLBCL/NCT02530125 ^a ANR) II (AML/NCT01096602 ^a ANR) I-II (MM/NCT02077959 ^a ANR)		
MEDI0680, AMP-514 (Amplimmune)	Hz IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	I-II (B-NHL, DLBCL/NCT02271945 ^a C)		
REGN2810 (Regeneron Pharmaceuticals)	Fh IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	I-II (B-NHL, HL/NCT02651662 ^a R) I-II (MM/NCT03194867 ^a NYR)		
PDR001 (Novartis)	Hz IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	II (DLBCL/NCT03207867 ^a NYR) I (AML/NCT03066648 ^a R) I (MM/NCT03111992 ^a R)		
BGB-A317 (BeiGene)	Hz IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	II (HL/NCT03209973 ^b R) I (B-NHL/NCT02795182 ^a R)		
SHR-1210 (Jiangsu Hengrui Medicine Co.)	Fh IgG4	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	II (HL/NCT03155425 ^b NYR)		
Js001 (Shanghai Junshi Biosciences)	Hz mAb	Blocks binding of PD-1 to PD-L1 and PD-L2 thus enhancing anti-tumor immunity.	I (Lymphoma/NCT02836834 ^b R)		

(Continued)

TABLE 4 | Continued

Target/expression	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)	References
PD-L1 (CD274 or B7-H1)/tumor cells	Atezolizumab, MPDL-3280A, RG7446 (Tecentriq/Genentech)	Hz IgG1 Glyco-Fc	Blocks PD-L1/PD-1 interaction thus enhancing antitumor immunity.	II (HL/NCT03120676 ^b R) II (CLL/NCT02846623 ^b R) I-II (DLBCL/NCT02926833 ^b R) I-II (AML/NCT02935361 ^a R) I-II (CML/NCT02935361 ^a R) I (FL/NCT02220842 ^a R) I (MM/NCT02784483 ^b R)	(89–91)
	BMS-936559, MDX-1105 (Medarex)	Fh IgG4	Blocks PD-L1/PD-1 interaction thus enhancing antitumor immunity.	I (HL, NHL, CML, MM/NCT01452334 ^b W)	
	Durvalumab, MEDI-4736 (Medimmune)	Fh IgG1 Glyco-Fc	Blocks PD-L1/PD-1 interaction thus enhancing antitumor immunity. ADCC	II (AML/NCT02775903 ^a R) II (DLBCL/NCT03003520 ^b R) II (MM/NCT03000452 ^b R) II (NK-T Lymphoma/NCT03054532 ^a NYR) I-II (CLL/NCT02733042 ^a R) I-II (NK-T Lymphoma/NCT02556463 ^a R) I-II (FL/NCT02401048 ^a ANR)	
	Avelumab, MSB0010718C (Bavencio/EMD Serono; Merck KGaA)	Fh IgG1	Blocks PD-L1/PD-1 interaction thus enhancing antitumor immunity.	III (DLBCL/NCT02951156 ^a R) II (T-NHL/NCT03046953 ^b NYR) I-II (AML/NCT02953561 ^a R) I-II (B-NHL/NCT03169790 ^a NYR) I (HL/NCT02603419 ^b R)	
CTLA-4 (Cytotoxic T lymphocyte-associated antigen 4, CD152)/T-cells, Treg, NK cells	Ipilimumab (Yervoy/Medarex)	Fh IgG1	Blocks the interaction of CTLA-4 with CD80/CD86 thus enhancing antitumor immunity.	II (AML/NCT02397720 ^a R) I-II (HL/NCT01896999 ^a R) I-II (MM/NCT02681302 ^b R) I (CML/NCT00732186 ^a W) I (B-NHL/NCT01729806 ^a ANR) I (B-ALL/NCT02879695 ^b R)	(83, 92, 93)
	Tremelimumab, CP-675,206 (Pfizer)	Fh IgG2	Blocks the interaction of CTLA-4 with CD80/CD86 thus enhancing antitumor immunity.	I-II (B-NHL/NCT02205333 ^a T) I (DLBCL/NCT02549651 ^a R) I (MM/NCT02716805 ^a R)	
KIR2D (killer inhibitory receptor 2D)/NK cells	Lirilumab, IPH2102 (Innate Pharma, Novo Nordisk)	Fh IgG4	Blocks the interaction of HLAC with KIR2D thus augmenting NK-cell activity.	II (AML/NCT01687387 ^b C) disc. II (CLL/NCT02481297 ^a ANR) I (MM/NCT02252263 ^a ANR)	(94, 95)
NKG2A (CD94)/NK cells, CTLs	Monalizumab, IPH2201 (Innate Pharma; Novo Nordisk)	Hz IgG4	Blocks the interaction of HLA-E with NKG2A thus augmenting NK cells and CTLs reactivity.	I-II (CLL/NCT02557516 ^a R) I (HMs/NCT02921685 ^a R)	(96, 97)
KIR3DL2 (killer inhibitory receptor 3DL2; CD158k)/Tumor cells	IPH4102 (University of Genoa/Innate Pharma)	Hz IgG1	ADCC ADCP Blocks KIR3DL2.	I (CTCL/NCT02593045 ^b R)	(98)
LAG3 (lymphocyte-activated gene-3, CD223)/Th cells, Treg	BMS-986016 (Bristol-Myers Squibb)	Fh IgG4	Blocks the binding of LAG3 to MHC-II thus decreasing tumor suppressive activity.	I/II (HL/NCT02061761 ^a R) I/II (DLBCL/NCT02061761 ^a R) I (CLL, MM/NCT02061761 ^b R)	(99)
TIM-3 (T-cell immunoglobulin- and mucin-domain-containing molecule 3)/T-cells, NK cells, monocytes	MBG453 (Novartis Pharmaceuticals)	Hz IgG4	Blocks TIM-3/Galectin-9 interaction thus enhancing Th1 responses and abrogating Treg suppressive functions.	I (AML/NCT03066648 ^a R)	(100)

(Continued)

TABLE 4 | Continued

Target/expression	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)	References
CD200 (OX-2)/Tumor cells	Samalizumab, ALXN6000 (Alexion Pharmaceuticals)	Hz IgG2/4	Blocks CD200/CD200R interactions, restoring CTLs functions and antitumor immunity.	I-II (AML/NCT03013998 ^a R) I-II (CLL and MM/NCT00648739 ^a C) disc.	(101)
Costimulatory receptors					
CD137 (4-1BB)/T-cells, Treg, DCs, NK cells, NKT cells	Urelumab, BMS-663513 (Medarex)	Fh IgG4	Mimicks activation of CD137 mediated by CD137L (4-1BBL) inducing CTLs and NK cells activation.	II (CLL/NCT02420938 ^a W) I-II (B-NHL/NCT02253992 ^a R) I (B-NHL/NCT01471210 ^b C) I (MM/NCT02252263 ^a ANR)	(102–104)
	Utomilumab, PF-05082566 (Pfizer)	Fh IgG2	Mimicks activation of CD137 mediated by CD137L (4-1BBL) inducing CTLs and NK cells activation.	III (DLBCL/NCT02951156 ^a R) I (FL/NCT01307267 ^a R)	
OX40 (CD134)/T-cells, Treg	MEDI6469 (AgonOx; Providence Cancer Center)	m IgG1	OX40 triggering stimulates T-cells and blocks/depletes Treg	I-II (B-cell lymphomas/NCT02205333 ^a T) Replaced by Tavolixizumab (MEDI0562)	(105)
CD27/T-cells, B-cells, NKs	Varlilumab, CDX-1127 (Celldex Therapeutics Inc.)	Fh IgG1	Mimicks CD27-CD70 interactions which accelerate NK-mediated tumor clearance while generating an adaptive immune response	II (DLBCL/NCT03038672 ^a NYR) I (B-NHL/T-NHL/NCT01460134 ^b ANR)	(106)
CD70/T-cells, B-cells, mDC, tumor cells	ARGX-110 (arGEN-X)	Hz IgG1 Glyco-Fc	ADCC ADCP CDC Blocks proliferation/survival of malignant cells. Inhibits activation/proliferation of CD27-positive T-reg.	I-II (AML/MDS/NCT03030612 ^a R)	(107, 108)
CD80 (B7-1)/APCs, tumor cells	Galiximab, IDEC-114 (Biogen Idec)	Pz IgG1	ADCC PCD Inhibition of CD80 signaling	III (FL/NCT00363636 ^a T, NCT00384150 ^a T) disc. II (B-NHL/NCT00516217 ^b C) disc.	(109, 110)
CD40/APCs tumor cells	Lucatumumab, CHIR-12.12, HCD-122 (Novartis; XOMA)	Fh IgG1	ADCC Antagonizes CD40L-mediated proliferation and survival	II (MM/NCT00231166 ^b C) disc. I-II (NCT00670592 ^a C) disc. I (CLL/NCT00108108 ^a T) disc. I (FL/NCT01275209 ^a C) disc.	(111–115)
	Dacetuzumab, SGN-40 (Seattle Genetics)	Hz IgG1	ADCC, ADCP Partial agonist that triggers both cellular proliferation and activation in APCs which subsequently activate B-cells and T-cells	II (DLBCL/NCT00435916 ^a C, NCT00529503 ^a C) I-II (CLL/NCT00283101 ^b C) I (MM/NCT00079716 ^b C, NCT00664898 ^a C)	
	SEA-CD40, SEA-1C10 (Seattle Genetics) Derived from dacetuzumab	Hz IgG1 Glyco-Fc	ADCC Agonist that triggers both cellular proliferation and activation in APCs which subsequently activate B-cells and T-cells	I (B-NHLs, HL/NCT02376699 ^a R)	
	ChiLob 7/4 (University of Southampton)	Ch IgG1	CDC, ADCC Agonist that triggers both cellular proliferation and activation in APCs which subsequently activate B-cells and T-cells	I (DLBCL/NCT01561911 ^b C)	

(Continued)

TABLE 4 | Continued

Target/Expression	mAb (commercial name/originator)	IgG class	MOA	Active indications in HMs (highest phase)	References
GITR (glucocorticoid-induced tumor necrosis factor receptor family-related gene)/NK cells, Th cells, CTLs, B-cells, APCs, Treg	GWN323 (Novartis)	Fh IgG1 ADCP	Agonist that induces signaling through GITR antagonizing Treg mediated suppression and enhancing Th, CTLs and NK cells proliferation and activation	I (B-cell lymphomas; NCT02740270 ^a R) (116, 117)	
CD47/phagocytes and DCs	Hu5F9-G4 (Stanford University) CC-90002, INBRX-103 (Inhibrx)	Hz IgG4 Hz IgG	Blocks CD47 Blocks CD47 ADCP?	I-II (B-NHL/NCT02953509 ^a R) I (AML/MDS/NCT02678338 ^a R, NCT03248479 ^b NYR) I (AML/MDS/NCT02641102 ^a R) I (B-NHL/NCT02367196 ^a R)	(118, 119)

^aAntibodies that reached clinical studies.

^bMonootherapy.

mAb, monoclonal antibody; MOA, mechanisms of action; HMs, hematological malignancies; Ch, human-mouse chimeric; Fh, fully human; Hz, humanized; P2, Human-primate chimeric; m, mouse; Glyco-Fc, glycoengineered Fc fragment; CDC, complement-dependent cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; PCD, programmed cell death; HL, Hodgkin's lymphoma; MM, multiple myeloma; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; B-NHL, B-cell non-Hodgkin's lymphoma; CML, chronic myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; NK-L, NK-cells lymphoma; MF, mycosis fungoïdes; SS, Sézary syndrome; MCL, mantle cell lymphoma; CTCL, cutaneous T-cell lymphoma; NCT, number of clinical trial (clinicaltrials.gov); C, completed; R, recruiting; T, terminated; ANR, active non-recruiting; NYR, not yet recruiting; S, suspended; W, withdrawn.

(349, 354). In addition, PD-L1 drives the differentiation of naive CD4⁺ T-cells into induced Tregs, which also express PD-1 on their cell surface, and maintain their suppressive function (355). The end result of the PD1 axis activation is T-cell exhaustion or anergy, dampening effector T-cell functions and leading to immune tolerance.

This situation prompted the development of different mAbs to target either the receptor or the ligand with the goal of “releasing the brakes” on effector T-cells preventing suppression of the antitumor response and causing tumor cytotoxicity. Similar to antibodies targeting PD-1 (nivolumab and pembrolizumab are the most widely marketed anti-PD-1 antibodies), anti-PD-L1 antagonists aim to restore effector T-cell and NKs activities while abrogating intra-tumoral Treg-mediated suppression (83). In addition, some anti-PD-L1 are able to mediate ADCC and other Fc-mediated functions. Four PD-L1 mAbs have demonstrated clinical activity in several solid tumors including atezolizumab, durvalumab, avelumab, and MDX-1105 (BMS-936559) (356). Activity of the IgG4 MDX-1105 and the low-ADCC inducer, Fc-engineered, humanized, IgG1 atezolizumab rely on blocking PD-L1. In contrast, durvalumab and avelumab combine two MOA: blocking PD-L1/PD-1 interactions, and directly killing PD-L1-positive tumor cells (356). Clinical studies involving both molecules are recruiting patients or just initiating in different hematological malignancies. Some of the diseases that could be targeted by anti-PD-L1 double MOA are: HL, B-ALL, FL, or MM (357–359). Nonetheless, anti-PD1 targeting in MM had low efficacy and, most notably, established MM therapies such as IMiDs are able to reduce PD-L1 on MM cells and could interfere with the outcome (360).

CD40

This glycoprotein is a member of the TNFR superfamily that is principally expressed on APCs, but also on several tumors, such as B-cell lymphomas and carcinomas (111). Through the binding to its ligand CD40L (or CD154) on CD4⁺ T-cells, CD40 plays a key role in a broad variety of immune and inflammatory responses, including T-cell-dependent immunoglobulin class switching, memory B-cell development, germinal center formation, functional maturation of DC, and upregulation of macrophage cytotoxic function. To date, different anti-CD40 mAbs have been developed including three agonistic and one antagonistic which are being investigated in a range of lymphoid and solid tumors (111).

Lucatumumab (HCD122/CHIR12.12) is a fully human antibody that antagonizes CD40L-mediated proliferation and survival on CLL and MM cells, and triggers ADCC (111, 112, 361). Lucatumumab has overall modest activity as single agent or in combined regimens in multiple clinical studies on B-cell tumors, including HL (NCT00283101) (362), CLL (NCT00108108) (363), and MM (NCT00231166) (364). Similar to lucatumumab, the humanized IgG1 dacetuzumab (SGN-40) has tumoricidal activities in cultured NHL cells through ADCC, ADCP and direct apoptosis via caspase-3 activation. In contrast to lucatumumab, dacetuzumab does not prevent CD40/CD40L interaction, and behaves as a partial agonist by augmenting effector CTL responses (365, 366). The efficacy and safety of dacetuzumab as a single agent to treat rrMM, rrNHL, or rrCLL was investigated in three

phase I studies, respectively (NCT 00079716, NCT00103779, NCT00283101), which demonstrated mild side effects but modest efficacy across the cancers tested (367–369). Nevertheless, combining dacetuzumab with chemotherapy and/or rituximab demonstrated synergistic activities in both preclinical and phase I clinical studies in rrDLBCL (NCT00529503, NCT00655837) (370–372).

Despite the limited activity of anti-CD40 antagonists, results with the partial agonist dacetuzumab and compelling evidence in preclinical models confirmed that CD40 agonists acting as CD40L could be a better venue to drive stronger antitumor responses (113, 373). Currently, two types of agonist anti-CD40 are available. The first type combines the activation of tumor-specific immune responses with a direct tumoricidal activity. In this group, we can include dacetuzumab along with the chimeric ChiLob7/4 and the human sugar-engineered SEA-CD40 antibodies (113–115, 365, 366). Upon binding to CD40, these drugs trigger both cellular proliferation and activation of APCs which activate innate and adaptive antitumor immunity (113, 373). In addition, these antibodies also directly kill CD40-expressing cancer cells through ADCC, and eventually inhibit proliferation and growth of CD40-expressing tumor cells. ChiLob7/4 has completed a phase I study in B-NHL showing a well-tolerated range of doses whereas SEA-CD40 is enrolling patients, at the time of writing, in a first phase I study in combination with pembrolizumab in solid tumors, B-NHLs and HL (NCT02376699).

A second type of CD40-directed antibodies triggers antitumor immune responses as sole MOA. Molecules such as APX005M, ADC-1013 or the IgG2 mAb CP-870,893 do not include FcR engagement as MOA and are being examined in solid tumors alone or in combination with immune checkpoint inhibitors (NCT03123783; NCT02482168; NCT02379741). Positive results may lead to the investigation to hematological malignancies. In both types, it is expected that antitumor efficacy highly depends on the CD40 status of the tumor infiltrate, mainly tumor-specific CTLs and possibly TAMs. Accordingly, the direct tumoricidal effects depend highly on the CD40 expression of the tumor.

Killer Cell Immunoglobulin-Like Receptor 3DL2 (KIR3DL2; CD158k)

This transmembrane glycoprotein belongs to the family of cell inhibitory receptors expressed by NK cells and subsets of CD8⁺ T-cells but not by most normal CD4⁺ T-cells. In contrast, KIR3DL2 expression is found in several CD4⁺ T malignancies, including SS, mycosis fungoides (MF) and anaplastic large cell lymphoma (ALCL) (98, 374). This receptor plays a dual role in the pathogenesis of these cancers: it acts as an inhibitory coreceptor which down-modulates CD3-dependent signaling events, and prevents programmed cell death. IPH-4102 is a humanized IgG1 antibody against KIR3DL2 whose potent antitumor properties *ex vivo* against SS and CTCL primary cells, and *in vivo* against KIR3DL2-positive tumor cells are achieved mainly through ADCC and ADCP. Only a minor contribution was attributed to the neutralization of the inhibitory receptor (98, 374). IPH-4102 advanced to phase I in 2015 (NCT02593045).

CD70

This member of the TNF family is a receptor transiently expressed on activated T- and B-cells and on mature DC (375). Its ligand is CD27, another costimulatory receptor found on the surface of T-cells, B-cell, and NKs (376). The interaction of both molecules accelerates NK-mediated tumor clearance while generating an adaptive immune response (377). For this reason these NSLAs are being investigated in oncoimmunology. Whereas anti-CD27 antibodies, such as Varilumab (CDX-1127), just boost innate and adaptive antitumor responses, anti-CD70 antibodies also target tumor cells inducing direct cell killing. CD70 is expressed in several hematological malignancies that activate NF-κB pathways leading to proliferation and survival of malignant cells (378–380). In addition, CD70 seems to be involved in the recruitment of CD27-positive Tregs to the TME thus allowing tumor evasion (381). Despite most anti-CD70 mAbs under development are immunoconjugates (Table 6), one classical antibody is under clinical evaluation. ARGX-110 is a defucosylated IgG1 mAb with several different MOA (107). By neutralizing CD70-CD27 interactions it deprives cell growth signaling in tumor cells while inhibiting the activation and proliferation of CD27-positive Tregs. In addition, ARGX-110 displays enhanced ADCC and ADCP while preserving a strong CDC (107). The first phase I study (NCT02759250) in patients with advanced solid tumors expressing CD70 provided evidence of good tolerability of ARGX-110 and antitumor activity at all dose levels (108). Currently, a phase I-II study is recruiting patients to evaluate ARGX-110 efficacy in AML (NCT03030612).

CD47 (Integrin-Associated Protein)

Phagocytosis is a complex process needed for programmed removal of apoptotic as well as IgG- or complement-opsonized cells that can be inhibited by the binding of the ubiquitous negative regulatory Ig receptor CD47 to the signal regulatory protein alpha (SIRP α), expressed on phagocytes and DCs (382, 383). CD47 was found to be universally expressed in human cancers where helps to prevent phagocytic elimination of tumor cells (118, 119). Notably, CD47 expression is preferentially found in AML-LSCs (384, 385) and negatively correlates with clinical outcome in AML, ALL, NHL, and MM (118, 119). The hypothesis that blocking CD47-SIRP α interactions would restore phagocytosis of tumor cells has been widely validated in primary human xenograft models treated with commercial and clinically developed anti-CD47 antibodies (118, 119, 384, 386–389). Based on this background, two novel anti-CD47 antibodies, Hu5F9-G4 and CC-900002, are being examined in several clinical studies (NCT02678338, NCT02953509, NCT02641002, NCT01410981, NCT02367196, NCT02663518), and many others have initiated clinical development, such as C47B222-(CHO).

CD47 is the first targeted receptor involved in phagocytosis, however, whether anti-CD47 MOA relies only in activation of immune cells or, in addition to immune cell activation, it strongly depends on Fc-mediated effector activities is a controversial issue. Based on preclinical investigations, it is assumed that these novel anti-CD47 mAbs impede CD47-SIRP α interactions leading to macrophage-mediated phagocytosis of B-NHL and AML cells,

TABLE 5 | Characteristics of ADCs and ARCs directed to LSAs.

Target	mAb (commercial name/orIGINator)	IgG class	Conjugate	Active indications in HMs (highest phase)	Reference
CD33	Gemtuzumab ozogamicin (<i>Mylotarg</i> /UCB)	Hz IgG4	Calicheamicin	Approved (AML, W) IV (AML/NCT00161668 °C) III (AML/NCT00136084 °C, NCT00049517 ^b ANR, NCT00372593 ^b C) II (CML/NCT01019317 °C) II (APL/NCT00413166 °C) I-II (CLL/NCT00038831 ^b C)	(120, 121)
	Vadastuximab talirine, SGN-CD33A (Seattle Genetics)	Hz IgG1	PBD	III (AML/NCT02785900 ^b T) I-II (MDS/NCT02706899 ^b S) I (APL/NCT01902329 ^b ANR)	(122)
	IMGN-779 (ImmunoGen)	N/A	DGN462	I (AML/NCT02674763 ^b R)	(123)
	AVE9633 (ImmunoGen)	Hz IgG1	DM4	I (AML/NCT00543972 ^b T) disc.	(124)
	²²⁵ Ac-HuM195, Lintuzumab-Ac225 (PDL BioPharma)	Hz IgG1	Actinium-225	III (AML/NCT00006045 ^b U) II (CML/NCT00002800 °C) II (APL/NCT00002609 °C) II (MDS/NCT00997243 ^b T) I (MM/NCT02998047 ^b R)	(125)
	Coltuximab ravidansine, SAR3419 (Sanofi, ImmunoGen)	Hz IgG1	DM4	II (DLBCL/NCT01470456 °C, NCT01472887 °C) II (B-ALL/NCT01440179 ^b T) disc. I (B-NHL/NCT00796731 ^b C)	(126)
	Denintuzumab mafodotin, SGN-CD19A (Seattle Genetics)	Hz IgG1	MMAF	II (DLBCL/FL/NCT02855359 ^b R) I (B-ALL/NCT01786096 °C)	(127)
CD19	131-I-tositumomab (Bexxar/Corixa Corp.)	m IgG1	Iodine 131	Approved (B-NHL, W) II (MCL/NCT00022945 ^b C) disc. III (FL/NCT00268983 ^b C) II (CLL/NCT00476047 ^b C)	(128)
	⁹⁰ Y-ibritumomab tiuxetan (Zevalin/Biogen Idec)	m IgG1	Yttrium 90	Approved/B-NHL III (FL/NCT02063685 ^b R) III (MCL/NCT01510184 ^a T) disc. III (DLBCL/NCT02366663 ^b T) disc. II (CLL/NCT00119392 ^a ANR) II (MM/NCT01207765 ^a T) disc.	(129)
	Inotuzumab ozogamicin, CMC-544 (Besponsa/Celltech Group)	Hz IgG4	Calicheamicin	Approved/ALL III (AML/NCT03150693 ^b R) III (DLBCL/NCT01232556 ^b T) disc. III (FL/NCT00562965 ^b T)	(130)
CD22	Pinatuzumab vedotin, RG7593 (Genentech)	Hz IgG1	MMAE	I-II (B-NHL/NCT01691898 ^b ANR) disc.	(131)
	Epratuzumab-SN38 (Immunomedics)	Hz IgG1	SN-38	Preclinical (B-NHL, B-ALL)	(132)
	Epratuzumab I-131 (Immunomedics)	Hz IgG1	Iodine 131	Preclinical (B-NHL) disc.	(133)
	Moxetumomab pasudotox, CAT-3888 (NCI)	Fv	PE-38	III (HCL/NCT01829711 ^a ANR) II (B-ALL/NCT02338050 ^a T) I/II (CLL/B-NHL/NCT01030536 ^b C) disc.	(134)
CD79b	Polatuzumab vedotin, RG-759 (Genentech)	Hz IgG1	MMAE	II (DLBCL/NCT01992653 ^b ANR) I/II (FL/NCT01691898 ^b ANR) I (CLL/NCT01290549 ^b C)	(135)
CD138	Indatuximab ravidansine, BT-062 (BioTest AG)	Ch IgG4	DM4	II (MM/NCT01638936 ^a ANR NCT01001442 ^a C)	(136)

Antibodies that reached clinical studies.

^aMonotherapy.^bCombined therapy.

ADCs, antibody-drug conjugates; ARC, antibody-radiionuclide conjugates; mAb, monoclonal antibody; HMs, hematological malignancies; Ch, human-mouse chimeric; Fh, fully human; Hz, humanized; m, mouse; Glyco-Fc, glycoengineered Fc fragment; N/A, not available; Fv, fragment variable; AML, acute myeloid leukemia; APL, acute prolymphocytic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; DLBCL, diffuse large B-cell lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; B-NHL, B-cell non-Hodgkin's lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; HCL, hairy cell leukemia; disc., discontinued in hematological malignancies; NCT, number of clinical trial (clinicaltrials.gov); C, completed; R, recruiting; T, terminated; ANR, active non-recruiting; NYR, not yet recruiting; T, terminated; S, suspended; W, withdrawn; U, unknown; PBD, pyrrolobenzodiazepine dimmers; MMA, monomethyl auristatin; PE-38 *Pseudomonas aeruginosa* exotoxin A; DGN462, a DNA-alkylating payload; DM4, a cytotoxic maytansinoid; SN-38, an active metabolite of irinotecan.

TABLE 6 | Characteristics of ADCs and ARCs directed to NLSAs.

Target	mAb (commercial name/originator)	IgG class	Conjugate	Active indications in HMs (highest phase)	Reference
CD25	ADCT-301, HuMax-TAC-PBD (ADC Therapeutics; Genmab)	Fh IgG1	PBD	I (AML/B-ALL/NCT02588092 ^a R) I (HL, B-NHL, PTCL, CTCL/NCT02432235 ^a R)	(137)
	IMTOX-25, RFT5-DGA (XOMA)	mlgG1	DGA	II (ATL/NCT01378871 ^a C) disc. II (T-NHL/NCT00667017 ^a C) disc.	(138)
	anti-Tac(Fv)-PE38, LMB-2 (NCI)	Fv	PE-38	II (HCL/NCT00321555 ^a ANR, NCT00923013 ^b R) II (CTCL/NCT00080535 ^a C) II (CLL/NCT00077922 ^a C) disc. I-II (ATLL/NCT00924170 ^a ANR)	(139)
CD30	Brentuximab vedotin, SGN-35 (Adcetris/Seattle Genetics)	Ch IgG1	MMAE	Approved (ALCL, HL) Preregistration (CTCL) III (CTCL/NCT01578499 ^a ANR) III (SS/NCT01578499 ^a ANR) III (T-cell lymphoma/NCT01777152 ^a ANR) II (DLBCL/NCT02734771 ^b R, NCT01925612 ^b T) II (AML/NCT01461538 ^b C) II (FL, MZL/NCT02623920 ^b W, NCT02594163 ^b ANR) II (MF/SS/PTCL/NCT01352520 ^a ANR, NCT03113500 ^b R) I-II (NK-T lymphoma/NCT03246750 ^b NYR)	(140)
CD37	AGS67E (Agensys)	Fh IgG2	MMAE	I (AML/NCT02610062 ^a R) I (B-NHL/NCT02175433 ^a R)	(79)
	IMGN529, K7153A (ImmunoGen)	Hz IgG1	DM1	II (DLBCL/NCT02564744 ^b R) I (CLL, B-NHL/NCT01534715 ^a C)	(141)
	177Lu-tetulomab, lilotomab (Betalutin/Nordic Nanovector)	Mouse N/A	Lutetium 177	I-II (B-NHL/NCT01796171 ^a R) I (DLBCL/NCT02658968 ^b R)	(142)
CD56	Lorotuzumab mertansine, IMGN-901 (ImmunoGen)	Hz IgG1	DM1	II (AML, NK-cell leukemia, B-ALL CML, MF/NCT02420873 ^a C) I (MM/NCT00991562 ^b C/NCT00346255 ^a C)	(143)
CD70	Voretuzumab mafodotin, SGN-75 (Seattle Genetics)	Hz IgG1	MMAF	I (B-NHL/NCT01015911 ^a C) disc. Lack of efficacy; second generation SGN-70A	(144)
	SGN-70A (Seattle Genetics)	Hz IgG1	PBD	I (DLBCL, MCL, FL/NCT02216890 ^a C) disc. (superseding SGN-75)	(145)
	MDX-1203 (Medarex)	Fh IgG1	MED-2460	I (B-NHL/NCT00944905 ^a C)	(146)
CD74	Milatuzumab-doxorubicin, IMMU-110 (Immunomedics)	Hz IgG1	Doxorubicin	I-II (MM/NCT01101594 ^a C)	(147)
CD117	LOP-628 (Novartis)	Hz IgG1	Maytansine	I (AML/NCT02221505 ^a T) disc.	

Antibodies that reached clinical studies.

^aMonotherapy.

^bCombined therapy.

ADCs, antibody-drug conjugates; ARC, antibody-radionuclide conjugates; mAb, monoclonal antibody; HMs, hematological malignancies; Ch, human–mouse chimeric; Fh, fully human; Hz, humanized; m, mouse; N/A, not available; Fv, fragment variable; AML, acute myeloid leukemia; HL, Hodgkin's lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; B-NHL, B-cell non-Hodgkin's lymphoma; PTCL, peripheral T-cell lymphoma; CTCL, cutaneous T-cell lymphoma; ATL, adult T-cell leukemia and lymphoma; HCL, hairy cell leukemia; CLL, chronic lymphocytic leukemia; ALCL, anaplastic large-cell lymphoma; SS, Sézary syndrome; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MZL, marginal zone lymphoma; MF, mycosis fungoidea; CML, chronic myeloid leukemia; MM, multiple myeloma; MCL, mantle cell lymphoma; disc., discontinued in hematological malignancies; NCT, number of clinical trial ([clinicaltrials.gov](#)); C, completed; R, recruiting; T, terminated; ANR, active non-recruiting; NYR, not yet recruiting; T, terminated; S, suspended; W, withdrawn; U, unknown; PBD, pyrrolobenzodiazepine dimers; DGA (deglycosylated ricin A-chain); PE-38 *Pseudomonas aeruginosa* exotoxin A; MMA, monomethyl auristatin; DM1, a cytotoxic maytansinoid; MED-2460, a DNA-alkylating payload.

including LSC cells (118, 119). However, recent evidence suggests that the Fc region of the murine IgG1 (B6H12.2) and human IgG4 (Hu5F9-G4) are able to bind human and murine FcRs and mediate effector functions (118, 390, 391); hence, whether the therapeutic effect observed is due to solely blocking CD47 or to an opsonizing effect combined with CD47 blocking activity remains unclear. In this sense, antileukemic activity of the IgG1 antibody C47B222-(CHO) does not rely on CD47 neutralization but, on the contrary, depends on robust Fc effector functions such as ADCP and ADCC (118). Importantly, fusion proteins containing the high-affinity human SIRP α (known as CV1) only have antitumor activity when fused to IgG4, but not as SIRP α monomer (392, 393). Similarly, a SIRP α -Fc molecule known as TTI-621 demonstrated potent antileukemic activity as IgG1 Fc conjugate, but not with a Fc mutated to avoid effector functions (394). Furthermore, mice harboring inactivating mutations at the SIRP α cytoplasmic tail show similar growth and metastasis of implanted syngeneic melanoma tumor cells as wild-type mice, suggesting again that disruption of CD47-SIRP α alone does not yield an effective antitumor response (393).

Finally, another concern related to antibody-based CD47 therapy is tolerability. Except for C47B222-(CHO), anti-CD47 antibodies have been reported to cause platelet aggregation and red blood cell hemagglutination (118, 119). Therefore, it is not clear whether an optimal dosing strategy could be achieved that provides a therapeutic window with limited toxicity. Results from ongoing clinical studies will shed light on this issue.

ANTIBODY-DRUG AND ANTIBODY-RADIONUCLIDE CONJUGATES (ADCs AND ARCs)

ADCs, along with ARCs, comprise the largest group of the non-canonical antibody formats in clinical studies for hematological malignancies. The principles of ADC/ARC activity and the considerations for their development, including the choice of antibody, drug and radionuclide, are beyond the scope of this review and have been discussed elsewhere (3, 395, 396). However, as the most advanced ADCs in the clinic are directed to hematological indications, a brief account of this landscape with some examples of ADCs which target LSAs and NLSAs are given in **Tables 5** and **6**, including the first ADCs developed and approved by the FDA: gemtuzumab ozogamicin, now discontinued, and brentuximab vedotin.

CONCLUSION

From rituximab, the first mAb approved in 1997 to treat cancer, to the recently approved daratumumab and elotuzumab to treat MM, several antibodies have changed the clinical practice and have transformed the therapeutic landscape of hematological malignancies. However, as we have shown, this number is extremely low compared with the total number of antibodies being studied in clinical trials. Today, most of the antibodies already approved target LSAs, whereas the majority of agents

in development for hematological malignancies targets NLSAs. This situation reflects a paradigm shift in the criteria followed to select a target to develop as novel immune therapy in blood malignancies care. The impact of this change is still unknown in disorders where mAbs targeting LSAs were not used, or resulted to be ineffective, such as MM, AML, or T-NHL. In this respect, novel antibodies directly targeting NLSAs in MM or AML are a profound change compared with earlier treatment approaches. Remarkably, today most of the therapeutic antibodies under development target both disorders. Nonetheless, in other diseases, such as T-cell or NK-cells malignancies, the scenario is not so promising since few candidates are in clinical development, and many of them show lack of activity in these entities. For example, while patients with B-NHL may benefit from immune checkpoint drugs, it is likely that patients with T-lymphomas will not. However, efforts must be maintained. NLSAs constitute a bigger group of molecules than LSAs, and for this reason the number of antibodies showing successful results is likely to be higher.

Most of the antibodies reviewed in this work, used as single agent or in combined regimens, are not better than standard treatments. In some cases the selected NLSA is not appropriate. For instance, targeting soluble factors is a successful approach in immunological disorders that seems to be ineffective against hematological malignancies, likely due to the disseminated nature of these cancers. In other cases, the antibody under evaluation may not display all its potential or may not trigger the right MOA. In this respect, directed modifications both in the Fv and Fc may reverse the lack of activity. Moreover, combining antibodies with other drugs, without previous strong supporting evidence, may negatively affect the outcome in clinical settings. Unfavorable combination regimens may impair the main MOA of a single antibody, or even highly pretreated patients may harbor the unfavorable background themselves. To evaluate a novel antibody, the selection of patients must take into account several factors including sensitivity and tolerability to prior treatments, disease stage and cytogenetic profile. Finally, one of the most important issues in the evaluation of antibodies targeting NLSAs expressed in different disorders is the selection of the optimal dose, which is not usually based on biological criteria. As we have learnt from some antibodies targeting LSAs, benefits within a wide range of identical lineage disorders cannot be obtained using the same dosing schedule. The uncertainty over the optimal dosing schedule is even higher when a particular mAb is examined in diseases differing not only in maturation stages but most importantly in different lineages or even, in different tissues.

Far beyond these explanations, the abundance of novel targeted agents draw a promising and consolidated landscape in hematological malignancies. Hopefully, in the near future, clinicians may consider different standard treatments for a given disease as different treatments may be available and may be tailored for molecularly defined responsive groups. In the coming years, unmet medical needs in a wide variety of conditions should be reduced and patient choices for antibody therapeutics should increase in short- as well as long-term. The optimal integration

of mAbs and other novel agents with current treatment strategies will require intelligent, rather than commercially driven, preclinical, and clinical design over the coming years.

AUTHOR CONTRIBUTIONS

CC-M reviewed the literature, prepared the tables, and wrote the manuscript. AA-S and BS-C reviewed the literature and drafted the work. CM-C reviewed the literature and wrote the manuscript.

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Therapeutic Antibodies to KIR3DL2 and Other Target Antigens on Cutaneous T-Cell Lymphomas

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KIR3DL2 is a member of the killer cell immunoglobulin-like receptor (KIR) family that was initially identified at the surface of natural killer (NK) cells. KIR3DL2, also known as CD158k, is expressed as a disulfide-linked homodimer. Each chain is composed of three immunoglobulin-like domains and a long cytoplasmic tail containing two immunoreceptor tyrosine-based inhibitory motifs. Beside its expression on NK cells, it is also found on rare circulating T lymphocytes, mainly CD8⁺. Although the KIR gene number varies between haplotype, KIR3DL2 is a framework gene present in all individuals. Together with the presence of genomic regulatory sequences unique to KIR3DL2, this suggests some particular functions for the derived protein in comparison with other KIR family members. Several ligands have been identified for KIR3DL2. As for other KIRs, binding to HLA class I molecules is essential for NK development by promoting phenomena such as licensing and driving NK cell maturation. For KIR3DL2, this includes binding to HLA-A3 and -A11 and to the free heavy chain form of HLA-B27. In addition, KIR3DL2 binds to CpG oligonucleotides (ODN) and ensures their transport to endosomal toll-like receptor 9 that promotes cell activation. These characteristics have implicated KIR3DL2 in several pathologies: ankylosing spondylitis and cutaneous T-cell lymphomas such as Sézary syndrome, CD30⁺ cutaneous lymphoma, and transformed mycosis fungoïdes. Consequently, a new generation of humanized monoclonal antibodies (mAbs) directed against KIR3DL2 has been helpful in the diagnosis, follow-up, and treatment of these diseases. In addition, preliminary clinical studies of a novel targeted immunotherapy for cutaneous T-cell lymphomas using the anti-KIR3DL2 mAb IPH4102 are now underway. In this review, we discuss the various aspects of KIR3DL2 on the functions of CD4⁺ T cells and how targeting this receptor helps to develop innovative therapeutic strategies.

Keywords: KIR3DL2, Sézary syndrome, mycosis fungoïdes, cutaneous T-cell lymphomas, tumor marker, monoclonal antibody

INTRODUCTION

Introduction of monoclonal antibodies (mAbs) has been a very successful breakthrough for the diagnosis and treatment of a number of tumors. First used for immunophenotyping to better identify and characterize the tumor cell pool, they became useful in the quantification of residual malignant cells during patient follow-up and in the evaluation of chemotherapeutic protocols. mAbs have been highly valuable in identifying therapeutic targets and initiated development of the use of specially designed mAb in cancer treatment. Humanized mAb alone, leaving aside

their applications as drug delivery systems, is most widely used for tumor-targeting immunotherapies. Rituximab (anti-CD20 mAb) was the first mAb approved for cancer therapy. It has significantly improved patient survival in several B-cell malignancies such as diffuse large-cell lymphomas with response rate of 60–80% (1, 2). Many efforts have been devoted to understanding the mechanisms of action of anti-CD20 antibody tumor depletion (2–4). Beside complement activation, FcR immune effectors [phagocytes and natural killer (NK) cells] play an essential role in the *in vivo* clearance of mAb-coated tumor cells (5–8). Another way of killing tumor cells by mAb is by F(ab')₂-dependent targeting of cell surface signaling receptors associated with apoptosis induction (9–12). In many cases, the therapeutic efficacy of a mAb relies on both Fc- and F(ab')₂-dependent mechanisms. However, because of the lack of efficient therapeutic targets and the resistance to chemotherapy, too many cancers are still resistant to treatment, particularly at advanced stages. In this review, we focus on a class of such tumors, the cutaneous T-cell lymphomas (CTCL), which require improved identification of tumor markers and more efficient treatment. The rapidly growing numbers of clinically approved tumor-targeting mAb enlarge the spectrum of potential treatments for these cases.

CUTANEOUS T-CELL LYMPHOMAS

Cutaneous T-cell lymphomas represent a group of rare and heterogeneous extranodal non-Hodgkin's lymphomas characterized by skin infiltration of malignant monoclonal T lymphocytes (13). Sézary syndrome (SS) and mycosis fungoides (MF) are the most common forms of CTCL, both being very difficult to treat at advanced stages. Their diagnosis is based on clinical, histopathological, molecular biological, and immunopathological features (14). However, the lack of unambiguous immunophenotypic or molecular biomarkers makes the differential diagnosis of CTCL with erythrodermic inflammatory dermatoses challenging (15). MF, accounting for around 65% of CTCL cases, usually presents with an indolent clinical course restricted to the skin, passing from macule and patch stage to infiltrated plaque stage. However, tumor-stage disease and cell transformation are associated with much poorer prognosis. SS is an aggressive leukemic variant of CTCL clinically defined by the classical triad of erythroderma, lymphadenopathy, and peripheral blood involvement. Detection of an identical malignant T-cell clone in the skin and the blood, based on T-cell receptor gene rearrangements, is a critical element for the diagnosis of SS. Staging for CTCL based on the TNM (tumor-node-metastasis) system has been extremely useful and remains the standard for the classification of MF/SS patients. Although progress has been made in the treatment of transformed MF and SS, there is still no cure for these diseases. Intensive chemotherapies are mostly inappropriate for CTCL due to the high risk of infection in patients with a compromised skin barrier (14).

As mentioned earlier, early diagnosis of SS can be challenging and evaluation of the tumor burden is difficult. A number of studies have attempted to identify characteristic immunophenotypic changes and molecular biomarkers in Sézary cells that could

be useful as additional diagnostic criteria (16–20). Using flow cytometry, the loss of cell surface markers such as CD7, CD26, and/or CD27 on CD4⁺ T cells is helpful to estimate the tumor mass and to orient the choice of therapy. However, the specificity and sensitivity of these tests to identify the malignant clone are to be considered with caution. Markers mostly expressed on NK cells, such as CD158k (KIR3DL2) and CD335 (NKp46), can be expressed on erythrodermic MF/SS T cells and can be considered as more reliable markers for the malignant clone detection (21–24). Despite the possible induction of partial or complete remission, the median survival of SS is 1–5 years, illustrating the need for novel targeted therapies. Promising targets include the C-C chemokine receptor type 4 (CCR4), CD30, programmed-death 1, and KIR3DL2 to which therapeutic mAb has been designed and are currently in the clinical phase of study.

KIR3DL2 IN BIOLOGY

A Biological Marker

In humans, the main NK cell receptors for major histocompatibility class I (MHC-I) molecules are the killer cell immunoglobulin-like receptors (KIRs) or CD158x. They have been named according to their biochemical structure, having either two (KIR2D) or three (KIR3D) extracellular Ig-like domains and either a long cytoplasmic tail (KIR-L) containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) or a short cytoplasmic tail (KIR-S) that associates with signaling molecules to transduce an activating signal (25). Interestingly, this important function of MHC-I recognition is shared in non-primate species by structurally different molecules, the lectin-like receptors (Ly49x). At the genomic level, KIRs are encoded in the leukocyte receptor cluster on chromosome 19q13.4, while the Ly49 genes in rodents are encoded in the NK complex on chromosome 6. Human haplotypes encoding KIRs have major differences in gene content and allelic polymorphism, with up to 14 genes and 3 framework genes, namely KIR2DL4, KIR3DL2, and KIR3DL3 (26, 27). Given that MHC-I and KIRs are encoded on different chromosomes, this results in a fascinating complexity of cognate KIRs/HLA class I genotypes.

Unlike T or B lymphocytes, NK cells do not generate their recognition repertoire through receptor gene rearrangements. Instead they use germline-encoded activating and inhibiting receptors, the resulting signals deciding the fate of the cellular response. Expression of a limited set of activating and inhibiting receptors in any given NK lymphocyte ensures the generation of a remarkable degree of cell diversity (28). Inhibiting KIR receptors also play an important role in the development of functional NK cells. The strength of the interaction between the inhibitory KIR and the MHC-I determines the threshold of activation of a given NK cell, a process known as NK cell education (29, 30).

Killer cell immunoglobulin-like receptors are also important in reproduction through the role of uterine NK cells in the process of decidualization (31). During this arterial remodeling process, appropriate KIR/HLA-C interactions between uterine NK cells and extra-villous trophoblasts are necessary to ensure reproductive success (32). For example, a strong maternal

inhibitory KIR repertoire associated with a fetal high affinity HLA ligand was found to be detrimental to healthy placentation (33).

In the peripheral blood of healthy humans, KIR3DL2 is not only expressed by about 20% of NK cells but also expressed by a small proportion of CD4⁺ (5%) and CD8⁺ (9%) T lymphocytes. An enriched KIR3DL2 expression on memory CD45RO⁺CD28⁻CCR7⁻CD62L⁻ T cells has also been reported (34). Similar to NK cells, KIR3DL2 is an inhibitory co-receptor on T lymphocytes. KIR3DL2 is unique among the KIR family in being expressed as a disulfide-linked homodimer (p140) (35). This characteristic may be important in terms of ligand-binding capacity.

Ligands

The large number of protein-encoding polymorphic variants of KIR3DL2 hinders definition of the complete list of ligands for this receptor, although the true contribution of allelic variations to ligand recognition is unknown. It was originally shown to bind specifically to HLA-A3 and -A11 (35, 36). It seems that association of the RLRAEAQVK EBV-peptide to HLA-A3 and -A11 is critical for binding to KIR3DL2 (37). The requirement for specific peptide association to the MHC complex is unclear as KIR receptors are more generally considered as MHC-I expression sensors on target cells. This peptide selectivity may confer NK cells with an additional recognition mechanism (38). KIR3DL2 also binds to the free heavy chain dimers of HLA-B27 (39). In addition to the classical β2m-associated heavy chain, HLA-B27 is expressed at the cell surface as dimers of free heavy chains due to the presence of a reactive cysteine at position 67. In contrast to the binding to HLA-A3 and -A11, KIR3DL2 recognition of HLA-B27-free heavy chains is independent of the bound peptide sequence (40).

KIR3DL2 also binds to CpG-oligodeoxynucleotides (CpG-ODN), and this ligation induces KIR3DL2 down-modulation from the cell surface and translocation to the endosome to deliver the CpG-ODN to the toll-like receptor 9 (TLR9) (41). CpG-ODNs belong to a class of ligands called pathogen-associated molecular patterns (PAMPs) recognized by TLRs. As mentioned earlier, certain TLRs are localized in the endoplasmic reticulum and endosomes where, upon ligation by their respective ligands, they can initiate signal transduction, promote cytokine release, and increase NK cell cytotoxicity (42–45). Recognition of CpG-ODNs or other PAMPs is also observed for KIR3D or KIR2D receptors encompassing a D0 Ig-like domain. This illustrates that KIRs not only function as HLA class I receptors but can also serve as receptors to mediate antimicrobial responses.

Role in Immune Response

KIR3DL2 belongs to the inhibitory receptor family like the other KIR-L and is characterized by the presence of ITIM/ITSM-like sequences in its cytoplasmic tail. It is indeed capable, upon ligation at the surface of NK cells, to inhibit IFNγ production and cytotoxic function. On T lymphocytes, KIR3DL2 ligation has no effect alone. It is a co-receptor that contributes to the response initiated via the TCR. In particular, KIR3DL2 ligation on activated T cells results in an antiapoptotic effect and the production of IL-17 (46). KIR3DL2 ligation by HLA-B27 also promotes the

survival of NK cells and inhibits their production of IFN-γ (34). Of note, KIR3DL2 expression is upregulated upon activation of NK and T cells. KIR3DL2 expressing T cells may therefore be enriched in Th17 cells, the T-cell subset producing IL-17, suggesting that it may have a role in the differentiation of this T-cell subset. Interestingly, while IL-17 can have anti-tumor effects as a pro-inflammatory cytokine, it has also been identified as exerting a promoting role in carcinogenesis, tumor metastasis, and resistance to chemotherapy of diverse types of cancers (47).

KIR3DL2 IN PATHOLOGY

KIR3DL2 and Ankylosing Spondylitis (AS)

The human leukocyte antigen HLA-B27 is strongly associated with the development of AS, with 94% of patients expressing HLA-B27, compared to 9.4% of healthy individuals (48). As mentioned earlier, KIR3DL2 recognizes HLA-B27 as a dimer of free B27 heavy chains. Proportions of KIR3DL2⁺ CD4⁺ T cells producing IL-17 are increased in the peripheral blood and synovial fluid of patients with AS (34). *In vitro*, KIR3DL2⁺ CD4 T cells stimulated with B27 heavy chain dimers or IL-23 and IL-1 produce more IL-17 when isolated from AS patients than from healthy individuals (46). A role for Th17 cells in the pathogenesis of AS has been suggested by the strong genetic linkage with IL-23R polymorphism (49). In AS, KIR3DL2⁺CD4⁺ T cells accounted for 60% of all IL-23R-expressing CD4⁺ T cells. These data suggest that the B27 interaction with KIR3DL2 could play a central role in AS and other HLA-B27-linked autoimmune diseases.

KIR3DL2 in CTCL

A Diagnostic Tool

As mentioned earlier, diagnosis and evaluation of the tumor mass in CTCL can be challenging. Histological examination of blood smears to determine the tumor mass, with Sézary cells defined by a cerebriform nuclear morphology, is widely used and valuable, while flow cytometry analysis of T-cell blood subsets provides a more objective and reproducible means to quantify and track circulating lymphocyte involvement in patients with MF/SS. For example, a CD4:CD8 ratio higher than 10 is observed in about 80% of patients with SS, whereas loss of CD7 (CD4⁺CD7⁻ ≥ 30%) or CD26 (CD4⁺CD26⁻ ≥ 40%) is found in about half of the SS patients (50–52). However, loss of CD7 or CD26 among CD4⁺ T cells can also be found in benign inflammatory erythroderma or rare healthy subjects. Even T-cell clonality can be detected in 34% of cases with benign inflammatory erythroderma (53). This illustrates the need for other specific markers for CTCL. Among the proposed potential markers, several belong to the NK cell lineage, raising the provocative question of a NK-cell reprogramming mechanism occurring in the transformation of some CTCL (54). Indeed, an abnormal expression of several NK receptors has been observed at the surface of SS cells. These include CD85j/Ig-like transcript 2 (ILT2)-receptor (55), the natural cytotoxicity receptor (NCR) NKP46/NCR1 (22), and KIR3DL2 (56).

Ig-like transcript 2 is an inhibitory receptor, analogous to KIRs, specific for the α3-domain epitope shared by some MHC-I

molecules and the UL18 antigen encoded by human cytomegalovirus (57). Although it is expressed on NK and some memory CD8⁺ T cells, ILT2 is absent from the surface of resting normal CD4⁺ T lymphocytes, allowing ILT2 expression to effectively identify circulating Sézary cells in SS patients (55).

NKp46, with NKp30 and NKp44, constitutes the NK cell NCRs family. It was shown that umbilical cord blood CD8⁺ T cells long-term cultured with IL-15 could express NCR (58). In this line, NCRs are also observed on the surface of intraepithelial T lymphocytes in celiac disease where they can drive TCR-independent cytotoxicity and cytokine production (59). It was still a surprise to find NKp46 expression not on cytotoxic effector T cells but on non-CTL malignant CD4⁺ T lymphocytes in SS patients (22). Expression of NKp46 is not observed on normal circulating CD4⁺ T cells, a clear indication that such ectopic expression is a consequence of malignant transformation. Indeed, in the circulating T cells from SS patients, expression of NKp46 is restricted to the clonal V β CD4⁺ population identifying the tumor cells. NKp46 expression also correlated with KIR3DL2 expression and reflected the clinical course of the disease with a lower expression in remission or post-treatment periods. Of note, NKp46 and KIR3DL2 are frequently co-expressed in transformed MF (23).

The Q66 mAb was the first to specifically recognize the p170 KIR now called CD158k or KIR3DL2 (35). Although SS cells are difficult to culture *in vitro*, IL-7 proved to be useful to generate some CTCL cell lines (60–62). Expression of KIR3DL2 detected by the Q66 mAb was initially demonstrated in the former cell lines and further confirmed on the corresponding patient's primary circulating cells and extended to seven other SS patients and to the skin of two patients with advanced MF (56). Expression of KIR3DL2 on SS cells is polymorphic but is not associated with a particular allele (63). It is increasingly clear that KIR3DL2, alone or associated with other markers, could efficiently delineate circulating Sézary cells and reduce diagnostic difficulties (21, 64, 65). Because KIR3DL2 is poorly expressed by normal T lymphocytes or reactive lymphocytes from benign erythrodermic inflammatory diseases, it is a highly specific marker for Sézary cells. For example, KIR3DL2 transcripts were found significantly overexpressed in skin biopsies from patients with SS compared to benign erythrodermic dermatoses (66). KIR3DL2 has progressed to become the best marker of SS after expression was reported in 30 of 34 (82%), 32 of 33 (97%), and 11 of 17 (65%) patients with SS in studies from three different groups (51, 64, 67). Availability of new IgG anti-KIR3DL2 mAb with higher affinity and avidity than the first IgM Q66 will increase the possibility of detecting SS cells with low CD158k/KIR3DL2 expression. KIR3DL2 mAb identifies the tumor cells in most CTCL patients analyzed and, therefore, represents a valid tool to dynamically evaluate the evolution of the tumor pool during disease evolution as well as the response to treatment (68).

A Prognostic Tool

During the course of the disease or after treatment, it is crucial to assess whether an increase in the CD4 population results from an expansion of the tumor cell population or of reactive T lymphocytes. In addition, the tumor burden in blood had a

prognostic value in patients with erythrodermic CTCL (69). It was shown that measure of the absolute CD3⁺CD158k⁺ circulating cells closely correlated with morphologically identified (with cerebriform-like nuclei) Sézary cell numbers in patients with SS under systemic therapy (21). When comparing KIR3DL2⁺ and KIR3DL2⁻ populations of CD4⁺ T cells, a decrease in KIR3DL2 expressing T cells is associated with the response to therapy when increased KIR3DL2⁻ CD4⁺ T cells are observed, highlighting the usefulness of this marker for the follow-up of SS patients (17). A recent study reported that 87% of a group of SS patients ($n = 64$) expressed KIR3DL2 (range: 7–98% of tumor T cells) at diagnosis. Analyzing the follow-up of these patients indicates that the presence of more than 85% of KIR3DL2⁺ cells among CD3⁺ T cells is the main prognostic factor at diagnosis for SS (68). This study also demonstrates that circulating CD3⁺CD4⁺KIR3DL2⁺ T-cell counts can be used to monitor treatment efficacy and relapse in SS patients. KIR3DL2 detection permits to estimate whether the ongoing treatment specifically targeted the malignant T-cell clone and, if so, to visualize the pool of residual tumor cells. KIR3DL2 therefore represents an early predictive marker of relapse or progression.

A Therapeutic Target

Based on the above data, it is clear that KIR3DL2 can be a therapeutic target for CTCL. However the remaining questions are: why do cutaneous malignant T cells express this marker and what is its function on these cells? As mentioned earlier, KIR3DL2 expression on CTCL cells may be the result of some kind of genetic remodeling that induces NK marker ectopic expression. In healthy individuals, KIRs were also expressed by a small proportion of cytotoxic CD8⁺ T cells (70). Unlike NK cells, where inhibitory KIRs play a role in education, KIRs on T cells are acquired at the memory stage, and the proportion of KIR⁺CD8⁺ T lymphocytes increases with age (70). The engagement of MHC class I inhibitory receptors has been shown to contribute to the down-regulation of T cell effector function in KIR⁺CD8⁺ T and to the negative control of activation-induced cell death (AICD) (71). Expression of KIRs (or ILT2) can proceed from a regulatory mechanism that would raise the activation threshold in cytotoxic T cells, thereby providing a safety mechanism to control these potentially harmful cells (72). Therefore, KIR3DL2 expression on Sézary cells may reflect the ability of neoplastic cells to better avoid antigen receptor-mediated cell death in an inflammatory environment of persistent antigenic stimulation of cutaneous T cells. Indeed, it has been established that accumulation of Sézary cells is not a result of increased proliferation but rather reflected a resistance to apoptosis and particularly to AICD (73). Thus, although KIR3DL2 has no independent receptor function, co-ligation of CD3 and KIR3DL2 induces a strong inhibition of the proliferation and apoptosis on Sézary cells when non-KIR3DL2 cells proliferated and reached apoptosis normally (74). Thus, expression of KIR3DL2 by malignant cutaneous T cells may be a key element to protect these cells from AICD in a highly stimulating environment, but what can trigger KIR3DL2 signaling in Sézary cells? There is no particular association of SS with the HLA-A3, -A11, or -B27 haplotype

but, as mentioned earlier, CpG-ODN has been reported to bind KIR3DL2 on NK cells leading to NK cell activation (41). When tested on Sézary cells, KIR3DL2 engagement by CpG-ODN promotes the internalization of the receptor and the generation of apoptotic signals in the malignant CD4⁺ cells (75). In phase I/II trials on CTCL patients, subcutaneous injection of class-B CpG-ODN has led to a clinical response rate of 32–36% and in the absence of major cytotoxic side effects (76, 77). These data suggest that, in addition to promoting the generation of an anti-tumor immune response, CpG-ODN might initiate a direct effect on Sézary cells through binding to KIR3DL2.

KIR3DL2 expression is not restricted to SS and MF tumor cells. Recently it has been shown that in primary anaplastic large-cell lymphoma, an aggressive CD30⁺ CTCL, tumor cells also express KIR3DL2 and can be the target of a potent anti-tumor activity *in vitro*, re-enforcing this marker as a therapeutic target for these patients (78). The restricted expression of KIR3DL2 in normal immune cells, and the possibility to selectively target the tumor cells in CTCL, led to the development of a humanized mAb for an effective treatment strategy. IPH4102, a humanized IgG1, is the selected anti-KIR3DL2 mAb candidate with potent depleting activity against primary Sézary patient cells (79, 80). In preclinical studies, *in vitro* assays using the Sézary cell line HuT 78 demonstrated that IPH4102 modes of action include antibody-dependent cell cytotoxicity (ADCC) involving NK cells, and antibody-dependent cell phagocytosis due to macrophage activation, but no killing *via* direct complement activation on target cells (79). Effective anti-tumor killing was also achieved *ex vivo* in co-cultures of patient's Sézary cells and autologous NK cells as effectors. Even with an effector/target ratio lower than 1:1, malignant cells from all patients tested were efficiently killed while NK effector survival was not compromised by the action of IPH4102, unlike the Alemtuzumab control, illustrating the selectivity of action of the anti-KIR3DL2 mAb (79). *In vivo* efficiency of the anti-tumor activity of IPH4102 was tested in SCID mice engrafted with KIR3DL2-transfected Raji cells. In this model, engrafted mice treated twice weekly with an isotype control mAb had a median survival of 17 days. On the other hand, mice treated with as little as 30 µg of IPH4102 had a median survival of 39 days, and 2 of the 30 mice remained alive at the end of the experiment (79). IPH4102 has also demonstrated a favorable preclinical safety profile in regulatory pharmacotoxicology experiments in non-human primates (80).

IPH4102 is currently being investigated in the first-in-human multicenter phase I study (NCT02593045) evaluating repeated administrations at escalating doses of single-agent IPH4102 in relapsed/refractory CTCL. A recent report of this ongoing study that includes 22 CTCL patients, including 19 SS patients, indicated that IPH4102 is very well tolerated in these patients (who have had extensive treatment) with no reported treatment-related death (81). The majority of adverse effects are low grade and typical for CTCL. Upon escalation and up to now, the best global overall response rate is 47% in SS patients, reaching 58% responses in the circulation. In addition, two complete responses were observed in skin and five complete responses were observed in blood. These preliminary data are encouraging to promote

IPH4102 as a new targeted treatment in patients with advanced CTCL.

OTHER mAb IN CTCL TREATMENT

Alemtuzumab (Campath; Anti-CD52 mAb)

Alemtuzumab is a humanized IgG1 kappa mAb specific for CD52, an antigen expressed by most T and B lymphocytes. The usual protocol of administration of 30 mg doses three times per week has led to a good outcome in SS, but much less convincing results in patients with MF. However it was associated with severe leukopenia, immune depletion and opportunistic infections that may require treatment discontinuation (82, 83). To minimize immune suppression and infections due to its wide expression in the immune system, protocols have been proposed with injection of 10 mg only if SS cells become higher than 1,000/mm³ (84). Although side effects are considerably reduced, such protocols are usually not curative on a long-term perspective.

Brentuximab Vedotin (Anti-CD30 mAb)

Brentuximab vedotin (SGN-35) is a chimeric anti-CD30 mAb conjugated to monomethyl auristatin E, a cytotoxic anti-tubulin agent. Thirty-two MF or SS patients at stage IB-IV were included in a phase II prospective study (85). The overall response rate was 70% with responses at all stages. However, the expression of CD30 by immunochemistry was very variable and patients with expression lower than 5% experienced decreased probability of response. Another prospective phase II study, targeting 48 patients with CD30⁺ CTCL and including 28 CD30⁺ MF, reported an overall response rate of 71% with a complete response in 35% of cases (86). In these studies, the most frequent adverse event is peripheral neuropathy, occurring in 66% of the patients and showing resolution during a 2-year time course.

Mogamulizumab (Anti-CCR4 mAb)

Mogamulizumab is a humanized anti-CCR4 monoclonal antibody with a defucosylated Fc region leading to increased antibody-dependent cellular cytotoxicity (87). CCR4 is expressed on Tregs and T helper cells and plays an important role in skin homing. In a phase I/II study, mogamulizumab induced an overall response rate of 47.1% in SS patients and 28.6% in MF patients (88). In a multicenter Japanese phase II study involving 37 patients with relapsed CCR4-positive tumors, mogamulizumab treatment induced 35% of objective response, including 5 patients (14%) experiencing complete response (89). The most common adverse effect of this treatment is lymphocytopenia (81%), and cases of severe Steven–Johnson–Lyell syndrome due to the induced immune deficiency of regulatory T cells have been reported (90, 91). An international phase III trial of mogamulizumab versus vorinostat is ongoing in previously treated CTCL patients.

CONCLUSION

There is no doubt that the development of targeted systemic biological therapies will benefit the management of CTCL. Several ongoing trials with therapeutic mAbs, including brentuximab

vedotin, mogamulizumab, and IPH4102, show interesting results in these patients with limited toxicity. KIR3DL2 is expressed irrespectively of disease stage in all subtypes of CTCL, with the highest prevalence in SS and transformed MF, two subsets with high and unmet therapeutic needs. The origin of its expression is unknown, but the presence of chronic bacterial stimulation may be responsible for increased KIR3DL2 expression on cutaneous T cells. The limited expression of KIR3DL2 on normal immune cells, in comparison with the ectopic expression on CTCL tumor cells, allows to selectively and efficiently kill malignant cells. KIR3DL2 can be targeted by both IPH4102 or by one of its ligands, CpG-ODN. The mAb acts *via* an ADCC and phagocytosis, while

CpG-ODN induces the internalization of KIR3DL2 receptor upon ligation and cell apoptosis *via* the Toll-like receptor activation pathway. CpG-ODN also efficiently activates key elements of the immune system that participate in the local anti-tumor rejection process. One can hope that the identification of novel targets and development of therapeutic mAbs will prove to be efficient and safe alternatives for the treatment of CTCL.

AUTHOR CONTRIBUTIONS

AB and AM-C are senior co-authors. AB, AM-C, and CS wrote the manuscript.

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92R Monoclonal Antibody Inhibits Human CCR9⁺ Leukemia Cells Growth in NSG Mice Xenografts

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CCR9 is as an interesting target for the treatment of human CCR9⁺-T cell acute lymphoblastic leukemia, since its expression is limited to immature cells in the thymus, infiltrating leukocytes in the small intestine and a small fraction of mature circulating T lymphocytes. 92R, a new mouse mAb (IgG2a isotype), was raised using the A-isoform of hCCR9 as immunogen. Its initial characterization demonstrates that binds with high affinity to the CCR9 N-terminal domain, competing with the previously described 91R mAb for receptor binding. 92R inhibits human CCR9⁺ tumor growth in T and B-cell deficient Rag2^{-/-} mice. *In vitro* assays suggested complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity as possible *in vivo* mechanisms of action. Unexpectedly, 92R strongly inhibited tumor growth also in a model with compromised NK and complement activities, suggesting that other mechanisms, including phagocytosis or apoptosis, might also be playing a role on 92R-mediated tumor elimination. Taken together, these data contribute to strengthen the hypothesis of the immune system's opportunistic nature.

Keywords: cancer, therapeutic antibodies, combinations, oncology, chemokine receptors

INTRODUCTION

Chemokine receptors and their ligands are crucial for organogenesis and lymphocyte trafficking, both in homeostasis and inflammation (1). The chemokine receptor 9 (CCR9) expression in normal cells is limited to immature T lymphocytes in the thymus (2–5), small bowel infiltrating cells (6), a small fraction of circulating memory T lymphocytes (CCR9⁺α4β7^{high}) (7), IgA secreting plasma B cells (1), and plasmacytoid dendritic cells (8). Up to now, the only known ligand for CCR9 is the chemokine CCL25 (3, 9). CCL25 is secreted in the thymus by epithelial and dendritic cells (4, 10) and also by the small intestinal crypt epithelium (6). The CCL25-CCR9 interaction controls migration of thymocytes within the thymus and homing of mature CCR9⁺ lymphocytes to the intestinal tract (7). In addition, there is a strong association between aberrant chemokine receptor expression on tumor cells (i.e., CXCR4 or CCR7) with cancer progression, poor prognosis, and organ-selective metastases (11–13). For CCR9 expression in tumor cells, the data are still limited, but CCR9 expression correlates with the ability of the tumor to generate metastasis in the small intestine (14–16), the main site, in addition to the thymus, of CCL25 secretion. CCR9 overexpression in acute and chronic T cell leukemia has been linked to disease aggressiveness (17). In addition, aberrant CCR9 expression in prostate tumors, breast cancer, or melanoma, has been correlated with *in vitro* invasiveness in response to CCL25 (14, 15, 17–23). Tumor cells-expressing

CCR9 have competitive advantages, since engagement of the CCL25 ligand enhances cell survival and provides resistance to apoptosis *via* the phosphatidylinositol 3-kinase/Akt pathway on several solid tumors (20, 21, 24–30); it activates the JNK1 antiapoptotic pathway in leukemic cells (31) and participates in Notch1-mediated cell proliferation (19).

Targeted therapies and immunotherapy have safety advantages over non-specific cytotoxic agents, since they are able to discriminate between normal and tumor cells. Therefore, their use for the treatment of cancer is in constant expansion (32). The described therapeutic tools that specifically target human CCR9⁺-tumors and have been tried in xenogeneic models are limited to the use of the CCR9-ligand coupled to a cytotoxic agent (CCL25-PE38 fusion protein) (33), the use of ligand-specific antibodies, alone or in combination with etoposide (25), or the mAb 91R that selectively inhibited growth of a human acute T lymphoblastic leukemia (T-ALL) cell line in Rag2^{-/-} xenografts (34). The first two strategies eliminate tumor cells by targeting the CCL25-CCR9 interaction, whereas the last directly targets the cells expressing CCR9. These data provide evidence of CCR9 as a potential target for cancer immunotherapy.

With the aim of selecting other anti-CCR9 mAb with (i) different specificities, (ii) different affinities for CCR9, (iii) provided of different mechanism(s) of action, and (iv) displaying high melting points, new hybridomas were generated and screened. mAbs with these properties could be more convenient to be used for therapeutic purposes. Here, we report the generation and characterization of 92R, an anti-CCR9 mAb able to selectively inhibit *in vivo* growth of human acute T-ALL cells transplanted into immunodeficient Rag2^{-/-} or NSG mice. This antibody has therapeutic potential for the targeted elimination of CCR9⁺-tumor cells, used either alone or in combination with other therapies.

MATERIALS AND METHODS

Cells and Reagents

Human embryonic kidney 293 (HEK-293, CRL-1573) cells and HEK-293 cells stably transfected with the human chemokine receptor CCR9, or the empty vector (pCIneo) were a kind gift of A. Zaballos (CNB-CSIC, Madrid, Spain), cells were cultured as described (3). MOLT-4 (CRL-182) and Jurkat (TIB-152) human T-ALL cell lines were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (complete medium). Neomycin-resistant stable HEK-293 transfectants were cultured in the presence of 1 mg/ml G418 (Sigma) and periodically tested for CCR9 expression (not shown). Recombinant human CCL25 and CXCL12 were purchased from Peprotech. We used the following antibodies: 3C3 (ATCC HB-12653), 112509, mouse mAb anti-hCCR9 (IgG2a; R&D) and M4, a serum pool generated by immunizing BALB/c mice with three intraperitoneal injections of 10⁷ MOLT-4 cells in PBS (days 1, 25, and 50); sera were collected on day 60.

Generation of Human CCR9-Specific mAb

Murine 91R and 92R anti-human CCR9 mAb were raised after immunization of BALB/c mice with a gene gun (Bio-Rad) particle-mediated DNA administration of the pCIneo plasmid bearing the human CCR9 cDNA, as previously described (34). Mouse sera were collected 7–10 days (d) after the last boost and tested for specific antibodies by flow cytometry using stably transfected hCCR9-HEK-293 cells, and pCIneo-HEK-293 cells as negative control. Selected mice were boosted intravenously with 10⁷ hCCR9-HEK293 cells 3 and 2 days prior to splenocyte fusion (35). Two weeks post-fusion, culture supernatants were screened by flow cytometry for CCR9-specific antibodies using hCCR9-HEK293 cells. Positive hybridomas were cloned, mAb purified from culture supernatants and antibody isotype determined by enzyme-linked immunosorbent assay (ELISA) (35).

Flow Cytometry

For staining, 2 × 10⁵ cells/well were centrifuged in V-bottom 96-well plates and washed with phosphate-buffered saline, pH 7.4 (PBS) supplemented with 0.5% bovine serum albumin (BSA), 1% FBS, and 0.1% sodium azide (PBSst). Non-specific binding of the mAb to the cell surface was blocked by preincubating the cells with 40 µg/ml rat IgG (Sigma) in a 100 µl final volume (20 min, 4°C). Cells were incubated with the primary mAb (30 min, 4°C), washed, and the binding was revealed with a secondary FITC- or PE-goat F(ab')2 anti-mouse IgG (H + L) antibody (Beckman Coulter; 30 min, 4°C). Samples were analyzed on an Epics XL or a Cytomics cytometer (Beckman Coulter). For competition analyses, cells were incubated with 50 µl of either the unlabeled antibody or an isotype-matched mAb (10 µg/ml, 40 min, 4°C), followed by 50 µl of an anti-CCR9 biotin-labeled antibody (0.5–2 µg/ml, 30 min, 4°C). After washing, FITC- or PE-conjugated streptavidin was added (30 min, 4°C). Cell staining was evaluated by flow cytometry.

Competitive ELISA

Microtiter plates (Maxi-sorb, Nunc) were used to coat the hCCR9(2–22) synthetic peptide (1 µg/ml in PBS), overnight at 4°C. Afterward, the unoccupied protein-binding sites in the wells were blocked with 0.5% BSA in PBS. Previously titrated biotin-labeled mAb was mixed with unlabeled mAb (2 µg/ml in PBS–0.5% BSA), added to the plate and incubated 1 h, at room temperature. Antibodies bound to the plate were detected with peroxidase-labeled streptavidin (Sigma) and revealed with o-phenylenediamine dihydrochloride (4 mg/ml in 0.15 M sodium citrate buffer, pH 5.0; Sigma). The reaction was stopped with 3N sulfuric acid and the O.D. 492 nm determined. Antibodies were biotinylated with Hydrazide-LC-Biotin (ThermoFisher Scientific) following the supplier's instructions.

Chemotaxis Assays

Migration assays were performed in transwell inserts (Costar) with a 5-µm pore diameter. MOLT-4 cells were re-suspended in RPMI with 1% BSA and 25 mM HEPES, pH 7.4 (10⁷ cells/ml), and 100 µl aliquots were loaded into the upper inserts. Samples of 0–300 nM human CCL25, prepared in 600 µl of the same

medium, were placed in the lower wells. After 2 h incubation at 37°C, 5% CO₂, inserts were removed and the number of cells that had migrated from the transwell insert to the well were counted on an EPICS XL flow cytometer. To analyze the antibody-blocking activity of CCL25-induced migration, MOLT-4 cells were preincubated with different amounts of anti-CCR9 mAb or irrelevant isotype-matched mAb before being loaded into the transwell. For these experiments, 200 nM human CCL25 was used as chemoattractant.

Peptide Synthesis

Linear peptides were synthesized in the Proteomic facility of the CNB, with an automated multiple-peptide synthesizer (AMS 422, Abimed) using a solid-phase procedure and standard Fmoc-chemistry. The synthesis of multiple peptides was performed simultaneously, on a cellulose membrane, by sequential conjugation of membrane-protected amino acids (aa), from their carboxy terminal ends. The application of the activated aa was carried out using the Auto-Spot Robot (ASP222, Abimed) (36). Two independent membranes were prepared with the same set of peptides (each 12 aa long, 10 aa overlap), spanning the complete hCCR9-A isoform (369 aa). In addition, a similar synthesis was carried out where to peptides corresponding to aa 8–19 of hCCR9-A, aa in positions 11–16, each one of them was substituted for each of the remaining proteinogenic aa. Membranes were blocked by incubation with 1% BSA in PBS for 1 h at room temperature, washed, and then incubated for 2 h with anti-CCR9 or isotype control antibodies (in PBS containing 1% BSA and 0.05% Tween-20). After three additional washes, membranes were incubated with a peroxidase-labeled goat anti-mouse IgG antibody (Sigma), for 1 h at room temperature, developed with a chemiluminescence system (GE Healthcare), and exposed to standard X-ray film. The densitometric quantification of the signal obtained at each spot was performed with the ImageJ software.

Surface Plasmon Resonance Analyses

Surface Plasmon Resonance experiments were carried out in a biosensor Biacore 3000 (Biacore, GE Healthcare), using HBS-EP (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, pH 7.4) as running buffer. At the end of each binding cycle, the sensor surface was regenerated with 10 mM glycine-HCl, pH 1.5, allowing resonance signals to return to baseline values. A synthetic peptide corresponding to the aa in positions 2–22 of the human CCR9 isoform A (hCCR9A, identifier: P51686-1), with an additional cystein residue in its N terminus (C-TPTDFTSPINMADDYGS) was immobilized on a carboxymethylated dextran CM5 sensor chip by an amine coupling reaction, as recommended by the supplier. A reference surface was generated in the same manner, except that all carboxyl groups were blocked in the absence of ligand. For kinetic analyses, immobilized antigen at low density was used to minimize mass transport effects and analyte rebinding. Anti-CCR9 mAbs were used as soluble analytes in HBS-EP buffer at concentrations ranking from 0.41 to 33 nM. The interaction analyses were carried out at 25°C with a flow rate of 30 µl/min. Data were collected for 90 s of association and 180 s of

dissociation. For competition analyses, a biotin-labeled peptide corresponding to aa 2–19 of hCCR9A (biotin-K-TPTDFTSPINMADDYGS) was captured on a streptavidin-coated chip (SA). Anti-CCR9 mAbs at a constant concentration (10 nM) were mixed with competitor synthetic peptides at different concentrations (0.01–1 µM). The antibody-peptide mixtures were preincubated for 30 min before they were injected into the biosensor. Sensograms were overlaid, aligned, and analyzed with BIA evaluation Software 4.1. The K_D was determined by fitting the data using a bivalent analyte model. All data set were processed using a double-referencing method (37).

Xenograft Assays

BALB/c Rag2^{-/-} mice (Taconic Bioscience) were bred in the CNB animal facility and used at ages ranging from 8 to 22 weeks. For *in vivo* experiments, MOLT-4 cells (2 × 10⁶) were inoculated sub-cutaneously (s.c.) in the flank of Rag2^{-/-} mice on day 0. In these experimental conditions, 80–90% of the cell inoculations gave rise to tumors. The animals carrying the MOLT-4 cells were divided into four groups, which were inoculated intraperitoneally (i.p.) with anti-hCCR9 91R, its isotype control (IgG2b), anti-hCCR9 92R, or its isotype control (IgG2a) on days 1, 8, 15, and 22 (4 mg/kg on day 1 and 8; 2 mg/kg on days 15 and 22). Tumor size was measured with a Vernier caliper (Mitutoyo) and tumor volume (mm³) calculated as V = [axial diameter length, mm] × [(rotational diameter, mm)²/2]. Mice were sacrificed and tumors were weighted and processed for histology. Tumor burden is expressed as percent tumor weight relative to that of isotype control-treated mice.

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ stock # 005557 (NSG mice, Jackson Laboratories, ME, USA) were bred in the CIB animal facility and used for similar experiments as described in the above paragraph. Animals were injected subcutaneously on day 0 with MOLT-4 cells (1 × 10⁶) in the flank. Two groups were inoculated intra-peritoneally with 4 mg/kg on day 2 and day 7 with anti-hCCR9 92R or its isotype control (IgG2b). All mice were sacrificed at the same time and tumors were weighted and processed for histology.

Complement-Dependent Cytotoxicity (CDC)

MOLT-4 cells (10⁵ cells in 100 µl) were plated on each well of a 96-well V-bottom plate. The cells were incubated with the indicated concentrations of 92R, 91R (anti-hCCR9) or isotype-matched control mAb (30 min, 37°C), centrifuged, and washed. Active or 56°C heat-inactivated baby rabbit complement (25%; AbD Serotec) was added in serum-free Dulbecco's modified Eagle's medium with 1% BSA (1 h, 37°C). The complement in M4 serum was also heat inactivated (56°C, 30 min). The number of non-viable cells was evaluated by flow cytometry after staining the cells with the viability exclusion marker 7-aminoactinomycin (7-AAD; BD Biosciences; 10 min, 4°C); each condition was analyzed in triplicate. Specific lysis was calculated as: 100 × (% dead cells with active complement – % dead cells with inactive complement)/(100% – % dead cells with inactive complement).

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Murine NK cells were isolated from spleens from BALB/c mice using the Auto Macs Pro negative selection system (Miltenyi). After purification, cells were analyzed for mCD3, mCD45 and mCD49b expression by flow cytometry. The purity of NK cells, defined as CD3⁻CD45⁺CD49b⁺, in all the preparations was at least 90%. Cells were cultured for 6–7 days in RPMI 1640 (Lonza), supplemented with 10% FBS, and 1,000 U/ml murine recombinant IL-2 (Peprotech). For cytotoxicity assays, target MOLT-4 cells labeled with Cell Trace CFSE (Invitrogen) were preincubated (30 min) with the indicated mAb concentrations. NK and target cells were cocultured (4 h) at a 20:1 ratio in RPMI-10% FBS, then stained with 7-AAD (10 min, 4°C) and analyzed by flow cytometry. Gating on 7-AAD-positive cells within the CFSE⁺ population indicated the proportion of dead target cells. Specific killing was calculated as: $100 \times [(\% \text{ dead target cells in sample} - \% \text{ spontaneous dead target cells}) / (100 - \% \text{ spontaneous dead target cells})]$. Target cells incubated without effector cells were used to assess spontaneous cell death.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 4 software. Statistical significance was established at $P < 0.05$ as evaluated by Student's *t*-test, unless otherwise indicated. Results are shown as mean \pm SEM.

RESULTS

Initial Characterization of the Anti-hCCR9 Chemokine Receptor mAb 92R

The murine anti-hCCR9 mAb 92R (IgG2a) was generated after gene gun immunization with the full-length hCCR9-A cDNA coding sequence, inserted in a eukaryotic expression vector (pCINeo), using the same strategy as described for 91R mAb (34). Specificity of the binding of 92R to hCCR9-A protein was assessed by flow cytometry analyses of HEK-293 cells stably transfected with the construct used for immunization, using as negative control the same cells transfected with the empty pCINeo plasmid (Figure 1A). In addition, we demonstrated that 92R, similarly to 91R also stained cells expressing the endogenous CCR9 protein in the MOLT-4 T-ALL cell line, but failed to stain the Jurkat T-ALL cells that do not express CCR9 on their cell surface (Figure 1B).

Furthermore, competition analyses demonstrated that 92R competes with itself and 91R for binding to the CCR9⁺ cells MOLT-4, but not with the 3C3 anti-hCCR9 mAb in flow cytometry. Similarly, 91R competes with itself and 92R but not with 3C3 mAb, as demonstrated by flow cytometry (Figure 2A). These data were corroborated by ELISA assays, where binding of biotinylated 91R to the hCCR9 (aa 2–22) synthetic peptide was competed by unlabeled 92R, but not by the isotype control antibody IgG2a. Similarly, binding of biotinylated 92R to the same peptide was competed by unlabeled 91R, but not by the isotype control antibody IgG2b (Figure 2B).

In addition, 92R fails, similarly to 91R (34), to inhibit CCL25-induced migration of CCR9⁺ MOLT-4 cells, unlike the anti-CCR9

mAb 3C3, described to inhibit the CCL25:CCR9 interaction. Indeed, we determined the migration response of MOLT-4 cells to different CCL25 concentrations, observing that it reached the maximum level between 200 and 250 nM CCL25 (Figure 3A). Then, using 200 nM CCL25, the percentage of migrating cells in the absence or presence of different antibodies anti-hCCR9 or their isotype controls was determined. The results show a migration inhibition only in the presence of 3C3 mAb, but neither 92R nor 91R (nor their isotype controls) inhibited this migration (Figure 3B).

Identification of the Critical aa for the Binding to the hCCR9 Epitope by 92R and 91R mAbs

Since 92R and 91R are able to cross-compete with each other for the binding of hCCR9 on the cell surface (Figure 2A), and both of them bind the same epitope, comprised by aa 2–22 of hCCR9-A (Figure 2B), we aimed to identify the energetically critical aa for the interaction "hotspots" within the epitope. For this reason, pepscan analyses were carried out using 180 overlapping synthetic peptides, covering the entire hCCR9 sequence. These experiments allowed to identify the hotspot residues within the epitope, energetically critical for high-affinity binding of each antibody. Indeed, both antibodies gave positive signals with the same peptides 3–6 (Figure 4A), allowing to identify aa 11–16 from hCCR9-A as the functionally critical residues on the epitope recognized by 92R and 91R (Figure 4B). It is worth to note that although the peptides identified by these mAb are the same, the signals obtained with 91R and 92R are somehow different from each other (Figure 4A). Subsequently, each one of the aa from hotspots (sequence PNMADD) was substituted for one of the 19 other aa, allowing to ascertain the relative relevance of each one of the energetically critical aa for the binding of 91R and 92R to the hCCR9 synthetic peptides (Figure 4C). For both 91R and 92R mAbs, turned out that in these assays, the binding of these antibodies to the hotspots are strictly dependent of the presence of an N residue in position 12. The only replacement allowed at position 16 is a D for an E, indicating the relevance of a negative charge at this position. A14 could be replaced by hydrophilic uncharged aa (N, Q, or S) with a significant reduction of signal intensity. The few allowed changes for P11, unlike for A14, are more evident for 91R. Conversely, M13 and D15 allow more changes, with appreciable signal differences between 92R and 91R. For both mAb, the substitution of D15 for K or P is not allowed. The substitution of D15 for R is allowed for 91R but 92R loses its binding capacity. Taken together, these data support the notion that 91R and 92R are different antibodies, since the allowed changes on each position were different. This was further corroborated by sequence analyses of the variable regions from the light and heavy chain cDNAs (Figure 5). Indeed, there are five aa differences on the heavy chain framework and ten on the light chain framework. In addition, on the heavy chain, there is an aa change in CDR1, and on the light chain, there are two aa changes in CDR1, one in CDR2 and another one in CDR3 (Figure 5).

Surface Plasmon resonance analyses using the synthetic surface-bound peptide hCCR9 (2–22) as molecule representative

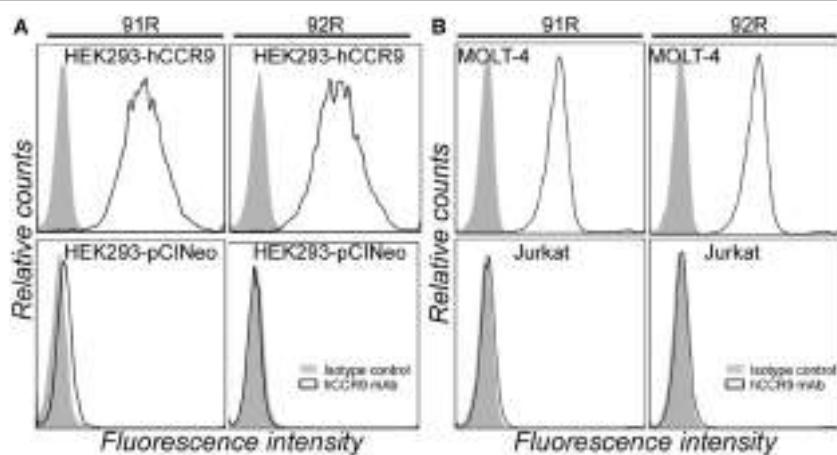


FIGURE 1 | 92R mAb identifies human chemokine receptor CCR9. **(A)** Representative flow cytometry staining of HEK-293 cells stably transfected with either pCINeo-hCCR9 or the empty pCINeo vector using the mAbs 91R, 92R (empty histograms), or isotype-matched control mAbs (IgG2b and IgG2a, respectively) (filled histograms). **(B)** MOLT-4 and Jurkat human leukemia cells were stained with the anti-human CCR9 mAb 91R and 92R (empty histograms) or isotype-matched control mAbs (filled histograms) and analyzed by flow cytometry.

of CCR9, allowed to determine the apparent affinity constants for these antibodies, which was estimated as K_D 3.6 nM for 91R and K_D 8.9 nM for 92R (Figures 6A,B). Competition experiments showed that 1 μ M of peptide hCCR9 (2–22) inhibited most of the binding of 92R to the surface and fully inhibited the 91R binding; whereas peptide hCCR9 (13–30), corresponding to the N-terminus of the B isoform, failed to inhibit mAb binding. The competition with the same concentration of the peptide hCCR9 (10–30), which also contains the hotspots, gave, however, a slightly different competition pattern between 92R and 91R, since for 92R, the sensograms were similar when the competitors were hCCR9(10–30) or hCCR9(2–22), whereas a 20% of the 91R signal was still detected at the end of the association phase in the presence of hCCR9(10–30) peptide (Figures 6C,D). Taken together, these data show that the two antibodies 91R and 92R are able to bind differentially to the same hCCR9 epitope with high affinity.

92R mAb Inhibits *In Vivo* the Growth of Human CCR9⁺-Tumors in Xenografts

The antitumor potential of 92R mAb was assessed in immunodeficient ($Rag2^{-/-}$, BALB/c) mice, after subcutaneous injection of 2×10^6 CCR9⁺ cells from the human T-ALL cell line MOLT-4. On days 1, 8, 15, and 22 after tumor cell injection, the animals were treated with either 92R, 91R, or their isotype controls (IgG2a or IgG2b, respectively) at 4 mg/kg (days 1 and 8), or 2 mg/kg (days 15 and 22). The size of developing tumors was measured until day 78, when mice were sacrificed. The differences between tumor size were significant on 91R-treated mice as compared to isotype-treated mice from day 60 ($P < 0.05$) (Figures 7A,C). On 92R, we failed to detect tumors, although they grew in their control isotype-treated mice (Figures 7A,D). At the time of sacrifice, tumors were removed and weighted. The mean tumor weight for IgG2b isotype-treated control was 1.23 ± 0.33 g and for IgG2a, 0.11 ± 0.06 g (Figure 7B) and were absent in animals treated with 92R (Figures 7B,D). As a positive control, 91R was used, where we found a reduction of 91% on tumor burden, in agreement with previously published data (34). As mentioned above, for 92R, no tumors were detected after sacrificing the animals, although 6/9 animals treated with its isotype control (IgG2a) had tumors (Figure 7D). These data support that 92R efficiently blocked the *in vivo* progression of acute T cell leukemia xenografts and suggest the possibility of using this antibody for therapeutic purposes in human CCR9⁺ tumors.

isotype-treated control was 1.25 ± 0.39 g, whereas for 91R-treated mice was 0.11 ± 0.06 g (Figure 7B) and were absent in animals treated with 92R (Figures 7B,D). As a positive control, 91R was used, where we found a reduction of 91% on tumor burden, in agreement with previously published data (34). As mentioned above, for 92R, no tumors were detected after sacrificing the animals, although 6/9 animals treated with its isotype control (IgG2a) had tumors (Figure 7D). These data support that 92R efficiently blocked the *in vivo* progression of acute T cell leukemia xenografts and suggest the possibility of using this antibody for therapeutic purposes in human CCR9⁺ tumors.

92R mAb Mediates Complement-Dependent and Antibody-Dependent NK Cell-Mediated Cytotoxicity

Complement-dependent cytotoxicity and ADCC are two of the main mechanisms *in vivo* for tumor cell elimination by therapeutic antibodies. For this reason, we tested the *in vitro* ability of 92R to induce lysis of MOLT-4 leukemia cells by either complement fixation, or by NK cell mediated cell cytotoxicity triggered by binding of 92R mAb to the NK cell surface Fc receptors.

For the CDC experiments, specific death of the MOLT-4 cells was evaluated by flow cytometry analyses of 7-AAD incorporation. Both 92R and 91R were able to promote complement-dependent cell lysis (46 ± 1 and $49 \pm 2\%$, respectively) with a much higher efficiency than the commercial anti-CCR9 antibody 112509 ($7.1 \pm 0.9\%$) (Figures 8A,B). As positive control, we used a mouse sera (M4) against MOLT-4, which gave a specific lysis of $60 \pm 0.8\%$. The minimal concentration of 92R mAb that gave a detectable specific cell lysis was 0.4 μ g/ml.

For ADCC experiments, MOLT-4 target cells labeled with the green fluorescent dye CFSE and precoated with either 92R, or isotype control (IgG2a, negative control) were combined with previously *in vitro* activated mouse NK cells (4 h at 37°C). The

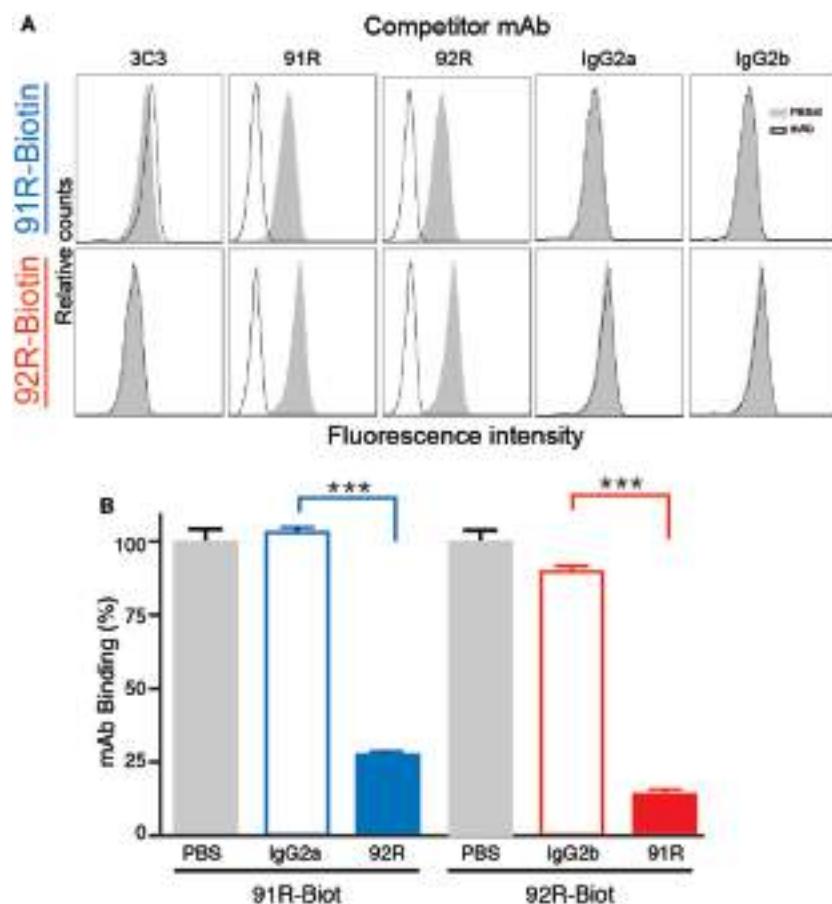


FIGURE 2 | 92R and 91R mAbs compete with each other for binding to hCCR9. **(A)** Competitive binding analyses to CCR9⁺ MOLT-4 cells. Cells were preincubated with unlabeled 3C3, 91R, 92R anti-CCR9 mAbs, or their isotype controls (IgG2a or IgG2b) and, without washing the antibody excess, stained with either biotinylated 91R (top row) or biotinylated 92R (bottom row). After washing, binding of the biotinylated antibodies to the MOLT-4 cells was revealed with streptavidin-FITC and analyzed by flow cytometry. **(B)** Enzyme-linked immunosorbent assay competitive binding analysis of anti-CCR9 mAbs to the hCCR9 (aa 2–22) peptide using biotin-labeled 91R and 92R in the presence of unlabeled competitors (IgG2a-isotype control, IgG2b-isotype control, 91R, or 92R) and revealed with peroxidase conjugated streptavidin. ***P < 0.001.

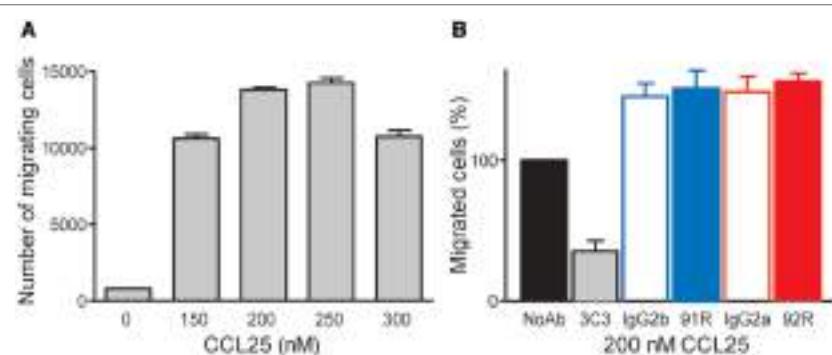


FIGURE 3 | 92R and 91R mAbs failed to inhibit CCL25-mediated migration of the CCR9⁺ MOLT-4 cells. **(A)** Migration response of MOLT-4 cells to different CCL25 concentrations. **(B)** 200 nM CCL25, in the absence of antibody was used to determine the highest number of migrating cells (defined as 100% migration) and to compare with similar experiments where 91R, 92R, or their isotype control antibodies (IgG2g or IgG2a, respectively) were added. In addition, the anti-CCR9 mAb 3C3, known to inhibit the CCL25:CCR9 interaction was used as positive control for migration inhibition.

incorporation of 7-AAD by the CFSE-labeled cells allowed to determine specific NK-mediated killing of MOLT-4 cells by flow cytometry. On these experiments, 91R mAb was used as positive

control. Both 92R and 91R were able to promote NK-dependent cell lysis of the cells (57.7 ± 1.1 and 54.9 ± 4.1 , respectively) with a much higher efficiency than the commercial anti-CCR9 antibody

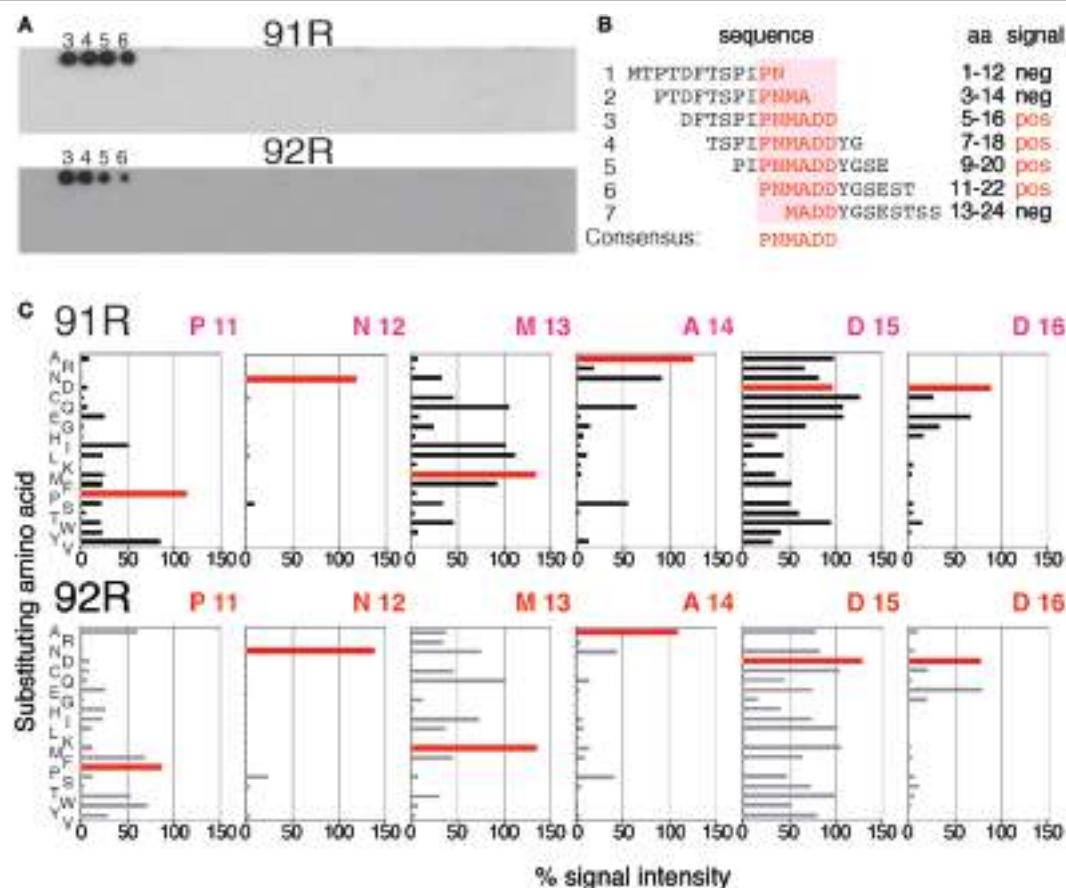


FIGURE 4 | Identification and characterization of the energetically critical amino acid (aa) sequence recognized by 91R and 92R. **(A)** Pepscan analyses were used to identify the hotspots recognized by 91R and 92R mAbs. One hundred and eighty overlapping peptides, 12 aa long each, covering the full sequence of the hCCR9-A isoform, were synthesized on a cellulose membrane. After blocking, the membrane was incubated with 91R or 92R mAbs. Binding of the antibodies was revealed with a peroxidase-coupled anti-mouse IgG antibody and ECL ($n = 2$). **(B)** The sequence of peptides 1–7, including the four giving positive signals for 91R and 92R mAbs are aligned, and the minimum sequence recognized described as it was the unique entire sequence present in all of these peptides (aa 11–16). **(C)** Densitometric quantification of pepscan assays where aa from positions 11–16 were individually substituted by the other 19 proteinogenic aa after binding to 91R or 92R.

112509 used as a control ($20.5 \pm 0.9\%$) (Figures 8C,D). As an additional positive control, we used the mouse serum M4 raised against MOLT-4, which gave a specific lysis of $91.1 \pm 1.1\%$. The 92R mAb concentration needed for a detectable response was $0.04 \mu\text{g/ml}$, whereas it was negligible in the absence of antibody. 91R was used as positive control for ADCC, obtaining similar results to the previously published (34).

Taken together, these results suggest a role for CDC and ADCC in the *in vivo* reduction of tumor growth observed in the xenograft model. To directly determine the *in vivo* role of CDC and ADCC in the antitumoral potential of 92R mAb, we generated subcutaneous xenografts by injection of 1×10^6 CCR9⁺ cells from the human T-ALL cell line MOLT-4 in NSG mice. We used this particular mouse strain because its complement is not functional (due to a mutation in the C5 gene), and their NK activity is highly diminished (Jackson Labs, Bar Harbor, MD, USA). Therefore, if 92R was able to inhibit tumor growth in this model, the mechanism(s) of *in vivo* tumor reduction would be different

from CDC and ADCC. On days 2 and 9 after tumor cell injection, the animals were treated with either 92R or its isotype control (IgG2a) at $4 \mu\text{g/kg}$. On day 31, mice were sacrificed (Figure 9A), tumors removed, and weighted. The mean weight of the tumors was smaller in 92R-treated mice ($0.256 \pm 0.22 \text{ g}$) as compared to control isotype-treated mice ($0.776 \pm 0.53 \text{ g}$) (Figure 9B); therefore, the total tumor burden, measured as the mean of the tumor weights for each group, was reduced by 67% on animals treated with 92R, as compared to control isotype-treated animals, which correlated with the pictures taken of the tumors (Figure 9C). These data support the notion that mechanisms distinct from CDC and ADCC may play a role on the inhibition of acute leukemia tumor growth mediated by 92R *in vivo* in xenografts generated by human CCR9⁺ tumors. However, *in vitro* experiments analyzing whether 92R could mediate the inhibition of tumor growth through apoptosis (Figure S1 in Supplementary Material) or phagocytosis (Figure S2 in Supplementary Material) gave negative results.

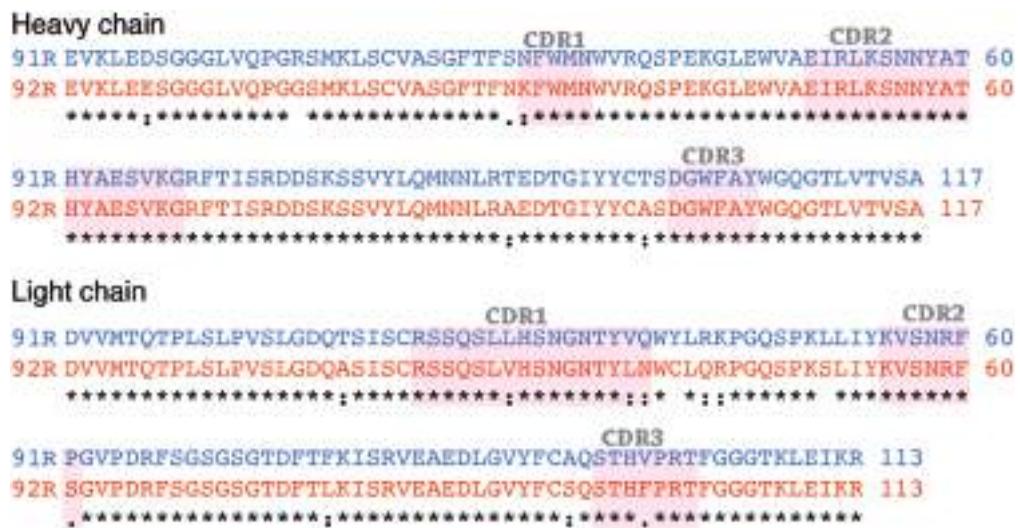


FIGURE 5 | Comparison of amino acid sequences of light and heavy chain variable regions of 91R and 92R. Alignment of light and heavy chain variable IgG regions using the Clustal program version 2.1. The CDR determinants, identified following Kabat's model are shaded. There are differences between 91R and 92R sequences both in the framework region and in the CDR determinants.

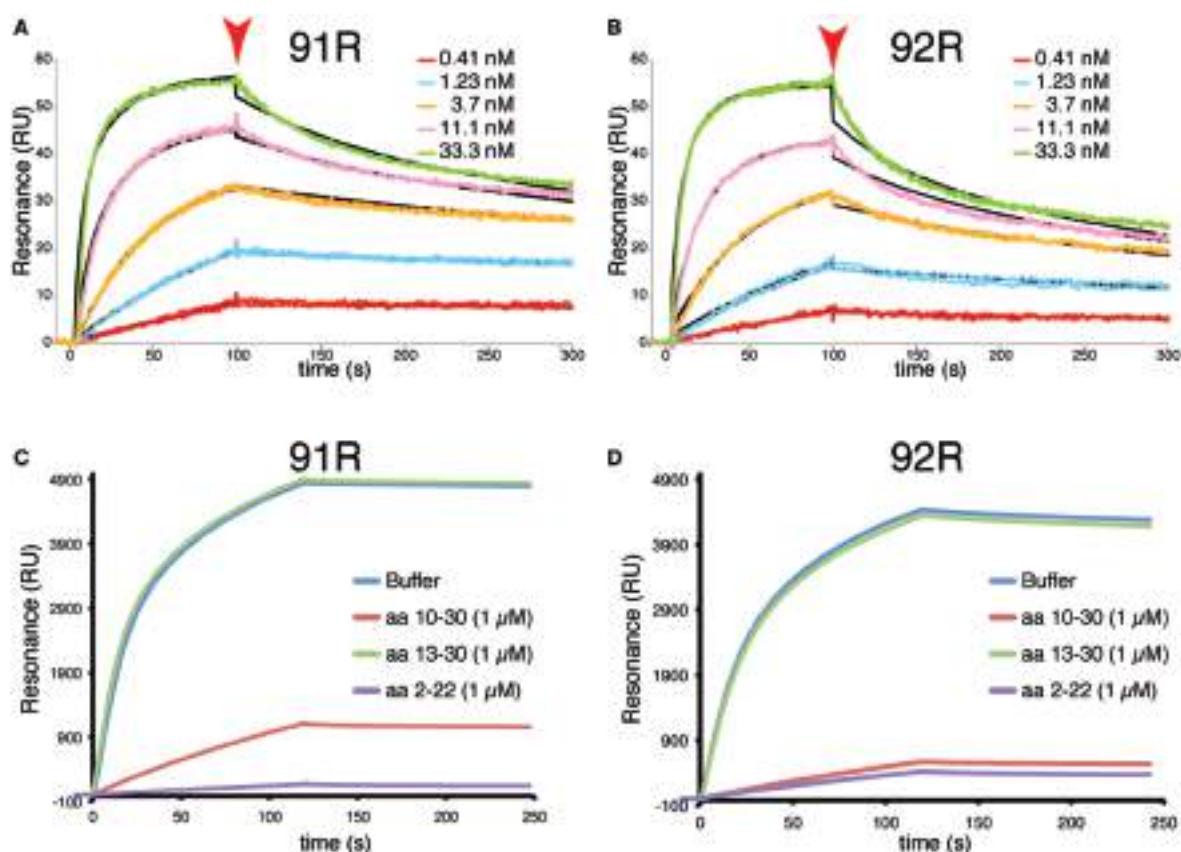


FIGURE 6 | Surface plasmon resonance analyses of the interaction between 92R or 91R and hCCR9 synthetic peptides. A synthetic peptide corresponding to hCCR9A aa 2–22 was immobilized on a carboxymethyl-dextrane CM5 chip by an amino coupling method. Afterward, different concentrations of 91R (**A**) or 92R (**B**) mAb were added, ranging from 0.41 to 33 nM. The black sensograms show the fitted curves obtained with the Biaevaluation 4 software. Competitive experiments were analyzed by binding of 91R (**C**) or 92R (**D**) to a biotinylated synthetic peptide hCCR9(2–19)-captured onto the surface of a SA sensor chip. As competitors, 1 μ M of peptides hCCR9(2–22), hCCR9(10–30) and hCCR9(13–30) were used. The differences between experimental and control flow cells is given in resonance units (RU).

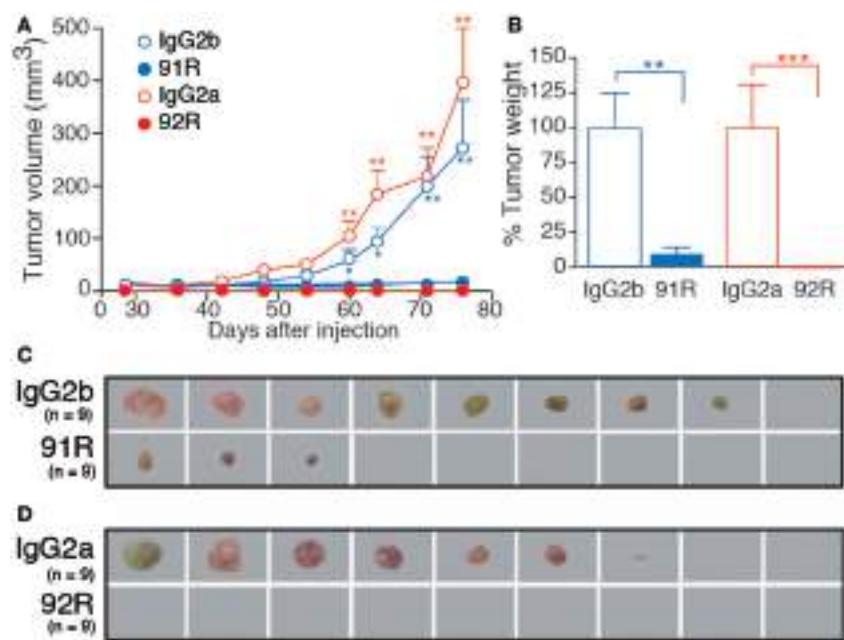


FIGURE 7 | Leukemia xenograft growth is reduced in mice treated with 92R or 91R mAb. For xenograft analyses, MOLT-4 cells (2×10^6) were inoculated subcutaneously in Rag2^{-/-} mice on day 0. Experimental groups received four intraperitoneal doses of 91R, 92R, or their isotype-controls (irrelevant IgG2b and IgG2a mAbs, respectively). First and second inoculations were with 4 mg/kg; whereas third and fourth inoculations were with 2 mg/kg. Tumor growth was measured with a Vernier caliper every 3 days. After mice were sacrificed on day 78, tumors were removed and weighed. **(A)** Tumor growth kinetics. Tumor volume was measured at times indicated and calculated as $V = [\text{axial diameter length, mm}] \times [\text{(rotational diameter, mm})^2/2]$ (9 mice/group). **(B)** Tumor weight (%) relative to IgG2b (or IgG2a) treatment on day 78. Mean \pm SEM ($n = 9$ mice/group). **(C)** Images of tumors from IgG2b-isotype control or 91R-treated mice at the time of sacrifice (day 78). **(D)** Images of tumors from IgG2a-isotype control or 92R-treated mice at the time of sacrifice (day 78). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

DISCUSSION

Patients affected of human T-lineage acute lymphoblastic leukemia (T-ALL) undergo chemotherapeutic treatment; however, a significant amount of them relapse or are refractory to the standard treatment and then should undergo bone-marrow transplantation subsequent to whole body irradiation, with uncertain results (the 5-year overall survival for young adults and adolescents is around 50%, while for children it is near 80%) (38, 39). Therefore, a less invasive therapy would be beneficial for these patients. We believe that the use of mAb-based therapies, as they are being used for other cancer types, would be a real improvement for them.

Overexpression of homeostatic chemokine receptors in tumor cells is linked to cancer progression, metastasis, and poor prognosis (22). Many reports describe a relevant role for the CCL25/CCR9 axis in cancer progression (26, 30), in particular, CCR9 expression has been associated with leukemia aggressiveness (17), its aberrant expression has been detected in several solid tumors (14, 15, 17–30, 40) and has been associated in organ selective metastasis of melanoma to small intestine (14–16), suggesting its potential as a target for cancer treatment. In this context, we have described the generation and characterization of mAb 91R, which inhibits CCR9⁺-tumor growth on *in vivo* subcutaneous xenografts of human ALL in immunodeficient Rag2^{-/-} mice. We continued this work to select other anti-CCR9 mAb with different

epitope specificities, affinities, mechanisms of action and if possible, with higher melting points.

Here, we report the identification and characterization of 92R mAb, an anti-hCCR9 antibody that was raised using as immunogen the full cDNA sequence of the human CCR9-A receptor, which is the main expressed isoform (41). As expected, 92R identifies hCCR9-transfected cells and endogenous CCR9 expressed in MOLT-4 cells. The observation that 92R and 91R cross-compete with each other for binding to the antigen in flow cytometry assays suggests that 92R recognizes an epitope present in the hCCR9 N-terminal domain. This assumption was confirmed by the ELISA data where 92R was able to bind to the synthetic peptide aa 2–22 of hCCR9-A. The energetically critical residues within the hCCR9 epitope required for 92R binding were determined by Pepscan, where a set of overlapping peptides that span all the CCR9-A aa sequence was used. Both 92R and 91R mAb identified as hotspots the aa sequence 11–16 of the receptor, although, the relative intensity signals for each of the recognized peptides was different. Strongly suggesting that 92R and 91R mAb bind differentially to the epitope. Fine mapping of the hotspot sequence shows the relevance of each of these aa, where N12 is strictly required for the interaction with both mAb (92R and 91R). However, the replacement of D15 for R is allowed for 91R, but not for 92R, and the few allowed changes for P11 are distinct for 91R and 92R. Taken together, these data suggest that 92R and 91R are two different antibodies. This was corroborated

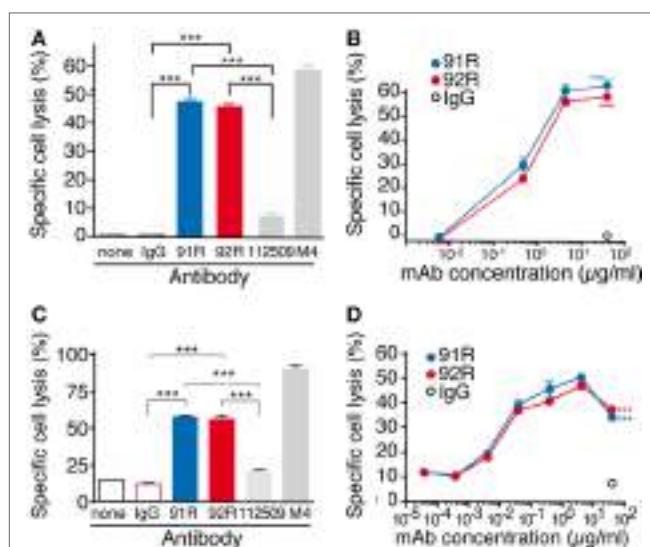


FIGURE 8 | 92R promotes complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity in MOLT-4 human leukemic cells. **(A)** MOLT-4 cells were opsonized with 91R, 92R, or isotype-matched mAb (40 µg/ml, 30 min, 37°C), washed, and incubated (1 h) with 25% active (37°C) or inactive (56°C) baby rabbit complement; cell viability was evaluated in a flow cytometer after 7-AAD staining. Specific complement lysis in the absence of antibody or with 91R, 92R, 112509, M4 or isotype-matched mAb (IgG2a or IgG2b). Each condition was analyzed in triplicate. Data show mean ± SEM of four independent experiments. **(B)** Dose-response curve for specific complement lysis using 91R or 92R and a control IgG2b mAb at indicated concentrations. Data show mean ± SEM of a representative experiment. **(C)** Specific NK-dependent cytotoxicity mediated by 91R, 92R, 112509, isotype-matched mAb (IgG2a or IgG2b) or positive control M4 serum. NK cells were isolated from BALB/c spleens and cultured for 6–7 days in medium containing mrl-2. CFSE-labeled MOLT-4 target cells were preincubated with 91R, 92R, 112509, isotype-matched mAb (IgG2a or IgG2b), or positive control M4 pooled sera (1:1,000) (30 min, 37°C). NK cells and labeled target cells were then cocultured at a 20:1 ratio (4 h, 37°C). Specific lysis was determined by staining dead cells with 7-AAD and analyzing the number of 7-AAD⁺ green cells by flow cytometry. Each condition was analyzed in triplicate. Data show mean ± SEM ($n = 5$ independent experiments). **(D)** Dose-response curve for specific complement lysis using 91R and a control IgG2b mAb at indicated concentrations. Data show mean ± SEM for duplicates from one representative experiment of four. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

by aa sequence analysis of the variable heavy and light chains of these antibodies.

Interestingly, on the heavy chain hyper-variable regions (CDR), there is only one aa difference between both mAb. In CDR1, 92R has a positive charged aa (K) that might justify the lack of interaction with the peptide when the negatively charged D15 is replaced by R. In CDR2, most of the aa are hydrophilic, and many of them charged, with a balance of positive charged aa. This suggests that the electrostatic interactions between these antibodies with CCR9 are very important. Furthermore, in the light chain CDRs, most of the aa differences between 92R and 91R are conservative, and do not seem to be responsible for the signal patterns observed in the Pepscan analyses.

SPR data shows that for both 92R and 91R, the apparent K_D of their interaction with the peptide hCCR9(2–22) are on

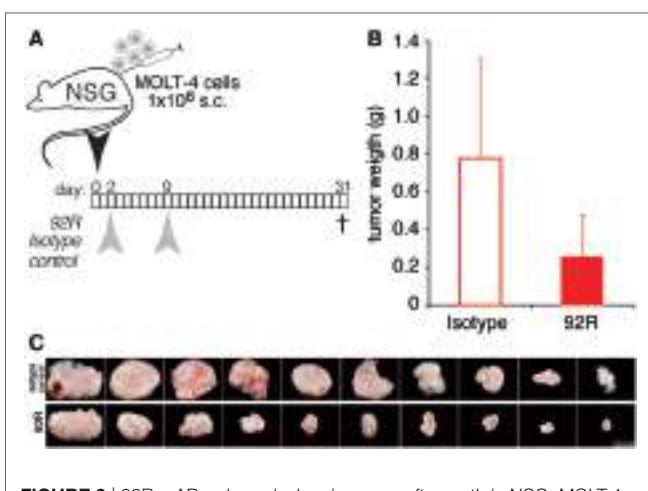


FIGURE 9 | 92R mAb reduces leukemia xenograft growth in NSG. MOLT-4 cells (1×10^6) were inoculated subcutaneously in NSG mice on day 0. Experimental groups received two intraperitoneal doses of 92R or its isotype-control (IgG2a mAb). **(A)** Antibody administration schedule, the animals received 4 mg/kg each on days 2 and 9 of either 92R or the control isotype IgG2a mAb. Mice were sacrificed on day 31, when tumors were removed and weighed. **(B)** Tumor weight in grams of IgG2a (isotype control) and 92R treated animals on day 31. Mean ± SEM ($n = 10$ mice/group). **(C)** Images of tumors from IgG2a-isotype control or 92R-treated mice at the time of sacrifice (day 31).

the nM range, although it is very likely that their affinities for hCCR9 are higher due to the absence in the synthetic peptide of the posttranslational modifications described for CCR9 and other chemokine receptors, such as sulfation or glycosylation (34, 42–44). Furthermore, SPR competition experiments demonstrate that these mAbs most likely do not identify the hCCR9-B isoform that starts in M13 of hCCR9-A, in full agreement with the lack of signal with peptide in position 7 on the Pepscan membranes. Moreover, 91R is able to discriminate between peptides hCCR9(2–22) and hCCR9(10–30) but, 92R does not. This implies that aa 2–9 from the hCCR9-A isoform, contained within the epitope recognized by these mAb, but outside from the defined hotspot sequences (aa 11–16) might also be required for the high affinity binding of 91R, but not for 92R, strengthening the notion that there are functional differences between these two mAb.

The functional differences between 92R and 91R were corroborated by the results of *in vivo* inhibition analyses of tumor growth in xenograft models, where in 91R-treated animals, 3 out of 9 developed tumors, whereas no tumors were detected on 92R-treated animals. We could not exclude the possibility that these differences were due to the different isotypes of the mAb (IgG2b for 91R, IgG2a for 92R). However, both antibodies were able to elicit CDC and ADCC *in vitro* against CCR9⁺ cells, without significant quantitative differences. To further dissect the mechanism(s) involved in 92R-mediated tumor growth inhibition, and to determine the *in vivo* relevance of CDC and ADCC, another strain of immunocompromised mice was used, characterized by an impaired complement and NK cell activities, in addition to lack of T and B lymphocytes (NSG mice). The data obtained in the experiments using NSG mice show a fundamental role for CDC and ADCC as 92R *in vivo* mechanisms of action,

since unlike on the experiments carried-out in Rag2^{-/-} mice, in this case, all the 92R-treated animals ($n = 10$) had tumors. However, it also points out that 92R can inhibit tumor growth through additional mechanisms. Indeed, 92R is able to reduce 67% the tumor burden in animals with impaired NK and complement activities. These additional mechanisms are not yet unraveled, and despite the *in vitro* data suggesting that apoptosis or phagocytosis do not play a role, we cannot formally exclude that 92R might use these mechanisms *in vivo* to inhibit MOLT-4 tumor growth.

Taken together, the data presented here suggests an antitumoral potential for 92R mAb, since in addition to its ability to inhibit tumor growth in xenografts, is able to kill the tumor cells through multiple mechanisms of action, making it an excellent therapeutic agent candidate against CCR9⁺-tumors.

ETHICS STATEMENT

Animal care and treatment were carried out in accordance with Spanish and EU laws. The CSIC Ethics Committee approved these experiments and the Community of Madrid Agriculture Department approved the use of experimental animals: PROEX 038/17 (to JG-S) and PROEX 164/16, PROEX 17/14 and PROEX 121/16 (to LK).

AUTHOR CONTRIBUTIONS

LK designed the immunizations, carried out the cell fusions, and the initial screening. BS-C, IC-G, and MV carried out the *in vitro* and *in vivo* experiments with Rag2^{-/-} mice. MM carried out the competition assays and affinity measurements with the Biacore

biosensor, SS carried out the *in vivo* experiments with the NSG mice. JG-S and LK were responsible for the overall concept and design of the study, data interpretation, and writing, together with BS-C the final manuscript. All authors contributed to drafting, revising, and approving the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/articles/10.3389/fimmu.2018.00077/full#supplementary-material>.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest. JG-S and LK are inventors of a patent application covering 91R and 92R mAbs, owned by the CSIC.

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Chondroitin Sulfate Proteoglycan 4 and Its Potential As an Antibody Immunotherapy Target across Different Tumor Types

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Overexpression of the chondroitin sulfate proteoglycan 4 (CSPG4) has been associated with the pathology of multiple types of such as melanoma, breast cancer, squamous cell carcinoma, mesothelioma, neuroblastoma, adult and pediatric sarcomas, and some hematological cancers. CSPG4 has been reported to exhibit a role in the growth and survival as well as in the spreading and metastasis of tumor cells. CSPG4 is overexpressed in several malignant diseases, while it is thought to have restricted and low expression in normal tissues. Thus, CSPG4 has become the target of numerous anticancer treatment approaches, including monoclonal antibody-based therapies. This study reviews key potential anti-CSPG4 antibody and immune-based therapies and examines their direct antiproliferative/metastatic and immune activating mechanisms of action.

Keywords: CSPG4, MCSP, HMW-MAA, NG2, melanoma, triple-negative breast cancer, immunotherapy, antibodies

INTRODUCTION

Recent advances in the field of cancer immunotherapy, involving specific targeting modalities like monoclonal antibodies or chimeric antigen receptor (CAR) T cell therapies, depend on the identification of appropriate surface antigens. The search for appropriate targets is especially important for certain malignancies such as triple-negative breast cancer (TNBC), which lack expression of the human epidermal growth factor receptor 2 (HER2/neu), estrogen or progesterone receptors, thus rendering them insensitive to available targeted therapies.

Chondroitin sulfate proteoglycan 4 (CSPG4) is a highly glycosylated transmembrane protein, and a member of the chondroitin sulfate group of glycosaminoglycans (GAGs). CSPG4, also referred to as melanoma-associated chondroitin sulfate proteoglycan (MCSP), high-molecular-weight melanoma-associated antigen (HMW-MAA), or neuron-glial antigen 2 (NG2), was first associated with malignant melanoma and subsequently implicated in the pathology of other solid tumors of

different origins, as well as of hematological cancers (1). It has been investigated as a potential immunotherapy target due to its restricted/low distribution in normal tissues and overexpression in certain tumors at different disease stages, and based on evidence for multiple roles in supporting tumor growth and dissemination. Together, long-emerging studies point to CSPG4 as a promising target for cancer therapies, including immunotherapies with monoclonal antibodies. In this review, we summarize reported functions of CSPG4 in cancer and we examine the development of ongoing immunotherapy strategies, most notably monoclonal antibodies that target CSPG4.

CSPG4 NORMAL TISSUE DISTRIBUTION, STRUCTURE, AND PHYSIOLOGICAL FUNCTIONS

Chondroitin sulfate proteoglycan 4 is heterogeneously expressed on normal tissues such as mesenchymal stromal cells—adult progenitor cells, which have been suggested to lose its expression during terminal differentiation (2). Early immunofluorescence/immunohistochemistry data on the distribution of CSPG4 in normal tissues suggest it is expressed in nevi, epidermis and hair follicles but not detected in brain, thyroid, thymus, lung, liver, ureter, testis, spleen, ovary, or peripheral nerves (3). In another early study, immunohistochemical analysis of fetal and adult human tissues suggest CSPG4 distribution in the adrenal cortex, liver, choroid and small intestine in the fetus and in adult peripheral nerves, liver, salivary glands, bladder, lung bronchial glands and sebaceous glands (4). In more recent studies, expression of CSPG4 and its rat ortholog has been reported at low or moderate levels on neuronal glial cells, arteriolar pericytes, smooth muscle cells, macrophages, melanocytes, articular chondrocytes, and others (5–11). At the RNA level, CSPG4 expression has been recently reported in skin, trachea, veins, lung, heart, muscle, diaphragm, adipose tissue, uterus, prostate, thymus, spleen, bone marrow, and gastrointestinal tissue, but importantly at 6.6 times lower levels than in tumors (12).

Its physiological functions are not completely understood and multiple studies report specific roles in different tissues throughout development. In placenta formation, the expression of CSPG4 on extravillous trophoblasts has been implicated in their differentiation and migration (13). CSPG4 is also proclaimed to be involved in angiogenesis and vascularization. Using murine *in vivo* models, NG2 was shown to induce *de novo* vascularization of otherwise the avascular corneal tissue, suggesting an important role in angiogenesis (14). Further reports suggest the involvement of CSPG4 in glial and oligodendrocyte formation and neuronal network regulation, epithelial keratinocyte replenishment, and epidermal stem cell positioning and homeostasis (15, 16).

Although a full understanding of the physiological roles of CSPG4 is still required, all reports suggest it is ubiquitously involved in multiple tissue development and homeostasis processes, and its roles may be differentially modulated based on the nature of the local tissue microenvironment (17). The regulation of CSPG4 expression is reported to be strongly affected by inflammatory cytokines such as TNF- α , interleukin (IL)-1 α , IFN- γ , and

TGF- β and hypoxia-induced mechanisms involving hypoxia-inducible factors. Furthermore, CSPG4 expression was described to depend on epigenetic pathways, certain transcription factors and microRNAs (see Ampofo et al. for review).

Its functional versatility could be explained by its protein scaffold structural characteristics (Figure 1). CSPG4 is a type I single pass transmembrane protein which exists as a core glycoprotein and chondroitin sulfate-decorated proteoglycan (18). Studies with the rat ortholog state CSPG4 consists of a large extracellular portion, a transmembrane domain and a short intracellular portion (19). The extracellular portion comprises three distinct domains. Located furthest from the membrane, D1 is composed of two laminin G-type subdomains and is abundant in disulfide bonds, important for the stability of tertiary structure. This domain is potentially involved in the interactions with the extracellular matrix (20). The middle domain, D2, comprises 15 CSPG4 specific repeats containing several potential glycosylation and chondroitin sulfate binding sites. The CS decoration may confer different attributes, including interaction with integrins and metalloprotease activation (21, 22). It is presently unclear whether CSPG4 is characterized with different glycosylation/glycation patterns in normal or cancerous tissues. The D2 domain has also been proposed to directly bind collagens (23, 24). Although CSPG4 has no reported enzymatic functions, murine ortholog studies suggest it may bind growth factors and present them to receptor tyrosine kinases (RTKs), thus acting as a RTK coreceptor (25, 26).

Domain D3 is the one proximal to the cellular membrane, and contains putative protease cleavage sites as well as carbohydrate decoration, suggesting potential interactions with lectins and integrins (27, 28). Proteolytic cleavage may also allow ectodomain shedding. In support, levels of soluble CSPG4 have been reported in the sera of healthy individuals and patients with melanoma (29). The presence of soluble CSPG4 within circulation has been proposed as a potential diagnostic biomarker to aid melanoma detection and classification at the vertical growth phase (29). Moreover, CSPG4 may undergo endocytic recycling mediated by the endocytic receptor Stonin1 (30). Thus, endocytosis and ecto-domain shedding of CSPG4 may point to different mechanisms involved in the turnover of membrane-bound protein.

The intracellular portion of CSPG4 is characterized by the presence of a threonine- and a proline-rich motif and a PDZ [postsynaptic density protein 95 (PSD-95)—*Drosophila* disc large tumor suppressor (Dlg1)—Zona occludens 1 (ZO-1)] domain (31, 32). The threonine motifs serve as kinase phosphoacceptor sites for protein kinase C α (PKC α) and extracellular signal-regulated kinase 1/2 (ERK1/2) (33, 34). The proline-rich domain and the PDZ domain most likely function as protein scaffolds for other intracellular proteins (31). The structural characteristics of CSPG4 may confer possible functions as a signaling mediator molecule, connecting the extracellular matrix (ECM) with two main intracellular signaling cascades—the integrin-focal adhesion kinase (FAK) axis through integrin interactions and the mitogen-activated protein kinase (MAPK) pathway through activation of RTKs and ERK1/2 (33, 35, 36). These may bestow functional attributes that could encompass promotion of cellular survival, proliferation, and motility. Importantly, studies with

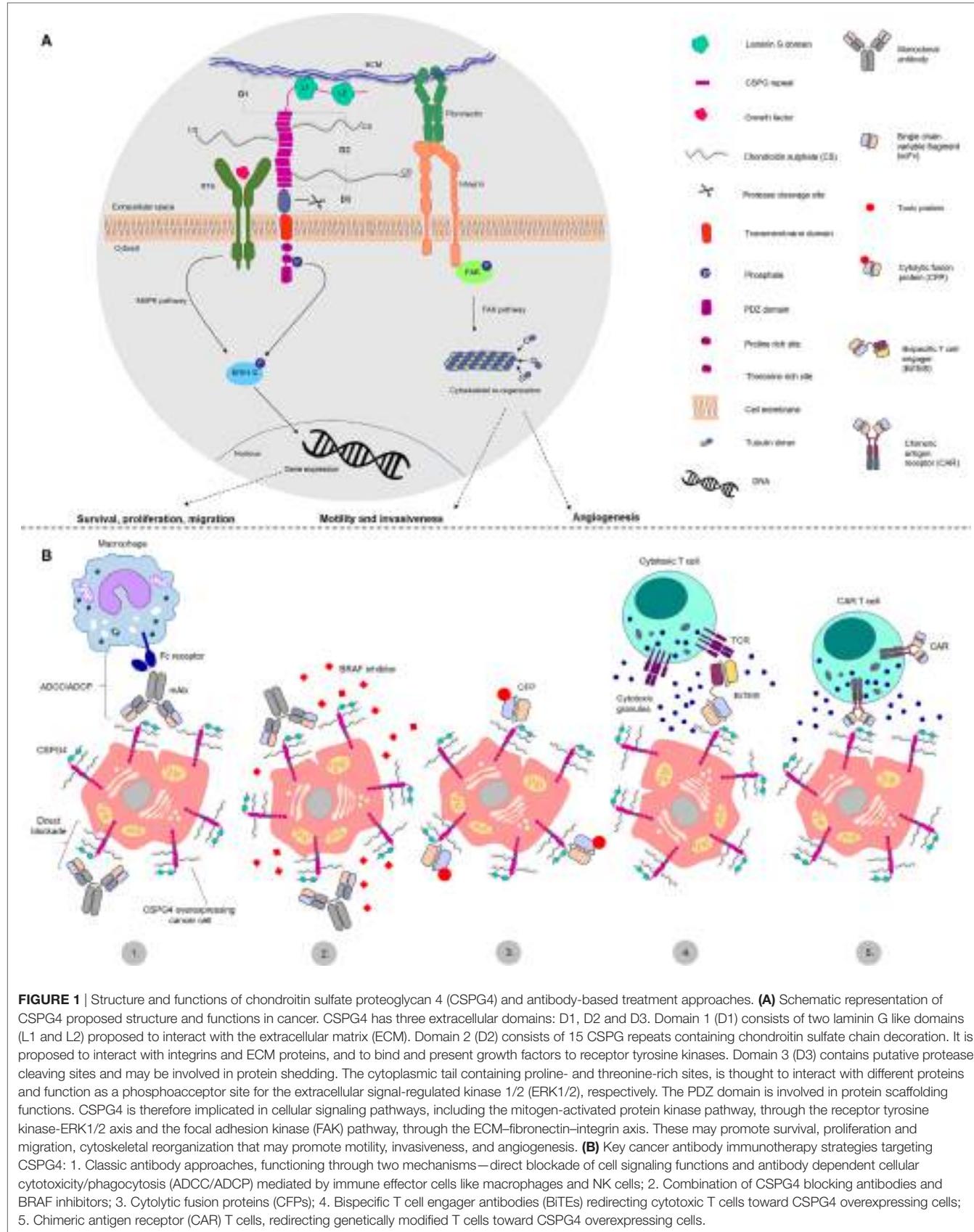


FIGURE 1 | Structure and functions of chondroitin sulfate proteoglycan 4 (CSPG4) and antibody-based treatment approaches. **(A)** Schematic representation of CSPG4 proposed structure and functions in cancer. CSPG4 has three extracellular domains: D1, D2 and D3. Domain 1 (D1) consists of two laminin G like domains (L1 and L2) proposed to interact with the extracellular matrix (ECM). Domain 2 (D2) consists of 15 CSPG repeats containing chondroitin sulfate chain decoration. It is proposed to interact with integrins and ECM proteins, and to bind and present growth factors to receptor tyrosine kinases. Domain 3 (D3) contains putative protease cleaving sites and may be involved in protein shedding. The cytoplasmic tail containing proline- and threonine-rich sites, is thought to interact with different proteins and function as a phosphoacceptor site for the extracellular signal-regulated kinase 1/2 (ERK1/2), respectively. The PDZ domain is involved in protein scaffolding functions. CSPG4 is therefore implicated in cellular signaling pathways, including the mitogen-activated protein kinase pathway, through the receptor tyrosine kinase ERK1/2 axis and the focal adhesion kinase (FAK) pathway, through the ECM-fibronectin-integrin axis. These may promote survival, proliferation and migration, cytoskeletal reorganization that may promote motility, invasiveness, and angiogenesis. **(B)** Key cancer antibody immunotherapy strategies targeting CSPG4: 1. Classic antibody approaches, functioning through two mechanisms—direct blockade of cell signaling functions and antibody dependent cellular cytotoxicity/phagocytosis (ADCC/ADCp) mediated by immune effector cells like macrophages and NK cells; 2. Combination of CSPG4 blocking antibodies and BRAF inhibitors; 3. Cytolytic fusion proteins (CFPs); 4. Bispecific T cell engager antibodies (BiTEs) redirecting cytotoxic T cells toward CSPG4 overexpressing cells; 5. Chimeric antigen receptor (CAR) T cells, redirecting genetically modified T cells toward CSPG4 overexpressing cells.

NG2 knockout mice suggest that deletion of this antigen is not lethal (37).

Therefore, research studies into CSPG4 point to stem cell origin, multiple functions in tissue development, together with regulation of expression by inflammation, hypoxia, decreased methylation mechanisms, as well as specific protein scaffolding structure and reported roles in cell signaling. These features may also translate to contributions in cancer pathology in several tumor types.

CSPG4 EXPRESSION AND PUTATIVE PATHOLOGICAL FUNCTIONS IN CANCER

Early reports associated overexpression of CSPG4 with malignant melanomas, and more recently its enhanced expression has been identified in other cancer types. Despite these developments, the exact involvement of CSPG4 in the etiology of cancer is widely unknown.

It remains unclear whether CSPG4 has a role in tumor initiation or its expression only accumulates in tumors as a secondary tumor-associated event. Overexpression of CSPG4 has not been reported to be a result of genetic aberrations such as gene amplifications or chromosome translocation, suggesting CSPG4 may not be a primary driver in the onset of cancer. Even though its expression is not connected to the onset of epithelial tumors or hematological cancers, it has been linked to the putative mesenchymal stem cell origin of sarcomas and the epithelial–mesenchymal transition in melanoma, thought to be important for malignant transformation (36, 38–40). Several reports point toward potential roles in cancer growth and dissemination. Expression of the CSPG4 rodent homolog has been shown to localize in the invasive front of the filopodia of oligodendrocytes, suggesting involvement in mediating tumor cell motility and cancer dissemination (38, 41). In support, immunohistochemistry data evaluating CSPG4 expression in human melanoma claim higher levels in metastatic lesions than in primary tumors, and CSPG4 mRNA expression was reported to be predictive of metastasis formation in soft tissue sarcoma patients (6, 42).

Furthermore, CSPG4 is believed to contribute to cancer growth and progression through promotion of angiogenesis. Using cell line and patient derived glioblastoma xenografts in nude mice, Wang et al. reported that NG2 RNA interference *in vivo* decreased tumor volume and vasculature (43). In addition, a retrospective study focused on germline polymorphisms related to the function of pericytes in colorectal cancer, identified a CSPG4 polymorphism to be predictive of lower progression-free survival in patients treated with the monoclonal antibody (mAb) bevacizumab—specific for the vascular endothelial growth factor (44). More evidence is required to clarify the exact mechanism through which CSPG4 promotes angiogenesis in cancer.

Disparate reports therefore point to potential contributions of CSPG4 in cancer growth, vascularization, dissemination, and metastasis (**Figure 1**). These may provide opportunities for therapeutic interventions targeting CSPG4 and multiple cancer-associated pathways this molecule may be involved in.

Expression in Neuroectodermal Cancers Malignant Melanoma

Among the neuroectodermal tumors, malignant melanoma is the most thoroughly characterized in terms of CSPG4 expression and functions (36). CSPG4 is the only well characterized cell surface melanoma-associated antigen and it has been examined as a potential target employing different therapeutic approaches. It is expressed in over 70% of melanomas, and its expression has been detected throughout different disease stages.

Multiple studies demonstrate the importance of a functional full length CSPG4 for the survival, growth, and motility of melanoma tumor cells *in vitro* and tumor formation *in vivo*. CSPG4 contributes to enhanced activation of the integrin-FAK pathway through interaction with ECM components, which leads to integrin clustering and subsequent downstream signaling, cytoskeletal reorganization and increased motility and invasiveness (14, 21, 27, 33, 35). On the other hand, CSPG4 facilitates the MAPK pathway through activation of ERK1 and ERK2. ERK1 and 2 can then regulate the microphthalmia-associated transcription factor, which then alters the levels of vital proteins—increasing the expression of fibronectin, but also repressing the expression of E-cadherin, both of which have been shown to be associated with metastasis (45–49).

Since a big proportion of melanomas feature constitutively active MAPK pathway due to mutations in the gene encoding the serine/threonine-protein kinase, BRAF, several groups have investigated the effect of CSPG4 in BRAF mutant melanoma *in vitro*. Full length CSPG4 is required for maximal activation of ERK1/2, and siRNA inhibition of CSPG4 leads to a reduction in ERK signaling (50). Importantly, *in vitro* studies report that specific small molecule inhibitors for mutant BRAF can synergize with the 225.28 anti-CSPG4 mAb in inhibiting cell growth and proliferation (51). On the other hand, ERK1/2 signaling blockade leads to reduced CSPG4-dependent cancer cell motility (50). These reports suggest that alongside constitutive activation of BRAF, CSPG4 may additionally promote MAPK pathway activation.

Glioma

Expression of CSPG4 is found in subsets of normal glial cells in developing and adult central nervous system (see Dimou and Gallo for a review) (52). Although less well examined than melanoma, CSPG4 expression has also been associated with gliomas that originate from astrocytes, such as glioblastoma multiforme (GBM) (53–55). In a study analyzing mRNA expression data sourced from The Cancer Genome Atlas, CSPG4 mRNA levels were reported to be elevated compared to normal tissue controls (54). As in melanoma, functions of CSPG4 in glioblastoma are believed to be related to malignant progression through facilitating tumor cell interactions with collagen and promoting angiogenesis (56, 57). Interestingly, as reviewed by Ampofo et al., the expression levels of both CSPG4 and the platelet-derived growth factor receptor alpha (PDGFR- α) in glioma are downregulated by micro RNA miR192-2 (17). Moreover, CSPG4 and PDGFR- α have been reported to interact and enhance cell proliferation upon PDGF stimulation. Therefore, miR192-2 has been proposed by Ampofo et al., to have therapeutic potential in glioma. CSPG4

has also been associated with pediatric brain tumors such as medulloblastoma (58–60).

Expression in Epithelial Cancers

Breast Cancer

Another cancer type of epithelial origin, whose associations with CSPG4 have attracted a surge of interest, is breast cancer. Expression of CSPG4 in triple-negative breast cancer (TNBC) in particular is of special value, since this subtype represents an area of unmet need for novel targeted therapies. TNBCs, comprising 15% of all breast cancers, lack expression of the estrogen, progesterone and the HER2. Their triple-negative status makes them insensitive to the current hormone and HER2 targeted therapies. TNBCs are more aggressive with worse prognosis compared with other breast cancer types with no targeted therapies available, and therefore, novel targets and treatment options are urgently required.

Chondroitin sulfate proteoglycan 4 expression was described in TNBC primary lesions and metastatic tumor cells from pleural infusions, including cancer stem cells (61). Although CSPG4 expression does not appear to be expressed exclusively by basal breast cancers including TNBCs, its expression may be associated with poor prognosis and relapse in breast cancers (62). Findings to-date indicate a link between the expressions of carbohydrate sulfotransferase-11 (encoded by CHST11) which is involved in decorating CSPG4 with chondroitin sulfate and the metastatic behavior of triple-negative breast cancer cells (63). Reportedly, CHST11 is overexpressed in aggressive breast cancers and facilitates the interaction between p-selectin and CSPG4. These observations are in line with the proposed role of CSPG4 as a mediator between the ECM and intracellular signaling pathways and a metastatic driver, P-selectin. The latter is thought to allow cancer cells to resist the immune response, and to support binding to endothelial cells, and thus hematogenous spread (64). Further research is required to elucidate the CSPG4 pathological contributions to breast cancer formation and progression.

Head and Neck Cancers

Head and neck squamous-cell carcinomas (HNSCC) normally have a poor prognosis, with a 5-year survival rate of around 40–50%. Warta et al. recently reported CSPG4 to be significantly overexpressed in HNSCC cells (65). Furthermore, high expression in patient lesions was found to correlate with poorer prognosis compared with low expression of CSPG4 (65). The study noted that few biomarkers are currently available to predict survival in HNSCC, and that CSPG4 could in future serve as a prognostic indicator.

Expression in Mesenchymal Cancers

A recent study using murine sarcoma models demonstrated that both malignant bone and soft tissue sarcomas, as well as benign desmoid tumors could originate from CSPG4-expressing pericyte cells (66). Nevertheless, the authors did not investigate the specific implications of CSPG4 expression in the tumor formation process. Another potential mechanism described by Cattaruzza et al. suggests an interplay between CSPG4 and type VI collagen in the progression of soft-tissue sarcoma (67). The same study

reports upregulation of both CSPG4 and collagen type VI in soft-tissue sarcoma lesions and suggests that the combination can predict metastasis and poor clinical prognosis. However, the role of CSPG4 in mesenchymal tumors is yet to be revealed.

Expression in Hematological Cancers

Chondroitin sulfate proteoglycan 4 expression has also been reported in acute myeloid leukemia and acute lymphoblastic leukemia (ALL), while it is not expressed on normal lymphocytes, granulocytes or hematopoietic progenitor cells (68–71). Remarkably, for both ALL and AML, CSPG4 expression strongly correlated with the 11q23 gene rearrangement of the KMT2A gene encoding the lysine methyltransferase 2A. The mechanisms behind this correlation are still unknown. Nicolosi et al., shared an unpublished observation that a potential function of CSPG4 in leukemic malignancies could be induction of drug resistance, based on CSPG4 *in vitro* knock-in experiments in mixed lineage leukemia gene bearing cells, resulting in increased drug transporter expression (38).

CSPG4 AND CROSS-TALK WITH THE IMMUNE SYSTEM

Several studies have investigated the interactions between the chondroitin sulfate chains of proteoglycans or their degradation products and components of the immune system.

It has been reported that CS could stimulate monocytes to secrete IL-1 β and to induce B cell proliferation *in vitro* (72). The effect of CS on B cell proliferation was corroborated by Aoyama et al., who demonstrated that CS could enhance murine B cell proliferation *in vitro* through PKC translocation and activation of protein kinase B (PKB, Akt) kinase (73). A role of CS in the maturation of dendritic cells was suggested with human monocyte-derived dendritic cells cultured *in vitro* in the presence of CS, hyaluronic acid, components of the ECM and human granulocyte-macrophage colony-stimulating factor (GM-CSF). CS-stimulated cells could differentiate faster than when cultured with GM-CSF and IL-4 (74). Another study utilizing splenocytes from ovalbumin (OVA)-immunized mice cultured *ex vivo*, showed that CS addition to culture stimulated secretion of Th1-type cytokines including IFN- γ , IL-2, and IL-12 and suppressed the secretion of Th2-type cytokines (IL-5 and IL-10) (75). Moreover, injection of BALB/c mice with CS and other glycosaminoglycans (GAGs) was shown to induce autoimmune conditions like rheumatoid arthritis through recruitment of CD4 $^{+}$ T cells (76). In addition, treatment of murine NK cells with chondroitinase or a proteoglycan biosynthesis inhibitor resulted in substantial decrease in IFN- γ secretion through interaction with murine IL-12. On the other hand, CSPG low-molecular weight disaccharide fragments could control the inflammatory response in a mouse model of autoimmune encephalitis, in a rat model of inflammation-mediated neuropathology of the eye, and in a delayed-type hypersensitivity model in Balb/c mice through reduced migration and activation of inflammatory T cells (77).

With regards to the role of chondroitin sulfate proteoglycans (CSPGs), an early study reported these located inside the granules

of human NK cells and their exocytosis during NK cell-mediated cytotoxicity of tumor cells (78). Furthermore, a study of primary cultured human macrophages, showed secreted CSPGs as metabolic products of macrophages and increased secreted CSPG4 following lipopolysaccharide (79) stimulation (80).

A more recent immune monitoring study identified that both healthy individuals and melanoma patients have circulating CSPG4-reactive CD4⁺ T cells. The study did not report a significant correlation between the T cell responses against a HLA-DR presented CSPG4 peptide, quantified through IFN- γ production, and the tumor burden of patients. The authors showed that a smaller proportion of the melanoma patients (11 out of 42) compared to healthy volunteers (11 out of 13) exhibited T cell reactivity to CSPG4 (81). Other *in vivo* studies demonstrated that LPS induced the expression of the murine CSPG4 ortholog by rat microglia cells. Further *in vitro* experiments showed that NG2 RNA silencing of LPS-treated microglia blocked the mRNA expression of nitric oxide synthase (Yajima et al.), and of proinflammatory cytokines including IL-1 β and tumor necrosis factor α (TNF- α), but not of chemokines like monocyte chemoattractant protein 1 and stromal cell-derived factor 1 α (SDF-1 α). Importantly, this study demonstrated that NG2 is not only expressed upon stimulation of the microglia, but it likely has a role in regulating expression of pro-inflammatory cytokines (82).

In summary, chondroitin sulfate proteoglycans (CSPGs), CS carbohydrate chains, as well as small molecular weight CS degradation products and CSPG4, each appear to influence the activation, maturation, proliferation and migration of different immune cell subsets. The definitive roles for CSPG4 in the immunology of cancer are however still widely unexplored. More research is required to clarify whether the interactions between CSPG4, its amino acid or carbohydrate moieties and different components of the immune system could be exploited to enhance patient response to CSPG4 targeted immunotherapy or to be counteracted to avoid potential negative immunomodulatory functions.

CSPG4 AS A TARGET FOR ANTIBODY THERAPIES IN CANCER

Since CSPG4 is found to be overexpressed in a number of malignancies and based on its low and restricted distribution in normal tissues, alongside emerging evidence for crucial roles in cancer growth and dissemination, much research has focused on the development of different therapeutic approaches, including monoclonal antibodies. Some of these antibody-based strategies focused on CSPG4 are discussed below.

Classic mAb Approaches

Published studies describe a limited number of mAb clones recognizing CSPG4, the most commonly cited of which is a murine clone, 225.28. One of the earliest efficacy studies reports the antitumor efficacy of the murine 225.28 conjugated to methotrexate in nude mice bearing human melanoma xenografts (83). Even though conjugated mAb had superior efficacy to methotrexate alone, the efficacy of the mAb alone was not investigated. Melanoma tumor-bearing SCID mice treated with

the murine mAb 225.28 bore smaller tumors compared with controls, and treatments were associated with modulation of various tumor suppressor-genes and genes involved in cancer metastasis (84). Besides melanoma, this murine mAb was also reported to inhibit the proliferation, adhesion and migration of TNBC cells, as well as to downregulate tumor-promoting signaling pathways *in vitro*. Moreover, the murine mAb was demonstrated to reduce tumor growth in two human TNBC cell line-derived lung metastasis models in SCID mice, and to decrease tumor angiogenesis and tumor recurrence after surgical removal in an orthotopic human TNBC cell line xenograft model in SCID mice (61). The murine antibody clone 225.28 was later also demonstrated to restrict tumor growth synergistically in combination with BRAF inhibitors *in vitro* (51). The anticancer potential of mAb 225.28 was tested against 11q23 ALL cells (71). The mAb on its own showed no direct effects on proliferation inhibition in ALL cells *in vitro*, but it increased the efficacy of the chemotherapy agent Cytarabine. The mAb 225.28 also showed some tumor growth restriction efficacy in a subcutaneous ALL model in SCID mice. One of the first studies reporting an antibody engineered with a human Fc region, was of a chimeric version of the mouse mAb 225.28. The study showed that this mAb could mediate antibody-dependent cellular phagocytosis (ADCP) by human monocytes *in vitro* and could restrict tumor growth *in vivo* in a melanoma NOD-SCID-Gamma (NSG) mouse model engrafted with human immune cells (85). Interestingly, anti-CSPG4 IgG4 was demonstrated to not only lack tumor inhibition properties *in vivo*, but to also impair the efficacy of its IgG1 analog when administered in combination. This finding highlights the importance of choosing appropriate Fc domain and antibody isotype when designing therapeutic mAbs. Moreover, the original murine anti-CSPG4 clone 225.28 has been reported to exhibit direct cancer cell proliferation inhibition properties. In this context, the data reported by Karagiannis et al. opens different avenues for discussion and future investigation, concerning the effect of chimerizing or humanizing mAbs on their direct blockade properties, as well as the magnitude of the immunologically induced anticancer effects engendered by mAb immunotherapy.

Chondroitin sulfate proteoglycan 4 has also been targeted in other malignant indications using different antibody clones. The murine mAb clone TP41.2 was used in malignant mesothelioma (86). TP41.2 showed *in vitro* antitumor effects by reducing cancer cell proliferation, adhesion, motility, migration and invasion. The antibody also reduced tumor growth *in vivo* and prolonged the survival of mesothelioma-bearing SCID mice. In a different study by Wang et al. the authors utilize a single-chain variable fragments (scFv) fused to a human IgG1 Fc portion—scFv-FcC21 and demonstrated growth and migration inhibition of a TNBC cell line *in vitro*, as well as reduction of lung metastasis in a melanoma cell line model in SCID mice *in vivo* (87). The scFv-FcC21 antibody was shown not to be able to induce antibody-dependent cellular cytotoxicity or phagocytosis *in vitro*. Furthermore, an antimouse CSPG4/NG2 antibody clone 9.2.27 was conjugated to polyethylene glycol to increase its affinity for rat Fc γ RIII on rat NK cells, and was used in combination with adoptive NK cells in glioblastoma engrafted athymic rats. The combination of

adoptive NK cell transfer and PEGylated mAb 9.2.27 could restrict tumor growth *in vivo* (88). Curiously, the authors accounted the therapeutic efficacy to macrophages recruited to the tumor, whose clodronate-mediated depletion abolished the antitumor effects of the mAb. The authors suggested that the adoptively transferred NK cell plus mAb treatment might be responsible for re-educating tumor-infiltrating macrophages to render antitumor functions, however, the exact mechanism remains unknown. Interestingly, the antigenic determinants of the monoclonal antibodies, described in the above section, have been suggested to recognize the CSPG4 core protein independently of the presence of chondroitin sulphate (89, 90). It has also been proposed that removal of the CS decoration from the core CSPG4 protein would not affect the reactivity towards CSPG4 of any of the murine clones, mentioned above. Nevertheless, these observations require further confirmation (89).

Even though certain anti-CSPG4 antibody clones showed promise for therapeutic application in different cancer types and in a number of *in vitro* and *in vivo* models, most of published studies were performed using antibodies with mouse Fc regions. With some encouraging findings against CSPG4-expressing tumors, the potential of anti-CSPG4 antibodies against cancer would benefit from further in-depth research using novel constructs including those engineered with human Fc regions.

Anti-idiotypic Antibodies

Early strategies targeting CSPG4 utilized the development of anti-idiotypic antibodies (anti-id), which target the binding sites of other anti-CSPG4 antibodies, essentially mimicking the tumor antigen's binding site on the antibody, and thus aiming to serve as immunogens or vaccines (91, 92). A clinical study reported increased survival and metastasis regression in patients with melanoma who developed anti-CSPG4 antibodies following administration of the anti-idiotypic mAb MK2-23 (93). Despite the promising clinical results, MK2-23 never reached clinical application as a therapy due to standardization issues and safety concerns linked to its administration together with the adjuvant Bacille Calmette-Guerin (BCG), deemed necessary for triggering effective adaptive immune responses (94). Further approaches to overcome those issues included fusing MK2-23 to human IL-2 (94) or utilizing DNA vaccines encoding MK2-23 scFv (95). In a phase I clinical trial, another anti-idiotypic antibody, MF11-30, induced complete remission in one advanced melanoma patient as well as conferred minor survival benefits in three other patients with advanced melanoma whilst no toxicities were reported as a part of Ref. (96).

Another approach concerns vaccine approaches, such as mimotope vaccination studies utilizing conformational CSPG4 epitopes recognized by the 763.74 or the 225.28S anti-CSPG4 mAb clones. Such strategies have been studied and reported induction of CSPG4-specific antibodies in mimotope-vaccinated animals, as well as promising antimelanoma activity of these antibodies *in vitro* through direct proliferation inhibition or in murine effector cell ADCC assays (97–99).

Even though anti-id antibody strategies or mimotope vaccines are currently not in the spotlight, these early studies indicate that CSPG4 may be a promising target for vaccine-based cancer immunotherapy.

Bispecific T Cell Engagers

Bispecific T cell engagers represent a novel therapeutic modality based on the fusion of two single-chain variable fragments (scFv), one of which binds the target antigen while the other engages with T cells *via* CD3. Unlike classic monoclonal antibodies, BiTEs are designed to activate cytotoxic T cells against tumor cells. In 2011, a new CSPG4-targeting BiTE antibody was shown to induce antitumor effects *in vitro* *via* redirected lysis (100). Following incubation with healthy donor PBMCs, all of the 23 melanoma cell lines utilized in the study were successfully lysed in a dose- and effector:target ratio-dependent manner. Furthermore, the BiTE antibody showed promise in a melanoma patient setting *in vitro* by triggering cytotoxicity by melanoma patient-derived T cells against allogeneic or autologous melanoma cells. Another study reports important findings about the design of CSPG4-targeting BiTE therapeutics linked to the epitope distance to the target cell (101). Anti-CSPG4 BiTE antibodies proved much more potent when binding epitopes located closer to the cell membrane. This was proposed to be linked to the large size of the CSPG4 antigen and represents an important factor to consider for the design of antibody therapeutic agents with maximal potency.

CAR T Cells

Chimeric Antigen Receptor T cells represent another promising T cell-based therapeutic approach utilizing monoclonal antibodies whose efficacy against CSPG4 is being investigated. CAR T cells are genetically modified to express a chimeric receptor based on the targeting moiety of a mAb (scFv) recognizing the antigen of interest, to re-direct cytotoxic T cells toward tumor cells. The robust clinical success in the treatment of ALL has attracted a lot of attention on CAR T cell approaches, with one anti-CD19 based therapy expected to soon be granted FDA approval for the treatment of pediatric ALL (102). One of the first studies investigating the potential of anti-CSPG4 CAR T cells showed *in vitro* cytolytic potency against a variety of solid tumor cell lines including breast cancer, melanoma, mesothelioma, glioblastoma and osteosarcoma (103). Another study investigating anti-CSPG4 CAR T cells announced promising efficacy outcomes against melanoma, breast cancer and head and neck cancer *in vitro* and *in vivo* using cell line xenografts in mice (104).

Even though preclinical data on anti-CSPG4 CAR T cell therapy are encouraging, it remains to be established whether the clinical responses observed with CAR T cell treatments against liquid tumors can be reproduced in solid malignancies. A prominent limitation of the efficacious re-targeting of T cells against solid tumors is the tumor stroma, often inhibiting T cell trafficking and potency. Multiple approaches have been designed to address this issue, including the expression of chemokine receptors by the CAR T cells or *in vivo* tumor modification to encourage the secretion of T cell chemoattractants (105).

Radioimmunotherapy

Monoclonal antibodies recognizing CSPG4 could be conjugated to a radioactive isotope for radioimmunotherapy, designed to target radiation directly and more specifically to tumor cells, with the aim of reducing non-specific exposure of normal cells to the radioactive isotope. Targeted radioimmunotherapy may

TABLE 1 | Antibody-based treatment approaches targeting CSPG4.

Treatment strategy	Clone/construct	Toxin conjugate	Treatment combination	Antibody species	In vitro model and indication	In vivo model and indication	Proposed mechanism(s) of action	Clinical trial	Key reference
Classic mAb	225.28S	MTX	N/A	Full mouse antibody	Melanoma cell line	Human melanoma cell line xenograft; nude mice	Growth inhibition, delivery of cytotoxic drugs to the tumor	N/A	(83)
Classic mAb	225.28S	N/A	N/A	Full mouse antibody	Melanoma, TNBC cell line	Human melanoma cell line xenograft; SCID mice	Disruption of the interaction between the cancer cells and the ECM	N/A	(84)
Classic mAb	225.28S	N/A	N/A	Full mouse antibody	TNBC cell line	Human TNBC cell line lung metastasis model; SCID mice Orthotopic human TNBC cell line xenograft; SCID mice	Direct effects (growth, adhesion, and migration inhibition)	N/A	(61)
Classic mAb	225.28S	N/A	PLX4032 (BRAF inhibitor)	Full mouse antibody	Human BRAF ^{V600E} mutant melanoma cell lines	N/A	Synergistic direct effects (growth, migration, survival inhibition); delayed BRAF inhibitor resistance	N/A	(51)
Classic mAb	225.28S	N/A	Cytarbine (chemotherapy)	Full mouse antibody	11q23 AML cell line	Human AML cell line subcutaneous model; SCID mice	No direct effects (mAb alone); synergistic antiproliferative effects with Cytarbine; no <i>in vivo</i> effects on tumor growth/animal survival	N/A	(71)
Classic mAb	225.28S	N/A	N/A	Chimeric antibody (mouse Fab, human Fc)	Melanoma cell lines	Human melanoma cell line xenograft; human immune cell engrafted NSG mice	Immune mediated effects (ADCC/ADCP)	N/A	(85)
Classic mAb	TP41.2	N/A	N/A	Full mouse antibody	Mesothelioma cell line	Human mesothelioma cell line xenograft; SCID mice	Direct effects (cell growth, adhesion, motility, migration, and invasiveness)	N/A	(86)
Classic mAb	scFv-FcC21	N/A	N/A	Recombinant scFv mAb with a human Fc region	TNBC cell line	Human melanoma cell line xenograft; SCID mice	No immune mediated effects; direct effects (cell growth and migration inhibition)	N/A	(43, 87)
Classic mAb	9.2.27	PEG	Adoptive NK cell transfer	Full mouse antibody	N/A	Human GBM cell line xenograft, patient derived GBM xenograft; athymic rats	Immune-mediated effects by macrophages	N/A	(88)
Classic mAb	Polyclonal mAbs	N/A	Vemurafenib (BRAF ^{V600E} inhibitor)	Full mouse antibody	Human BRAF ^{V600E} mutant melanoma cell lines	N/A	Synergistic direct effects (proliferation and migration inhibition)	N/A	(117)
Anti-idiotypic mAb	MK2-23	N/A	BCG	Full mouse antibody	N/A	N/A	Induction of adaptive humoral immune response	Advanced melanoma patients; Phase I/II	(93)

(Continued)

TABLE 1 | Continued

Treatment strategy	Clone/ construct	Toxin conjugate	Treatment combination	Antibody species	In vitro model and indication	In vivo model and indication	Proposed mechanism(s) of action	Clinical trial	Key reference
Anti-idiotypic mAb	MK2-23	IL-2	N/A	Full mouse antibody	N/A	BALB/c mice	Induction of adaptive humoral immune response	N/A	(94)
Anti-idiotypic mAb	MF11-30	N/A	N/A	Full mouse antibody	N/A	N/A	Induction of adaptive humoral immune response	Advanced melanoma patients; 2x phase I	(96)
BiTE	MCSP-BiTE	N/A	N/A	Recombinant BiTE construct	Human melanoma cell lines; melanoma patient-derived samples	N/A	Cytotoxic T cell-mediated tumor cell killing	N/A	(100)
BiTE	MCSP120, MCSP128, MCSP113, MCSP70	N/A	N/A	Recombinant BiTE construct based on mouse hybridoma-derived mAbs	CHO cells expressing CSPG4 domain portions	N/A	Cytotoxic T cell-mediated tumor cell killing	N/A	(101)
CAR	CAR constructs based on mAbs 225.28; TP41.2; 149.53	N/A	N/A	Recombinant CAR construct, includes mAb scFv region based on mouse mAbs	Human melanoma, breast cancer, mesothelioma, glioblastoma and osteosarcoma cell lines	N/A	Cytotoxic CAR T cell-mediated tumor cell killing	N/A	(103)
CAR	CAR construct based on mAb 763.74	N/A	N/A	Recombinant CAR construct, includes mAb scFv region based on mouse mAbs	Human, melanoma, HNSCC and breast cancer cell lines	Human, melanoma, HNSCC and breast cancer cell line xenografts; NSG mice	Cytotoxic CAR T cell-mediated tumor cell killing	N/A	(104)
CAR	CAR construct based on mAb 225.28	N/A	N/A	Recombinant CAR construct, includes mAb scFv region based on mouse mAbs	Human melanoma cell lines	N/A	Cytotoxic CAR T cell-mediated tumor cell killing	N/A	(119)
Radioimmunotherapy	(213)Bi-DTPA-9.2.27 (based on mAb 9.2.27)	(213)Bi-cDTPA	N/A	Full mouse antibody	N/A	N/A	Radiotherapy induced targeted cytotoxicity	Advanced melanoma patients, phase I	(108)
Radioimmunotherapy	225.28S	212Pb	N/A	Full mouse antibody	Human TNBC cell line	Human TNBC cell line xenograft; nude mice	Radiotherapy induced targeted cytotoxicity	N/A	(109)
CFP	α CSPG4(scFv)-MAP	MAP	N/A	Recombinant construct, scFv mAb genetically fused to MAP	Human TNBC cell line	Human TNBC cell line xenograft; BALB/c mice	MAP induced targeted cytotoxicity	N/A	(112)

(Continued)

Treatment strategy	Clone/construct	Toxin conjugate	Treatment combination	Antibody species	In vitro model and indication	In vivo model and indication	Proposed mechanism(s) of action	Clinical trial	Key reference
CFP	Anti-MCSP TRAIL (based on mAb 9.2.27)	N/A	Fully mouse mAb genetically fused to TRAIL	Human melanoma cell lines	TRAIL induced targeted cytotoxicity	Human melanoma cell line xenograft, athymic mice	N/A	N/A	(114)
Phototoxic immunotherapeutics	Anti-CSPG4 (scFv)-SNAP-tag	IR700 (photosensitizing agent)	Anti-EGFR (scFv)-SNAP-tag and anti-EpCAM(scFv)-SNAP-tag	Recombinant construct, scFv mAb genetically fused to SNAP tag	Human TNBC cell lines	N/A	Phototherapeutic activity	N/A	(118)

mAb, monoclonal antibody; MTX, methotrexate; PEG, polyethylene glycol; TNBC, triple-negative breast cancer; SCID, severe combined immunodeficiency; ECM, extracellular matrix; NSG, NOD scid gamma; GBM, glioblastoma multiforme; scFv, single-chain variable fragments; BiTE, bispecific T cell engager; CAR, chimeric antigen receptor; MAP, microtubule associated protein; TRAIL, TNF-related apoptosis-inducing ligand; HNSCC, head and neck squamous-cell carcinoma; CAR, chimeric antigen receptor; EGFR, epidermal growth factor receptor; EpcamP, epithelial cell adhesion molecule.

be promising with regards to CSPG4, based on overexpression in advanced tumors, restricted distribution and lower levels of expression in normal tissues (106). So far, radioimmunotherapy has only been successfully applied for the treatment of patients with lymphoma, while most clinical trials in solid tumors have rarely reached phase III, often due to cost, or to stringent patient inclusion criteria (107). Nevertheless, the outcomes of a phase I clinical trial of (213)Bi-cDTPA-9.2.27 (based on the anti-CSPG4 mAb clone 9.2.27) in advanced melanoma indicated no toxicities, a 10% objective partial response rate (108). Furthermore, a more recent preclinical study evaluating mAb 225.28 radiolabeled with 212Pb showed efficacy against triple negative breast cancer cells expressing CSPG4, both *in vivo* and *in vitro* (109).

Based on these findings, radioactive isotope-conjugated anti-CSPG4 antibodies may yet hold promise for patients with CSPG4-expressing tumors.

Cytolytic Fusion Proteins (CFPs)

Cytolytic fusion proteins or immunotoxins, are classified as protein toxins, most commonly of plant or bacterial origin, genetically fused or conjugated to another protein (often an antibody or an antibody fragment), recognizing a cell surface target molecule and delivering the payload to the cancer cell (110).

The microtubule-associated protein tau, recently investigated as a toxin, primarily functions as a microtubule stabilizer and a regulator of cell division (111). Targeting CSPG4 using a scFv fused to MAP against TNBC cell lines *in vitro* and against human TNBC cell line xenografts in Balb/c mice showed similar antitumor efficacy to an anti-CSPG4 scFv conjugated to a chemotherapeutic agent, with no toxic effects *in vivo* (112).

Another interesting approach involves an anti-CSPG4 scFv conjugated to the TNF-related apoptosis-inducing ligand (TRAIL), a soluble protein ligand able to induce apoptosis through binding the cell surface-anchored TRAIL receptor. In a study investigating a TRAIL-fused anti-CSPG4 scFv (based on the mAb 9.2.27), the novel therapeutic candidate showed potent *in vitro* activity against melanoma cell lines, but no off-target effects on normal melanocytes (113, 114). Moreover, it restricted the growth of a human melanoma xenograft in athymic mice.

Both CFP examples suggest that this may represent a promising targeted delivery alternative to chemotherapy, especially for antigens such as CSPG4 with high expression in tumors and restricted expression in normal tissues.

COMBINATION THERAPIES AND OTHER APPLICATIONS

BRAF inhibitors, now approved for the treatment of patients with melanomas bearing mutant forms of BRAF, are often only effective for a short time before cancer recurs, due to intrinsic and acquired pathway resistance (115). Therefore, alternative treatments and treatment combinations that may overcome resistance mechanisms are desirable (116). The anti-CSPG4 mAb 225.28 combined with a BRAF inhibitor exhibited synergistic antitumor effects and enhanced efficacy against BRAF^{V600E} mutant melanoma cells *in vitro* compared to either agent alone (51). Furthermore, the mAb was shown to delay the development of resistance by

melanoma cells. More recently, Pucciarelli et al. showed that combining polyclonal anti-CSPG4 antibodies, induced by mimotope vaccination, with the BRAF inhibitor vemurafenib synergistically reduced the proliferation and migration of melanoma cells *in vitro* (117). These preliminary findings suggest that combining anti-CSPG4 antibodies with pathway inhibitors may enhance the restricted success of BRAF inhibitors in melanoma.

Cancer theranostic agents, combining both diagnostic and therapeutic treatment in one targeted molecule are an emerging modality. As with other antibody therapeutic applications, CSPG4 has been recognized as a promising candidate for theranostic applications, based on high expression by tumor cells and low expression by healthy cells. In an *in vitro* photo-immunotheranostic study, single-chain variable fragment (scFv) antibodies recognizing TNBC targets, including CSPG4, as well as epidermal growth factor receptor (EGFR) and epithelial cell adhesion molecule (EpCAM), were conjugated to a potent photosensitizing agent and were used to target TNBC cell lines and tumor biopsy samples (118). The conjugated scFvs demonstrated high quality imaging capacity, and triggered apoptosis of cancer cells *via* induction of reactive oxygen species. Moreover, combinatorial administration of all three conjugated scFv antibodies together, increased cytotoxic activity against breast cancer cells *in vitro* by up to 40% compared with treatment by each individual agent alone. Further findings are awaited to confirm and provide further efficacy insights on these encouraging outcomes in future *in vivo* studies.

CONCLUSION AND FUTURE DIRECTIONS

Despite advances in immunotherapy such as the emergence of checkpoint inhibitors for melanoma, mAb, or CAR T cell strategies that specifically target melanoma cells are still lacking. Localized overexpression in several aggressive tumor types and in tumor vasculature, combined with low and restricted distribution in normal tissues, as well as evidence for important functions to support cancer growth, angiogenesis and dissemination, represent important attributes that identify CSPG4 as a promising target for therapeutic approaches, including monoclonal antibodies (**Table 1**). Importantly, in order to develop more successful therapeutics, a better understanding of the functions of CSPG4 in cancer and its interaction with the immune system and the tumor immune stroma are urgently needed.

While many treatment strategies centered on CSPG4 appear to have had success both *in vitro* and *in vivo* in rodent models, the next steps require in-depth studies with humanized or human antibodies, in disease-relevant and in clinically congruent models of cancer, including animal models engrafted with components of human immunity. These will permit mechanistic and efficacy evaluations in systems better able to recapitulate the patient setting.

An exciting prospect for targeting CSPG4 is the observed synergy between anti-CSPG4 monoclonal antibodies and BRAF inhibitors. In melanoma, a proportion of patients' tumors have constitutively activated BRAF. Small molecule inhibitors recognizing mutant forms of BRAF have proved very effective. However, clinical responses are often short-lived due to the emergence of resistance. Combinatorial studies of monoclonal anti-CSPG4 antibodies with BRAF inhibitors have demonstrated enhanced

effects and delayed the occurrence of resistance. Further understanding of the mechanisms that underpin the efficacy of these and other combinatory strategies may offer important clues that stand to improve current treatments.

Additionally, targeting CSPG4 may lead to targeted therapy for triple-negative breast cancer patients who do not benefit from therapies apart from standard chemotherapy. Therefore, further research and translation into clinical trials could be especially beneficial for the TNBC patient group.

As with all therapeutic approaches, the benefits of treatment must be balanced with the likelihood and severity of adverse effects. CSPG4 is expressed at low levels in some normal tissues; therefore, it is important to evaluate and mitigate any on-target, off-tumor toxic effects of CSPG4-specific targeted therapy. Encouragingly, a phase I clinical trial investigating anti-CSPG4 radioimmunotherapy with a mAb (9.2.27) conjugated to an α -particle-emitting radioisotope which was administered systemically in patients with melanoma reported no adverse events while some clinical benefits were observed (108). Furthermore, CSPG4-based immunotherapy strategies would benefit from the development of more effective methods of treatment delivery, such as hypobaric pressure skin delivery, which would limit potential off-target effects and reduce the cost of the therapy (120).

The emergence of novel antibody-based approaches offers fresh optimism that aggressive cancers, such as TNBC, glioma and head and neck carcinomas, which do not benefit from currently available therapies, but for which CSPG4 expression and its tumor-promoting functions have been reported, may become responsive to treatments based on this target. Therefore, renewed focus on CSPG4 may in future translate into significant benefits for patients with cancer.

AUTHOR CONTRIBUTIONS

KI, SK, and AT conceived and designed the study. KI, AC, SM, GC, SC, MG, MN searched and studied the literature. KI, MG, SK and AT wrote the manuscript. AC, SM, GC, MN, JS, ST, KE discussed and interpreted the literature findings and helped to edit the manuscript. SK supervised the study.

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Will a mAb-Based Immunotherapy Directed against Cancer Stem Cells Be Feasible?

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The cancer stem cell (CSC) hypothesis suggests that within a tumor, there is a small subpopulation of cells with stem cell properties responsible for tumor maintenance and metastasis generation. This hypothesis also implies that new antitumor drugs, rather than targeting the bulk of the tumor mass, would be more effective if they directly targeted the CSC subpopulation. The CSCs from several types of tumors have been identified with mAbs recognizing surface antigens in these cells; however, antigens specifically or exclusively expressed in the CSC population have not yet been identified. Thus, questioning the possibility of using therapeutic antibodies directed against the CSCs. Here, we review the possibilities of using antibodies directly targeting the CSCs as therapeutic agents in the form of naked antibodies, antibodies conjugated to nanoparticles, or antibody cocktails.

Keywords: cancer stem cells, therapeutic antibodies, immunotherapy, effective cancer therapies, cancer genetics

INTRODUCTION

Although the most frequently used anticancer treatments still are chemotherapy and radiotherapy, it is clear by now that monoclonal antibodies have emerged on the last 20 years as the most relevant new type of anticancer drugs with clinically proven therapeutic value. Concomitantly, this has generated an enormous interest, which has led to a burst of new approaches and clinical trials, where monoclonal antibodies represent the key element (1). However, most of the current anti-cancer treatments, including antibodies or other molecular interventions, increase the survival and improve the quality of life on patients, but do not necessarily cure.

It is obvious that antibodies against HER2, CD20, VEGF, EGFR, or CD52 have shown their clinical therapeutic value as anticancer drugs (1). In addition, antibodies that enhance the immune response by either blocking the PD-1/PD-L1 axis (2); antibodies anti-CTLA-4 (3, 4); or antibodies that block inhibitory receptors of NK cells (5, 6); or even CAR T cells (variable antibody regions engineered TCR-carrying T cells) (7), have proven also very useful. Indeed, they are able to redirect the antitumor immune response and allow envisaging the possibility of a cure for cancer patients. Obviously, the cure for cancer patients might come from the use of more or less complex combinations of antibodies that will include other drugs or cells (8).

Thus, the remaining questions are as follows: *Is this the best we can do to cure cancer patients? Are we hitting the right targets?* In this review, we would like to discuss the characteristics of the cancer stem cells (CSCs) that make them ideal targets, and the possible strategies of using antibodies to directly target the CSC population as the best option to cure cancer patients.

ADULT STEM CELLS AND CSCs

One of the concepts that have largely changed our understanding about tumor biology was the CSC hypothesis (9). Stem cells are defined as cells with the ability of self-renew (perpetuate themselves) and to differentiate, generating mature cells of a particular tissue. Adult (or tissue-specific) stem cells are rare cells that have been identified in many tissues, including the hematopoietic stem cells (HSCs) in the bone marrow (10, 11), the mammary stem cells in the mammary gland (12, 13), neural stem cells in the nervous system (14, 15), and the intestine stem cells in the intestine (16), among others. In several cases, a hierarchical structure has been demonstrated, where adult stem cells generate the appropriate cells from that tissue and maintain its homeostasis. The adult stem cell is able to undergo either symmetric cell divisions, generating two daughter stem cells, or asymmetrically, where the stem cell gives rise to a daughter stem cell and another cell committed for differentiation (17). From the committed cell, a common progenitor will be generated lacking self-renewal ability, but able to generate all the cell types of the differentiated tissue. The common progenitor will in turn generate more committed progenitors; each one of them will be able to generate one or two differentiated cell types from the tissue (Figure 1). This differentiation process is concomitant with cell expansion, explaining the reason why in many cases the frequency of adult stem cells is below 1% (18).

The CSC hypothesis proposes for tumors a hierarchical structure similar to the described for adult tissues. A small fraction of cells within the tumor harbor stem-cell like characteristics (referred to as CSCs), with an indefinite self-renewal potential

and able to drive tumorigenesis, being able to develop into a heterogeneous, more differentiated population, which constitutes the tumor mass (9). The CSCs were initially identified in acute myeloid leukemia (19) and prospectively identified in solid tumors including the mammary gland (20), the brain (21), and many others. The existence of CSC has been unequivocally demonstrated *in vivo* in glioblastomas, intestine, melanomas, and mammary tumors (22–25). One of the predictions of the CSC hypothesis was that more effective cancer therapies would target the CSC, instead of the bulk of the tumor (9). This is supported by the finding that CSC, such as normal stem cells, are more resistant to conventional chemotherapy and radiotherapy than more differentiated tumor cells (26), suggesting that effective therapies against the CSC would target self-renewal and/or differentiation of these cells (27).

Interestingly, it has been demonstrated in glioblastomas that therapies directly targeting the CSC are more effective than the ones targeting the tumor mass. In fact, standard chemotherapy was able to kill the bulk of the glioblastoma, but not the CSC, and the tumors quickly returned. When, in addition to chemotherapy, the CSC population was depleted in mouse glioblastoma models using a genetic trick, the tumors shrank back into “residual vestiges” that did not resemble glioblastomas (22). Thus, these data suggest that the predictions of the CSC hypothesis are true and that therapies directed to the CSC will turn out to be more effective.

CSC MARKERS

Once established that the CSC represents a distinct tumor cell population, involved in tumor formation and maintenance, the

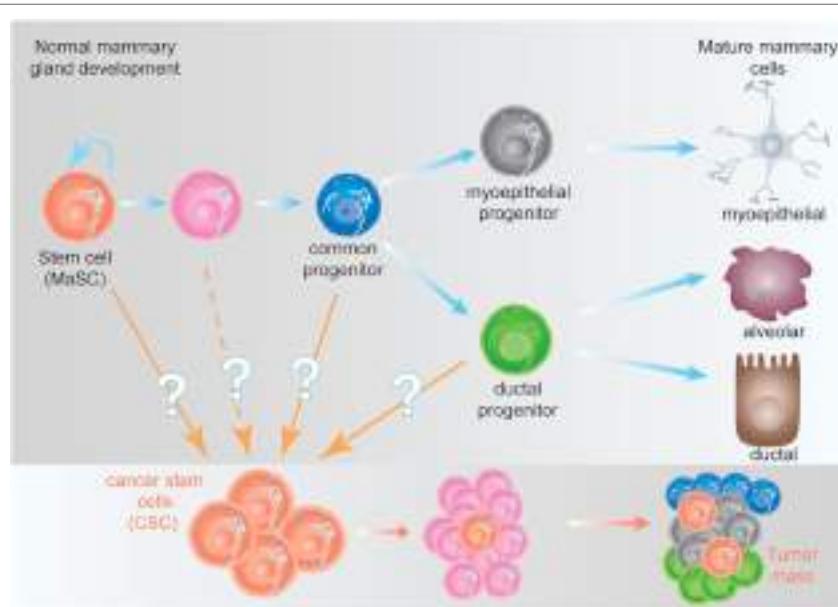


FIGURE 1 | Hypothetical model of the mammary epithelial hierarchy and its relationship with cancer stem cells (CSCs). (Top) The mammary stem cell (MaSC) differentiates through a common progenitor into either a myoepithelial or a ductal progenitor, which are committed to generate mature myoepithelial or ductal and alveolar cells, respectively. During this process, the MaSC and its progeny undergo at least nine cell divisions to generate the fully differentiated cells (not represented here), giving a ratio 1:500 MaSC:differentiated cells (18). (Bottom) CSCs, independent of their origin, are malignant-transformed cells with stem cell characteristics. They are able to generate a tumor (or metastases), although they represent a small fraction of the tumor mass (9).

identification of their specific markers has been a priority. First, for the isolation of the CSC and a more detailed analysis on their biology, but also for the possibility of using some of these markers as putative therapeutic targets. In many cases, the combination of positive and negative expression of surface markers allowed the identification of CSC populations. For example, on the identification of CSC in acute myelocytic leukemia (AML), where the cells were fractionated on the basis of CD34 and CD38 expression, demonstrating that only the CD34⁺CD38⁻ cells, but not the CD34⁺CD38⁺ or CD34⁻ cells, were able to engraft immunocompromised mice, replicating many aspects of human AML (19). Similarly, combinations of other surface markers, such as CD24, CD44, ESA, and CD133, allowed the identification of CSC in tumors from breast (20), liver (28), brain (21), lung (29), colon (30), prostate (31), pancreas (32, 33), head and neck squamous carcinoma (34), multiple myeloma (35), melanoma (36), among others (Table 1). It should be noted that in many cases, the surface markers used to identify CSC also identify adult stem cells on the corresponding normal tissues, or are surface markers shared by other cell types (Table 2).

The available data allow raising the question of whether there are specific CSC markers. Although at this time we cannot formally exclude their existence, since the CSC possess the same genetic information as the rest of the tumor (there are no additional mutations in the CSC as compared to the tumor mass), it is more likely that the phenotypic differences on CSC are due to differential gene expression. Indeed, both phenotypic and genetic analyses have failed so far, to pinpoint a single marker specific of any CSC population. In this context, genetic analyses aiming to understand self-renewal, a hallmark of stem cells and cancer, allowed pinpointing two genetic programs, one of them expressed by embryonic stem cells (ESC), and the other by adult tissue stem cells. When analyzing expression of these programs in human cancers, it was observed that in tumors where the ESC-like transcriptional program was activated, strongly predicted

metastasis and death, whereas expression of the adult tissue stem cells program led to a better prognosis (64). These types of analyses might allow to identify differentially expressed genes in the CSC as compared with the tumor mass and consequently be highly relevant for the identification of new CSC markers (cell surface markers, secreted proteins, intracellular proteins, or transcription factors). It should be noted that therapeutic antibodies can be generated, in addition to surface marker proteins, also against intracellular proteins, including transcription factors (65).

The next question that can be raised is whether the markers used for the identification of the CSC can also be used as therapeutic antibody targets. There is no straight answer to this question. Obviously, only mAbs that positively identify the CSC population could be used for therapeutic purposes. Returning to the example of the myelocytic leukemia, the combination of the CD34 and

TABLE 2 | Distribution of frequently used cancer stem cell phenotypic markers.

Phenotypic marker	Tumor type	Reference ^a	Normal tissue expression ^b
CD133 ⁺	Brain, liver, lung, colon, prostate, pancreatic, and ovary	(21, 31, 37–41, 43–45, 48–52, 54, 56, 59, 60)	1, 2, 4, 5, 6, 7, 8, 11, 12, 14, 16, 17, 18, 19, 20, 21, 22, 23, 26, 27
ESA1	Breast	(20, 42)	All tissues high
CD44 ⁺	Breast, colon, prostate, pancreas, and head and neck	(20, 31, 34, 42–45, 59, 60)	5, 10, 11, 16, 19, 20, 22, 24, 26, 27
EpCAM ⁺	Colon and pancreatic	(43–45, 59, 60)	1, 2, 4, 5, 6, 8, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 26, 27
CD20	Melanoma	(36)	1, 3, 5, 6, 8, 9, 10, 11, 12, 14, 16, 18, 19, 20, 22, 23, 26, 27
CD49f ⁺	Breast and liver	(20, 42, 48, 49)	1, 8, 12, 14, 15, 16, 17, 27
CD34 ⁺	Leukemia	(47)	5, 15, 16, 17, 19, 20, 21, 23
CD123 ⁺	Leukemia	(47)	5, 10, 11, 19, 20
CD24 ⁺	Colon and pancreatic	(43–45, 59, 60)	n.a. ^c
BCRP1 ⁺	Brain	(38–41)	n.a.
ABCG2	Lung	(50, 51)	1, 3, 5, 6, 7, 12, 14, 16, 17, 19, 21, 23, 25, 27
CD138 ⁺	Multiple myeloma	(35, 55)	1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 17, 19, 20, 21, 22, 24, 27
CD90 ⁺	Liver	(48, 49)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27
CD166 ⁺	Colon	(62)	5, 7, 21, 25

^aReference on the expression of the phenotypic marker in different tumor types.

^bEach number corresponds to a normal tissue with expression levels >10-fold over background. The data have been obtained from Fagerberg et al. (63). The code number for each tissue is as follows—1: colon; 2: kidney; 3: liver; 4: pancreas; 5: lung; 6: prostate; 7: brain; 8: stomach; 9: spleen; 10: lymph node; 11: appendix; 12: small intestine; 13: adrenal gland; 14: duodenum; 15: adipose tissue; 16: endometrium; 17: placenta; 18: testis; 19: gall bladder; 20: urinary bladder; 21: thyroid gland; 22: esophagus; 23: heart; 24: skin; 25: ovary; 26: bone marrow; and 27: salivary gland.

^cn.a.: data for protein expression of this gene in normal tissues are not available in reference (63).

TABLE 1 | Phenotypic markers used to identify cancer stem cell (CSC).

Tumor type	Phenotype of CSC	Reference
Brain	CD133 ⁺ CD133 ⁺ BCRP1 ⁺ A2B5 ⁺ SSEA-1 ⁺	(21, 37) (38–41)
Breast	CD44 ⁺ CD24 ^{low} ESA ⁺ ALDH-1 ^{high}	(20, 42)
Colon	CD133 ⁺ CD44 ⁺ CD166 ⁺ EpCAM ⁺ CD24 ⁺	(43–45)
Head and neck	CD44 ⁺	(34)
Kidney	CD105 ⁺	(46)
Leukemia	CD34 ⁺ CD38 ⁺ HLA-DR-CD71 ⁺ CD90 ⁺ CD117 ⁺ CD123 ⁺	(47)
Liver	CD133 ⁺ CD49f ⁺ CD90 ⁺	(48, 49)
Lung	CD133 ⁺ ABCG2 ^{high} CD133 ⁺ Sca1 ⁺ CD45 ⁺ PECAM ⁺ CD34 ⁺ Musashi-1 ⁺ CD34 ⁺ CD21 ⁺ cKIT ⁺ p63 ⁺ OCT-4 ⁺	(50, 51) (52) (53)
Melanoma	CD20 ⁺ CD133 ⁺ CD166 ⁺ Nestin ⁺	(36) (54)
Multiple myeloma	CD138 [–]	(35, 55)
Ovarian	CD133 ⁺ CD133 ⁺ CD117 ⁺ CD44 ⁺ CD24 ⁺ ALDH1A1 ⁺	(56) (57, 58)
Pancreas	CD133 ⁺ CD44 ⁺ EpCAM ⁺ CD24 ⁺	(59, 60)
Prostate	CD133 ⁺ CD44 ⁺ α2β1 ^{high}	(31)
Retinoblastoma	CD44 ⁺ CD133 ⁺ CXCR4 ⁺ CD90 [–]	(61)

CD38 markers has been useful for the identification and isolation of the CSC (19). But since the CSCs are CD34⁺CD38⁻ cells, the CD38 antibody cannot be used for therapeutic purposes (the CSCs are negative for this marker), although the CD34 mAb could.

POSSIBLE STRATEGIES TO TARGET CSC

A priori, the strategies to directly target the CSC population would tackle (i) differences in surface marker expression; (ii) interfere with signaling pathways relevant for their function; (iii) inhibit their function; (iv) interfere with metastasis formation; or (v) a combination of the above. In the following paragraphs, we will try to dissect these strategies (see Figure 2).

Therapies Targeting CSC Surface Markers

It turns out, from the data on Table 1, that CD133 (prominin-1) has been established as a marker of CSC on many solid tumors including brain, colon, liver, lung, ovarian, pancreatic, and prostate tumors. The role of CD133 as a CSC marker has, however, been questioned, for example, using the lung cancer cell lines A549 and H446, where more than 45% of the cells represent bona-fide CSC, it has been reported that both CD133⁺ and CD133⁻ cells are able to form tumors with the same efficiency (29). In addition, CD133 exhibits several splice variants and different poorly characterized glycosylated isoforms (38), and as shown on Table 2, this antigen is broadly expressed on normal tissues (63). Thus, making it questionable whether CD133 represents a specific CSC marker and a therapeutic target for antibody-mediated elimination of CSC.

Targeting the adhesion molecule CD44 with monoclonal antibodies in xenografts of AML allowed to demonstrate that this treatment eradicated the leukemic CSC (66). Similarly, an antibody specific for the membrane IL-3Ra receptor (CD123) overexpressed in leukemia CSC (see Table 1) has been used to specifically target leukemia CSC in human AML. The treatments

decreased leukemogenicity and eradicated CSC in mice (67, 68). In addition, an antibody targeting CD47 has demonstrated its ability to eliminate human acute lymphoblastic leukemia in xenograft transplants (69). The T cell immunoglobulin mucin-3 (TIM-3) was also identified as a surface molecule expressed on leukemia stem cells in most types of AML except for acute promyelocytic leukemia, but not on normal HSCs. TIM-3⁺ but not TIM-3⁻ AML cells engrafted, replicating in immunodeficient mice, many of the aspect on human AML. Furthermore, antibodies specific for TIM-3 dramatically diminished their leukemic burden (69). It should be noted that these experiments were carried out in xenotransplants, where the only cells expressing CD44, CD123, CD47, or TIM-3 were the transplanted tumor cells, thus any putative toxic effects on other body cells expressing these markers CD44⁺, CD123⁺, CD47⁺, or TIM-3⁺ could not be assessed in these models. However, a possibility, discussed in details in another review from this issue is to use combinations of antibodies (8), where even if the antibodies mentioned earlier for the treatment of AML used separately could be also toxic for the normal tissues, their combination (CD44, CD123, CD47, and TIM-3) could use smaller doses of each one of them, avoiding the concentrations required to induce toxicity in normal cells, but still be effective killing the CSC on AML. This is one of the possibilities that should be investigated for the treatment of AML and other types of cancer.

Another possibility of combination of antibodies against surface marker that can be investigated from the data on Table 1 deal with liver tumor CSCs, which are CD133⁺CD49f⁺CD90⁺ (48, 49). Each one of these markers is broadly expressed in normal tissues (63) as seen in Table 2. The use of antibodies against any of these markers as therapeutic tools might not be sufficiently selective for CSC and be toxic to healthy tissues. However, it might turn out that a strategy combining antibodies against the three molecules, using lower doses of each one of them, may still be effective while avoiding the unwanted toxicity with these lower doses.

Therapies targeting CSC surface markers can be exemplified by a clinical trial on untreated multiple myeloma using the anti-CD19 mAb MEDI-551 in combination with dexamethasone and lenalidomide. The rational of the trial is to determine whether the treatment with MEDI-551 decreases the number of CSC in these multiple myeloma patients (NCT01861340) (70).

In some cases, although the mAb identifies a target present on both adult stem cells and CSC, the antibody could be used to target the CSC. This would be the case for the mAb Nilo1, identifying mouse embryonic radial glia, adult neural stem cells, and also a subpopulation of mouse and human glioblastoma cells (71, 72), allowing to suggest that it might identify the CSC population (73). If Nilo1 indeed identifies the CSC, it could be envisaged that this antibody conjugated, for example, to gold nanoparticles would be able to photo-ablate Nilo1⁺ cells after these targeted cells absorb near infrared light. This would result in increased local temperature at the selected location, destroying the target cells (74). This approach would be feasible since the adult neural stem cells are restricted to their niche (subventricular zone), an expected different location from the tumor. However, in other tumor types, such as hematopoietic tumors

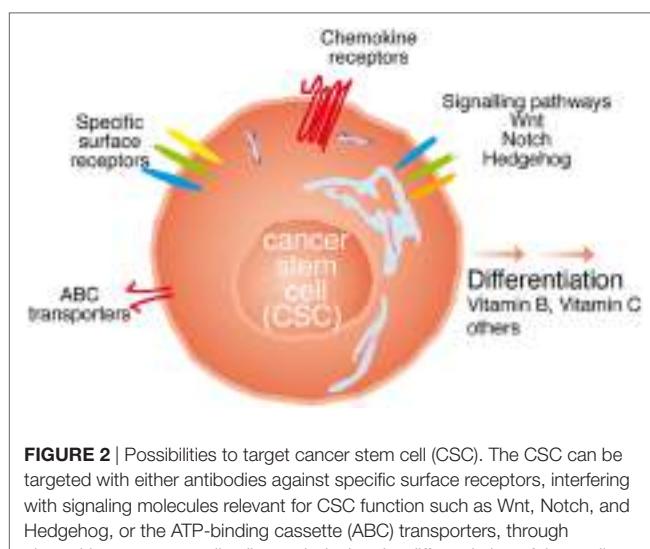


FIGURE 2 | Possibilities to target cancer stem cell (CSC). The CSC can be targeted with either antibodies against specific surface receptors, interfering with signaling molecules relevant for CSC function such as Wnt, Notch, and Hedgehog, or the ATP-binding cassette (ABC) transporters, through chemokine receptor antibodies, or inducing the differentiation of these cells.

or tumors of the mammary gland, this approach would be much more difficult to apply.

Another possible approach tackles the observation that both adult stem cell and CSC express higher levels of the ATP-binding cassette (ABC) transporters on their cell membranes. The ABC transporters have been proposed to contribute to multidrug resistance, because they allow to pump out of the cytoplasm many antitumor drugs, resulting in lower intracellular drug concentrations (35, 50), allowing the CSC to become more resistant to chemotherapeutic drugs (50, 75). However, some experiments using inhibitors of the ABC transporter have been successfully carried out (76). It seems that the generation of ABC transporter-blocking antibodies might inhibit ABC-transporter functions, without many of the negative toxic effects of the inhibitors, and therefore this will make the CSC more sensitive to chemotherapeutic drugs.

Finally, a clinical trial aims to determine the CSC load of HER2⁺ breast cancer tumors treated with the anti-HER2 antibody trastuzumab, in combination with adjuvant, doxorubicin hydrochloride, or cyclophosphamide followed by paclitaxel (NCT01424865).

Targeting Signal Pathways

The signaling pathways involved in stemness, both in adult stem cells and CSC, including Notch, Hedgehog, and Wnt representing relevant therapeutic targets for CSC (9). Indeed, monoclonal antibodies against Notch are able to reduce the CSC population in colorectal tumors (77) and also in breast cancer cell lines (78). Similarly, antibodies against the Wnt-1 signaling pathway induce apoptosis in human colorectal cancer cells (79).

Small molecule Hedgehog antagonists have also been successfully used to inhibit systemic metastases in xenografts with tumors derived from human pancreas (80), but in this case, as far as the authors are aware, blocking antibodies have not yet been used. In fact, inhibitors of Wnt, Notch, and Hedgehog activities are being investigated in a clinical trial on esophageal cancer patients (NCT02221245). Other clinical trials use therapeutic antibodies against DLL4 to inhibit Notch signaling (presumably targeting Notch expressed on the CSC) in combination with paclitaxel in ovarian, peritoneal, and fallopian tube cancer (NCT03030287); in combination with FOLFIRI (irinotecan, folic acid, leucovorin, and fluorouracil), in metastatic colorectal cancer (NCT01189942); or the anti-DLL4 antibody demcizumab in combination with Gemcitabine Abraxane on metastatic pancreatic cancer (NCT01189929). Other examples use either a bispecific DLL4/VEGF antibody (OMP-305B83) in metastatic colorectal cancer, combined with the chemotherapeutic agents FOLFIRI (NCT03035253); or in combination with the chemotherapeutics carboplatin and pemetrexed for lung cancer (NCT01189968). Finally, another clinical trial uses the anti-DLL4 antibody demcizumab, in combination with the anti-PD-1 antibody pembrolizumab (immune checkpoint) in metastatic solid tumors (NCT02722954), aiming to inhibit Notch and simultaneously busting the antitumor immune response by inhibiting the PD-1/PD-L1 immune checkpoint.

A different approach used was to combine the Hedgehog inhibitor IPI-926 with the anti-EGFR antibody cetuximab in head and neck cancer patients (NCT01255800).

Other signaling pathways relevant in oncology include the tyrosine kinase family. The tyrosine kinase inhibitor lapatinib has been combined with the anti-HER2 antibody trastuzumab in a clinical trial in breast cancer patients (NCT00524303), where the authors want to analyze changes in CSC load.

Trigger Differentiation

An additional possibility is to trigger the differentiation of the CSC. This will imply that they are not able to self-renew anymore, and therefore they would be more sensitive to regular chemotherapy and radiotherapy. In fact, several agents, such as retinoic acid (RA) [i.e., 13-cis RA (isotretinoin)], are used to modify cell expression patterns inhibiting proliferation and inducing cell differentiation and apoptosis (81–83). In addition, vitamin C has also been shown to trigger differentiation of CSC on leukemia, enhancing their sensitivity to PARP inhibition (84). It seems clear that these compounds will be used in combinations with antibodies and/or other drugs.

An example of therapeutic interest on triggering CSC differentiation is shown by a current clinical trial, aiming to analyze the role of the vitamin B derivate Fursultiamine on the differentiation of CSC in squamous cell carcinomas (NCT02423811) (76, 85).

Others

The effects of any anti-CSC antibody can be potentiated if it is used in combination with antibodies inhibiting immune-checkpoints negative signals. These include antibodies binding to the PD-1 receptor on the T cells (nivolumab and pembrolizumab), to PD-L1 on the tumor cells (atezolizumab, durvalumab, and avelumab) or to CTLA-4 on T cells (ipilimumab) (86). This strategy will be relevant since the anti-checkpoint antibodies are able to switch the antitumor response from an immunosuppressed status, to another that allows to attack the tumor.

Since the CSCs are, in addition to the tumor-initiating cells, the unique cells that can form metastasis, as they are only cells within a tumor with a strong proliferation potential, able to generate the more differentiated tumor cells, which form the tumor mass, and at the same time a strong self-renewal potential through symmetric cell divisions (9). The use of any antibody or drug against the CSC, in combination with anti-chemokine receptor antibodies such as CXCR4, CCR7, and CCR9 (85, 87–96), would inhibit the migration of the CSC, their migration, invasion, and seeding of the metastatic cells, therefore improving the patient's health.

Another possibility is to combine any antibody or drug specific for CSC with antibodies inhibiting tumor neo-vascularization, such as VEGF or VEGFR. In this context, there is a clinical trial that combines the preoperative treatment with the anti-VEGF antibody bevacizumab and chemotherapy in patients with breast cancer (NCT01190345), where they aim to determine the CSC activity (measured by the amount of aldehyde dehydrogenase 1/ALDH1⁺ cells before and after treatment).

Here, we have pinpointed some of the ongoing trials and pre-clinical experiments being carried out aiming to directly target

CSC; however, there are many more possibilities to be carefully analyzed.

CONCLUSION

The existence on many tumors of a subpopulation of cells with stem cell characteristics (the CSC population) it is clear by now. Furthermore, the concept that new anticancer treatments will be more effective if they directly target the CSC population, seems settled in the scientific community. The number of clinical trials targeting the CSC is, however, relatively small. Furthermore, from the 86 clinical trials found with the keywords “cancer stem cells,” only 12 of them use monoclonal antibodies as therapeutic agents. This is due, at least in part, to the lack of CSC-specific markers. We are optimistic, however, and believe that in the near future, this number will greatly increase. The new clinical trials will involve several combinations of antibodies, antibodies and chemotherapeutic drugs, small drug molecules, or the discovery of molecules able to differentiate the CSC. These will make a large advance in oncologic treatments specifically designed to destroy or kill CSCs.

Taken together, this does not mean that the work ahead will be easy, in particular since examples have been described where not only the CSC give rise to daughter CSC and non-CSC but also where the non-CSC population can, in some situations, give rise to some CSC (97). Thus, advances in the field of antibody immunotherapy directly targeting the CSC will require combinations of genetic analyses to identify differentially

expressed genes in the CSC population, and an improved knowledge on the biology of the CSC (98), together with the use of complex algorithms to determine effective concentrations of different antibodies and drugs, to avoid adult stem cells harm. Thus, strategies using antibodies directly targeting the CSC population, while bursting the antitumor immune response and inhibiting neo-vascularization may represent an unparalleled opportunity to cure cancer.

AUTHOR CONTRIBUTIONS

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New Strategies Using Antibody Combinations to Increase Cancer Treatment Effectiveness

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Antibodies have proven their high value in antitumor therapy over the last two decades. They are currently being used as the first-choice to treat some of the most frequent metastatic cancers, like HER2⁺ breast cancers or colorectal cancers, currently treated with trastuzumab (Herceptin) and bevacizumab (Avastin), respectively. The impressive therapeutic success of antibodies inhibiting immune checkpoints has extended the use of therapeutic antibodies to previously unanticipated tumor types. These anti-immune checkpoint antibodies allowed the cure of patients devoid of other therapeutic options, through the recovery of the patient's own immune response against the tumor. In this review, we describe how the antibody-based therapies will evolve, including the use of antibodies in combinations, their main characteristics, advantages, and how they could contribute to significantly increase the chances of success in cancer therapy. Indeed, novel combinations will consist of mixtures of antibodies against either different epitopes of the same molecule or different targets on the same tumor cell; bispecific or multispecific antibodies able of simultaneously binding tumor cells, immune cells or extracellular molecules; immunomodulatory antibodies; antibody-based molecules, including fusion proteins between a ligand or a receptor domain and the IgG Fab or Fc fragments; autologous or heterologous cells; and different formats of vaccines. Through complementary mechanisms of action, these combinations could contribute to elude the current limitations of a single antibody which recognizes only one particular epitope. These combinations may allow the simultaneous attack of the cancer cells by using the help of the own immune cells and exerting wider therapeutic effects, based on a more specific, fast, and robust response, trying to mimic the action of the immune system.

Keywords: cancer, antibody combinations, oncology, therapeutic antibodies, immunotherapy

INTRODUCTION

Nowadays, the therapeutic activity of antibodies in oncology has been widely demonstrated (1–6), being these proteins, after chemotherapy, radiotherapy, and small molecule inhibitors, one of the most used drugs for oncological treatments (7, 8). Most of the antibodies used on antitumor immunotherapies had positive health effects as long as the antibody is present in the patient's blood. The clinical use of antibodies directed against antigens not present on the tumor cells, but on cells of the immune

system (i.e., anti-immune checkpoint antibodies), evidenced the beneficial effects of the treatment, which persisted even after it was finished (9, 10). These findings allowed to demonstrate that the anti-checkpoint antibodies were able to reprogram the organism's response, re-directing the antitumor immune response, and skewing the balance on the tumor microenvironment toward immune destruction of the tumor. Thus, allowing to envisage a cure for cancer.

The aim of this review is to discuss the information on the possible anti-cancer treatments using monoclonal antibodies (mAbs; in clinical trials or already in the market) in combinations, either with other antibodies or with other biological agents (11–18). The clinical trials are mentioned throughout this review only as examples of the different types of combinations being currently analyzed for cancer treatment. Thus, for most of the studies details and results will be shown on Supplementary Tables S1 and S2. The use of radiation therapy, chemotherapy, small-molecule compounds, or stem cell autotransplants will be mentioned only, if the information is strictly necessary in our attempt to dissect the reasons behind the use of these combinations. Similarly, there is no specific section describing combinations including checkpoint inhibitory mAbs, since this subject has been recently reviewed (19–24), including the reviews by Xu-Monette and Young and Aris et al., in this issue (25, 26). Similarly, combinations directed against cancer stem cells will not be discussed since they have been treated elsewhere in this research topic (27).

Antibodies are generated by the immune system's adaptive arm to defend the organism from pathogens and malignant cells. The antibodies are basically secreted molecules involved in mediating interactions on the extracellular compartment; they are made by a variable part that gives its binding specificity, and a constant region that is able to interact with other molecules or cells of the innate and adaptive immune system, to give them molecular information regarding their interaction with antigen. Thus, it should come to no surprise that antibodies' functions promote health and that treatments based on antibodies might, therefore, be curative (6, 28, 29).

Very few therapeutic antibodies are able to directly kill the tumor cells, either by interacting with a signal pathway (i.e., as a receptor antagonist or sequestering the ligand), or by direct triggering of apoptosis. Most of them kill the tumor cells through the interaction with other molecules or cells of the immune system, acting as molecules mediating interactions on the extracellular compartment. Although they originated as receptors on the surface of cells from the acquired immune system, they became secreted on mature B cells, and through either engagement with Fc-receptor-bearing cells or by interaction with the complement system, they can exert a broad spectrum of effector functions, coordinating the immune response (see Figure 1).

A current goal of antitumor immune therapies is to trigger, from the beginning, all the possible host body defense mechanisms. Aiming to destroy, as early as possible, the highest number of tumor cells, decreasing the possibilities of the tumor developing escape mechanisms, to obtain a more effective therapy. The defense mechanisms include (i) to directly kill the tumor cells; (ii) to switch the immune system from an antitumor

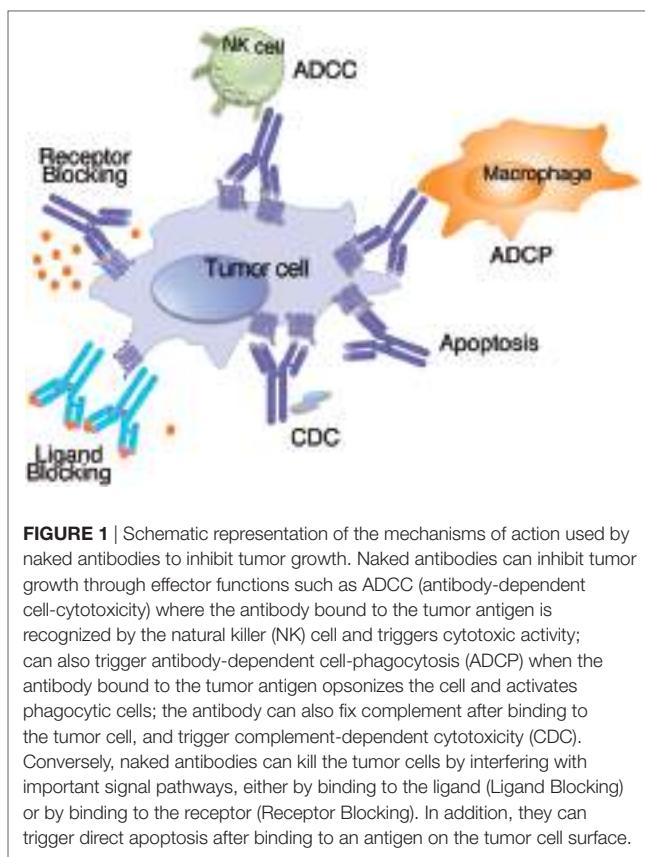


FIGURE 1 | Schematic representation of the mechanisms of action used by naked antibodies to inhibit tumor growth. Naked antibodies can inhibit tumor growth through effector functions such as ADCC (antibody-dependent cell-cytotoxicity) where the antibody bound to the tumor antigen is recognized by the natural killer (NK) cell and triggers cytotoxic activity; can also trigger antibody-dependent cell-phagocytosis (ADCP) when the antibody bound to the tumor antigen opsonizes the cell and activates phagocytic cells; the antibody can also fix complement after binding to the tumor cell, and trigger complement-dependent cytotoxicity (CDC). Conversely, naked antibodies can kill the tumor cells by interfering with important signal pathways, either by binding to the ligand (Ligand Blocking) or by binding to the receptor (Receptor Blocking). In addition, they can trigger direct apoptosis after binding to an antigen on the tumor cell surface.

immunosuppressed status to another that allows to attack the tumor, i.e., through stimulating the secretion of cytokines or modulating cell to cell interactions; (iii) to attract the immune system cells to the tumor; (iv) to decrease the tumor-directed neo-vascularization; and (v) to inhibit migration, metalloprotease secretion, and tumor cell invasion, among others.

The current trends for the use of antibodies in oncology as therapeutic agents are to employ them either alone or, more often, as combinations with (i) cytotoxic agents; (ii) radiotherapy; (iii) molecularly targeted drugs interfering with tumor cell survival or proliferation; (iv) other antibodies against the same target; (v) other antibodies against molecules implicated in the same signaling pathway; (vi) other antibodies, each one of them specific for unrelated targets (including targets in immune system cells and neo-vascularization); (vii) vaccines or oncolytic virus; (viii) cells that would either act as immunogens or as effector cells; or (ix) adjuvants, liposomes, nanoparticles, etc.

For the treatment of cancer, the FDA and the EMA (United States and European Union Drug Administrations), have approved (or are reviewing) a total of 32 therapeutic antibodies or their derivatives. Interestingly, the number has doubled between 2012 and 2017 (Tables 1 and 2), concomitant with a 100% increase in phase III clinical trials using mAb on a similar time-period (30). Twenty of these antibodies are indicated for treatment of patients with solid tumors (Table 1), identifying 13 different targets; whereas 12 are indicated for neoplasias of hematological origin (Table 2), identifying eight different targets. The targets identified by these antibodies are described in Table 3. Other therapeutic

TABLE 1 | Antibodies approved (or in review) by the FDA and/or EMA for the clinical treatment of solid tumors.^a

Approved indications	Target	International non-proprietary name	Brand name	Format	Proposed mechanism(s) of action	EU/US First approval year	Sponsor	Reference
Soft tissue sarcoma	PDGFR α	Olaratumab	Lartruvo	Human IgG1	Binds to PDGFR- α , blocks ligand binding and receptor signaling	2016/2016	Eli Lilly and Co.	(31–34)
Breast cancer	HER2	Pertuzumab	Perjeta	Humanized IgG1	Inhibits HER dimerization, prevents the formation of ligand-induced heterodimers of HER2 with other family members. Induces ADCC	2013/2012	Genentech	(35–38)
Breast cancer and gastric cancer	HER2	Trastuzumab	Herceptin	Humanized IgG1	Inhibits HER dimerization, prevents the formation of ligand-induced heterodimers of HER2 with other family members. Induces ADCC and phagocytosis	2000/1998	Genentech	(39–43)
Breast cancer	HER2	Ado-trastuzumab emtansine	Kadcyla	Humanized IgG1 conjugated to emtansine (ADC)	Inhibits HER dimerization, prevents the formation of ligand-induced heterodimers of HER2 with other family members. Induces ADCC and phagocytosis. Transports emtansine (microtubule inhibitor) to HER2-positive tumors	2013/2013	Genentech	(44–47)
HNSCC	Epidermal growth factor receptor (EGFR)	Necitumumab	Portrazza	Human IgG1	Binds to EGFR, blocks ligand binding and triggers EGFR internalization and degradation. Induces ADCC	2015/2015	Eli Lilly and Co.	(48–53)
Colorectal cancer	EGFR	Panitumumab	Vectibix	Human IgG2	Binds to EGFR, competitively inhibits the binding of its ligands, blocking receptor signaling	2007/2006	Amgen	(54–58)
HNSCC, Colorectal cancer	EGFR	Cetuximab	Erbitux	Chimeric IgG1	Binds to EGFR, blocks ligand binding and triggers EGFR internalization and degradation. Induces ADCC	2004/2004	ImClone LLC	(59–63)
Breast cancer, colorectal cancer, non-squamous NSCLC, RCC, cervix carcinoma, ovarian or fallopian tube cancer, primary peritoneal cancer, and glioblastoma	VEGFA	Bevacizumab	Avastin	Humanized IgG1	Binds to VEGFA, prevents interaction with its receptors and their subsequent activation	2005/2004	Genentech	(64–68)
NSCLC, gastric cancer, and colorectal cancer	VEGFR2	Ramucirumab	Cyramza	Human IgG1	Binds to VEGFR2, inhibits the binding of its ligands, blocking receptor signaling	2014/2014	Eli Lilly and Co.	(69–73)
NSCLC and urothelial carcinoma	PD-L1	Atezolizumab	Tecentriq	Humanized IgG1	Blocks the interaction of PD-L1 with programmed cell death protein 1 (PD-1) and CD80. Blocks the immune checkpoint inhibition. Contains a modified Fc region to limit ADCC or CDC	2017 ^b /2016	Genentech	(74–78)
Urothelial carcinoma	PD-L1	Durvalumab	Imfinzi	Human IgG1	Blocks the interaction of PD-L1 with PD-1 and CD80. Blocks the immune checkpoint inhibition. Does not induce ADCC	NA/2017	AstraZeneca	(79–82)

(Continued)

TABLE 1 | Continued

Approved indications	Target	International non-proprietary name	Brand name	Format	Proposed mechanism(s) of action	EU/US First approval year	Sponsor	Reference
Merkel cell carcinoma	PD-L1	Avelumab	Bavencio	Human IgG1	Blocks the interaction of PD-L1 with PD-1 and CD80. Blocks the immune checkpoint inhibition	2017 ^b /2017	Merck/Pfizer	(83–86)
Melanoma, RCC, NSCLC, HNSCC, cHL and urothelial carcinoma	PD-1	Nivolumab	Opdivo	Human IgG4	Binds to PD-1, blocks its interaction with PD-L1 and PD-L2. Blocks the immune checkpoint inhibition. Does not induce ADCC	2015/2014	Ono Pharma (Japan)/Bristol-Myers Squibb (Worldwide)	(87–91)
Melanoma, NSCLC, HNSCC, and cHL	PD-1	Pembrolizumab	Keytruda	Humanized IgG4	Binds to PD-1, blocks its interaction with PD-L1 and PD-L2. Blocks the immune checkpoint inhibition. Does not induce ADCC	2015/2014	Merck	(92–96)
Melanoma	CTLA-4	Ipilimumab	Yervoy	Human IgG1	Binds to CTLA-4, blocks its interaction with CD80 and CD86, increasing T cell activation and proliferation	2011/2011	Bristol-Myers Squibb	(97–100)
Bone metastases from solid tumors, Increase of bone mass	RANK-L	Denosumab	Prolia Xgeva	Human IgG2	Binds to RANK-L, blocks its interaction with RANK, and prevents bone loss	2010/2010	Amgen	(101–105)
Colorectal cancer	IL-1 α	MABp1 ^c	Xilonix	Human IgG1	Binds to IL-1 α , blocks its interaction with IL1-R	In review/NA	XBiotech	(106–108)
Neuroblastoma	GD2	Dinutuximab	Unituxin	Chimeric IgG1	Binds to the TAA GD2. Activates CDC and ADCC	2015 ^{d, e} /2015	United Therapeutics	(109–112)
EpCAM ⁺ -Colon cancer	EpCAM	Edrecolomab	Panorex	Murine IgG2a	Engages immune effector cells. Activates CDC, ADCC, and phagocytosis	1995 ^{d, e} /NA	GlaxoSmith Kline	(113–116)
EpCAM ⁺ -carcinomas related ascites	EpCAM/CD3	Catumaxomab	Removab	Rat/mouse bispecific monoclonal antibody	Attracts immune cells to the tumor proximity, promoting T cell activation and effector functions. Activates CDC, ADCC and phagocytosis	2009 ^e /NA	Trion Pharma/Biotech	(117–121)

^aAdapted from Janice M. Reichert, PhD, The Antibody Society; last update July, 2017.

ADC, antibody-drug conjugate; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; cHL, classical Hodgkin lymphoma; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; TAA, tumor-associated antigen.

^bCountry-specific approval, 20 July 2017.

^cInternational non-proprietary name pending.

^dEMA initial authorization.

^eWithdrawn or marketing discontinued for the first approved indication.

NA, not approved or in review in the EU, not approved or information on review status not available in the US.

Color shades corresponds to structurally related target molecules.

TABLE 2 | Antibodies approved (or in review) by the FDA and/or EMA for the clinical treatment of hematologic neoplasias.^a

Approved indication	Target	International non-proprietary name	Brand name	Format	Proposed mechanism(s) of action	EU/US First approval year	Sponsor	Reference
ALL	CD19/ CD3	Blinatumomab	Blinacyto	Murine bispecific tandem scFv	Binds CD19 on tumor B cells and puts them in close contact with T cells through the CD3 (TCR complex), activates them, and results in redirected tumor cell lysis	2015/2014	Amgen	(122–126)
CLL and follicular lymphoma	CD20	Obinutuzumab	Gazyva Gazyvaro	Humanized IgG1; Glycoengineered	Lyses B cells by effector-cell recruitment. Enhanced CDC, ADCC, and ADCP, contains a modified Fc region with increased binding affinity for FcgammaRIII. Mutation (R7159) enhances apoptosis	2014/2013	Roche	(127–132)
CLL	CD20	Ofatumumab	Arzerra	Human IgG1	Binds to CD20 and engages immune effector cells, mediates B-cell lysis. Activates CDC, ADCC, and ADCP	2010/2009	Novartis	(133–136)
NHL	CD20	Tositumomab- ¹³¹ I	Bexxar	Murine IgG2a linked to ¹³¹ I	Binds to CD20 and engages immune effector cells, mediates B-cell lysis. Activates CDC, ADCC, and ADCP, induces apoptosis. Ionizing radiation kills CD20 ⁺ cells	NA/2003 ^a	GlaxoSmithKline	(137–140)
NHL	CD20	Ibritumomab-tiuxetan	Zevalin	Murine IgG1 linked to ⁹⁰ Y-tiuxetan	Binds to CD20, the tiuxetan moiety binds ⁹⁰ Y, the beta emission induces cell damage. Activates CDC, ADCC, and apoptosis	2004/2002	Spectrum Pharmaceuticals	(141–144)
NHL and CLL	CD20	Rituximab	MabThera-Rituxan	Chimeric IgG1	Binds to CD20 and engages immune effector cells, mediates B-cell lysis. Activates CDC, ADCC, and ADCP	1998/1997	Roche Biogen/Genentech	(145–150)
ALL	CD22	Inotuzumab ozogamicin	Besponsa	Humanized IgG4 linked to N-acetyl-gamma-calicheamicin (ADC)	Binds to CD22 ⁺⁺ cells. After internalization, the toxin induces double-stranded DNA breaks and apoptosis	2017/2017	Pfizer	(151–155)
Hodgkin lymphoma and systemic anaplastic large cell lymphoma	CD30	Brentuximab vedotin	Adcetris	Chimeric IgG1 linked to monomethyl auristatin E (MMAE; ADC)	Binds to CD30 ⁺⁺ cells. After internalization, the toxin MMAE, disrupts microtubules and induces apoptosis	2012/2011	Seattle Genetics	(156–161)
Acute myeloid leukemia	CD33	Gemtuzumab ozogamicin	Mylotarg	Humanized IgG4 linked to N-acetyl gamma calicheamicin (ADC)	Binds to CD33 ⁺ cells. After internalization, the toxin induces double-stranded DNA breaks and apoptosis. Does not activate ADCC	In review/in review; 2000 ^a	Wyeth	(162–165)
Multiple myeloma	CD38	Daratumumab	Darzalex	Human IgG1	Binds to CD38 ⁺ cells. Activates CDC, ADCC, and ADCP	2016/2015	Janssen Biotech	(166–170)
Multiple myeloma	SLAMF7	Elotuzumab	Empliciti	Humanized IgG1	Binds to SLAMF7. Activates ADCC	2016/2015	Bristol-Myers Squibb	(171–176)
CLL	CD52	Alemtuzumab	Campath	Humanized IgG1	Binds to CD52 ⁺ lymphocytes. Activates ADCC and CDC	2001/2001	Genzyme	(177–179)

^aAdapted from Janice M. Reichert, PhD, The Antibody Society; last update July, 2017.

ADC, antibody-drug conjugate; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ALL, acute lymphoblastic leukemia; CDC, complement-dependent cytotoxicity; CLL, chronic lymphoblastic leukemia; MAE, monomethyl auristatin E; NHL, non-Hodgkin lymphoma; scFv, single-chain variable fragment.

^bWithdrawn or marketing discontinued for the first approved indication.

NA, not approved or in review in the EU, not approved or information on review status not available in the US.

Color shades corresponds to structurally related target molecules.

TABLE 3 | Characteristics of the main target molecules identified by therapeutic antibodies used in oncology.

Antibody target	Nature of the target	Function	Expression	Monoclonal antibody effects in cancer therapy	Reference
PDGFR α	Platelet-derived growth factor receptor alpha	Protein of the tyrosine kinase family	Cell proliferation, differentiation and migration	Ubiquitous, highly expressed on endothelial cells	Cell proliferation inhibition (180, 181)
HER2	Human epidermal growth factor receptor 2	Glycoprotein of the tyrosine kinase family	Enhances cell proliferation and favors survival	Epithelial cells, highly expressed on many tumors	Cell proliferation inhibition (182, 183)
EGFR	Epidermal growth factor receptor	Glycoprotein of the tyrosine kinase family	Cell proliferation and differentiation	Epithelial cells	Cell proliferation inhibition (184, 185)
VEGFA	Vascular endothelial growth factor A	Glycoprotein of the PDGF/VEGF family	Proliferation and migration of endothelial cells	Hypoxic cells, highly expressed on many tumors	Angiogenesis inhibition (186, 187)
VEGFR2	Vascular endothelial growth factor receptor 2	Cell surface receptor of the tyrosine kinase family	Proliferation and migration of endothelial cells	Vascular and lymphatic endothelial cells	Angiogenesis inhibition (186, 187)
PD-L1	Programmed cell death-1 ligand 1	Protein of the immunoglobulin superfamily	Inhibits T cell activation and cytokine production	Myeloid and lymphoid lineage cells, highly expressed on certain cancer cells	Immune checkpoint inhibition (188, 189)
PD-1	Programmed Cell Death-1	Protein of the immunoglobulin superfamily	Inhibits T cell activation and cytokine production	B and T lymphocytes	Immune checkpoint inhibition (188, 189)
CTLA-4	Cytotoxic T-lymphocyte antigen 4	Protein of the immunoglobulin superfamily	Inhibits T cell activation and cytokine production	B and T lymphocytes	Immune checkpoint inhibition (188–190)
RANK-L	Receptor activator of nuclear factor κ B ligand	Ligand of the tumor necrosis factor superfamily	Activates osteoclast through NF- κ B activation	Osteoblasts and T lymphocytes	Inhibition of bone destruction (191–193)
IL-1 α	Interleukin-1 alpha	Cytokine of the interleukin-1 family	Pleiotropic effects, including inflammatory response and apoptosis	Secreted by activated macrophages and monocytes	Cell growth inhibition and anti-inflammatory (194)
GD2	Glycolipid disialoganglioside	Cell surface glycolipid receptor	Attachment of tumor cells to extracellular matrix	Nervous system cells and melanocytes, highly expressed on neuroblastomas and melanomas	Activates CDC and ADCC (195, 196)
EpCAM	Epithelial cell adhesion molecule	Cell surface glycoprotein	Cell adhesion	Epithelial tissues, highly expressed on carcinomas	Activates CDC, ADCC, and ADCP (188, 189, 197, 198)
CD3	CD3 subunit of the T cell receptor complex	Cell surface glycoprotein of the immunoglobulin superfamily	T cell receptor signal transduction	T lymphocytes	Activates CDC and ADCC (188, 189)
CD19	B cell Receptor CD19	Surface antigen of the immunoglobulin superfamily	B cell differentiation and activation	B lymphocytes and DC	Activates CDC and ADCC (188, 189)
CD20	B cell receptor CD20	Cell surface antigen of the MS4A family	B cell development and activation	B lymphocytes and a subset of T cells	Activates CDC, ADCC, and ADCP (188, 189)
CD22	B cell receptor CD22	Surface antigen of the immunoglobulin superfamily	B cell signaling and adhesion	B lymphocytes	ADC (188, 189)
CD30	Tumor necrosis factor receptor superfamily member 8	Cell surface antigen of the TNF-receptor superfamily	Pleiotropic effects, including lymph proliferation, differentiation, and activation	T and B lymphocytes and natural killer (NK) cells	ADC (188, 189)
CD33	Platelet endothelial cell adhesion molecule	Cell surface lectin	Cell adhesion and apoptosis	Myeloid lineage cells	ADC (188, 189)

(Continued)

TABLE 3 | Continued

Antibody target	Nature of the target	Function	Expression	Monoclonal antibody effects in cancer therapy	Reference
CD38	ADP-ribosyl cyclase 1	Cell surface glycoprotein	Cell activation and adhesion	Activates CDC, ADCC, and ADCP NK cells, DC, and macrophages, highly expressed on B cells and activated T cells	(188, 189)
SLAMF7	Signaling lymphocytic activation molecule family member 7	Cell surface receptor of the CS2 family	NK cells activation	T and B lymphocytes, NK cells, DC, and monocytes	(188, 189)
CD52	CAMPATH-1	Cell surface glycoprotein	Modulates lymphocytes activation and adhesion	T and B lymphocytes, NK cells, monocytes, macrophages, epithelial, and sperm cells	Activates CDC and ADCC (188, 189)

ADC, antibody-drug conjugate; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; DC, dendritic Cells; MS4A, membrane-spanning 4-domains subfamily A; NK cells, natural killer cells.
Color shades corresponds to structurally related target molecules.

antibodies, not yet approved, but mentioned on this review are summarized on **Table 4**.

From the antibodies approved (or under review) for the treatment of solid tumors, seven of them recognize tumor cell surface tyrosine kinase receptors involved in proliferation and survival pathways. These receptors (see **Table 3**) are PDGFR α (targeted by the antibody olaratumab), HER2 (pertuzumab, trastuzumab and emtansine, ado-trastuzumab) and epidermal growth factor receptor (EGFR; necitumumab, panitumumab, and cetuximab). Two antibodies inhibit tumor angiogenesis by binding to the soluble ligand VEGF (bevacizumab) or to the endothelial cell receptor VEGFR2 (ramucirumab). Six of the mAb disrupt inhibitory immune checkpoint signals by binding to the programmed cell death protein 1 (PD-1) receptor on the T cells (nivolumab and pembrolizumab), to PD-L1 on the tumor cells (atezolizumab, durvalumab, and avelumab) or to CTLA-4 on T cells (ipilimumab). Two of these mAb block the binding of cytokines that are involved in the growth of some tumors, including antibodies against RANK-L (denosumab) and IL-1 α (MABp1). The last three mAb recognize antigens overexpressed on the surface of tumor cells. They identify GD2 (dinutuximab) and EpCAM (edrecolomab and catumaxomab) (**Table 1**).

From the antibodies approved (or under review) for the treatment of hematopoietic neoplasias, eight antibodies recognize B cell antigens. Among those, one recognizes CD19 (blinatumomab), five mAb recognize CD20 (obinutuzumab, ofatumumab, rituximab, ibritumomab tiuxetan, and 131I tositumomab), one mAb binds to CD22 (inotuzumab ozogamicin), and the last one recognizes CD52 (alemtuzumab). Other two antibodies identify antigens expressed by B cells and by other cells of the immune system, including an anti-CD30 mAb (shared between B and T cells, brentuximab vedotin) and an anti-SLAMF7 (present on activated B cells and natural killer (NK) cells among others, elotuzumab). In addition, there are two antibodies against other immune cells, an anti-CD33 (myeloid lineage, gemtuzumab ozogamicin); and the non-lineage-restricted CD38 (daratumumab) (**Table 2**).

We will describe in the following paragraphs a set of clinical trials using antibodies in combination for oncological treatments, giving a systematic description of the antibody combinations with biological agents and their rationale. Describing the current aims of antibody-mediated cancer therapy and to envisage where its future lies. In addition, there will be a section where the therapeutic effects and toxicities for selected clinical trials will be discussed, which will help us to envisage the future of therapeutic antibodies for cancer treatments. Before starting with this systematic analysis, we will describe, with one example, in this case for the treatment of GD2 $^+$ -neuroblastomas, the complexity of the clinical trials being carried out.

EVOLUTION OF TREATMENT COMPLEXITY WITH ANTIBODY IN COMBINATIONS

In this section, we will discuss, as an example, the use of anti GD2 antibodies for the treatment of GD2-positive solid tumors, including neuroblastoma (266–269). Near 50 clinical assays have

TABLE 4 | Summary of the therapeutic antibodies not yet approved for clinical treatments.^a

Antibody name	Molecular format	Company or Institute	Target/main characteristics	Reference/Clinical Trial Identifier
3H1 (CEA-Vac)	Mouse IgG1	Titan Pharmaceuticals	Anti-idiotype antibody that mimics an epitope of the carcinoembryonic antigen (CEA)	(199)/NCT00033748
5B1 (MVT-5873)	Human IgG1	MabVax Therapeutics	Carbohydrate determinant 19-9 (CA19-9, carbohydrate antigen sialyl-Lewis A)	(200)/NCT03118349
11D10 (TriAb)	Mouse IgG1	Titan Pharmaceuticals	Anti-idiotype antibody that mimics a human milk fat globule membrane epitope	(201)/NCT00033748, NCT00045617
A27.15	Mouse IgG1	University of Arizona	Anti-transferrin receptor (TfR) antibody that blocks the binding of transferrin	(202)/NCT0003082
Abagovomab	Mouse IgG1	CellControl Biomedical Laboratories; Menarini	Anti-idiotype antibody that mimics an epitope of the ovarian cancer tumor-associated antigen CA-125	(203)/NCT00058435, NCT01959672
Andecaliximab (GS-5745)	Humanized IgG4	Gilead Sciences	Anti-matrix metalloproteinase 9 antibody that inhibits its enzymatic activity	(204, 205)/ NCT02864381
B-701	Human IgG1	BioClin Therapeutics	Fibroblast growth factor receptor 3. Antagonist	(206)/NCT03123055
Basiliximab (Simulect)	Chimeric mouse–human IgG1	Novartis; Cerimon Pharmaceuticals	Interleukin-2 receptor alpha-subunit (IL-2Ralpha, IL2Ra, CD25). Antagonist	(207)/ NCT00626483
BMS-986148	ADC	Bristol-Myers Squibb	Mesothelin (MSLN). Antibody conjugated to an undisclosed cytotoxic drug	(208)/NCT02341625
BMS-986179	Bristol-Myers Squibb	Bristol-Myers Squibb	Ecto-5'-nucleotidase (CD73)	(209–211)/ NCT02754141
BTH1704	Humanized IgG1	Cancer Research UK; Biothera	Mucin-1 (MUC1). Antagonist	(212)/NCT02132403
Cabralizumab (FPA008)	Humanized IgG4	Five Prime Therapeutics; Bristol-Myers Squibb; Ono Pharmaceutical	Colony-stimulating factor 1 receptor (CSF1R). Antagonist	(213)/NCT02526017, NCT03158272
Canakinumab (ACZ885)	Fully human IgG1	Novartis Pharmaceuticals	Interleukin-1 beta (IL-1beta, IL-1b). Antagonist	(214)/NCT02900664
Carotuximab (TRC105)	Chimeric mouse-human IgG1	Roswell Park Cancer Institute; Santen Pharmaceutical; TRACON Pharmaceuticals	Endoglin (CD105). Inhibitor	(215)/NCT03181308
CC-90002	Humanized IgG	Inhixx; Celgene Corporation	Leukocyte surface antigen CD47. Antagonist	(216)/NCT02367196
CDX-1401	Human antibody fusion protein	Ludwig Institute for Cancer Research; Celldex Therapeutics Inc	Dendritic and epithelial cell receptor DEC205. Antibody linked to the tumor-associated antigen NY-ESO-1	(217)/NCT02129075, NCT02495636
Cergutuzumab amunaleukin (CEA-IL2v, RG7813)	Immunocytokine	Roche	CEA. Antibody fused to a single IL-2 variant moiety with abolished CD25 binding	(218)/NCT02350673
CJM112	Fully human IgG1	Novartis	Interleukin-17A (IL-17, IL-17A). Antagonist	(219)/NCT03111992, NCT02900664
Conatumumab (AMG 655)	Fully human IgG1	Amgen; Takeda	Tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2, DR5). Agonist	(220)/NCT01327612
Darleukin (L19-IL2)	Immunocytokine, fusion protein	Philogen; Bayer HealthCare Pharmaceuticals	Extra-domain B domain of fibronectin. A human single-chain variable fragment (scFv) antibody fragment fused to interleukin-2 (IL-2)	(221)/NCT02076633
Demcizumab (OMP-21M18)	Humanized IgG2	OncoMed Pharmaceuticals	Delta-like ligand 4 (DLL4). antagonist	(222)/NCT02722954
Drozitumab (PRO95780)	Fully human, IgG1	Genentech	TRAIL-R2 (DR5). Agonist	(223)/NCT00851136

(Continued)

TABLE 4 | Continued

Antibody name	Molecular format	Company or Institute	Target/main characteristics	Reference/Clinical Trial Identifier
E2.3	Mouse IgG1	Salk Institute	Anti-TfR antibody that blocks the binding of transferrin to the receptor	(202, 224)/NCT00003082
Emactuzumab (RO5509554)	Humanized IgG1	Roche	CSF1R (CD115). Antagonist	(225)/NCT02760797
EMD 525797 (DI17E6)	Humanized IgG2	EMD Serono; Merck Serono	Integrin alpha-V subunit (CD51). Antagonist	(226)/NCT01008475
Emebetuzumab (LY2875358)	Humanized IgG4	Eli Lilly	c-Met receptor tyrosine kinase (c-MET; MET; hepatocyte growth factor receptor; c-Met proto-oncogene)	(227)/NCT02082210
Epratuzumab (AMG 412)	Humanized IgG1	Immunomedics	Anti-CD22 antibody that mediates antibody-dependent cellular cytotoxicity (ADCC)	(228)/NCT00941928
Ficlatuzumab (AV-299, SCH 900105)	Human IgG1	AVEO Pharmaceuticals	Hepatocyte growth factor (HGF). Inhibitor	(229)/NCT02277197
Ganitumab (AMG 479)	Fully human IgG1	Amgen; NantWorks; Takeda	Insulin-like growth factor 1 receptor. Antagonist	(230)/NCT00788957, NCT01327612
GD2Bi-aATC (Hu3F8Bi-armed ATC)	Humanized bispecific	National Cancer Institute; Barbara Ann Karmanos Cancer Institute	CD3 and disialoganglioside GD2	(231, 232)/NCT02173093
Imalumab (BAX69)	Fully human	Cytokine PharmaSciences; Shire	Macrophage migration inhibitory factor. Inhibitor	(233)/NCT02448810
IMC-CS4 (LY3022855)	Human IgG1	ImClone Systems	CSF1R (C-FMS; CD115)	(234)/NCT03153410
Intetumumab (CINTO 95)	Fully human IgG1	Centocor; BeiGene	Anti-Integrin alpha-V subunit (CD51) antibody that blocks both alpha-v beta-3 and alpha-v beta-5 integrins	(235)/NCT00888043
Lirilumab	Humanized monoclonal antibody (mAb) IgG4	Bristol-Myers Squibb	KIR (killer-cell immunoglobulin-like receptors)	(236)/NCT01714739
m170	Mouse IgG1	University of California, Davis	MUC1	(237)/NCT00009750
MEDI3617	Human IgG1	MedImmune	Angiopoietin 2. Antagonist	(238)/NCT01248949, NCT02141542
Milatuzumab (hLL1)	Humanized IgG1	Immunomedics	CD74	(239)/NCT00989586
Mirvetuximab soravtansine (IMGN853)	Chimeric mouse-human, ADC	ImmunoGen	Folate receptor 1. Antibody conjugated to the maytansinoid DM4 (N2'-Deacetyl-N2'-(4-mercaptop-4-methyl-1-oxopentyl)-maytansine)	(240)/NCT02606305
MM-111	Human bispecific	Merrimack Pharmaceuticals	ErbB receptors ErbB2 and ErbB3. Inhibitor	(241)/NCT01097460
MNRP1685A	Fully human IgG1	Genentech; Roche	Neuropilin-1. Inhibitor	(242)/NCT00954642
MOXR0916	Humanized IgG1	Genentech	OX40. Antagonist	(243)/NCT02410512
Navicixizumab (OMP-305B83)	Bispecific Humanized IgG2	OncoMed Pharmaceuticals	Delta-like ligand 4 (DLL4) and vascular endothelial growth factor A (VEGF). Inhibitor	(244)/NCT03030287, NCT02298387
Nimotuzumab (TheraCim hR3, BIOMAb EGFR, Theraloc)	Humanized IgG1	Center of Molecular Immunology; CIMYM	Epidermal growth factor receptor (EGFR). Inhibitor	(245)/NCT02947386
Otlertuzumab (TRU-016)	Recombinant single-chain polypeptide	Aptevo Therapeutics	CD37 Potential immunostimulatory and antineoplastic activities	(246)/NCT01317901
Parsatuzumab (MEGF0444A)	Humanized IgG1	Genentech	Epidermal growth factor-like domain 7. Inhibitor	(247)/NCT01399684
PD-0360324	Humanized IgG2	Pfizer	Cytokine CSF1 (CSF-1, macrophage colony-stimulating factor, M-CSF). Inhibitor	(248)/NCT02554812

(Continued)

TABLE 4 | Continued

Antibody name	Molecular format	Company or Institute	Target/main characteristics	Reference/Clinical Trial Identifier
PDR001	Humanized IgG4	Novartis	Programmed cell death protein 1 (PD-1). Inhibitor	(13)/NCT02900664, NCT03111992
PF-04518600	Fully human IgG2	Pfizer	OX40. Agonist	(249)/NCT02554812
Pidilizumab (CT-011, MDV9300)	Humanized IgG1	CureTech; Medivation	PD-1, Inhibitor	(250); NCT01067287
Relatlimab BMS-986016	Human mAb IgG4	Bristol-Myers Squibb	LAG-3 (human lymphocyte activation gene 3 protein) Potential immune checkpoint inhibitory and antineoplastic activities	(251)/NCT01968109, NCT02061761
Rilotumumab (AMG102)	Fully human IgG2	Amgen	Human hepatocyte growth factor (HGF, c-Met). Inhibitor	(252)/NCT00788957
RO6958688	Bispecific	Roche	CD3 and CEA. Inhibitor	(253, 254)/NCT02650713
RO7009789 (CP-870,893)	Fully human IgG2	Roche	CD40. Agonist	(255)/NCT02665416, NCT02760797
Rovalpituzumab tesirine (SC16LD6.5)	Humanized ADC	Stemcentrx	Delta-like protein 3 (DLL3). Antibody conjugated to tesirine, a pyrrolobenzodiazepine dimer	(256)/NCT03026166
SGN-LIV1A	Humanized ADC	Seattle Genetics	Zinc transporter LIV-1 (SLC39A6). Antibody conjugated to maleimidocaproylvaline-citrulline-p-aminobenzoyloxycarbonyl-MMAE (vcMMAE)	(257)/NCT01969643
SS1 (dsFv) PE38 (CAT-5001)	Immunotoxin	National Institutes of Health (USA)	MSLN. Single-chain antibody linked to Pseudomonas exotoxin PE-38	(258)/NCT01051934
Tigatumumab (CS-1008)	Humanized IgG1	Daiichi Sankyo Company; University of Alabama at Birmingham	TRAIL-R2 (DR5). Agonist	(259, 260)/NCT01307891
Urelumab (BMS-663513)	Fully human IgG4 mAb	Bristol-Myers Squibb	Anti-CD137 Potential immunostimulatory and antineoplastic activities	(261)/NCT01471210, NCT01775631, NCT02110082, NCT02253992
Utomilumab (PF-05082566)	Human IgG2	Pfizer	CD137 (4-1BB). Agonist	(262)/NCT02554812
Vanucizumab (RG7221)	Bispecific Humanized	Roche	Angiopoietin 2 (ANG2, ANGPT2) and vascular endothelial growth factor (VEGF). Inhibitor	(263)/NCT01688206, NCT02665416, NCT02715531
Varlitumab (CDX-1127)	Fully human IgG1	Celldex Therapeutics	CD27. Agonistic.	(16)/NCT02410512
Veltuzumab (IMMU-106, hA20)	Humanized	Immunomedics	Anti-CD20 antibody that triggers complement-dependent cell lysis and antibody-dependent cell-mediated cytotoxicity (ADCC)	(264)/NCT00989586
VGX-100	Fully human IgG1	Circadian Technologies Limited	Vascular endothelial growth factor C (VEGF-C or Flt4 ligand). Inhibitor	(265)/NCT01514123

^aOnly clinical trials included in this review.

been started using two different mouse antibodies and their corresponding chimeras or humanized antibodies. The clinical use of dinutuximab (ch14.18) was approved in 2015, whereas the therapeutic efficacy of the other antibody, 3F8 has been demonstrated with many patients (270–272). These antibodies, recognizing the neuroblastoma tumor-associated antigen GD2, are being used to (i) kill the tumor with either the naked antibody alone, apparently through Fc-mediated effector actions [antibody-dependent cell cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cell phagocytosis (ADCP)] or apoptosis (NCT00002458, NCT00072358, NCT01418495, NCT01419834, NCT01704872, NCT02258815, NCT02743429); (ii) kill the tumor by the naked antibody in combination with chemotherapeutic agents (busulfan, carboplatin, cisplatin, cyclophosphamide, doxorubicin, etoposide, lomustine, melphalan, or vincristine), small molecule drugs (crizotinib), external radiation, and/or conventional surgery (NCT03098030, NCT03126916); (iii) directly transport a radioelement toward the tumor, by conjugating the radioelement to the anti-GD2 mAb. This will induce radiolysis of the tumor cells, minimizing the effects on normal cells (NCT00058370, NCT00445965, NCT03126916); (iv) use them in combination with agents that modify cell expression patterns, inhibiting proliferation and inducing cell differentiation and apoptosis [i.e., isotretinoin (13-cis retinoic acid or RA)] (NCT00003022, NCT00030719, NCT01183416, NCT01183429, NCT01183884, NCT01526603, NCT01711554, NCT02100930, NCT03033303); or (v) use them in combination with agents able to burst the host immune response against the tumor. These include, granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF) (NCT01704716, NCT01767194, NCT02484443, NCT02502786, NCT03189706); which increase the number of innate immune response cells by triggering the proliferation of granulocytes and macrophages; increasing both innate and adaptive responses by inducing the maturation/proliferation of NK cells and T lymphocyte proliferation with interleukin-2 (IL-2) alone or in combination with GM-CSF and/or RA (NCT00005576, NCT00026312, NCT01041638, NCT01592045, NCT01662804, NCT02169609, NCT02641782); regulating the threshold of the immune response with an adjuvant, changing the secreted cytokine expression pattern of cells bearing certain pattern recognition receptors (i.e., beta glucan that binds the C-type lectin receptor Dectin-1) (NCT00037011, NCT00492167, NCT0089258); increasing the pool of cytotoxic cells able to fight the tumor with allogeneic NK cells (NCT00877110, NCT01857934, NCT02573896, NCT02650648); using *in vitro* activated T cells coated with bispecific OKT3-hu3F8 mAb, together with IL-2 and GM-CSF to redirect T lymphocyte cell lysis (NCT02173093); and combining the anti-GD2 antibody with nivolumab, an anti-immune checkpoint (PD-1) mAb able to block the immunosuppressor activity induced by the tumor (NCT02914405).

From these “basic” aims further combinations arose, for example one where the aim is to induce radiolysis of the tumor cells with ^{131}I -3F8, simultaneously bursting the innate immune response with filgastrim (G-CSF), inhibiting neo-vascularization with bevacizumab (anti-VEGF), together with autologous stem cell rescue of irradiated patients (NCT00450827).

We believe that this example gave a rough idea of the complexity that clinical trials for one antibody (two in this case) can reach. The chimeric, human-murine, anti-GD2 mAb dinutuximab has been approved in combination with GM-CSF, IL-2, and retinoic acid for the treatment of pediatric patients with high-risk neuroblastoma (273). Interestingly, the overall survival and event-free survival of patients treated with dinutuximab increased 2 years when compared to standard treatment during phase III clinical trials (273).

COMBINATION OF ANTIBODIES WITH NON-BIOLOGICAL AGENTS

Chemotherapeutic drugs are cytotoxic agents affecting unspecifically cell proliferation and survival, which inhibit topoisomerases I or II (doxorubicin, etoposide, irinotecan, topotecan, etc.), produce DNA breaks interfering with DNA replication, RNA transcription and cell division through changes in DNA alkylation, DNA methylation, and DNA cross-linking or intercalating between base pairs in the DNA helix (busulfan, melphalan, cyclophosphamide, carboplatin, cisplatin, lomustine, thiotepa, etc.). These chemotherapeutic drugs are being used in combination with mAbs for many cancer treatments (274).

In addition to surgery, treatment with antibodies and external irradiation has also been used. Localized external irradiation allows, by destroying tumor cells, better exposure of the tumor antigens to the immune system cells, this combination is also working well and is being used in numerous clinical trials (275–279).

Small molecule drugs that inhibit molecular interactions or enzymatic activity of proteins involved in cell signaling, or inhibitors of protein kinases overexpressed in tumor cells (including erlotinib, ibrutinib, imatinib, lapatinib, olaparib, regorafenib, ruxolitinib, sorafenib, sunitinib, etc.), are also being used in combination with antibodies (280, 281). There are numerous examples of treatments with this type of combinations that, by simultaneously inhibiting ligand-receptor interactions and kinases belonging to the same signaling pathway, have led to very positive therapeutic results (282–286).

COMBINATION OF ANTIBODIES WITH BIOLOGICAL AGENTS

These are therapies that use a combination of antibodies or antibody-based molecules with other biological substances, for example, recombinant proteins, genetic material, virus, bacteria, and cells (16). Most of these strategies are designed to stimulate the host immune system to act against the cancer cells.

In the following paragraphs, we describe antibodies in combinations, where (i) one of the antibodies identifies a tumor-associated antigen (an antigen overexpressed in tumor cells), used either naked, as an antibody-drug conjugate (ADC) or as an immunotoxin; (ii) antibodies against the tumor cell are used in combination with cytokines or immunocytokines to burst the immune response against the tumor, or conversely use anti-cytokine antibodies when the expressed cytokines can be

harmful for the antitumor response, aiming to disrupt their balance; (iii) the antibodies directly target the angiogenesis process, aiming to inhibit new vascularization required for tumor growth; (iv) the mAb can also be combined with effector cells to increase the immune response against the tumor; or (v) combined with antibodies against immunomodulatory or immunostimulatory proteins to disrupt the inhibitory signals sent by the tumor to the host immune system to inhibit the antitumor response. Although several of the examples we will describe could be included more than one sub-heading, each one of them is described only in one of them.

Antibodies Against Tumor-Associated Antigens

The rationale of using antibodies as therapeutic agents was to kill the tumor cells either directly or through activating the patient's immune system effector functions (ADCC, CDC, or phagocytosis) with antibodies specific for tumor-associated antigens. Mucin 1 (MUC-1), an antigen present on the surface of many adenocarcinomas, which is recognized by mAb m170 (237). This mAb has been used radiolabeled as ¹¹¹In-m170 or ⁹⁰Y-m170, in combination with chemotherapy and the immunosuppressor cyclosporine to treat patients with metastatic prostate cancer that did not respond to hormone therapy. This treatment was followed by peripheral stem cell transplantation (NCT00009750). The rationale is to kill the tumor with the combination of chemotherapy and the mAb coupled to the radioisotope in the presence of cyclosporine. Afterward peripheral stem cell transplantation will allow to refurbish the hematopoietic compartment.

Other approaches have been used on hematological neoplasias, one of them, a combination of two anti-transferrin receptor (TfR) antibodies A27.15 and E2.3 (202, 287) was used for the treatment of chronic myeloproliferative disorders (NCT00003082). The anti-TfR mAb block the binding of (Fe^{3+})-2-transferrin to TfR, resulting in decreased tumor cell growth. Other targets used in hematopoietic malignancies are CD20 and CD74. In this case, a combination of the anti-CD20 mAb veltuzumab (IMMU-106) (288) and milatuzumab (anti-CD74) (239) was used to treat relapsed or refractory B cell non-Hodgkin lymphoma (NCT00989586). CD74, a surface receptor of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) (289–291), is an MHC class II chaperone and an accessory-signaling molecule (292). Milatuzumab induces apoptosis, related to inhibition of CD74 activation by MIF, ADCC, or CDC (293), while veltuzumab triggers CDC and ADCC in cells that overexpress CD20 (288). Other combinations include rituximab (anti-CD20) in combination with CC-90002 (anti-CD47) (294, 295) for the treatment of advanced solid and hematologic cancers (NCT02367196). CC-90002 selectively binds to CD47 expressed on tumor cells, blocks CD47 interaction with signal regulatory protein alpha (SIRPa), a protein expressed on phagocytic cells, which prevents CD47/SIRPa-mediated signaling and abrogates the CD47/SIRPa-mediated inhibition of phagocytosis. The result is an induction of pro-phagocytic signaling, resulting in macrophage activation and the specific phagocytosis of tumor cells. In addition, CD47 signaling blockade activates both, an antitumor T lymphocyte immune response and T cell-mediated killing of CD47-expressing tumor cells. CD47, also called

integrin-associated protein (IAP), is a tumor-associated antigen (TAA) expressed on normal, healthy hematopoietic stem cells (HSC) and overexpressed on the surface of a variety of cancer cells. Expression of CD47 and its interaction with SIRPa leads to the inhibition of macrophage activation and protects cancer cells from phagocytosis, resulting in cancer cell proliferation (294, 296–298).

ADC and Immunotoxins

Some antitumor treatments use, rather than naked antibodies, antibody-toxin fusion proteins (immunotoxins) or antibodies linked to drugs (299–302). In some cases, to increase the cell-killing potential of antibodies, they can be covalently linked to potent cytotoxic or cytostatic agents, including small molecule drugs or inactive forms of a biological toxin. The antibody directs the toxin toward the tumor cell. When the cell endocytoses the ADC, it undergoes enzymatic cleavage and the drug is released, gets activated, and exerts its cytotoxic action, killing the tumor cell. The endocytic process works for antigens that can be internalized. Most of the current antibodies that are being used as ADC identify cell surface receptors that are efficiently endocytosed. However, many cell surface proteins are not internalized and a large amount of work is being carried out to develop alternatives, such as making an ADC where the antibody is coupled to the drug through a linker that can be cleaved by tumor cell-surface proteases, when in close contact with the tumor cell; conversely the antibody may carry a tumor receptor antagonist to direct it toward the tumor cell surface (303, 304).

One example includes a combination of an antitumor-associated antigen mAb and an ADC. Trastuzumab in combination with an antibody against the zinc transporter LIV-1 (SLC39A6) conjugated to the cytotoxic agent monomethyl auristatin E (MMAE) (257) for the treatment of patients with metastatic breast cancer (NCT01969643). In this particular combination, trastuzumab inhibits the tyrosine-kinase receptor HER2, while through the potent microtubule disrupting agent MMAE, which is coupled to the anti-LIV-1 antibody, induces cell cycle arrest in the G2/M phase and apoptosis of LIV-1⁺ cells (257). This type of combination can be made more potent by adding to the equation, antibodies against immune-checkpoints to burst antitumor immune responses. For example, the combination of nivolumab, ipilimumab, and rovalpituzumab tesirine has been used in extensive-stage small cell lung cancer (SCLC) (NCT03026166). Rovalpituzumab tesirine is an anti-delta-like protein 3 (DLL3) antibody conjugated to the cytotoxic pyrrolobenzodiazepine dimer D6.5 (256, 305). This antibody recognizes the membrane protein DLL3, which is overexpressed in certain tumors, binds to Notch receptors, and regulates Notch-mediated signaling (256). Thus, this combination should kill the cells overexpressing DLL3, while the anti-checkpoint antibodies nivolumab and ipilimumab redirect the host immune response to attack the tumor.

Another example shows the combination of mAb CR011, against the transmembrane protein GPNMB (glycoprotein non-metastatic B) coupled to MMAE (306). This ADC was used in combination with anti-PD-1 mAb nivolumab or pembrolizumab and varilimumab (307, 308), an agonistic anti-CD27 mAb, for the treatment of advanced melanoma (NCT02302339). The rationale

is, in addition to targeting the GPNMB⁺ cells with the antibody-coupled to the toxin, the anti-PD-1 antibodies suppress the tumor-promoted inhibition of the antitumor immune response, while the anti-CD27 triggers an activation of the cytotoxic T lymphocytes (CTL).

The following example combines the ADC mirvetuximab soravtansine with either bevacizumab (VEGF) or pembrolizumab (PD-1) in primary peritoneal, fallopian tube, or endometrial cancer (NCT02606305). Mirvetuximab soravtansine is an immunoconjugate consisting of a folate receptor 1 (FOLR1) mAb (M9346A) conjugated to the cytotoxic maytansinoid DM4 (240). DM4 is released after internalization, binds to tubulin, and disrupts microtubule dynamics. FOLR1 is a member of the folate receptor family, overexpressed on a variety of epithelial-derived cancer cells (240, 309). Other studies combine nivolumab (anti-PD-1) with the ADC BMS-986148 (208, 310), composed of a mAb against the cell surface glycoprotein mesothelin (MSLN), conjugated to an as of yet undisclosed cytotoxic drug, for the treatment of mesothelioma, NSCLC, ovarian cancer, pancreatic cancer, and gastric cancer (NCT02341625). The rationale here is to block with the anti-PD-1 mAb the binding of PD-L1 (present on the tumor cells) to its receptor PD-1 (present on T cells), avoiding the suppression of antitumor responses triggered by the PD-L1/PD-1 interaction, while targeting mesothelin⁺ cells with the BMS-986148 mAb. Since the mAb is an ADC, upon internalization the cytotoxic agent kills the tumor cells. The mAb also activates ADCC. Another combination, for the treatment of MSLN-expressing NSCLC uses a combination of the anti-VEGF antibody bevacizumab with the single-chain anti-MSLN mAb SS1 (dsFv) linked to the exotoxin PE-38 from Pseudomonas (258) (NCT01051934). Since MSLN is not shed in significant amounts into the bloodstream, the dsFv-toxin can be concentrated onto the tumor cell surface. Once the dsFv toxin is internalized, the toxin is released and inactivates eukaryotic translation elongation factor 2, disrupting tumor cell protein synthesis. Concomitantly, the anti-VEGF antibody inhibits angiogenesis (258).

Antibodies Combined with Cytokines and Immunocytokines

Another way to burst the host immune response against the tumor involves the use of either exogenous cytokines or fusion proteins that include a cytokine, administered either systemically or directly in the tumor. In some cases, due to cytokine toxicity, it could be envisaged to directly couple the cytokine to a mAb specific for a tumor-associated antigen, as a recombinant fusion protein (311, 312). These combinations allow, decreasing the dose, to reach higher local concentrations at the tumor site and to exert its therapeutic function avoiding systemic toxicity, while increasing the cytokine's half-life, since it is coupled to the antibody, which prevents renal clearance (313).

In another example for the treatment of advanced or metastatic solid tumors, a combination of nivolumab and the Aldesleukin Prodrug NKTR-214 was used (NCT02983045). NKTR-214 is a recombinant human IL-2 conjugated to six releasable polyethylene glycol chains (PEG) (314). When the cytokine is released, binds to CD122 (IL-2 receptor beta subunit) and the mAb may act synergistically with NKTR-214 by blocking PD-1

activation through the mAb and simultaneously stimulating growth and cytotoxic activity against the tumor of the patient's T and NK cells by the exogenous IL-2. The advantages of using this conjugated form of the IL-2 are that, on the one side, is released in a controlled way in the tumor's proximity, avoiding systemic toxic effects; and on the other side, PEG conjugation prevents IL-2 binding to the IL2Ralpha subunit (and the subsequent activation of CD4-positive regulatory immunosuppressive T cells), while IL2Rbeta activation plays a key role on the proliferation and activation of effector T cells (314). In another clinical trial, the NKTR-214 immunocytokine was also administered, using a similar therapeutic strategy for the treatment of patients with metastatic urothelial bladder cancer or metastatic NSCLC, in combination with atezolizumab (74–76) (NCT03138889). Another trial for advanced or metastatic solid tumors expressing the carcinoembryonic antigen (CEA), combined atezolizumab and cergutuzumab amunaleukin [CEA-IL-2 variant (IL2v)] (218), alone or together with a pretreatment with the anti-CD20 mAb obinutuzumab (NCT02350673). The immunocytokine CEA-IL2v is a fusion protein between a recombinant IL2v, unable to bind CD25, fused to the C-terminus of a high affinity, bivalent CEA-specific antibody (218). The strategy is to inhibit the PD-1/PD-1L checkpoint, increasing locally IL-2 activity on the tumor cells, allowing its binding to CD122. A similar strategy uses nivolumab in combination with the superagonist ALT-803 for the treatment of advanced and unresectable NSCLC (NCT02523469). ALT-803 is a fusion protein containing a mutated IL-15 (IL-15N72D) cytokine and a soluble, dimeric IL-15 receptor alpha Fc fusion protein (IL-15Ra-Fc) (315). The rationale of this study is to suppress the signaling through negative checkpoints, while activating and increasing NK levels and memory CD8⁺ T cells, through the binding of ALT-803 to the IL-2/IL-15 receptor beta gamma chain, strengthening the patient's immune response.

Another example is the anti-HER2 mAb trastuzumab, which has been used in combination with IL-12 in treating patients with recurrent solid tumors [breast cancer, endometrial carcinoma, gastric cancer, non-small cell lung cancer (NSCLC), SCLC, and ovarian epithelial cancer] (NCT00028535). IL-12 stimulates IFN-gamma production and enhances T- and NK-cell proliferation, differentiation, and activation (316). A more complex example combines two mAb-cytokine fusion proteins, consisting of L19, a human single-chain variable fragment directed against the extra-domain B (ED-B) of fibronectin, linked to either the human pro-inflammatory cytokine tumor necrosis factor alpha (TNFa, L19-TNF) or to human IL-2 (L19-IL-2). These combinations have been used on patients with malignant melanoma (317–320) (NCT02076633). The rationale of the trial is that the L19 moiety binds to the ED-B domain of a fibronectin isoform selectively expressed in the tumor neovasculature during neo-angiogenesis. TNFa may locally induce an immune response against ED-B⁺ tumor cells, while the IL-2 moiety may locally activate CTL, NK cells, and macrophages.

In another clinical trial, atezolizumab was combined with (i) ipilimumab, (ii) interferon alfa-2b, (iii) PEG-interferon Alfa-2a, (iv) bevacizumab and PEG-interferon alfa-2a, and (v) obinutuzumab, for the treatment of locally advanced or metastatic solid tumors (NCT02174172). The aim is to compare on the

same trial the results from these different strategies. The rationale is to restore the antitumor immune response by blocking immune checkpoints, while inducing cell cycle arrest, apoptosis or differentiation, which will lead to tumor growth inhibition, concomitant with T cell and NK cell activation, inhibition of angiogenesis and induction of cytokine expression, through the administration of interferon alpha (321).

Anti-Cytokine Antibodies

On the previous section, the aim was to provide exogenous cytokines to burst the antitumor immune response. Here, the aim is to disrupt the balance of other cytokines such as IL-17 or IL-1, that may hinder the antitumor immune response.

An example, for the treatment of patients with multiple myeloma, uses the anti PD-1 mAb PDR001 in combination with the mAb CJM112 (targeting IL-17) or with the Smac Mimetic LCL161 drug (an IAP inhibitor) (NCT03111992). This strategy aims to restore the cellular immune response inhibiting checkpoint signaling, changing the cytokine balance by decreasing the available IL-17 and favoring tumor cell apoptosis. A more complex clinical trial was used for the treatment of colorectal cancer, triple-negative breast cancer, NSCLC and adenocarcinoma, where the anti-PD-1 mAb PDR001 (13) was used in combination with either (i) the anti-interleukin-1 beta (IL-1b) mAb canakinumab (214), (ii) the anti-IL-17 mAb CJM112 (219), (iii) the small molecule inhibitor trametinib (MAPKK1 and MAPKK2 inhibitor) or (iv) the EGFR antagonist nazartinib (NCT02900664). The aim is to inhibit the immune checkpoint, while either simultaneously suppressing the inflammatory responses (blocking IL-1b or IL-17), or inhibiting tumor cell proliferation with mitogen-activated protein kinase and EGFR inhibitors.

Antibodies Targeting Angiogenesis

Unlike to what happens with hematologic tumors, the growth of a solid tumor is concomitant with a local increase in nutrient and oxygen consumption and secretion of metabolites, requiring neovascularization for its growth. Therefore, some of the antitumor therapies aim to interfere with the neo-vascularization process, either by including antibodies against the soluble ligands, or against their receptors present in the cell surface of endothelial cells (322).

The anti-VEGF-A mAb bevacizumab, able to inhibit angiogenesis is currently being tested in combination with other therapeutic agents to determine its usefulness for cancer treatment. These combinations include: cetuximab, in advanced lung cancer (NCT00368992); MEDI3617 (anti-Ang-2) (238), for advanced solid malignancies (NCT01248949); the mAb drozitumab (PRO95780) (223) against death receptor 5 (DR5/TRAIL-R2), in metastatic colorectal cancer (NCT00851136); NK immunotherapy, in recurrent solid tumors (NCT02857920); and atezolizumab (NCT03038100, NCT02659384), nivolumab (NCT02873962), or pembrolizumab (NCT02853318) for the treatment of ovarian, fallopian tube, or primary peritoneal cancer. Bevacizumab has also been combined with MNRP1685A (242, 323), a mAb against membrane-bound endothelial cell co-receptor neuropilin-1 (NRP1), overexpressed in certain tumor cells, for advanced or metastatic solid tumors (NCT00954642).

MNR1685A prevents angiogenesis by blocking binding of VEGF, VEGF-B, and placental growth factor 2 to neuropilin-1, resulting in vessel immaturity. Other combinations of bevacizumab include parsatuzumab (247), a mAb against the vascular-restricted extracellular matrix protein epidermal growth factor-like domain multiple 7 (EGFL7), upregulated during angiogenesis and overexpressed on the cell surface of different solid tumors, for the treatment of metastatic colorectal cancer (NCT01399684). Parsatuzumab inhibits vascular development regulated by EGFL7, affecting to the survival and migration of endothelial cells during angiogenesis. An additional combination used bevacizumab and anti-VEGFC/Flt4 (VGX-100) (265) for metastatic solid tumors (NCT01514123). The rationale is to simultaneously inhibit vascular and lymphatic endothelial cell proliferation and angiogenesis.

A different approach is to target angiogenesis with the Anti-VEGFR-2 mAb ramucirumab, in combination with the anti-c-MET [hepatocyte growth factor receptor (HGFR)] mAb emibetuzumab (227), on advanced refractory solid tumors (NCT02082210). The rationale of the trial is to inhibit angiogenesis and MET signaling on the tumor cells (227). A similar strategy has been used where instead of using an anti-HGFR mAb, uses fclatuzumab (229, 324), a mAb against the c-MET ligand (HGF), in combination with cetuximab (NCT02277197).

A similar strategy targets molecules with an expression highly restricted to the vascular endothelium. An example is the combination of pembrolizumab with demcizumab (222), a mAb that blocks the interaction of anti-delta-like ligand 4 (DLL4) with Notch-1 and Notch-4, inhibiting Notch-mediated signaling and gene transcription, impairing the productive growth of new blood vessels (325) (NCT02722954). Pembrolizumab avoids the immunosuppression by immune checkpoint signaling while demcizumab prevents angiogenesis.

In K-ras wild-type metastatic colorectal cancers, cetuximab mAb was used in combination with the alphaVbeta3 (vitronectin receptor) integrin inhibitor EMD 525797, an anti-alphaV integrin subunit mAb (226) (NCT01008475). AlphaVbeta3 integrin is a cell adhesion and signaling receptor expressed on the surface of tumor endothelial cells, with a crucial role in their adhesion and migration. The aim of the trial is to inhibit angiogenesis and endothelial cell interaction(s) with other cells or with the extracellular matrix, required for tumor angiogenesis and metastasis. A similar study in solid tumors combines bevacizumab with intetumumab (235, 326), a pan alpha-v human mAb that blocks both alpha-v beta-3 and alpha-v beta-5 integrins, resulting in inhibition of integrin-mediated tumor angiogenesis and tumor growth (NCT00888043).

The remaining clinical trials on this section, all of them combine an anti-checkpoint antibody (either anti-CTLA-4, PD-1 or PD-L1) with anti-angiogenic antibodies such as the anti-Angiopoietin 2 (Ang-2) mAb (MEDI3617) in metastatic melanoma (NCT02141542); with the antibody carotuximab (TRC105) (215) that recognizes the endothelial cell surface protein endoglin, essential for angiogenesis, in metastatic NSCLC (NCT03181308); with antibodies specific for Ang-1 and Ang-2, which prevent their interaction with their target tie2 receptors (NCT00861419); with vanucizumab (bispecific anti-VEGF/

Ang-2 antibody) (263), in advanced or metastatic solid tumors (NCT01688206). The bispecific mAb targets both VEGF-A and Ang-2, which are upregulated in a variety of tumor cell types, play key roles in tumor cell proliferation, angiogenesis and metastasis. The anti-VEGF-A arm is based on bevacizumab and the anti-Ang-2 arm is based on the anti-Ang-2 antibody LC06 (263). It simultaneously binds and neutralizes both VEGF-A and Ang-2. This prevents the activation of both VEGF-A/VEGFR- and Ang-2/Tie2-mediated signaling pathways, resulting in the inhibition of proliferation of VEGF-A- and/or Ang-2-overexpressing tumor cells (263, 327).

Another strategy is to combine the anti-EGFR mAb panitumumab with an anti-hepatocyte growth factor mAb rilotumumab (252) or ganitumab (230), an anti-insulin-like growth factor 1 receptor (IGF-1R) mAb in metastatic colorectal cancer with wild-type KRAS (NCT00788957) (328). The rationale here is to simultaneously inhibit strong proliferative signals triggered by EGFR and c-MET.

Antibodies Combined with Effector Cells

In some patients, the number of cells from the innate or adaptive immune system could be decreased by the effects of previous treatments. In these cases, treatments with antibodies, whose mechanisms of action depend on immune system cell effector functions (i.e., ADCC, ADCP, etc.), could be compromised. In these cases, either autologous (harvested prior to the treatment) or allogeneic cells (NK cells, T cells, CTL cells, dendritic cells (DC), etc.) can be administered concomitantly with the therapeutic antibodies.

A combination of mAb and cells for the treatment of hematological malignancies combines the anti-CD22 mAb epratuzumab (228) with haploididentical NK cells and low-dose exogenous IL-2, for the treatment of relapsed acute lymphoblastic leukemia (NCT00941928). CD22 is a cell surface glycoprotein present on mature B cells and on many B cell malignancies. In this example, since epratuzumab action involves ADDC, the exogenous administered haploididentical NK cells strengthens its effects. The exogenous IL-2 induces NK cell proliferation, activates cytotoxic immune responses against the tumor and induces expression of certain cytotoxic cytokines, such as interferon-gamma (IFNgamma) and transforming growth factor-beta. In another example, NK cells were used in combination with nivolumab for the treatment of recurrent solid tumors (NCT02843204), strengthening the endogenous immune response with the anti-checkpoint antibodies and increasing the NK cell load. Another combination used for the treatment of recurrent solid tumors uses NK cells in combination with bevacizumab (NCT02857920), increasing the NK cell load and simultaneously targeting tumor neo-vascularization. The last combinations to be mentioned with effector cells use pembrolizumab, administrated with autologous dendritic cells-cytokine induced killer cell (DC-CIK), for advanced solid tumors (NCT03190811), or the anti-PD-1 mAb, that was used *in vitro* to activate and expand DC-CIK from the patient's peripheral blood, before infusion (NCT02886897). These clinical trials aim to target the immune checkpoint and increasing the load of cytolytic cells with the DC-CIK.

Bispecific Antibodies

Nowadays the FDA and EMA allow clinical trials where the therapeutic agent is a combination of two antibodies. Bispecific antibodies may be considered as a particular combination where both antibodies are in a single molecule. This type of antibodies allows to put in close proximity the tumor cell with an effector cell, a cytokine, etc., or to re-direct the immune response of cytotoxic T cells bypassing antigen recognition through the TCR (329).

On all the trials on hematological tumors using bispecific mAb reported here, the same bispecific mAb blinatumomab (bispecific CD19-CD3) was used in different combinations. An example combines blinatumomab with the anti-CD20 mAb rituximab, for non-receptor tyrosine kinase (ABL)-negative B lineage acute lymphoblastic leukemia (NCT02003222). The rationale is to target the CD20⁺ B cells, while putting in close contact T cells (CD3⁺) with CD19⁺ B cells, to mount a strong cytotoxic T cell response. The rest of the trials from this group, all of them combine mAb targeting immune-checkpoints (CTLA-4, PD-1, or PD-L1) with the bispecific antibodies. These include blinatumomab in relapsed or refractory precursor B-lymphoblastic leukemia (NCT02879695) or for relapsed or refractory B cell acute lymphoblastic leukemia (NCT03160079). The rationale for these trials is to avoid the suppression of antitumor responses, while putting in close contact T cells (CD3⁺) with CD19⁺ B cells.

Bispecific antibodies in combination have also been used for the treatment of solid tumors. For most of the examples, one of the arms of the bispecific mAb identifies a tumor-associated antigen, such as the bispecific antibody RO6958688 (bispecific CD3-CEA) combined with atezolizumab, for the treatment of advanced and metastatic solid tumors (NCT02650713). The bispecific antibody RO6958688 (253, 254) recognizes, on the one side, the CD3 molecule of the TCR and, on the other side, the CEA, an antigen overexpressed in several tumors. The rationale of the trial is to block the binding of PD-L1 to its receptor avoiding the suppression of antitumor responses, while putting in close contact T cells (CD3⁺) with the CEA⁺ tumor cells, inducing a strong T cell activation which may result in a potent antitumor CTL response. Similarly, solid tumors were treated with atezolizumab in combination with bevacizumab or with vanucizumab, a bispecific mAb that simultaneously targets VEGF and Ang-2 (NCT02715531). This treatment aims to block the immune checkpoint suppression while inhibiting angiogenesis.

Another example of targeting tumor-associated antigens is the use of *in vitro*-activated T cells armed with GD2Bi-aATC, a bispecific antibody that recognizes CD3 and GD2 (231, 232), in combination with IL-2 and GM-CSF in patients with neuroblastoma or osteosarcoma (NCT02173093). The rationale of this study is to generate *in vitro* activated T cells that are infused in the patient after binding to the bispecific mAb, which will direct them to the tumor, generating a potent CTL response to kill tumor cells. Exogenous IL-2 and GM-CSF are added to maintain these cells and generate an inflammatory environment surrounding the tumor. In addition, another approach that has been used is to combine the anti-HER2 antibody trastuzumab

with a bispecific MM-11 mAb anti-ErbB2/anti-ErbB3 mAb (241) for the treatment of HER2⁺ breast cancer (NCT01097460). The aim is to simultaneously inhibit signaling through this family of tyrosine kinase receptors.

Another approach is to use bispecific mAb to inhibit angiogenesis in solid tumors. The two examples we describe use the bispecific mAb vanucizumab (VEGF-A/Ang-2). In one of them, it is used in combination with RO7009789 (255), an antibody with immunostimulatory effects that recognizes CD40, a member of the TNF receptor superfamily, for the treatment of metastatic solid tumors (NCT02665416). In the other example, it is used either alone or in combination with atezolizumab (PD-L1) in advanced or metastatic solid tumors (NCT01688206). The rationale for both cases is to either use an anti-CD40 agonist or an anti-checkpoint antibody to burst the antitumor immune response, while inhibiting simultaneously angiogenesis by blocking the VEGF-A/VEGFR- and Ang-2/Tie2-signaling pathways.

Antibodies with Immunomodulatory Effects

In a clinical trial for metastatic colorectal cancer, imalumab (BAX69) (233), a mAb that identifies MIF is used in combination with panitumumab (NCT02448810). BAX69 abrogates MIF signaling and MIF-mediated secretion of cytokines (IL-1 β , TNF- α , etc.) and inhibits proliferation of MIF overexpressing tumor cells, together with the antiproliferative effects of panitumumab (anti-EGFR mAb).

Other examples, used for solid tumors, combine nivolumab with cabirizumab, an anti-colony-stimulating factor 1 receptor (CSF1R) mAb, which inhibits binding of its ligands (CSF-1 and IL-34), blocking the production of inflammatory mediators by macrophages and monocytes and preventing osteoclast activation (NCT02526017; NCT03158272). The aim is to inhibit the tumor-induced immune suppression with nivolumab, and to block with cabirizumab the recruitment of CSF1R-dependent tumor-associated macrophages (TAMs). Cabirizumab also enhances T cell infiltration and antitumor T cell immune responses.

Another immunomodulatory antibody, anti-CD73 (BMS-986179) (209–211), has been used in combination with nivolumab on advanced or spread solid cancers (NCT02754141). In this case, the use of an antibody against the cell surface enzyme CD73 turns out to be very interesting. CD73 is overexpressed in many tumors and catalyzes the conversion of extracellular nucleotides into nucleosides, generating adenosine (211). The anti-CD73 antibody prevents the conversion of AMP to adenosine, which releases the inhibition of T cell, DC, and NK activities, induces the activation of macrophages, and reduces the activity of both myeloid-derived suppressor cells and regulatory T cells (209–211). This treatment was designed to abrogate the immunosuppressor effects of both, the immune checkpoint with nivolumab and the metabolic checkpoint with BMS-986179.

In addition to antibodies that release the inhibitory effects of immune-checkpoints, there are other antibodies that are able to directly activate the immune response. The following examples

represent clinical trials where these immunostimulatory antibodies are used. One of them combines avelumab, which suppresses the signaling through negative immune checkpoints, with either the anti-cytokine antibody PD-0360324 (anti-CSF-1 mAb) (248); or with the immunostimulatory antibodies PF-04518600 (anti-OX40 mAb), or utomilumab (an anti-CD137 mAb) (262) (NCT02554812); whereas another combines the anti-OX40 mAb (MOXR0916) with atezolizumab in locally advanced or metastatic solid tumors. (NCT02410512). The aim is to inhibit the PD-1/PD-L1 axis (avelumab or atezolizumab), simultaneously bursting the immune response through OX-40, CD137, or blocking TAMs generation with the anti-CSF-1 (Aspelagh, 2016). A similar strategy uses atezolizumab in combination with varlilumab (an agonistic anti-CD27 mAb), which results in an increase of the CTL response against CD27 ligands expressed on tumor cells (NCT02543645).

The anti-CD40 mAb RO7009789 activates and triggers proliferation of antigen-presenting cells (APC) and activates B and T cells, resulting in an enhanced immune response. When CD40 is expressed in solid tumor cells, RO7009789 leads to apoptosis and decreased tumor growth. This antibody in combination with the CSF1R inhibitory antibody emactuzumab has been used for the treatment of advanced solid tumors (225) (NCT02760797). Related examples combine anti-CD40 mAb either with nivolumab for the treatment of metastatic pancreatic adenocarcinoma (NCT03214250) or with the bispecific antibody vanucizumab (anti-VEGF-A and anti-Ang-2) (NCT02665416). The rationale is to activate the immune response through CD40 while inhibiting the angiogenesis blocking the binding of VEGF-A and Ang-2 to their receptors.

Adjuvants and other Immunostimulatory Agents

Another possible strategy is to combine therapeutic antibodies with molecules carrying repeated structural motifs that cannot be synthesized by vertebrates, and bind to pattern recognition receptors present in cells from the innate arm of the immune system (i.e., beta glucan that binds the C-type lectin receptor Dectin-1). These molecules are able to regulate the threshold of the immune response as an adjuvant, changing the secreted cytokine expression pattern. A particular example of this type of agents is the use of an attenuated preparation of the BCG (Bacille Calmette–Guerin) strain of *Mycobacterium bovis*, with potential immunostimulatory activity for the treatment of patients with bladder cancer (330).

An example, combining mAb and adjuvants is the use of BTH1704 (212), an mAb against MUC1, an aberrantly glycosylated antigen overexpressed on the surface of a variety of cancer cells, in combination with a polysaccharide beta 1,3/1,6 glucan derived from the cell wall of *Saccharomyces cerevisiae* (PGG Beta-Glucan), for the treatment of patients with advanced pancreatic cancer (NCT02132403). The rationale of this trial is to directly target the tumor with the anti-MUC1 mAb, while unspecifically stimulate the immune response with beta glucan by binding to an alternate site on the neutrophil complement receptor 3 (CR3), priming the neutrophil to become cytotoxic after binding to

complement on tumor cells *via* CR3. In addition, this agent may induce hematopoietic progenitor cell mobilization.

Antibodies in Combination with Vaccines

Tumor cells carry antigens which can be recognized as non-self by the immune system. In some cases, however, the microenvironment in which these tumor antigens are presented do not allow to evoke an immune response. There is a plethora of possibilities to burst the antitumor immune response, one of them is to use tumor antigens as a vaccine. In the context of antitumor therapies, anti-idiotypic antibodies represent a particular type of vaccines.

Since the interaction of antibodies with other molecules is based on structural complementarity, antibodies that recognize the region of an antibody that interacts with its antigen (anti-idiotypic antibodies) might mimic the structure of this antigen (i.e., a tumor marker). Thus, the anti-idiotypic antibodies might act as an antitumor vaccine able to trigger a host immune response to kill tumor cells. Combining chemotherapy and radiation therapy with vaccine therapy may help to kill tumor cells more effectively.

An example of anti-idiotypic antibodies used as vaccines in antitumor therapy is abagovomab, an IgG1 anti-idiotype mAb, that functionally mimics the 3D structure of a specific epitope on the ovarian cancer tumor-associated antigen CA-125 (203). Its variable region acts as a surrogate antigen for CA-125, bestowing potential antineoplastic activity; it has been used in ovarian epithelial, fallopian tube, or peritoneal cancer (NCT00058435). Another example of vaccine therapy combines two anti-idiotypic antibodies, 11D10 (201) an mAb that mimics an epitope of the high molecular weight human milk fat globule glycoprotein, expressed at high levels by human breast and other tumor cells and 3H1 an mAb that mimics an epitope of the tumor-associated protein CEA (199). This combination has been used for the treatment of colorectal cancer metastatic to the liver (NCT00033748). The 11D10 mAb has also been used in other combinations, for example, with a GD2 anti-idiotypic mAb vaccine, together with chemotherapy and radiotherapy, for the treatment of limited-stage SCLC (NCT00045617).

A study combining the anti-idiotypic mAb abagovomab, which mimics a specific epitope on CA-125 with stereotactic body radiation therapy (SBRT), chemotherapy, and the synthetic antiviral agent nelfinavir mesylate, which selectively binds to and inhibits human immunodeficiency virus protease, has been used for the treatment of locally advanced pancreatic cancer (NCT01959672).

Currently, other types of vaccines are being used with mAb in combination with agents that allow to evoke an immune response against tumor antigens. The mAb in these combinations may strengthen the evoked immune response. For the examples described below, the combination contains antibodies that either disrupt the PD-1/PDL-1 axis or that block the binding of B7-1 and B7-2 to CTLA-4 allowing T cell co-stimulatory signals and activation, unless otherwise specified. One example is the treatment with a peptide from Wilms tumor 1 antigen on recurrent ovarian cancer (NCT02737787). Another, more complex example is to administrate the peptide vaccine

PVX-410 (derived from X-box-binding protein 1-unspliced XBP1-US, XBP1-spliced syndecan-1, and CS1), to treat triple-negative breast cancer tumors (NCT02826434). A third example uses a HER2 intracellular domain peptide in combination with the polysaccharide-K as adjuvant, in HER2⁺ recurrent breast cancer patients, which are receiving pertuzumab or trastuzumab (NCT01922921). The rationale for this trial is to combine the effects of the anti-HER2 mAb with using a HER2 peptide to switch the B and T cell responses through APC activation. Otherwise, peptides could be presented as a fusion protein, such as in CIMAvax vaccine (EGF-rP64K/Montanide ISA 51), which triggers a strong humoral immune response against EGF and has been used in NSCLC (NCT02955290). There are also personalized neoantigen cancer vaccines, such as NeoVax for the treatment of high-risk renal cell carcinoma (NCT02950766).

Another approach to generate vaccines is to use modified virus such as Ad-CEA vaccine, an oncolytic adenovirus encoding an epitope of human CEA (331), used for the treatment of patients with previously untreated metastatic colorectal cancer (NCT03050814). This vaccine may induce both humoral and cellular immune responses against CEA⁺ tumor cells.

A different example uses the CV301 (CEA-MUC-1-TRICOM Vaccine) viral vaccine, which contains a version of the recombinant vaccinia viral vector and a recombinant fowlpox viral vector encoding both CEA and MUC-1, in combination with TRICOM [co-stimulatory molecules, B7-1, intracellular adhesion molecule 1 (ICAM-1), and LFA-3] (332). It may enhance presentation of CEA and MUC-1 to APC and subsequently a CTL response against the tumor cells, it has been used in previously treated NSCLC (NCT02840994). A similar approach has been evaluated for the treatment of prostate cancer using a recombinant vaccinia virus encoding a modified peptide of the prostate-specific antigen and TRICOM (NCT00113984). This viral vaccine may enhance antigen presentation and may activate a CTL response.

Attenuated bacteria have also been used as carriers for antitumor vaccines. For example, the attenuated Listeria ADXS11-001 encoding a papillomavirus type 16 E7 fused to a non-hemolytic listeriolysin O protein, it has been used for the treatment of cervical and Head and Neck Cancer (HNSCC) (NCT02291055). The rationale is to mount a CTL response against cancer cells overexpressing the cell surface glycoprotein HPV 16 E7, overexpressed in the majority of cervical cancer cells.

More sophisticated approaches use tumor vaccines, such as GM.CD40L, which is a cell-based vaccine composed of irradiated tumor cells transduced with GM-CSF and CD40-ligand (CD40L) genes (333). Upon administration, this vaccine may stimulate an antitumoral DC-mediated immune response, it has been used in lung adenocarcinomas (NCT02466568). Another example that does not use anti-checkpoint antibodies but combines the anti-HER2 mAb trastuzumab with a cell-based vaccine, consisting of two irradiated allogeneic mammary carcinoma cell lines genetically modified to secrete human GM-CSF, has been used for the treatment of HER2⁺ metastatic breast tumors (NCT00399529). An additional example of cell-based vaccines uses GVAX (334), a pancreatic cancer vaccine and IMC-CS4, a macrophage targeting

mAb (CSF1R inhibitor) for the treatment of pancreatic adenocarcinomas (234). GVAX is composed of irradiated, whole tumor cells (autologous or allogeneic), genetically modified to secrete GM-CSF (NCT03153410).

The immune response against the tumor can be boosted using also DNA, RNA, or liposome-based vaccines. As an example, triple-negative breast cancers have been treated with neoantigen DNA vaccine combined with anti-immune-checkpoint antibodies (NCT03199040). Other vaccines use autologous DC loaded *in vitro* with Cytomegalovirus pp65-lysosomal-associated membrane protein mRNA as a vaccine, in combination with the anti-IL2R alpha mAb basiliximab (207), in glioblastoma multiform (NCT00626483). The rationale is to restore the number of immunosuppressive T regulatory cells during recovery from therapeutic temozolomide-induced lymphopenia, together with a synergistic enhancement of vaccine-driven CTL responses. Another study describes the use of the immuno-modulating mAb varlilumab (anti-CD27, TNFR family) in combination with a liposome-based vaccine consisting of two peptides from MUC1 and the toll-like receptor 4 encapsulated in liposomes (NCT02270372). This immunization stimulates both cellular and humoral responses.

Another clinical trial uses trastuzumab and an allogeneic GM-CSF-secreting whole cell breast cancer vaccine for HER-2⁺ breast tumors. This study will also test whether cyclophosphamide can eliminate the suppressive influence of regulatory T cells. The vaccine consists of two irradiated allogeneic mammary carcinoma cell lines genetically modified to secrete human GM-CSF (NCT00399529).

Pidilizumab (anti-PD-1) (250) in combination with a DC fusion vaccine, following autologous stem cell transplantation has been used on multiple myeloma (NCT01067287). The rationale is to disrupt the PD-1/PD-L1 axis, while vaccinating the patients with de DC fusion vaccine, which consists of DC fused to the patient's myeloma, where the myeloma antigens will be presented by HLA class I to CD8⁺ T cells, allowing their activation and mounting a CTL response. This is done on patients after an autologous transplantation with HSCs. A similar approach uses nivolumab vaccine with autologous DCs pulsed with tumor lysate antigen, in patients with recurrent glioblastoma (NCT03014804).

Another study combines lymphodepletion with anti-CD45 mAb with a vaccine generated from autologous DC and Epstein Barr virus (EBV)-infected lymphoblastoid cell lines transduced with an LMP1/LMP2-expressing adenoviral vector, which are irradiated, and then used to stimulate and expand autologous CTL to produce LMP1-/LMP2-specific CTL *ex vivo*, for the treatment of EBV⁺-nasopharyngeal carcinoma (NCT00515957). The rationale of this trial is to deplete autologous CD45⁺ cells, then generate a cell-based vaccine to activate *ex vivo* specific CTL that are then infused into the patient.

Other Strategies

In some approaches, a fusion protein between the antibody and a tumor antigen is used in combination with other therapies. For example, on the treatment of NY-ESO 1⁺ NSCLC, where atezolizumab was combined with both the adjuvant poly-ICLC

(a synthetic complex of carboxymethylcellulose, polyinosinic-polycytidylic acid and poly-L-lysine double-stranded RNA) and DEC-205/NY-ESO-1 [CDX-1401, a fusion protein between a mAb directed against the endocytic DC receptor DEC-205, linked to the tumor-associated antigen (NY-ESO-1)] (NCT02495636) (217). Atezolizumab will inhibit immune checkpoints negative signals, while the internalization by DC of the mAb-antigen fusion protein may specifically deliver the NY-ESO-1 molecule and trigger a CTL response against cancer cells expressing this antigen. Simultaneously, the adjuvant may stimulate the release of cytotoxic cytokines by inducing IFNgamma production. A similar approach, for the treatment of melanoma patients, using CDX-1401 combined with a neoantigen-based melanoma-poly-ICLC vaccine and a recombinant Flt3 Ligand (CDX-301) (NCT02129075). This treatment should boost the immune system to mount a CTL response against cancer cells expressing NY-ESO-1. In addition, the adjuvant may induce IFNgamma production and the recombinant Flt3 ligand may stimulate the proliferation and mobilization of bone marrow precursor cells, including CD34⁺ cells, and DCs.

An additional strategy would be to use scavengers of the ligand with low immunogenicity. For example, sEphB4-HAS, a human serum albumin (HAS) fused with the extracellular domain of tyrosine kinase ephrin type-B receptor 4 (sEphB4) is combined with pembrolizumab, for the treatment of NSCLC or HNSCC (NCT03049618). Pembrolizumab inhibits negative immune checkpoint signals, whereas EphB4-HSA is expected to decrease angiogenesis and cell growth of Efnb2 and/or EphB4 overexpressing tumor cells, while the albumin moiety will avoid renal clearance of the fusion protein, increasing its half-life without affecting immunogenicity.

Another strategy would be to target matrix enzymes required for tumor invasiveness. An example is the use of nivolumab combined with andecaliximab (GS-5745) (204, 205), an inhibitory mAb of matrix metalloproteinase 9 (MMP-9) in recurrent gastric or gastroesophageal junction adenocarcinomas (NCT02864381). Since MMP-9 activity is associated with tumor invasion and metastasis (335), the rationale is that andecaliximab will inhibit extracellular matrix protein degradation and angiogenesis, while nivolumab will interfere with the PD-1/PD-L1 axis.

Pembrolizumab (PD-1) was used in combination with CVA21 (CAVATAK™), coxsackievirus A21, a naturally occurring enterovirus with potential antitumor activity. This combination was used for advanced NSCLC (NCT02824965). CVA21, intra-tumor administered, targets and binds the ICAM-1 and decay acceleration factor, cell surface molecules, both overexpressed on certain malignant cells (336, 337). After entering the cells, the virus replicates causing cell lysis. This, together with the inhibition of the immune checkpoint, results in a reduction of tumor cell growth.

Another strategy combines conatumumab (220), an agonist mAb directed against the extracellular domain of human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2), also known as DR5, with the anti-IGF-1R mAb ganitumab, in patients with advanced solid tumors without disease progression whose previous studies were closed (NCT01327612). TRAIL-2 and IGF-1R are expressed by a variety of solid tumors

and cancers of hematopoietic origin. Conatumumab mimics TRAIL activity, activating caspase cascades and inducing tumor cell apoptosis, while ganitumab inhibits IGF-1 binding and, therefore, the PI3K/Akt pathway. This treatment may result in the inhibition of tumor cell proliferation and the induction of tumor cell apoptosis. Another clinical trial combining tigatuzumab, a mAb targeting the death receptor TRAIL-R2 with abraxane, an albumin-stabilized nanoparticle containing paclitaxel, non-covalently coated with the anti-CD20 mAb rituximab, in patients with metastatic, triple-negative breast cancer (NCT01307891). The relevance of the trial is that combines an anti-TRAIL-R2 mAb that induces death, while albumin stabilizes the complex, whereas rituximab allows to target paclitaxel to CD20⁺ cells, minimizing toxicity on normal cells. A strategy being used on EGFR⁺ tumors is to combine an anti-immune checkpoint antibody with the anti-EGFR mAb necitumumab (NCT02451930) or nimotuzumab (245) (NCT02947386). Or the same strategy, but using B-701, a neutralizing mAb directed against the fibroblast growth factor receptor type 3, in combination with atezolizumab in urothelial cell carcinoma (NCT03123055).

One of the most sophisticated clinical trials combines chemotherapy, bevacizumab, avelumab, ALT-803 (IL-15 super agonist), aNK (allogenic human NK-92 cell line, expressing CD16 and IL-2), and GI-4000 (a heat-killed recombinant *Saccharomyces cerevisiae* yeast transfected with mutated forms of Ras) with the NANT pancreatic cancer vaccine (ETBX-011) containing a replication-defective adenoviral vector encoding a CEA epitope Ad5-CEA(6D), used for pancreatic cancer (NCT03136406). A clinical trial for colorectal cancer uses a similar combination of chemotherapy, nivolumab, avelumab bevacizumab, cetuximab, SBRT, haNK, ALT-803, and a cocktail of vaccines: ETBX-011, ETBX-021, ETBX-051, ETBX-061, GI-4000, GI-6207, and GI-6301 (NCT03169777). The rationale for these two clinical trials is to hit the tumor simultaneously with a wide spectrum of the available tools against the tumor, where the concentration of each one of the agents can be decreased to minimize the unwanted effects on normal cells.

QUESTIONS AND QUERIES RAISED BY THE COMBINATIONS

We hope that we have been able to depict up to here, the huge complexity inherent to the use of therapeutic antibodies in combination with other biological agents for the treatment of cancer. Since most of the clinical trials described in this review are relatively recent (started during the last 7 years), many of them lack results in public databases, including the clinical trials database from NCI, or as published scientific manuscripts. This burst of clinical trials using antibodies in combinations with other biologicals is based on the positive results found by some combinations (anti-HER2 or anti-GD2 mAb), although the complexity increase in these combinations also implies an increase in the possibility of adverse side effects/increased toxicity or lack of additive or synergistic effects of the therapeutic agents. Initially, the therapeutic doses used for combinations were taken from the monotherapeutic trials, although in many cases, the non-toxic concentrations used in monotherapy, turn to be toxic

in combinations, generating new toxicity profiles (338). This is of particular relevance when antibodies able to burst the antitumor immune response are used (either to inhibit the immune checkpoint proteins, to block inhibitory NK receptors or to trigger NK cells through activating receptors, etc.), which might lead to a dis-regulation of the immune response, an uncontrolled inflammatory response, and autoimmunity. This problem can also be related to an apparent lack of additive or synergistic effects of the therapeutic agents, where the potential clinical benefits of the combination could be overlooked by the initial toxicity of the mixture. Dose and schedule changes, however, can overcome the toxicity effects, allowing to demonstrate the enhanced clinical benefits of a particular combination (339). The use of CTLA-4 and PD-1 inhibitor antibodies in combination (nivolumab and ipilimumab), improved the treatment efficacy in advanced melanoma, as compared to monotherapies (340). Indeed, this combination has been approved in 2016 by the US FDA for the treatment of metastatic melanoma (341), despite the higher frequency and severity of adverse reactions of the combination, as compared to the corresponding monotherapies (340, 342–344).

It is interesting to note that a combination of TRC105 (carotuximab, anti-endoglin antibody) with bevacizumab was used on a clinical trial for the treatment of patients with advanced cancer, where the combination was well tolerated and clinical activity was observed in a VEGF inhibitor-refractory population (NCT01332721) (345), the same combination failed to improve progression-free survival on patients with refractory metastatic renal cell cancer (NCT01727089) (346). These data clearly suggest that the problem does not strictly lie with the antibody combination, but rather it might be related to the tumor microenvironment, tumor type, the therapeutic approach used, or the clinical history of the patient.

Several examples of clinical trials where antibodies that have been used in combination with other biologicals, which were well tolerated and showed additive or synergistic therapeutic responses have been selected. These include a clinical trial for melanoma patients with low tumor infiltrating lymphocytes, an anti-PD-1 non-responsive phenotype. The combination of pembrolizumab with an intratumoral electroporation of a plasmid coding for interleukin 12 cDNA (pIL-12) showed a 40% clinical response with associated positive immune-based biomarker data and a safety profile (NCT02493361) (347), where the combination of pembrolizumab with the plasmid pIL-12 renders half of the patients responsive to the anti-PD-1. Another example that combines mAb with cytokines is a study of immune activation and antitumor activity in renal cancer of PEGylated human IL-10 (AM0010) in combination with pembrolizumab or nivolumab, the combinations were well tolerated, and CD8⁺ T cell activation was detected (NCT02009449) (348).

Other examples include a phase Ib study, otlertuzumab (TRU-016, an anti-CD37 mAb) in combination with rituximab and bendamustine, which was well tolerated and induced therapeutic responses in the majority of patients with relapsed indolent B-non-Hodgkin lymphoma (NCT01317901) (349). Similarly, the combination of pidilizumab plus rituximab was well tolerated and therapeutically active in patients with relapsed follicular lymphoma (350).

On a phase Ib study of utomilumab (PF-05082566, a 4-1BB/CD137 agonist), in combination with pembrolizumab (MK-3475) in patients with advanced solid tumors had a confirmed complete or partial response in 26.1% of them. Pharmacokinetics and immunogenicity of both mAb were similar when administered alone or in combination. A trend toward higher levels of activated memory/effector peripheral blood CD8⁺ T cells was observed in responders versus non-responders, supporting further investigation of this combination (NCT02179918) (262).

Other combinations include the anti-checkpoint antibody nivolumab in combination with an antibody that blocks the KIR inhibitory receptors in NK cells. On a phase I/II study of the NK-targeted antibody lirilumab (a fully human mAb that blocks inhibitory KIRs on NK cells) in combination with nivolumab in advanced HNSCC demonstrated, in preliminary results, clinical benefit, with deep and durable responses in some patients. This combination demonstrated a manageable safety profile similar to that observed with nivolumab monotherapy (NCT01714739) (347).

Another combination that might be interesting for the future of the field is the combination of oncolytic virus with anti-checkpoint antibodies. Indeed, preliminary results from several clinical trials using the oncolytic virus coxsackievirus A21 (CVA21, CAVATAK) in combination with ipilimumab (NCT01636882) (351) or pembrolizumab (NCT02043665) (348), for the treatment of patients with advanced cancer, showed that these combinations were generally well tolerated and induced antitumor activity. A phase II trial using intratumoral injection of the HF10 oncolytic virus, an attenuated, replication-competent mutant strain of herpes simplex virus type 1, and ipilimumab in patients with unresectable or metastatic melanoma showed therapeutic activity and the treatment was well tolerated (NCT02272855) (352).

For other clinical trials, on early phases, the combination is well tolerated, such as a dose escalation study of the OX40 agonist MOXR0916 and atezolizumab (anti-PD-L1 mAb) in patients with advanced solid tumors, using each agent at its recommended monotherapy dose, was well tolerated. (NCT02410512) (353).

Other clinical trials, including anti-checkpoint antibodies detect clear tumor regression, although with a toxicity higher than reasonable. This is the case for a clinical trial where BMS-986016 (anti-LAG-3 mAb) in combination with nivolumab was administered to patients with hematologic and solid malignancies. Preliminary results demonstrated objective tumor regressions, concomitant with the toxicity characteristic of immune checkpoint blockers (NCT02061761, NCT01968109) (348).

On another group of clinical trials, the main characteristic is that although they are well tolerated in general, they failed to provide significant additive/synergistic therapeutic effects. This is the case of a clinical trial where urelumab (a CD137 agonist), in combination with nivolumab was used for the treatment of hematologic and solid tumor malignancies. This combination did not provide significant additive/synergistic clinical benefits at the doses evaluated (NCT01471210, NCT02253992) (351). In another, urelumab in combination with rituximab or cetuximab was used in patients with refractory lymphoma or selected advanced solid tumors. Although the combinations were safe and

well tolerated, with minimal evidence of liver toxicity, they did not demonstrate substantial enhancement of clinical responses or lead to intratumoral immune modulation in these tumor settings (NCT01775631, NCT02110082) (348).

Finally, there are a couple of selected clinical trials using combinations of antibodies and other biologicals that were toxic or had to be terminated on overall benefit-risk assessment. These include a clinical trial with patients with advanced solid tumors, which were treated with a combination of MDX-447 [a bispecific mAb directed to Fc_YRI (CD64) and EGFR] with G-CSF, although the bispecific mAb alone was well tolerated, the combination was not well tolerated and precluded meaningful dose escalation on a phase I clinical trial (354). A second example is a phase II study of imalumab [BAX69, an anti-oxidized macrophage MIF (oxMIF)] and 5-FU/Leucovorin or Panitumumab (anti-EGFR mAb), versus the standard of care in metastatic colorectal cancer patients, which was terminated (February, 2017) based on overall benefit-risk assessment (NCT02448810), although it was initially reported that this combination was generally safe and well tolerated (355).

The problems that arose with using therapeutic antibodies in combinations has led the Society for Immunotherapy of Cancer to name a Combination Immunotherapy Task Force to identify and prioritize the most promising prospects for combinatorial approaches as well as to address the challenges associated with developing these strategies (339). Furthermore, it seems clear by now, that an improved understanding of pharmacodynamic effects of each agent within a combination will support the rational development of immune-based combinations for cancer treatment (356).

CONCLUSION

The broad variety of clinical trials summarized here presents the overwhelming complexity of the use of antibody in combinations for cancer treatments. The antibodies used either interfere with a ligand-receptor interaction, blocking a signaling pathway relevant for tumor growth, or identify tumor-associated antigens, where they somehow induce the death of the tumor cells by ADCC, CDC, and ADCP or directly inducing apoptosis. If the antibodies by themselves cannot kill the tumor cells, they can be conjugated to cytotoxic drugs to exert this function, or directly coupled to radio-labeled agents, where they trigger radiolysis. These antibodies can be combined with other antibodies that rather than directed against the tumor cells, identify targets on the tumor environment. These include antibodies that inhibit tumor-induced vascularization, or antibodies against cells or molecules involved in the immune response. A turning point was the use of mAb that block PD-L1/PD-1 interaction, or anti-CTLA-4 mAb; all of them disrupting the tumor-induced suppression of antitumor immune responses. In addition, mAb are used in combination with vaccines with the aim of evoking an antitumor response, either against a single tumor antigen or against a broad spectrum of antigens, for example when using irradiated tumor cells expressing pro-inflammatory cytokines.

It is obvious that there are many challenges to be solved regarding antibodies in combinations as antitumor therapeutic

agents. One of the most relevant challenges is the possible increase of toxicity of the combinations, concomitant with an increased complexity of the trials. This has to be carefully evaluated for each combination to identify the conditions giving the highest efficacy/toxicity ratio. This is of particular interest when the aim is to burst the antitumor host immune response, where a small response could fail to kill the tumor, but an over-response could lead to unwanted inflammatory or autoimmune processes. Another relevant challenge to be solved is the identification of new/additional biomarkers that would allow a personalized follow-up of the patient's status, which would be required due to the tumor and the patient's genotypic and phenotypic differences.

The current clinical trials suggest that in the future anti-cancer therapies will combine antibodies that block signaling cascades, or identify tumor-associated antigens with others that disrupt the tumor-induced immuno-suppression, together with vaccines that evoke an antitumor immune response, activated effector cells or CAR T cells. These combinations could also contain anti-cytokine antibodies, or cytokines, to burst the immune response. The aim will be to directly target tumor antigens or signal pathways, and at the same time interfere with the "immune history" of the organism, to make the patient's own body aware of the tumor presence and simultaneously help it to make a strong antitumor response. Thus, it will be the patient's immune response against the tumor the ultimate mechanism responsible for the cure of cancer.

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SUPPLEMENTARY MATERIAL

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APPENDIX

Key Concepts

Antibody-dependent cell cytotoxicity (ADCC): the binding of an antibody to a cell surface antigen promotes the interaction of the natural killer cell with the Fc antibody fragment and triggers its cytotoxic response.

Adaptive immune response: its main function is to eliminate pathogens and fight cancer. Specifically recognizes antigen through receptors on the surface of T and B lymphocytes. It creates immunological memory, after the initial response to a given antigen, leads to an exacerbated response to subsequent encounters with the same antigen. This is the basis of vaccination.

Antibody-dependent phagocytosis (ADCP): the binding of an antibody to a cell surface antigen allows the opsonization of the cell and promotes its phagocytosis.

Combined therapy: therapy that combines different therapeutic approaches (i.e., chemotherapy, radiotherapy, small molecule drugs, vaccines, antibodies, etc.).

Complement-dependent cytotoxicity (CDC): the binding of an antibody to a cell surface antigen activates the complement cascade, resulting in the cell's death.

Immune checkpoint: there are checkpoints that receive negative signals from the tumor, inhibiting the antitumor immune response. These include the PD-1/PD-L1 axis (PD-1 receptor on the T cells and to PD-L1 on the tumor cells) and CTLA-4 (on T cells). Antibodies against these molecules (nivolumab, pembrolizumab, atezolizumab, durvalumab, avelumab, and ipilimumab) are able to inhibit these negative signals and promote the antitumor immune response.

Immunotherapy: therapeutic treatment that takes advantage of the immune system response.

Innate immune response: provides immediate defense against infection, on a non-specific manner, is not long-lasting and recognizes pathogens through generic receptors.

Therapeutic antibody: an antibody that can be used for therapeutic purposes, which is able to either act as antagonist, guide a drug toward the tumor cell, where it will act, or kill the tumor cell either directly or by activating immune response mechanisms (ADCC, CDC, and ADCP).

Vaccine: compounds inoculated in an organism to evoke a primary immune response, generating immunological memory able to protect against the antigen.



Interplay between Natural Killer Cells and Anti-HER2 Antibodies: Perspectives for Breast Cancer Immunotherapy

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Overexpression of the human epidermal growth factor receptor 2 (HER2) defines a subgroup of breast tumors with aggressive behavior. The addition of HER2-targeted antibodies (i.e., trastuzumab, pertuzumab) to chemotherapy significantly improves relapse-free and overall survival in patients with early-stage and advanced disease. Nonetheless, considerable proportions of patients develop resistance to treatment, highlighting the need for additional and co-adjuvant therapeutic strategies. HER2-specific antibodies can trigger natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity and indirectly enhance the development of tumor-specific T cell immunity; both mechanisms contributing to their antitumor efficacy in preclinical models. Antibody-dependent NK cell activation results in the release of cytotoxic granules as well as the secretion of pro-inflammatory cytokines (i.e., IFN γ and TNF α) and chemokines. Hence, NK cell tumor suppressive functions include direct cytolytic killing of tumor cells as well as the regulation of subsequent antitumor adaptive immunity. Albeit tumors with gene expression signatures associated to the presence of cytotoxic lymphocyte infiltrates benefit from trastuzumab-based treatment, NK cell-related biomarkers of response/resistance to HER2-specific therapeutic antibodies in breast cancer patients remain elusive. Several variables, including (i) the configuration of the patient NK cell repertoire; (ii) tumor molecular features (i.e., estrogen receptor expression); (iii) concomitant therapeutic regimens (i.e., chemotherapeutic agents, tyrosine kinase inhibitors); and (iv) evasion mechanisms developed by progressive breast tumors, have been shown to quantitatively and qualitatively influence antibody-triggered NK cell responses. In this review, we discuss possible interventions for restoring/enhancing the therapeutic activity of HER2 therapeutic antibodies by harnessing NK cell antitumor potential through combinatorial approaches, including immune checkpoint blocking/stimulatory antibodies, cytokines and toll-like receptor agonists.

Keywords: natural killer cells, breast cancer, human epidermal growth factor receptor 2, trastuzumab, pertuzumab, antibody-dependent cell-mediated cytotoxicity, immunotherapy

INTRODUCTION

Breast cancer is a major health-care problem worldwide, with an estimated 1.67 million women diagnosed annually.¹ Human epidermal growth factor receptor 2 (HER2, also known as ErbB2 or HER2/neu) is a transmembrane receptor with tyrosine kinase activity, capable of activating several pro-survival intracellular signaling pathways (1). HER2 overexpression occurs in approximately 15–20% of breast tumors and is associated with aggressive disease and decreased survival (2). Addition of HER2-targeted therapeutic monoclonal antibodies (mAb) to chemotherapy improved overall survival in patients with early-stage and advanced disease (3). Currently, two complementary anti-HER2 therapeutic mAbs, trastuzumab, and pertuzumab, and the antibody-drug trastuzumab-emtansine (T-DM1) are approved for clinical use. Combination of chemotherapy with dual HER2 targeting with trastuzumab and pertuzumab are the prevailing therapeutic approaches for HER2⁺ tumors in the neoadjuvant setting and in the first-line treatment of metastatic disease; trastuzumab and lapatinib (a dual EGFR/HER2 tyrosine kinase inhibitor small molecule) can also be used in refractory patients with advanced disease (4, 5); T-DM1 has been approved for treating advanced HER2⁺ breast cancer patients with progressive disease following trastuzumab/pertuzumab and chemotherapy regimens (6). Despite significant improvement in the clinical outcome of HER2⁺ breast cancer since the introduction of these anti-HER2 drugs, there are patients with early disease that eventually relapse and disease progression inevitably occurs due to *de novo* or acquired resistance to treatment in metastatic patients (7). Potential tumor cell-intrinsic mechanisms of resistance to anti-HER2 mAb treatment have been identified, yet their clinical relevance remains uncertain (8).

All currently approved anti-HER2 mAbs are immunoglobulins (Ig) of the G1 subclass (IgG1) and, in addition to block HER2 oncogenic signaling, share the capability of triggering antitumor immune function by engaging specific receptors expressed by immune cells (Fc γ R family, **Box 1**) through their constant domain (Fc). Several publications indicate that NK and tumor-specific T lymphocytes significantly influence disease development and response to treatment with anti-HER2 mAbs (9–12). In addition to considerable data supporting the importance of T cells in immunosurveillance (9), a role for NK cell function in preventing early tumor development and metastatic spread is being increasingly appreciated (13, 14).

In this review, current understanding of antitumor immune responses driven by anti-HER2 mAbs will be discussed from the NK cell perspective, integrating a conceptual framework for the combinatorial use of anti-HER2 antibodies and several immunotherapy approaches enhancing NK cell function/survival in breast cancer.

REGULATION OF NK CELL ANTITUMOR FUNCTION

Natural killer cells are cytotoxic members of the innate lymphocyte cell family, important in the defense against virus-infected

BOX 1 | Antibody structure and Fc γ R family.

Antibodies (Abs) or immunoglobulins (Ig) display two functionally different domains: a variable Fab region which determines specificity and affinity for a particular antigen and a constant region or Fc fragment which can engage a diversity of cellular receptors in immune cells. Immunoglobulins of the G subclass (IgG) can interact with distinct Fc γ R family members, respectively, displaying activating and inhibitory signaling capacity. Human activating Fc γ Rs include Fc γ RI (CD64), Fc γ RIIA (CD32A), Fc γ RIIC (CD32C), and Fc γ RIIIA (CD16A), whereas Fc γ RIIB (CD32B) is the counterpart with inhibitory function. Fc γ R in mouse includes Fc γ RI, Fc γ RIII, and Fc γ RIV with stimulatory potential and the inhibitory Fc γ RIIB. Human NK cells primarily express Fc γ RIIIA in the absence of inhibitory Fc γ R; B cells exclusively express the inhibitory Fc γ RIIB; human dendritic cells express both the activating and the inhibitory forms of Fc γ RII A and B. Distinct monocyte/macrophage subpopulations have been shown to express diverse combinations of activating and inhibitory Fc γ R, including Fc γ RI, Fc γ RIIA, Fc γ RIIB, and Fc γ RIIIA. It is nowadays recognized that the Fc fragment of therapeutic antibodies elicits several of their effector mechanisms. Engagement of activating Fc γ R results in antibody-dependent cellular cytotoxicity and phagocytosis (ADCC and ADCP). With the exception of Fc γ RI, remaining Fc γ R show intermediate/low affinity for IgG and will bind to immune complexes or IgG-coated targets, resulting in receptor crosslinking and triggering of cellular responses. Human IgG2 and IgG4 isotypes display a poor interaction with Fc γ R whilst human IgG1 and IgG3 interact more strongly (15, 16).

and transformed cells. NK cell activation leads to the polarized release of cytolytic molecules, such as granzyme B and perforin stored in preformed granules, causing target cell death (14, 17, 18). NK cells can also trigger perforin-independent apoptosis by FasL- and TRAIL-mediated engagement of death-inducing receptors on target cells (19). Time-lapse imaging has revealed that a single activated NK cell can make serial contacts with multiple targets and kill an average of four tumor cells *in vitro* (20, 21). In addition, activated NK cells secrete IFN γ , TNF α , and chemokines (i.e., MIP1 α , MIP1 β , RANTES), boosting the recruitment of other immune effectors and the development of subsequent antitumor T cell immunity (14, 17, 18).

The importance of NK cell function for early tumor immune surveillance is supported by studies showing increased cancer risk in individuals with low NK cell activity (22), including several genetically predisposed cases (i.e., NKG2D haplotypes LNK1/LNK1) (23). On the other hand, correlation between tumor NK cell density/function and prognosis has been reported for a number of cancer types (e.g., colorectal, hepatocellular, gastric carcinomas, lung adenocarcinoma, and renal cancer), supporting their importance for metastasis control *in vivo* (13, 24, 25).

Natural killer cell activation is regulated by an array of germline encoded surface receptors with stimulatory or inhibitory function. NK cells use inhibitory receptors to prevent the killing of healthy cells, whereas crosslinking of activating receptors is required to initiate an immune response against transformed cells (26). NKG2D, NKp46 and NKp30, together with the co-stimulatory molecule DNAM-1, are considered the main activating receptors involved in direct tumor cell recognition (27–29). NKG2D recognizes stress-induced self-molecules, such as MICA/B and the ULBP family, upregulated in most neoplastic cell types (30); natural cytotoxicity receptors (NKp30 and NKp46) can recognize self-molecules exposed in damaged cells (i.e., BAT3, MLL5) or induced by inflammatory stimuli (i.e., B7-H6) (31, 32); and DNAM-1 specifically recognizes

¹<http://globocan.iarc.fr/old/FactSheets/cancers/breast-new.asp>.

CD155 (PVR) and CD112 (Nectin-2), overexpressed in a variety of tumor types (33). NK cell tolerance to self depends on inhibitory receptors specific for HLA class I molecules (HLA-I), which suppress NK cell activation against healthy cells expressing normal levels of surface HLA-I. Downregulation of surface HLA-I expression, in some virus-infected and transformed cells, allows for rapid NK cell responses against these targets (34). HLA-I specific NK cell receptors comprise killer cell immunoglobulin-like receptors (KIRs; **Box 2**) specific for distinct sets of HLA-I molecules (HLA-A, -B, -C); the CD94/NKG2A receptor specific for the HLA-I class Ib molecule HLA-E; and the leukocyte immunoglobulin-like receptor B1 (LILRB1) interacting with a broad spectrum of HLA-I molecules, including HLA-G. KIR and NKG2 receptor families also include members with activating function which, in some cases, can interact with HLA-I molecules albeit with lower affinity than their inhibitory counterparts (i.e., KIR2DS1 and CD94/NKG2C) (18).

Besides direct recognition, Fc γ RIIIA (CD16A) triggers NK cell activation against antibody-opsonized cells by a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells and certain T lymphocyte subsets (i.e., $\gamma\delta$ T cells) are the only immune cells expressing the activating CD16A, in the absence of other members of the Fc γ R family with inhibitory function (15) (**Box 1**). Among all activating NK cell receptors, CD16A was described as the only one capable of triggering resting NK cell activation in the absence of co-stimulation (37) and of increasing the killing frequency per NK cell (38).

Natural killer cells also express functional toll-like receptors (TLRs) (i.e., TLR2, TLR3, TLR5, TLR7/8, and TLR9), which sense the presence of microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) in the microenvironment, priming NK cell effector function (39, 40).

Overall NK cell antitumor efficacy depends on the combination of activation, effector function, proliferation, and survival, all these modulated by cytokines. IL-2 and IL-15 signaling through

BOX 2 | KIR receptors and their ligands.

The KIR receptor family includes six inhibitory receptors (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR3DL2, and KIR2DL5), six activating receptors (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, and KIR3DS1), and one, KIR2DL4, harbouring an ambiguous signaling motif. Inhibitory KIRs are characterized by a long cytoplasmatic tail containing ITIM motifs whereas activating KIRs have a short cytoplasmic tail and interact with DAP-12 for transducing stimulatory signals. Inhibitory KIR recognize specific epitopes on HLA-A, -B, and -C molecules, determined by polymorphisms within residues 77–83 of the $\alpha 1$ helix. KIR2DL2/L3 and KIR2DL1 respectively recognize the C1 and C2 epitopes, found in mutually exclusive subsets of HLA-C alleles. KIR3DL1 binds to the Bw4 epitope, carried by subsets of HLA-A and HLA-B alleles whereas KIR3DL2 interacts with the A3/11 epitope, restricted to HLA-A3 and A11 molecules. The HLA class I specificity of activating KIRs is still a matter of study. KIR2DS1 has been shown to recognize the C2-epitope, whereas KIR2DS4 can interact with groups C1 and C2 HLA-C alleles and HLA-A11. Inhibitory KIR2DL1, KIR2DL2/L3, and KIR3DL1 are highly polymorphic. Allelic variants display distinct avidity and/or specificity of the ligand-binding site, level of cell-surface expression, and signal transduction capacity. Combinations of particular KIR and HLA class I have been associated to differential susceptibility to a wide range of diseases (e.g., infectious and autoimmune syndromes) and can influence hematopoietic cell transplantation outcomes (34–36).

STAT5 promotes NK cell survival as well as increased IFN γ secretion, cytotoxicity, and proliferation (41); IL-12 and IL-18 signaling through STAT4 enhances NK cell cytotoxicity and cytokine production whereas type I IFNs (IFN α/β) are strong stimuli regulating NK cell cytotoxicity through the upregulation of perforin and FasL and promoting IFN γ secretion (42, 43). Conversely, TGF β has been shown to repress the mTOR pathway in NK cells, consequently reducing their proliferation, the abundance of various activating receptors and cytotoxic activity (44).

Similar to T lymphocytes, NK cells can express several activation-induced co-receptors with stimulatory (e.g., CD137, OX40, NKp44) or inhibitory (e.g., PD1, TIGIT) function which constitute yet another layer of regulatory elements for NK cell activation (45).

NK CELL-MEDIATED ADCC AS MECHANISM OF ACTION OF ANTI-HER2 ANTIBODIES

Natural killer cell recognition of HER2-overexpressing target cells involves a number of receptors that can determine natural cytotoxicity upon direct recognition or influence the magnitude of ADCC in the presence of HER2-specific mAbs (**Figure 1**).

HER2 signaling was shown to downregulate HLA-I and promote MICA and MICB protein expression in breast cancer cell lines *in vitro*, enhancing their susceptibility to NKG2D-mediated NK cell recognition and elimination (46–49). Indeed, an inverse relationship between HER2 and HLA-I expression was corroborated by immunohistochemistry (50) and concordant mRNA signatures in HER2 $^+$ tumors (51). As a matter of fact, gene expression signatures associated to cytotoxic lymphocytes are enriched in the stroma of good prognosis HER2 $^+$ tumors (52), suggesting that HER2 $^+$ breast carcinomas might be permissive to NK cell infiltration, at least at early stages of tumor development.

Anti-HER2 therapeutic mAbs introduced a novel ground by which NK cells could contribute to breast tumor control. Preclinical and clinical observations indicate that triggering of NK cell-mediated ADCC is one of the mechanisms accounting for anti-HER2 mAb therapeutic activity (53). Trastuzumab activity against xenografted tumors was severely attenuated in mice deficient in activating Fc γ R receptors (54) and trastuzumab F(ab') $_2$ fragments (lacking Fc domain) showed marginal antitumor activity *in vivo* despite retaining their anti-proliferative and pro-apoptotic effects *in vitro* (55). More precisely, NK cell depletion abolished anti-HER2 mAb therapeutic activity in preclinical mouse models of HER2 $^+$ breast cancer (56–59).

Indirect evidence also points to a significant contribution of NK cells to the clinical success of anti-HER2 mAb in breast cancer patients. Numbers of tumor-infiltrating leukocytes, particularly NK cells, were reported to increase after trastuzumab-docetaxel (60, 61) and T-DM1 treatment (62), suggesting that anti-HER2 mAb promoted NK cell tumor homing or *in situ* expansion. Remarkably, immune-gene expression signatures reflecting an increased recruitment of activated NK and T cells in breast tumors (i.e., CD8A, CD247, CD3D, GZMA) have been shown to be predictive of clinical benefit from preoperative and adjuvant

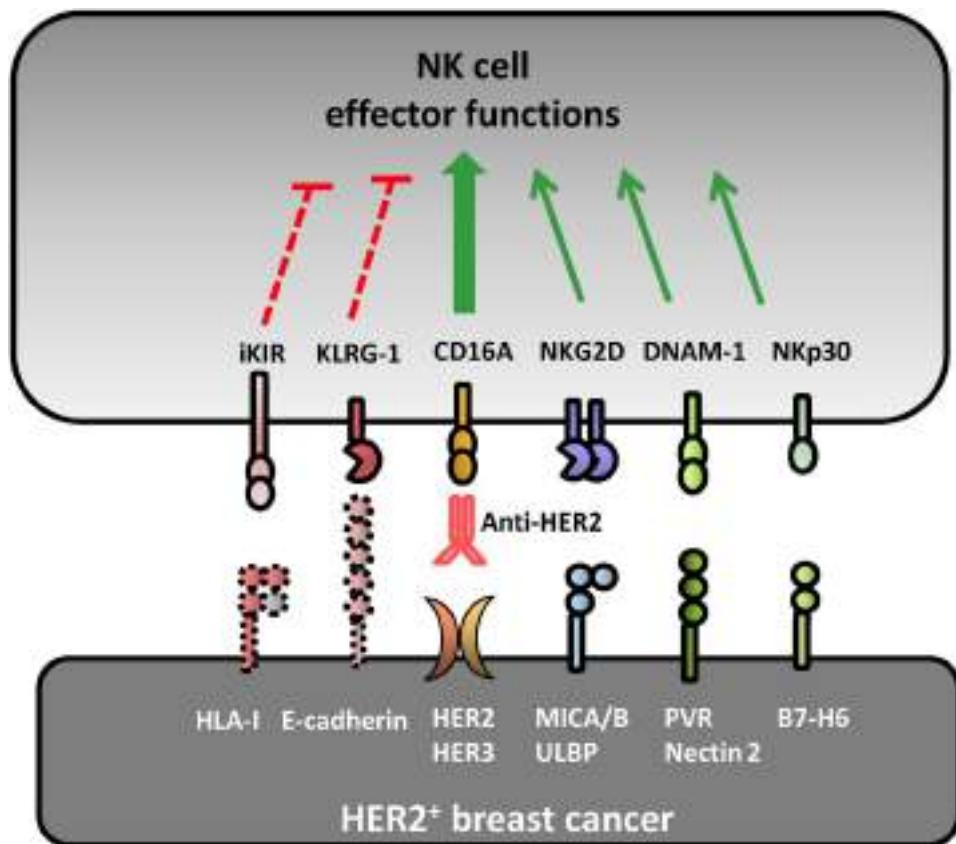


FIGURE 1 | Receptor-ligand pairs involved in natural killer (NK) cell recognition of HER2⁺ breast cancer cell lines. Several receptor-ligand pairs are involved in the crosstalk between breast cancer (BC) cells and NK lymphocytes. Natural cytotoxicity against HER2⁺ BC is mainly driven by NKG2D, DNAM-1, and NKp30 activating receptors upon interacting with their cognate ligands MICA/B, PVR/Nectin-2, and B7-H6, respectively. Human epidermal growth factor receptor 2 (HER2)-dependent downregulation of surface HLA-I expression impairs KIR-mediated inhibition facilitating NK cell recognition of BC cell lines. Anti-HER2 therapeutic monoclonal antibodies elicit a strong NK cell-mediated antibody-dependent cell-mediated cytotoxicity response against HER2⁺ BC cells upon interaction with the activating CD16A receptor. E-cadherin expression can be recognized by KLRG1 inhibitory receptor expressed by some NK cell subsets, modulating their direct and antibody-dependent cytotoxicity.

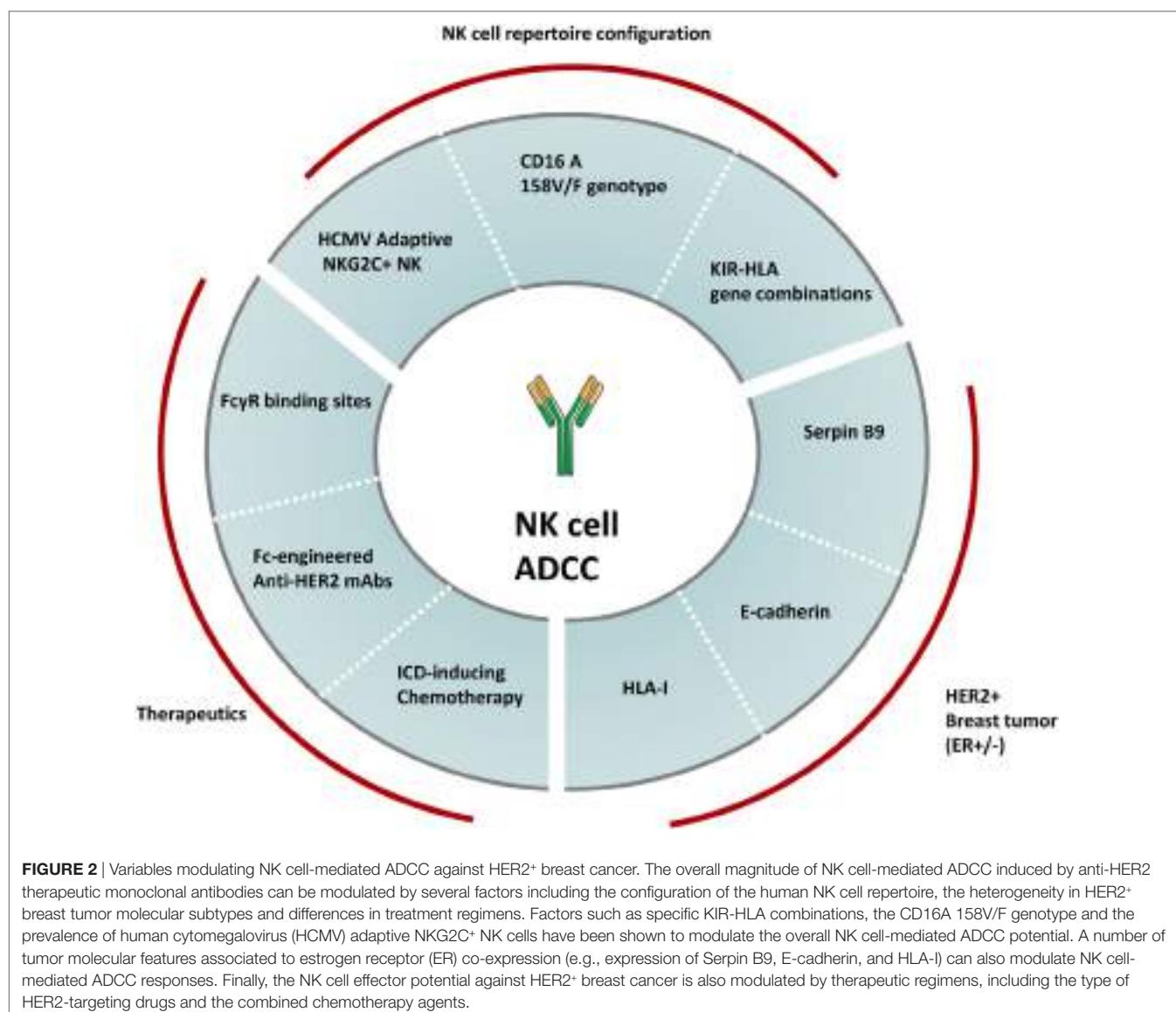
trastuzumab-based treatment (52, 63, 64). On the other hand, peripheral blood NK cells from patients undergoing complete or partial remission upon trastuzumab plus chemotherapy displayed high ADCC activity in *in vitro* lysis assays, whereas impaired NK cell-mediated ADCC responses correlated with therapy failure (65, 66). Of note, a number of factors, including the disparity in markers used for precise NK cell enumeration in tumor sections (e.g., CD57, CD56, GzmB) and the absence of standardized functional read-outs, have hindered the development of NK cell-related biomarkers of response to anti-HER2 therapeutic mAbs.

VARIABLES POTENTIALLY MODULATING NK CELL-MEDIATED ADCC IN HER2⁺ BREAST CANCER

The specific contribution of NK cell-mediated ADCC on the clinical benefit of anti-HER2 mAb in breast cancer patients could be modulated by several NK cell-, tumor cell- and therapy-related variables (Figure 2).

Influence of the NK Cell Repertoire Configuration on the Magnitude of ADCC

In healthy adults, approximately 90% of NK cells in peripheral blood belong to the cytotoxic CD56^{dim}CD16⁺ subpopulation capable of developing ADCC responses. A second major NK cell subpopulation, defined by a CD56^{bright} phenotype and the absence of the CD16A receptor, accounts for 10% of circulating NK cells, prevails in secondary lymphoid organs and lacks ADCC potential. Among CD56^{dim}CD16⁺ NK cells, several subsets displaying different NK cell receptor combinations are found at variable frequencies. Interindividual variability on the NK cell receptor repertoire is dictated by genetic and environmental factors. Major genetic factors include KIR and HLA-I genotypes. The KIR locus contains a variable number of genes, which together with their allelic diversity, determine the existence of a substantial number of distinct KIR haplotypes distributed in the world population (34, 35). KIR genes are stochastically expressed along NK cell differentiation, generating NK cell clones with discrete KIR combinations (34, 67). Only NK cell clones expressing at least one inhibitory receptor specific for self-HLA-I achieve functional

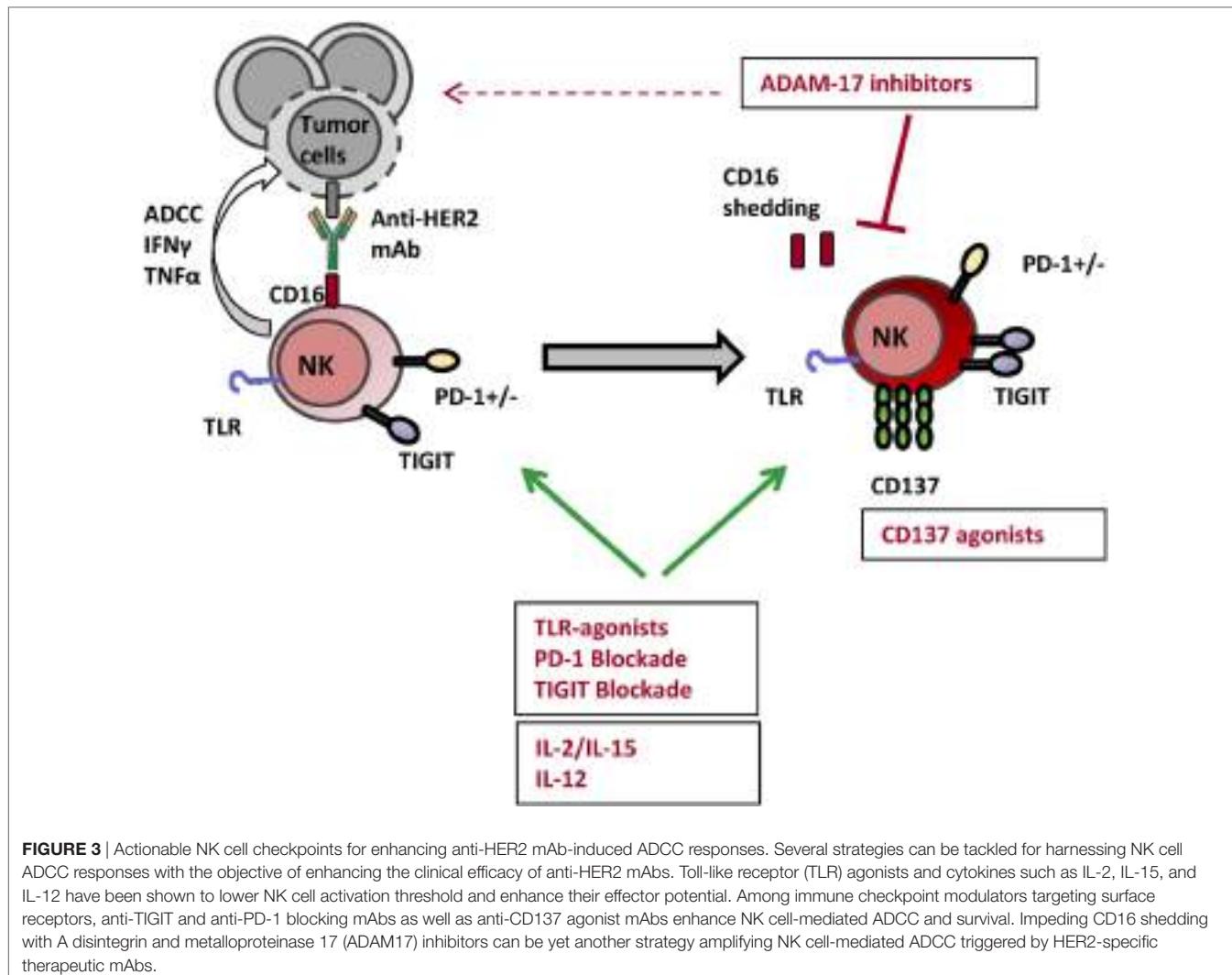


maturity. Thus, KIR-HLA-I interactions contribute to set the overall functional potential in the patient NK cell repertoire (68–70). Whether certain KIR-HLA-I gene combinations can modulate the efficacy of anti-HER2 mAbs in breast cancer patients remains unaddressed, yet associations between distinct paired KIR/KIR-ligands and clinical responses to other tumor antigen-specific mAbs, such as anti-GD2 dinutuximab, anti-CD20 rituximab, or anti-EGFR cetuximab have been reported (71, 72).

Another genetic factor known to modulate antibody-dependent NK cell activation is the CD16A (Fc_γRIIIA) 158V/F allelic dimorphism encoding for two receptor variants harboring either a phenylalanine (F) or valine (V) at amino acid position 158 in the receptor IgG-binding domain (73). Presence of a V residue defines receptors with high affinity for IgG1 (73). An initial association between the high affinity CD16A 158V/V genotype and complete clinical responses to trastuzumab-based treatment was described in a retrospective analysis of a small cohort of metastatic breast

cancer patients (74). Nonetheless, the association of CD16A 158V/F dimorphism with time to relapse and overall survival in larger patient cohorts receiving trastuzumab in adjuvancy remains controversial (75–77). Possible caveats accounting for the different results in these studies have been discussed in critical reviews (9, 78).

Environmental factors challenging the immune system, such as autoimmune or chronic inflammatory diseases and infections, can also shape the configuration of the NK cell compartment. In this regard, infection by human cytomegalovirus (HCMV) promotes, in some individuals, a persistent adaptive expansion of long-lived NK cells hallmark by the elevated expression of the CD94/NKG2C activating receptor (79–81). Adaptive NKG2C⁺ NK cells are functionally mature and have been associated with the control of HCMV infection in kidney transplant recipients (82, 83) as well as with protection from leukemia relapse upon hematopoietic stem cell transplantation



(84). Remarkably, NKG2C⁺ NK cells display enhanced effector function upon antibody-driven recognition of virus-infected targets and rituximab-coated B lymphoblastoid cell lines *in vitro* (85–87).

Influence of HER2 Breast Cancer Molecular Subtypes on NK Cell-Mediated ADCC

Hormone receptor status differentiates two HER2⁺ breast tumor subgroups with distinct pathological response rate and overall survival upon anti-HER2 mAb treatment (88). The benefit of anti-HER2 therapy is highest in estrogen receptor (ER)-negative tumors and progressively decreases in tumors with increased ER expression (89). Globally, many immune parameters in HER2⁺ breast tumors (i.e., TILs, CD8⁺ infiltrate) are inversely correlated with ER or progesterone receptor expression (90), and it is tempting to propose a possible relationship between decreased clinical benefit of ER⁺ tumors to anti-HER2 mAbs and their increased resistance to NK cell-mediated ADCC. E-cadherin

expression associated to ER⁺ breast carcinomas (91, 92) dampens trastuzumab-dependent ADCC through its specific interaction with the inhibitory killer cell lectin-like receptor G1 (KLRG1) on NK cells in preclinical *in vitro* and *in vivo* models (93, 94). Remarkably, resistance to trastuzumab-based treatment has been associated to E-cadherin expression in tumors from patients with HER2⁺ metastatic breast cancer (94). In addition, estrogens regulate the transcription of SerpinB9/proteinase inhibitor 9, a granzyme B inhibitor shown to decrease the susceptibility of ER⁺ breast cancer cells to NK and CD8⁺ T cell cytotoxicity *in vitro* (95, 96). Estrogens also upregulate HLA-I transcription through a cis-regulatory element in breast cancer cell lines (97–99), potentially modulating their susceptibility to NK cell-mediated ADCC. The relationship between ER and HLA-I expression has been confirmed by HLA-I immunohistochemical score in ER⁺/HER2⁺ as compared to ER⁻/HER2⁺ tumors (90). Whether other molecular features underlying breast carcinoma heterogeneity (e.g., mutations in PI3K, PTEN, p53, or p95HER2) (8) may modulate the susceptibility to NK cell-mediated ADCC remains uncertain.

Therapeutic Strategies Modulating NK Cell-Mediated ADCC

HER2 dual targeting with trastuzumab in combination with pertuzumab is nowadays the gold standard therapeutic approach for HER2⁺ breast cancer in the neoadjuvant setting and in the first-line treatment of metastatic disease. Patients that have progressed to prior trastuzumab, pertuzumab, and T-DM1 are treated with lapatinib. Both therapeutic strategies augment the coating of HER2⁺ tumors with IgG1 increasing the possibilities for NK cell-mediated ADCC antitumor responses. Simultaneous binding of pertuzumab and trastuzumab to HER2 increases the density of Fc_YR binding sites on HER2⁺ tumors; lapatinib does so, by preventing HER2 phosphorylation and internalization, hence increasing HER2 availability for trastuzumab (100–103).

Genetic engineering of the antibody Fc domain for optimizing Fc_YR engagement is one of the current strategies explored for enhancing the clinical success of several tumor antigen-specific mAbs (104). Margetuximab, an Fc-optimized HER2-specific mAb in clinical development, displayed increased binding to CD16A and elicited enhanced ADCC in breast cancer preclinical models (105). Promising single-agent activity of margetuximab has been recently reported for HER2⁺ breast and gastric cancer patients with advanced disease (106). Results of an ongoing two-arm open-label Phase 3 clinical trial in front of trastuzumab (NCT02492711) will reveal whether margetuximab displays superior efficacy, particularly for patients homozygous for the CD16A 158F/F low affinity genotype, in whom margetuximab showed the highest enhancement of NK cell-mediated ADCC in preclinical studies (105).

In addition to anti-HER2 mAbs, concomitant chemotherapy regimens may significantly impact on NK cell ADCC responses. Several chemotherapeutic agents currently combined or sequentially administered with anti-HER2 mAbs (i.e., anthracyclines, cyclophosphamide, taxanes) elicit a particular type of apoptosis, known as immunogenic cell death (ICD), that is accompanied by the coordinated release of DAMPs (e.g., ATP, and HMGB1) (107). DAMPs released along ICD activate a panel of pattern-recognition receptors (e.g., TLRs, P2RX7) and promote type I IFN release from cancer cells and the secretion of pro-inflammatory cytokines by immune cells (107, 108). Among DAMPs released along ICD, HMGB1 has been shown to enhance NK cell activation and recruitment to the tumor in a TLR2/4-dependent manner in preclinical models (109, 110) whereas type I IFNs have been shown to be necessary for the therapeutic efficacy of anti-HER2 mAb in MMTV-ErbB-2 transgenic mouse model (58). Indeed, a type I IFN signature predicted clinical responses to anthracycline-based chemotherapy in several independent cohorts of patients with breast cancer (108). In addition, *in vitro* treatment with anthracyclines and taxanes enhanced anti-HER2 mAb-induced ADCC by promoting endoplasmic reticulum-stress and the upregulation of NKG2D-ligands in breast carcinoma cells (111–113). Contravening the traditional view that chemotherapeutic drugs suppress patient immunity, anthracyclines- and taxanes-based treatments associated to enhanced NK cell function in breast cancer patients (60, 113–116).

On the whole, studies integrating information on the patient NK cell repertoire, NK cell receptor ligands on tumor cells and concomitant treatments might shed light on putative resistance mechanisms to anti-HER2 mAbs in HER2⁺ breast cancer patients.

NK CELL-MEDIATED ADCC AND THE VACCINE-LIKE EFFECT INDUCED BY ANTI-HER2 mAbs

Recent data highlight the importance of a vaccine-like effect by which antitumor mAb treatment facilitates the subsequent development of tumor-specific T cell responses, contributing to tumor elimination (117, 118). Antigen-presenting cells [i.e., macrophages and dendritic cells (DC)] use Fc_YR-mediated phagocytosis of immune complexes for enhancing tumor antigen processing and presentation, which can result in tumor-specific T-cell immunity (16, 117–119). Certainly, several evidences support the importance of antitumor T cell immunity for the clinical benefit of anti-HER2 mAb in breast cancer patients (115, 120–124).

Tumor cell cytotoxicity and cytokine/chemokine secretion upon antibody-dependent NK cell activation might directly and indirectly contribute to the vaccine-like effect induced by HER2-specific mAbs. On one hand, NK cell tumor cytolytic activity increases the availability of tumor antigen-containing immune complexes for antigen processing and presentation by DC and macrophages present in the tumor microenvironment. Independently of anti-HER2 mAbs, NK cell-DC crosstalk, involving cell-cell contacts and IFN γ , has been shown to prime DC polarization for IL-12 secretion, enhancing cross-presentation of tumor antigens to cytotoxic CD8⁺ T cells and the polarization of tumor-specific Th1 CD4⁺ T cells in preclinical models (59, 125–129). Moreover, activated NK cells are presumably capable of selectively killing immature DC while sparing activated DC, owing to their differential levels of surface HLA-I expression (130), thus selecting for immunogenic DC, effective inducers of antitumor T cells (127, 131). In patients, evidence of the participation of NK cell-mediated DC “editing” to the development of tumor-specific T cell immunity remains elusive. On the other hand, anti-HER2 mAb-dependent NK cell activation results in the production of IFN γ and chemokines (MIP1 α , MCP-1, RANTES, IL-8) (132), which might contribute to the recruitment and functional polarization of myeloid and T cells with antitumor potential. Noteworthy, coordinated NK and tumor-specific T cell responses have been detected in HER2⁺ breast cancer patients achieving pathological complete response to trastuzumab (133).

NK CELL EVASION IN BREAST CANCER

Neoplastic cells can develop a wide array of strategies to subvert NK cell recognition and cytotoxic function along tumor evolution (134, 135). Indeed, NK cell selective pressure contributes to tumor immunoediting leading to the emergence of evasive tumor cell clones (136–139). Generally, strategies hijacking NK cell function can be grouped into four categories: (i) shedding of ligands for NK cell activating receptors from tumor cells which act as decoy molecules leading to NK cell functional impairment

(e.g., MICA/B, B7-H6) (140, 141); (ii) upregulation of ligands for inhibitory NK cell receptors (e.g., HLA-I molecules; PD-L1) (142, 143); (iii) dysregulated expression of molecules conferring resistance to NK cell-mediated cytotoxicity (e.g., Bcl-2; Bcl-xL, cFLIP, caspase 8, Fas) (144); and (iv) immune suppressive cytokines (e.g., IL-10, TGF β) and metabolites (e.g., PGE2, adenosine) leading to NK cell dysfunction (135, 145).

Among all these strategies, increased levels of soluble MICA/B have been described in breast cancer patients (146) as well as overexpression of HLA-E, HLA-G in HER2 $^{+}$ tumors as determined by immunohistochemistry (147, 148). In addition, Fas downregulation in breast tumors has been correlated with shorter patient survival (149). Hence, several NK cell-evasive strategies operating along breast tumor progression may hamper the antitumor efficacy of anti-HER2 mAbs.

In concert with the development of an immune suppressive microenvironment in the progressing tumor, NK lymphocytes infiltrating advanced and metastatic breast carcinomas displayed an altered phenotype and reduced cytotoxic potential (150). According to data from distinct tumor types, NK cell infiltrates included high proportions of CD56 $^{\text{bright}}$ NK cells with increased expression of inhibitory CD94/NKG2A and decreased expression of activating NKp30, NKG2D, and DNAM-1 receptors (150). NK cells isolated from breast tumors also displayed reduced degranulation and IFN γ and TNF α production upon direct or antibody-dependent activation (150). Likewise, stratification of breast cancer patients by local and invasive disease, evidenced a progressive functional impairment of circulating NK cells associated to phenotypic alterations (150). Remarkably, CD16 expression on circulating NK cells was rather preserved, and cytotoxic responses induced by trastuzumab against the HER2 $^{+}$ breast cancer cell line SKBR3 were only affected at low trastuzumab doses in NK cells from patients with locally advanced or metastatic tumors (51, 151).

ENHANCING NK CELL-MEDIATED ADCC THROUGH IMMUNOTHERAPY IN HER2 BREAST CANCER

Only two mAbs, trastuzumab and pertuzumab and the antibody-drug conjugate T-DM1, are currently approved for breast cancer treatment. Strengthening NK cell-mediated ADCC responses through immunotherapy appears a suitable option for enhancing their clinical efficacy (45, 152, 153). In the following paragraphs, several approaches will be discussed based on data referring to HER2 $^{+}$ breast cancer (Figure 3).

Immunomodulatory mAbs Targeting Constitutive and Inducible Receptors in NK Cells

Several observations provide the rationale for combinatorial approaches including anti-HER2 mAbs and antibodies targeting surface NK cell receptors or co-receptors with activating and inhibitory function, termed immune checkpoints modulators. Nonetheless and despite promising results in preclinical

models, clinical trials combining anti-HER2 mAbs and immune checkpoint-targeting antibodies are currently lacking.

IFN γ secretion by NK cells has been shown to contribute to the tumor adaptive immune resistance response (154) by upregulating the expression of HLA-I and PD-L1 in HER2 $^{+}$ breast cancer cells *in vitro* and *in vivo* (58, 155, 156). HLA-I and PD-L1 can be, respectively, recognized by KIR, CD94/NKG2A, LILRB1, and PD-1 inhibitory receptors, modulating the subsequent recognition of transformed cells by NK and T lymphocytes.

Blocking mAbs targeting HLA-I-specific inhibitory receptors with constitutive expression in NK cells include an anti-NKG2A (monalizumab, IPH2201) and an anti-KIR (lirilumab, IPH2101, BMS-986015) (45). Both antibodies are currently in early clinical development being tested for safety and efficacy mostly for the treatment of hematological malignancies.² No clinical trials combining anti-HER2 mAbs and blocking agents targeting KIR or CD94/NKG2A are being developed, yet the safety and early efficacy of monalizumab and cetuximab (anti-EGFR) combination is being tested for the treatment of head and neck cancer (NCT02643550). Of note, an unexpected NK cell unresponsiveness consequent to treatment with lirilumab associated with treatment limited clinical efficacy in multiple myeloma patients (157, 158) warned about the undesired consequences of chronic targeting of HLA-I-specific NK cell receptors.

An alternative strategy, with unprecedented success as stand-alone treatment for several cancer types, is the blockade of the immune cell inhibitory PD-1/PD-L1 axis. Though generally considered a T cell co-receptor, PD-1 is also expressed by human exhausted NK cells (159) and circulating PD-1 $^{+}$ NK cell subpopulations were reported to be enriched in individuals with chronic viral infections as well as in cancer patients (159–161). PD-1 $^{+}$ expression is restricted to mature CD56 $^{\text{dim}}$ CD16 $^{+}$ NK cells and interferes with activation *via* NKp30, NKp46, or CD16 receptors (159). PD-L1 expression was preferentially detected in HER2 $^{+}$ breast tumors showing a strong cytotoxic local immune response (162) and the numbers of PD-1 $^{+}$ tumor-infiltrating lymphocytes were associated with poor prognosis in HER2 $^{+}$ breast cancer (163, 164). Remarkably, combination of HER2-specific mAbs with blocking antibodies targeting the PD-1/PD-L1 showed greater efficacy in preclinical models (58, 62). These observations support the suitability of combining anti-HER2 mAbs with immunotherapy targeting the PD1/PD-L1 axis. Several clinical trials assessing the benefit of mAbs targeting the PD1-PD1-L axis as monotherapy or in combination with chemotherapy, radiotherapy or hormone therapy are currently being developed for ER $^{+}$ or triple-negative breast tumors (see text footnote 2); likewise, combinatorial approaches with anti-HER2 mAbs are warranted.

TIGIT, a nectin-binding inhibitory co-receptor showing overlapping ligand specificity with the activating DNAM-1, is another inducible receptor with the capacity to modulate NK cell ADCC responses (165, 166). Both receptors recognize CD155 (also known as PVR) and CD112 (also known as Nectin-2), ubiquitous cell-adhesion molecules (167) overexpressed in

²<http://clinicaltrials.gov>

HER2⁺ breast cancer cell lines (51). Besides CD8⁺ T cells, TIGIT is preferentially expressed on CD16⁺ NK cells and upregulated upon activation *via* ADCC (168, 169). TIGIT blockade has been shown to enhance trastuzumab-triggered antitumor response by human NK cells *in vitro* (169). Currently, an anti-TIGIT blocking mAb (OMP-313M32) is in early clinical development being tested for safety as standalone treatment in patients with locally advanced or metastatic solid tumors (NCT03119428).

Another immune checkpoint shown to synergize with anti-HER2 mAb in xenotransplant models of breast cancer is CD137 (58, 170). CD137 (4-1BB; TNFRSF9) is a co-stimulatory receptor induced in activated leukocytes, originally described for its capacity to enhance antitumor T cell responses (171, 172). CD137 expression following CD16 ligation has been shown in murine and human NK cells (173) and CD137 upregulation has been well documented on *ex vivo* circulating NK cells from breast and head and neck cancer patients upon tumor antigen-specific mAb infusion (170, 174). Two agonistic anti-CD137 mAb are currently in clinical development (urelumab and utomilumab), being tested alone or in combination with anti-PD-1 mAbs in advanced solid and hematologic tumors (45).

Of note, since NK and some T lymphocyte subsets share many receptor/ligand pairs involved in their functional regulation (e.g., PD-1, TIGIT, 4-1BB/CD137, and CD94/NKG2A), combinations between anti-HER2 therapeutic mAbs and distinct immune checkpoint modulators would promote antitumor immunity by dual targeting T and NK cell functional exhaustion.

Anti-HER2 mAb Combination with Cytokines

Several attempts to potentiate NK cell antitumor function by systemic treatment with recombinant cytokines have also been carried out. Besides their effects on T cells, IL-2, and IL-15 signaling through STAT 5 enhance NK cell antitumor function (41, 175, 176).

IL-2 enhanced NK cell-mediated ADCC triggered by anti-HER2 mAb against breast cancer cell lines *in vitro* and *in vivo* (177, 178). However, clinical trials including combined administration of IL-2 with trastuzumab did not show improved disease outcome in metastatic HER2⁺ breast cancer patients (179, 180). Caveats of systemic IL-2 administration include treatment-associated toxicity, its rapid clearance *in vivo* and IL-2 pro-tumor effects through the concurrent activation of CD4⁺ regulatory T cells. Nonetheless, low-dose IL-2 is currently included in a number of clinical trials to support cellular adoptive approaches with combined infusions of NK cells and trastuzumab in HER2⁺ breast cancer patients (NCT02030561, NCT02843126).

IL-15 is an essential cytokine for human NK cell homeostasis; nonetheless, early clinical assays including systemic IL-15 were withdrawn due to concurrent adverse events and dose-limiting toxicities (181). Similarly, IL-15 enhanced the antitumor activity of trastuzumab, yet causing fatal side effects in a humanized tumor mice model (182). Current research efforts include the development of cytokine variants with extended *in vivo* half-life and targeted action on precise lymphocyte subsets and tumor

sites (i.e., engineered IL-2 “superkine,” IL-15R α Sushi-Fc fusion protein; IL-15 tri and tetraspecific killer engagers) (183–186).

IL-12 has been shown to enhance the antitumor actions of trastuzumab *via* the enhancement of NK cell IFN- γ production in mouse models (56, 57). In a clinical trial in which IL-12 was combined with trastuzumab and paclitaxel, increased levels of IFN- γ and several chemokines were detected in sera from patients with clinical benefit, but not in patients with progressive disease (187). Currently, two clinical trials are ongoing including IL-12 and trastuzumab combined treatment (NCT00004074, NCT00028535). Preclinical studies are focused on the development of approaches for targeting cytokine expression in the tumor site to avoid toxicities associated to systemic treatment (i.e., tumor-targeting immunocytokines, gene therapy with loco-regional injections of cytokine-encoding plasmid) (188).

Immunotherapy with TLR Ligands

Toll-like receptor TLR ligands have been shown to improve both the quality and the magnitude of host antitumor innate and adaptive immune responses (189). TLR2, TLR3, TLR8, and TLR9 agonists have been shown to prime NK cell effector function (39, 40) and to synergize with anti-HER2 mAb therapy in a type I and II IFNs-, NK-, and CD8⁺ T cell-dependent manner in preclinical models (190–192). In the context of breast cancer, TLR ligands are being tested as adjuvants in diverse HER2-peptide vaccination strategies (i.e., TLR9-ligand CpG ODN in NCT00640861; TLR7 agonist imiquimod in NCT02276300; AS15 mixture in NCT02364492, NCT00058526, NCT00140738; TLR3 agonist Hiltonol in NCT01532960), including trastuzumab in some instances (i.e., the TLR9-ligand PF03512676-CpG 7909 or agatolimod-: NCT03512676, NCT00043394, NCT00031278). Strategies for delivering TLR agonists into the tumor site would likely potentiate NK cell-mediated ADCC synergizing with anti-HER2 mAbs antitumor function.

ADAM Inhibitors

One of the consequences of CD16-mediated NK cell activation is the shedding of CD16 extracellular domain by the induced action of the A disintegrin and metalloprotease 17 (ADAM17), thus limiting subsequent CD16A receptor engagement and NK cell activation (193). Intriguingly, ADAM10 (with constitutive activity) and ADAM17 (inducible) also control the release of ligands for EGFR/HER receptors (194) and promote the shedding of B7-H6 and MICA/B ectodomains, amplified and overexpressed in breast tumors (195, 196) limiting NKp30- and NKG2D-mediated NK cell activation (140). In fact, ADAM10 and ADAM17 levels have been associated with poor responses and shorter relapse-free survival after trastuzumab treatment (197, 198). In this scenario, inhibition of ADAM17/10 could improve NK cell-mediated ADCC triggered by anti-HER2 mAb, preventing CD16 and B7-H6 shedding as well as enhancing HER2 surface availability. ADAM17 specific inhibitor prevented CD16 shedding and improved NK cell-mediated ADCC responses *in vitro* (199). Two clinical trials tested the combination of an ADAM17 inhibitor (INCB7839) with trastuzumab (NCT01254136, NCT00864175) yet the development of the compound was suspended by the sponsor corporation and no results were published. Currently,

the possibility of enhancing NK cell-mediated ADCC by combining ADAM17 inhibitor (INCB7839) and tumor antigen-specific antibodies is being tested in combination with rituximab (NCT02141451).

CONCLUDING REMARKS

Activation of NK cell effector functions by anti-HER2 therapeutic antibodies can directly contribute to tumor control by their direct cytolytic activity against transformed cells, but also indirectly by their effects on the tumor microenvironment, eventually favoring the development of antitumor adaptive immunity. Multiple strategies are being developed for enhancing NK cell-mediated antibody-dependent antitumor activity, while simultaneously targeting other immune cells which contribute to the control of tumor growth and spreading. Understanding which variables underlie breast cancer heterogeneity in terms of lymphocyte infiltration and susceptibility to immune surveillance, as well as how the heterogeneity in the NK cell repertoire can influence on the clinical benefit of HER2-targeting mAbs, will aid in the design of tailored strategies to broaden their therapeutic window.

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Therapeutic Antibodies against Intracellular Tumor Antigens

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Monoclonal antibodies are among the most clinically effective drugs used to treat cancer. However, their target repertoire is limited as there are relatively few tumor-specific or tumor-associated cell surface or soluble antigens. Intracellular molecules represent nearly half of the human proteome and provide an untapped reservoir of potential therapeutic targets. Antibodies have been developed to target externalized antigens, have also been engineered to enter into cells or may be expressed intracellularly with the aim of binding intracellular antigens. Furthermore, intracellular proteins can be degraded by the proteasome into short, commonly 8–10 amino acid long, peptides that are presented on the cell surface in the context of major histocompatibility complex class I (MHC-I) molecules. These tumor-associated peptide–MHC-I complexes can then be targeted by antibodies known as T-cell receptor mimic (TCRm) or T-cell receptor (TCR)-like antibodies, which recognize epitopes comprising both the peptide and the MHC-I molecule, similar to the recognition of such complexes by the TCR on T cells. Advances in the production of TCRm antibodies have enabled the generation of multiple TCRm antibodies, which have been tested *in vitro* and *in vivo*, expanding our understanding of their mechanisms of action and the importance of target epitope selection and expression. This review will summarize multiple approaches to targeting intracellular antigens with therapeutic antibodies, in particular describing the production and characterization of TCRm antibodies, the factors influencing their target identification, their advantages and disadvantages in the context of TCR therapies, and the potential to advance TCRm-based therapies into the clinic.

Keywords: T-cell receptor mimic antibody, intracellular antibody, intrabody, MHC class I presented peptide, T-cell epitope, cancer immunotherapy, therapeutic antibody, T-cell receptor-like antibody

INTRODUCTION

Historically the consensus in the immunotherapy field has been that antibody therapy is amenable to targeting only extracellular antigens that are accessible for antibody binding. This is due to the fact that the high molecular weight of antibodies prevents them from crossing the cell membrane to access intracellular targets. Consistent with this train of thought, the targets of approved antibody therapies are predominantly extracellular antigens (1). By contrast, small molecules have been used to target those intracellular antigens with a functionality that is suitable for drug screening. In comparison to antibodies, small molecules tend not to be as selective for their targets. They can exhibit unpredictable off-target activities, which consequently lead to adverse side effects and may require a more individualized clinical development pipeline.

More recently, there are three broad approaches whereby antibodies have been used to target intracellular antigens.

- (1) It is possible for antibodies (or their derivatives) to target antigens that are normally intracellular but become externalized (for example, during disease).
- (2) It is also possible to engineer antibodies or antibody fragments that penetrate into cells, or those that are directly expressed within cells using a gene therapy style approach.
- (3) Antibodies can also be generated that bind cell surface major histocompatibility complex class I (MHC-I)-presented peptides that are derived from intracellular proteins.

With further developments in this field, it is becoming clear that the dichotomy between the antibody targeting of intracellular and extracellular targets is not as rigid as originally thought. Antibodies with novel mechanisms of action are challenging this belief and are re-defining the selection of suitable targets for antibody therapy. Antibodies that target intracellular antigens could open the door to a whole new realm of therapeutic targets, with potentially immense clinical benefits. While antibodies targeting intracellular antigens have broad clinical potential, this review will focus primarily on their application for cancer therapy.

ANTIBODIES TARGETING EXTERNALIZED ANTIGENS

Intracellular antigens can become externalized on the cell surface or secreted and can, therefore, be targeted by antibodies. The Zeng group has further explored the possibility of developing antibodies to intracellular oncoproteins. After an initial proof-of-concept study investigating intracellular proteins targeted by both antibody and vaccine therapy, they focused on phosphatase of regenerating liver 3 (PRL-3) and developed a humanized anti-PRL-3 antibody (2, 3). PRL-3 is a cancer-related phosphatase (4) that is reported to be involved in malignant transformation and metastasis, as well as its expression correlating with poor prognosis (5). It is undetectable in most normal human tissues, is involved in colorectal cancer and uveal melanoma, and is overexpressed in 85% of gastric cancers (but not patient-matched normal gastric tissue), which is the cancer model that has been further studied (3). Importantly, intracellular PRL-3 can be externalized by tumor cells, thus enabling its targeting using classical antibody technology.

It is not the first time that secreted or externalized intracellular proteins have been observed on cancer cells or within the tumor microenvironment and identified as potential therapeutic targets. One such example is the intracellular melanosomal membrane glycoprotein, gp75, which is normally expressed in the melanosome, a specialized organelle present in melanocytes. In melanoma, gp75 is expressed on the cell surface of malignant melanocytes and can be targeted by antibodies in mouse melanoma models (6). In addition, heat-shock proteins 70 and 90 are chaperone proteins, which are further examples of targets that are intracellular in normal cells but become presented on

the cell surface, or secreted into the extracellular environment, in transformed cells (7, 8). Tumor cells have been previously shown to shed intracellular material into the tumor microenvironment and extracellular space. This is believed to be a consequence of the inflammatory reaction that surrounds tumor tissues, where immune surveillance can provoke apoptosis and necrosis of tumor cells, thus releasing intracellular components into the extracellular space (9). It has also been suggested that typically intracellular antigens can also be externalized through unconventional secretion pathways (10). This is corroborated by the observation that antibodies against gp75 can reject tumors where there is no necrosis, suggesting an alternative pathway enabling antigen externalization (6). It is the restricted expression profile and the secretion and externalization of PRL-3, by cancer cells, that make it possible to selectively target this oncoprotein with antibody therapy. In this context, it is possible to target an intracellular oncoprotein, which has become externalized onto the cell surface, with an antibody in the same manner as targeting a classical cell surface target.

Several observations have been made on the possible mechanisms of action that mediate the therapeutic effect of targeting extracellularized antigens with a non-neutralizing antibody. It is postulated that, *in vivo*, the Fc portion of these antibodies can be recognized by immune effector cells that have immunoglobulin (Ig) receptors (FcRs), such as macrophages, B cells, and natural killer (NK) cells (11). Therefore, the mechanisms of action could involve a combination of the following:

- (1) antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells,
- (2) antibody-dependent cellular phagocytosis by macrophages,
- (3) secreted antigens bound to antibody can form immune complexes that can be processed by dendritic cells, which then proceed to activate NK cells (12).

The importance of immune effector cells to the therapeutic efficacy and the aforementioned hypotheses are corroborated by the Zeng group's previous findings, which showed that anti-PRL-3 antibodies have no therapeutic activity in immunocompromised SCID mice or *in vitro* against PRL-3-expressing cancer cells where no effector cells are present (2, 13). Such engagement with innate immune effectors is a common mechanism of action of therapeutic antibodies that do not modify the activity of the target antigen, including those against cell surface targets.

INTRACELLULAR ANTIBODIES

Intracellular antibodies, which may also be called intrabodies, are antibodies that are produced in the cell, and bind an antigen within the same cell. This is a different delivery strategy from antibodies that are produced extracellularly, and are engineered to then penetrate the cell to access their intracellular target.

Antibodies are soluble proteins that are normally found circulating the body within the serum. They are synthesized in the endoplasmic reticulum (ER) of B cells as separate heavy chain and light chains, which are then linked by disulfide bonds in the mature Ig. However, the full-length antibody is not functional

in the cytosol, prior to secretion, due to its reducing conditions, which affect protein folding and the intramolecular disulfide bonds that are required to maintain the antibody's conformation and stability (14). Fortunately, the complementarity-determining regions that endow an antibody with its exceptional target specificity are located in the variable regions of both the heavy and light chains. Therefore, it is possible to use antibody fragments incorporating the specificity-providing regions within a single-chain variable fragment (scFv), which can be further engineered for cytosolic stability, to target intracellular antigens (15, 16). The scFv is a single polypeptide, which is a favorable characteristic for *in vivo* expression, and it has been studied as a therapeutic for viral infections and cancer, among other diseases.

Furthermore, the variable (V) region domain can be used by itself to form a domain antibody or Dab (17). These can be engineered from conventional human IgGs, or also from those from camelids (camel or llama) and cartilaginous fish (carp or nurse sharks), whose immune systems were found to have evolved high-affinity V-like domains fused to a conserved framework that is reflective of the constant Fc region found in human Ig (18, 19). It has been reported that single heavy chain V regions or light chain V regions can be expressed inside cells. These are referred to as intracellular domain antibodies, which do not require intramolecular disulfide bonds for stability, hence representing the smallest format of the antibody that retains target specificity while minimizing size—a crucial factor for intracellular targeting (20).

There are several critical aspects to generating functional intracellular antibodies. The first is designing an antibody format that will retain its stability and antibody binding capacity within the cell and the second is the ability to introduce or express the antibody within cells. Furthermore, as intracellular antibody fragments do not possess an Fc region (and full length intracellular antibodies cannot recruit extracellular immune effector cells from within the cell), different strategies must be employed to equip them with effector functions unless they have directly neutralizing activity against the target. Examples include ER targeting to cause degradation of the target protein, antibody–antigen interaction-dependent apoptosis that is used to induce programmed cell death through the activation of caspases, and suicide intrabody technology that causes proteolysis of the target protein (21, 22).

In cancer, some of the proteins that are key players in signaling pathways leading to malignant transformation have thus far been inaccessible to small molecule inhibitors (23). In particular, some of these are large, intracellular proteins that act as molecular scaffolds and function primarily through facilitating protein–protein interactions (PPIs). Due to their size, small molecules cannot physically block the large surface of such proteins, nor interfere in the protein–protein interfaces they form, which are typically hydrophobic, flat surfaces, presenting few possibilities for small molecule anchorage (24). This is where technologies that enable the use of antibodies within the cell can bridge the gap between small molecule inhibitors and large protein targets. In this context, the proteins themselves are not the target, but it is the interactions they form with other proteins or nucleic acids that are the therapeutic targets as they contribute to the diseased

state. One example is the use of an anti-RAS intrabody, which is composed of a single variable heavy region domain that targets activated GTP-bound RAS. This antibody competitively blocks RAS-effector functions within the tumor cell and while able to prevent *in vivo* tumor initiation and further tumor growth in murine models, it was not curative (25, 26). Thus, some antibodies may enable control of tumor growth and require combinations with additional agents to potentially achieve a cure.

Intrabodies can also be used to characterize the expression of their target proteins and study the *in vivo* knockdown of protein function, and can represent an alternative to generating gene knockout animal models. There are different types of intrabodies that can be tailored to target proteins within subcellular compartments, primarily the cytoplasm or the ER, but the addition of a signal peptide also allows targeting to the mitochondria or the nucleus. This can be used to confer additional subcellular specificity on their intracellular targeting. Importantly, antibodies retained in the ER do not experience the problems with conformation that are caused by the reducing conditions within the cytosol and can be active without neutralizing function against their target (27). For example, using intrabodies targeted to the ER (using a “KDEL” or “SEKDEL” sequence) allows the knockdown of proteins that are passing through the ER, thus abrogating their downstream function in a similar way to RNA interference and providing an alternative strategy for silencing gene products. It has also been proposed that ER-targeting intrabodies may maintain silencing more effectively than short interfering RNA (siRNA) and their specificity may be easier to predict than the off-target effects of an siRNA. An intradiabody that simultaneously enabled the knockdown of VEGF-R2 and Tie-2 was able to reduce both tumor growth and angiogenesis *in vivo* (28). Intrabody technology is overviewed in depth by recent reviews including Marschall and Dübel (29).

Delivery of Antibodies to the Intracellular Compartment

Despite the general consensus that antibodies can only be used to target extracellular or secreted antigens, the cellular uptake of antibodies (by processes such as endocytosis) has been observed both clinically and experimentally in the case of autoimmune disease. It has been reported that once autoantibodies bind their intracellular target, they can cause apoptosis of the cell (30–32). Therefore, the idea of using intracellular antibodies therapeutically represents a logical expansion of such observations. At present, the use of intracellular antibodies is still limited by the technology needed for antibody delivery and they are used primarily as research tools. A number of different methods are being investigated for the delivery of antibodies to the intracellular compartment within target cells. Some of these strategies are illustrated in **Figure 1** and they fall into two broad strategies:

- (1) The first is a type of “gene therapy” approach using vectors that enable expression of the intracellular antibodies within the target cell—these can be either viral vectors or plasmids.
- (2) The second is direct administration of the antibody-based therapeutic—either alone, using electroporation or with

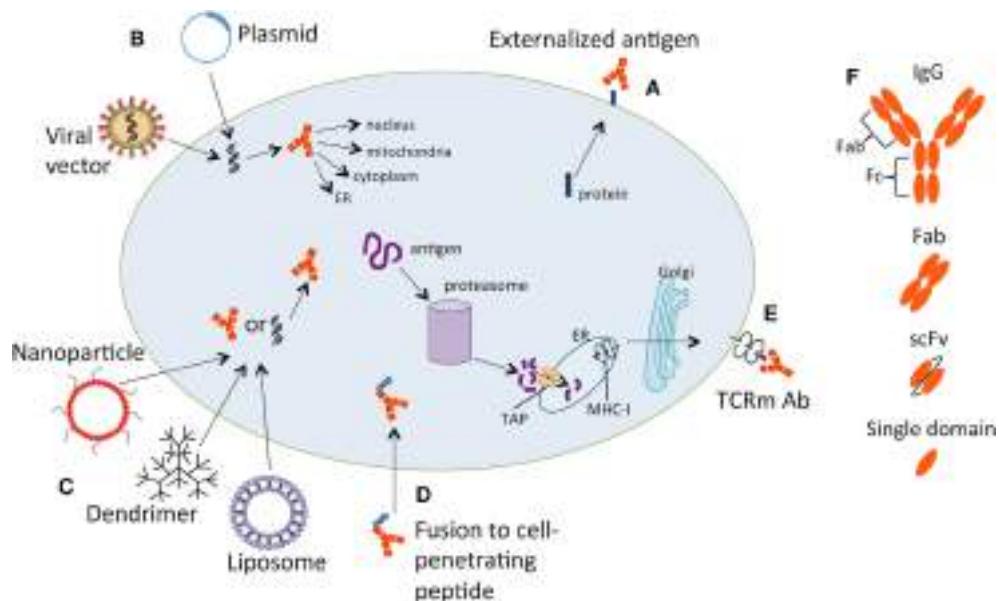


FIGURE 1 | Strategies for targeting intracellular tumor antigens with antibody therapy. Some of the methods for targeting intracellular tumor antigens are illustrated. (A) Intracellular antigens can be externalized on the cell surface or secreted, allowing targeting by antibodies. (B) Plasmids or viral vectors can be used to deliver antibody-encoding genes into the cell. Once internalized, the DNA is transcribed into the targeting antibody, which can be designed to translocate to the nucleus, mitochondria, endoplasmic reticulum (ER), or cytoplasm. (C) Nanoparticles, dendrimers, or liposomes can be used to deliver an antibody or an expression vector encoding the intracellular antibody into the target cell. (D) Antibodies can be fused to cell-penetrating peptides, which allow internalization of the antibody. (E) T-cell receptor mimic (TCRm) antibodies can be used to target peptides bound to major histocompatibility complex class I (MHC-I) molecules on the cell surface. The peptides are derived from intracellular proteins, which have been degraded by the proteasome into short peptides. Peptides are loaded onto MHC-I molecules in the ER, transported through the Golgi apparatus, and finally presented on the cell surface. (F) The antibody depicted on the diagram could represent a full-length IgG, a Fab fragment, scFv or a single domain antibody.

dendrimers, liposomes, nanoparticles, or by fusing the antibody to protein-transduction domains that enable it to penetrate the cell (33).

Viral vectors that can be used to deliver the genetic information for expression of intracellular antibodies include adenovirus, adeno-associated virus (AAV), and retrovirus (including lentivirus), which have all been studied extensively in the pre-clinical setting as gene therapy delivery vehicles (34). Retroviruses integrate the antibody fragment expression cassette into the host genome, allowing long-term expression of the intracellular antibody fragment. Despite this advantage, a safety concern with the use of lentiviruses is the risk of integration of the expression cassette in the proximity of an oncogene in the host genome, thereby triggering secondary cancers. By contrast, AAV releases the DNA as an episome, avoiding such safety concerns; however, there is always the possibility of loss of expression, which means relatively shorter term expression.

A non-viral strategy for delivering genes or proteins to the intracellular compartment involves the encapsulation of DNA or proteins in cationic lipid structures called liposomes (35–37). Liposomes form a closed, spherical particle that is amphiphilic and composed of one or more lipid bilayers with an aqueous center. In addition to delivering antibodies, they can also be coated with antibodies that bind cell surface proteins on the target cells (38). Thus, they are targeted to a specific cell type and

can deliver an antibody, or an expression vector encoding the intracellular antibody, to the target cell without employing a viral delivery method. Liposomes are internalized *via* endocytosis following interaction with the plasma membrane, which is based on multiple factors, including particle size and charge interactions (39). Nanoparticles are an alternative non-viral method for delivering DNA or antibodies intracellularly (40, 41). They are made of polymers such as poly lactic-co-glycolic acid (PLGA), which is an FDA-approved polymer that has been studied extensively for therapeutic applications (42). PLGA-based nanoparticles have been used to improve the endocytic cellular uptake of antibody fragments such as 3D8 scFv (43). Similarly, antibody-coupled delivery can be used, wherein the expression vector DNA is coupled to the C-terminus of an antibody that binds a cell surface target. The vector DNA is internalized upon internalization of the delivery antibody, and the therapeutic antibody it encodes is then expressed intracellularly (44). Expression vectors and antibodies can also be conjugated to dendrimers (synthetic polymers with a branching tree-like structure) for delivery into target cells (45).

Fusion to cell-penetrating peptides may be an alternative method for delivering antibody fragments into cells through protein transduction. Antibodies that have cell-penetrating peptides fused to them can be referred to as TransMabs (46). The first TransMab that was generated was composed of an anti-caspase-3 antibody fused to a 17 amino acid peptide that

could translocate the antibody across the plasma membrane of target cells (47). This then blocked events related to apoptosis, such as caspase-3 activity and DNA fragmentation. Using this method, the antibody-peptide fusion protein enters the cell through endocytosis (48). However, it is difficult to predict whether sufficient macrodrug will enter the cell in order for it to mediate a therapeutic effect. Particularly as cell-penetrating peptides fused to macromolecules have been reported to be at risk of being trapped within endosomes (49). Another disadvantage of this method is that antibody fragments will undergo degradation in the intracellular compartment, as would any protein, therefore, continuous re-administration would be required to maintain any therapeutic activity. A cell-penetrating IgG1 antibody targeting activated GTP-bound RAS (RT11) has recently been shown to block oncogenic signaling and inhibit tumor growth in mouse xenograft models with mutated but not wild type Ras. This iMab (internalizing and PPI interfering monoclonal antibody) has successfully blocked the activity of a highly desirable oncogenic target that lacks effective small molecule inhibitors (50).

T-CELL RECEPTOR MIMIC (TCRm) ANTIBODIES

Immunotherapies targeting intracellular proteins can also exploit the immune system's own intracellular surveillance mechanism. Intracellular proteins are degraded by the proteasome to form short peptides of specific lengths. These peptides are then presented on the cell surface of most nucleated cells, in a complex with MHC-I molecules (51). CD8⁺ T cells recognize peptide-MHC-I complexes through their clonotypic T-cell receptor (TCR) and become activated to kill malignant or virus-infected cells that present tumor or viral peptides (51). Significantly these MHC-presented peptides do not have a functionality that would make them suitable targets for small molecule drug screening.

Antibodies targeting disease-associated peptide-MHC-I complexes, the so-called TCRm antibodies or TCR-like antibodies, are similar to the TCR in that they bind both the peptide and the MHC-I molecule and, therefore, their binding is both peptide-specific and MHC-restricted (52, 53). TCRm antibodies have expanded the range of targetable antigens to include intracellular proteins without the delivery complications associated with intracellular antibodies. Another advantage of TCRm antibodies is that they combine the intricate tumor specificity of TCRs with the biological properties of antibodies, which do not succumb to immune regulatory mechanisms that obstruct T-cell function in the tumor microenvironment (54). Like conventional monoclonal antibodies, TCRm antibodies have been shown to cause tumor killing through antibody-dependent mechanisms such as cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (55, 56). Furthermore, studies have shown a TCRm antibody to cause apoptosis in breast cancer cells through a caspase-dependent pathway (55). In addition to the success of using naked TCRm antibodies, there have also been reports of anti-tumor activity when they are conjugated to toxins (57, 58). The ability of TCRm Abs to target intracellular antigens has also

been applied to cellular therapies in the development of chimeric antigen receptor T cells (59, 60).

TCRm Antibodies Published to Date

Since the advent of the necessary techniques and technologies, there has been an increase in the production of TCRm antibodies and constructs derived from them. The target peptides of such reagents have typically derived from either viral antigens (including HIV and Hepatitis B antigens) or cancer antigens, and they are commonly presented by either the HLA-A*0201 or the HLA-A*2402 MHC-I haplotype (61, 62). While TCRm antibodies can be used for therapeutic purposes, they are also widely used as research tools for the study of antigen presentation and recognition, as well as for structural studies. Some of the TCRm antibody therapeutics have shown promise in both *in vitro* and *in vivo* studies, however, none of them have advanced to clinical studies. Information on the TCRm and TCR-like antibodies generated to date is summarized in **Table 1**.

Production of TCRm Antibodies

T-cell receptor mimic antibodies are not as commonly available as traditional antibodies; this may be a consequence of the difficulty of their production in addition to the technology being less established. Recently, there has been an increase in the generation of TCRm antibodies targeting a variety of cancer or viral T-cell epitopes due to advances in the necessary technologies and techniques. TCRm antibodies have been produced either by immunization or by phage display, with both strategies presenting their respective pros and cons. One of the main limitations in the production of TCRm antibodies by both strategies was the correct refolding of recombinant peptide-MHC complexes and their purification (53). Recombinant peptide-MHC complexes are made by using bacterial expression to generate inclusion bodies containing the extracellular domains of the heavy chain of human leukocyte antigen (HLA) and β2-microglobulin. These are then refolded with the MHC-restricted peptide to generate correctly refolded monomers of high purity, in quantities that are sufficient for downstream applications. The correct refolding can be verified by structural and functional experiments, and the monomers can then be biotinylated for specificity and affinity characterization, and for antibody isolation (86–88).

Initially, TCRm antibodies were produced using hybridoma technology. The immunization methods used in these experiments limited the successful generation of TCRm antibodies. Antigen-presenting cells harboring immunogenic peptides in the groove of their MHC molecules were used as immunogens (89, 90). Obtaining TCRm antibodies of the correct specificity by employing this method yielded very few antibodies and many efforts proved to be unsuccessful (91). Since then, more successful attempts have been made by using recombinant peptide-MHC complexes, such as tetramers, in the immunization protocol, followed by high-throughput screening in order to isolate specific TCRm antibodies out of a pool of thousands of clones (62, 71, 81). This requires stable peptide-MHC-I binding and has resulted in the production of TCRm against tumor and viral T-cell epitopes.

TABLE 1 | TCRm antibodies for cancer immunotherapy.

Target	Epitope sequence	MHC haplotype	TCRm antibody name	Isotype/format	Cancer indications investigated	Isolation method	Reference
MAGEA1	EADPTGHSY	HLA-A*0101	Fab-G8	Fab	Melanoma	Phage	(63)
MAGEA1	EADPTGHSY	HLA-A*0101	Fab-Hyb3	Fab	Melanoma	Phage	(64)
GP100	KTWGQYWQV	HLA-A*0201	G2D12, G3G4	Fab	Melanoma	Phage	(65, 66)
GP100	IMDQVPFSV	HLA-A*0201	1A9, 1C8, 1A11, 1A7	Fab	Melanoma	Phage	(65, 66)
GP100	YLEPGPVTV/A	HLA-A*0201	2F1, 2B2, 2C5, 2D1	Fab	Melanoma	Phage	(65, 66)
GP100	IMDQVPFSV	HLA-A*0201	G1	scFv-PE38	Melanoma	Phage	(57)
GP100	ITDQVPFSV	HLA-A*0201	GPA7	sdAb-CAR	Melanoma	Phage	(60)
hTERT	ILAKFLHWL	HLA-A*0201	4A9, 4G9	Fab	Melanoma, prostate	Phage	(67)
hTERT	RLVDDFLV	HLA-A*0201	3H2, 3G3	Fab	Melanoma, prostate	Phage	(67)
MUC1	LLT VLTWV	HLA-A*0201	M2B1, M2F5, M3A1, M3B8, M3C8	Fab	Breast	Phage	(68)
NY-ESO-1	SLIMWMITQC	HLA-A*0201	3M4E5	Fab	Melanoma	Phage	(69)
MAGE3	FLWGPRAVLV	HLA-A*0201	7D4, 8A11, 2G12, 9E6	–	–	Hybridoma	(70)
hCG β	GVLPALPV	HLA-A*0201	RL4B/3.2G1	mlgG2a	Ovarian, colon, breast	Hybridoma	(71)
hCG β	GVLPALPV	HLA-A*0201	1B10	IgG1	Ovarian, colon, breast	Hybridoma	(72)
hCG β	TMTRVLQGV	HLA-A*0201	3F9	IgG1	Ovarian, colon, breast	Hybridoma	(72)
Her2/Neu	KIFGSLAFL	HLA-A*0201	1B8	IgG1	Breast, colon	Hybridoma	(73)
Melan-A/MART-1	EAAGIGILTV/ELA	HLA-A*0201		Fab	Melanoma	Phage	(74)
Melan-A/MART-1	EAAGIGILTV	HLA-A*0201	CAG10, CLA12	Fab-PE38	Melanoma	Phage	(58)
TARP	FLRNFSLML	HLA-A*0201	Fab-D2	Fab-PE38	Breast, prostate	Phage	(75)
p53	LLGRNSFEV	HLA-A*0201	I3.M3-2A6	–	–	Hybridoma	(76)
p53	RMPEAAPV	HLA-A*0201	T1-116C	IgG1	Breast	Hybridoma	(56)
p53	RMPEAAPV	HLA-A*0201	T1-29D, T1-84C	IgG1, IgG2b	–	Hybridoma	(77)
p53	GLAPPQHLIRV	HLA-A*0201	T2-108A, T2-2A, T2-116A	IgG1, IgG2a, IgG1	–	Hybridoma	(77)
Tyrosinase	YMDGTMMSQV	HLA-A*0201	TA2	Fab	Melanoma	Phage	(78)
p68	YLLPAIVHI	HLA-A*0201	RL6A	mlgG2a	Breast	Hybridoma	(79)
MIF	FLSELTQQL	HLA-A*0201	RL21A	IgG2a	Breast	Hybridoma	(80)
Proteinase 3	VLQELNVTV	HLA-A*0201	8F4	IgG2a	AML	Hybridoma	(81)
WT1	RMFPNAPYL	HLA-A*0201	ESK1	hlgG1	Mesothelioma, leukemia, ovarian, colon	Phage	(82)
WT1	RMFPNAPYL	HLA-A*0201	F2, F3	Fab	Leukemia	Phage	(59)
WT1	RMFPNAPYL	HLA-A*0201	Clone45	scFv	Leukemia	Phage	(83)
HA-1H	VLHDDLLEA	HLA-A*0201	#131	scFv, scFv-CAR	Leukemia	Phage	(84)
PRAME	ALYVDSLFFL	HLA-A*0201	Pr20	hlgG1	Leukemia, lymphoma, melanoma, breast, colon	Phage	(85)

Published TCRm antibodies targeting cancer antigens are summarized.

PE38, 38 kDa immunotoxin, which is a truncated form of *Pseudomonas* exotoxin that can be conjugated to a TCRm Ab. sdAb, single domain antibody that has a single antigen binding domain originating from llama VH₁. CAR, chimeric antigen receptor.

While the traditional strategy for making TCRm antibodies is hybridoma technology, in the mid 1990s, it was shown that phage display technology could also be used to isolate antibodies (87). In this method, libraries of phage particles are generated, where each phage displays a unique antibody (a scFv or a Fab fragment) as a fusion protein on their surface. Each phage particle has the genes that encode the particular antibody that is expressed on its cell surface. Therefore, it is possible to select different phage particles by assessing whether they bind a target and thereby isolate the antibodies that have the desired specificity. The bound phages are then eluted and amplified in bacteria. Phage display technology has been used to isolate various TCRm antibodies against cancer antigens (63, 69, 92, 93).

Most TCRm antibodies published thus far have used phage display libraries for antibody production. Investigators argue that the main advantage of phage display is that it is efficient while being a relatively fast method (53). On the other hand, hybridoma technology is a relatively slower strategy, and it requires the immunogenic peptide and the MHC complex to bind with high affinity and form a very stable complex in order for the

complex to persist throughout the immunization and *in vivo* IgG maturation. Nevertheless, the advantages of hybridoma technology include the isolation of antibodies that have a high affinity (in the low nanomolar range) for the peptide–MHC complex. This is due to the fact that antibodies undergo multiple antigen challenges and affinity maturation *in vivo*. Whereas affinities of TCRm antibodies produced through phage display tend to lie in the moderate nanomolar range (\approx 50–300 nM) and many require further *in vitro* affinity maturation (94, 95).

Furthermore, the antibodies produced through hybridoma technology are bivalent IgG isotype antibodies, whereas antibodies isolated using phage display are either scFv or Fab fragments (i.e. in the monovalent form with no Fc region). The Fc portion of the antibody is crucial in recruiting components of the immune system for cytotoxic effects mediated through ADCC and CDC. Antibodies in the monovalent form have reduced avidity (functional affinity) and increased turnover rates, which are undesirable when targeting epitopes that may be expressed at low densities, such as epitopes on tumor-associated peptide–MHC complexes (53). To circumvent this difficulty, further engineering

can be undertaken to address these limitations. For example, scFv or Fab tetramers can be generated through biotinylation, thereby increasing their avidity or antibodies can be engineered to have a classical Fc region. On the other hand, monovalent antibody fragments are ideal for studies of epitope presentation and structure, as well as being used as the targeting moieties that deliver a conjugated toxin to target cells. One advantage over immunization of mice to generate antibodies (unless using those genetically engineered to have a human B-cell repertoire) is the possibility to generate fully human antibodies from display libraries.

Considerations for Selecting TCRm Antibody Targets

The ideal target for a TCRm antibody would be a disease-specific peptide–MHC complex that is present at high density on the target cell surface while being absent from other normal cells. When considering TCRm antibodies against tumor targets, such peptides are most likely to arise from overexpressed proteins, which have a short half-life and, hence, a high turnover rate (96). Targeting an antigen with a functional role in tumor biology will also help avoid loss of the antigen under subsequent therapeutic selection pressure. The peptides must also have a high affinity for the patient's MHC and form a stable complex that persists on the cell surface, allowing recognition by TCRm antibodies.

Antigens that could be promising therapeutic targets include peptides processed from mutated proteins, which are tumor specific, such as KRAS G12V/D or oncogenic fusion proteins (97, 98). Over-expressed genes, cancer testis antigens, and re-expressed oncofetal proteins are also potential tumor targets, for example, CEA and WT1 (99). The expression of these targets on normal healthy tissues must be considered when developing these as therapeutics (100). TCRm antibodies could also have use in targeting cells of the tumor microenvironment, such as regulatory T cells, tumor-associated macrophages, or cells with a role in angiogenesis (101, 102).

A key factor that needs to be considered when choosing a target antigen for TCRm antibody therapy includes the epitope expression on the cell surface. It is important to consider that it is the presentation of the epitope, and not expression of the antigen *per se*, that will determine the availability of antibody binding sites. Epitope density of TCRm antibody targets has been reported to be as low as 100–1,000 sites per cell, which is significantly lower than some epitope densities reported for traditional mAb cell surface targets at 20,000–500,000 sites per cell. Nevertheless TCRm can activate ADCC against low-density targets (82, 103, 104). Before being presented on the MHC molecule, the peptide undergoes various steps of processing from its original protein. Therefore, events at any of these steps could affect the epitope density observed at the cell surface, including the level of protein expression and its half-life, the peptide processing, the MHC levels, and the presentation of the peptide in the context of MHC at the cell surface. Proteins must be stable and translated in sufficient quantities to allow peptide processing, and it has been shown that proteins with shorter half-lives are more likely to be presented than ones with longer half-lives (105). Furthermore, it has been reported that tumors downregulate their surface MHC expression as an immune

evasion mechanism, suggesting that such tumors will be less susceptible to TCRm therapy (106, 107). The possibility of this evasion mechanism must be considered when selecting both target antigens and disease indications.

Target Epitope Discovery

Progress in our understanding of peptide processing and presentation on MHC has facilitated the discovery and evaluation of novel peptide–MHC epitopes. Initially, expression profiling was used to identify epitopes on tumor-associated antigens (TAAs) found on tumor cells—a process also called “direct immunology.” Using this method, the isolation of tumor-specific CTLs from melanoma patients led to the discovery of the first tumor-specific CTL epitope, which was encoded by the MAGE-1 gene. A cDNA library of the melanoma was generated, and melanoma-specific CTLs were used to identify the cDNA that encoded the CTL epitope (108, 109). Since this initial discovery, the use of direct immunology has led to the identification of other epitopes, including ones from the MAGE, BAGE, and GAGE families, as well as Melan-A/MART-1, tyrosinase, and gp100.

Bioinformatics techniques using algorithms to predict peptide binding to specific MHC molecules are often used to predict TAA epitopes. This process is known as “reverse immunology” and is a systematic method of identifying TAA epitopes from a defined antigen that has emerged from the recent progress in genome sequencing and *in silico* techniques. It involves a prediction phase, where potential epitopes are predicted *in silico* using algorithms. The prediction of epitopes is based on proteasome processing, binding to MHC and TAP translocation. This is followed by the validation phase, where the predicted epitopes must then be verified by MHC-I peptide binding assays or mass spectrometry to confirm that they are found on the cell surface (110).

There are a significant number of peptides that while being capable of binding MHC-I are either not presented on cancer cells or are altered, for example, by post-translational modification. Thus, there has been considerable interest in performing cancer HLA peptidome analysis to identify MHC-I bound peptides within both normal and malignant cells and tissues (111). In this approach, the HLA-complexes are immunoaffinity purified, the bound peptides are isolated and then analyzed by mass spectrometry. By comparing the MHC-I bound peptides in normal and diseased tissues, it is possible to prioritize those epitopes that are most suitable for therapy. Interestingly a meta-analysis of the HLA peptidomes from 83 mass spectrometry-based datasets from four major hematological malignancies found very few common “pan-leukemia” epitopes and these exhibited low presentation frequencies within each cohort of patients (112). Thus, in hematological malignancy, the epitopes selected for therapy are likely to be disease specific and, thus, multiple TCRm antibodies will be needed to exploit this therapeutic approach.

TCRm Antibodies and TCR-Based Therapies

Both TCRm antibodies and recombinant TCRs can bind MHC-I presented peptides. Traditionally, those employing TCR-based therapies have compared their technology to the

desirable qualities of antibodies but commented on the inability of antibodies to target intracellular antigens. Those generating TCRm/TCR-like antibodies have promoted antibodies having higher affinity and specificity than TCRs (82, 113) and an easier development route and lower cost than TCR-targeted cellular therapies. However, advances in the engineering and production of soluble high-affinity TCRs and the production of TCRm antibodies have now made these approaches much more interchangeable.

T-cell receptor mimic antibodies can be used in place of a TCR as the targeting moiety for cellular therapies, such as CAR T cells (59, 60). Alternatively, a TCR can be fused to an Ig Fc region to enable TCR-directed antibody-dependent cytotoxicity (114). ImmTACs (immune-mobilizing monoclonal TCRs against cancer) are engineered high-affinity soluble TCRs bispecifically linked to anti-CD3 that can drive an anti-tumor T-cell response (115). Some studies have reported that the orientation of binding is similar for TCRs and TCRm antibodies, with both binding their peptide–MHC target in a diagonal orientation (116, 117). TCRm antibodies can also bind in additional conformations, gaining access to epitope regions that are not naturally targeted by TCRs (94, 118).

It seems likely that both TCRm antibodies and TCRs will be used to effectively target intracellular antigens using both soluble drugs and cellular therapies. The specificity of binding of high-affinity TCRs and TCRm antibodies to the target peptide presented by MHC-I is likely to be a crucial determinant of the suitability of individual reagents for therapy. Comparative studies using TCRs to the tumor-associated antigen survivin effectively highlighted the importance of specific peptide binding. High-affinity TCRs against a survivin peptide presented by HLA-A2 isolated from an allogeneic HLA-mismatched TCR repertoire lacked the ability to distinguish high levels on tumor cells from low expression in normal tissues. This included activated T cells, leading to fratricide when the engineered T cells targeted each other for destruction. However, an autologously derived TCR to the same survivin peptide targeted tumor cells but did not cause fratricidal toxicity (119). Molecular modeling of TCR-peptide–HLA complexes and alanine scanning of the survivin peptide demonstrated that maximal peptide recognition was critical for TCR selectivity for tumor cells. Thus, the specificity of the peptide–MHC binder could be as critical as the choice of target peptide.

Future Directions for TCRm Antibodies

T-cell receptor mimic antibodies have not yet entered the clinic, although Novartis have partnered with Eureka Therapeutics and Memorial Sloan Kettering Cancer Center to develop their ESK1 TCRm targeting WT1. Several key factors have the potential to improve the development of TCRm antibodies further with the prospect of undertaking clinical studies and ultimately establishing them as cancer therapeutics. These include epitope expression, production methodology, specificity validation and mechanism of action. It is also important to consider that TCRm antibodies may represent theranostics, combining diagnostic utility to determine target epitope presentation and therapeutic activity within a single agent.

The low epitope density of peptide–MHC complexes on the cell surface poses some limitations for TCRm antibody-based therapy. This might be addressed by choosing target epitopes that do not have low cell surface expression, by increasing MHC-I expression in tumors, by making TCRm antibodies more sensitive to low density epitopes or by choosing effector mechanisms that do not require high epitope density for cytotoxicity.

High-affinity, peptide-specific TCRm antibodies have proven difficult to produce in large numbers by either traditional phage or hybridoma approaches. Enhanced display technologies, particularly those capable of isolating fully human antibodies within a short period of time, offer some exciting opportunities to accelerate future TCRm antibody discovery. Having a wider array of antibodies for characterization will improve the chances of identifying those with the necessary affinity and specificity for further development.

It is crucial to ensure that the TCRm antibody does not recognize the MHC-I alone, as this molecule is found on most nucleated cells. Therefore, the TCRm antibody must be specific for the peptide–MHC complex, which also highlights that it should not cross-react with other processed peptides. As the TCRm antibody recognizes only few amino acid residues in the peptide, it will be crucial to assess which other processed peptides possess the same amino acids at those positions and whether there would be any risk of cross-reactivity. The importance of this is exemplified in a clinical trial of an affinity-enhanced TCR, which targeted a MAGE-A3 epitope (120). Following administration of the therapy, it was discovered that the TCR also recognized an epitope on the unrelated protein titin that is expressed in cardiac tissue. The cardiac toxicity led to two patient deaths. This cross-reactivity was not observed in normal tissue screening and the titin peptide was not conserved in mice. However, a limitation of *in silico* screening by amino acid substitution is that it may identify a wider variety of potentially cross-reactive peptides than can be functionally evaluated. Furthermore, even potentially cross-reactive peptides shown to bind MHC-I in T2 presentation assays may not be processed endogenously or presented on the cell surface in normal tissues.

One of the key limitations of TCRm antibody therapy is the MHC-restricted nature of the therapy—although this is crucial to enable the recognition of intracellular proteins. Most studies to date focus on the HLA-A*0201 haplotype, which is prevalent in up to 40% of Caucasians, and in up to 20% of populations of different ethnicities, which covers a large proportion of the world's population. There are other dominant HLA alleles worldwide, including HLA-A*2402 and HLA-A*1101 in Oriental populations. Although TCRm antibodies are HLA-restricted, it has been proposed that antibodies to three HLA alleles for a particular target antigen would cover >96% of the world's population (53). Structural analysis of the TCRm antibody ESK1 shows that it binds multiple HLA-A*02 variants and not only the HLA-A*0201 subtype, which it is designed to target (121). This is due to the fact that ESK1 binds a portion of the MHC molecule that is conserved among the various HLA-A2 subtypes, thereby suggesting that the certain TCRm could target a larger population of patients with a variety of

HLA subtypes. In addition, designing TCRm antibodies that target different antigens or different epitopes on the same antigen and using a combination of these as a therapeutic regimen could increase the chances of successful tumor eradication and minimize escape variants.

The manufacture and regulatory approval pathways for TCRm antibodies are likely to have similarities to that for classical monoclonal antibodies and share commonalities with TCR-based therapies. The latter being the lack of availability of suitable animal models to study agents targeting a dual epitope where potentially neither the MHC-I or target peptide is conserved. One opportunity potentially available for TCRm antibodies would be to use *in vivo* imaging studies to study the biodistribution of a subtherapeutic dose of the TCRm antibody in early clinical safety studies.

CONCLUSION

The generation of antibodies that can target intracellular antigens offers an unparalleled opportunity to expand the repertoire of therapeutic antibodies that are available to treat human disease. When coupled with advances in genomic sequencing

technologies, proteomic investigations and the increasing numbers of antibodies being made available to the research community, new disease-related proteins and their variants (post translational modifications, splice variants, mutations, etc.) that are suitable for antibody targeting will continue to be identified. Further developments in the production technology, delivery, and regulatory approval pathways for antibodies targeting intracellular antigens should also contribute to the introduction of many new exciting antibodies into the clinic in the future.

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Conflict of Interest Statement: AB and DL are inventors, and IT is a contributor, on a patent application describing the production and characterization of TCR mimic antibodies targeting p53:HLA-A2.

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Targeting Malignant Brain Tumors with Antibodies

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Antibodies have been shown to be a potent therapeutic tool. However, their use for targeting brain diseases, including neurodegenerative diseases and brain cancers, has been limited, particularly because the blood–brain barrier (BBB) makes brain tissue hard to access by conventional antibody-targeting strategies. In this review, we summarize new antibody therapeutic approaches to target brain tumors, especially malignant gliomas, as well as their potential drawbacks. Many different brain delivery platforms for antibodies have been studied such as liposomes, nanoparticle-based systems, cell-penetrating peptides (CPPs), and cell-based approaches. We have already shown the successful delivery of single-chain fragment variable (scFv) with CPP as a linker between two variable domains in the brain. Antibodies normally face poor penetration through the BBB, with some variants sufficiently passing the barrier on their own. A “Trojan horse” method allows passage of biomolecules, such as antibodies, through the BBB by receptor-mediated transcytosis (RMT). Such examples of therapeutic antibodies are the bispecific antibodies where one binding specificity recognizes and binds a BBB receptor, enabling RMT and where a second binding specificity recognizes an antigen as a therapeutic target. On the other hand, cell-based systems such as stem cells (SCs) are a promising delivery system because of their tumor tropism and ability to cross the BBB. Genetically engineered SCs can be used in gene therapy, where they express anti-tumor drugs, including antibodies. Different types and sources of SCs have been studied for the delivery of therapeutics to the brain; both mesenchymal stem cells (MSCs) and neural stem cells (NSCs) show great potential. Following the success in treatment of leukemias and lymphomas, the adoptive T-cell therapies, especially the chimeric antigen receptor-T cells (CAR-Ts), are making their way into glioma treatment as another type of cell-based therapy using the antibody to bind to the specific target(s). Finally, the current clinical trials are reviewed, showing the most recent progress of attractive approaches to deliver therapeutic antibodies across the BBB aiming at the specific antigen.

Keywords: antibody, glioma, bispecific Ab, blood–brain barrier, receptor-mediated transcytosis, cell-penetrating peptides, single-chain fragment variable, chimeric antigen receptor-T cell

INTRODUCTION

Approximately 27,000 new cases of malignant glial tumors are diagnosed in Europe every year. The most common are glioblastoma multiforme (50%) and anaplastic glioma (10%) (1). They are associated with high morbidity and mortality because they are highly invasive and neurologically destructive (2). Gliomas penetrate throughout the brain and extend far beyond the tumor mass that is visible with neuroimaging, making them difficult to treat (3). Despite surgical resection,

radiotherapy, and chemotherapy, the median survival time is only 14–15 months for patients with glioblastoma (4) and 2–5 years for those with anaplastic gliomas (2). New approaches to treatment are needed to improve the prognosis. A promising one is antibody (Ab; in this review, the acronym Ab is used for all forms of antibodies and their fragments, unless stated otherwise) therapy, which is discussed in this review.

Targeting brain diseases such as brain cancer and neurodegenerative diseases with therapeutics is especially challenging because of the presence of the blood–brain barrier (BBB). BBB has an extremely low permeability, which helps to maintain brain homeostasis (5). In the case of brain tumors, the BBB faces some abnormalities where, besides the morphological changes in the barrier, its permeability increases because of disrupted junctions in the layer of endothelial cells. However, increased permeability during some pathological processes still does not suffice for the passage of larger molecules such as biologicals. Crossing the BBB would facilitate the Abs to reach their targets and execute their therapeutic potential. The permeability of BBB can be achieved through invasive and non-invasive methods. Invasive methods (e.g., focused ultrasound, osmotic disruption, biochemical disruption) pose certain risks of infections, toxicity, and damage to the brain. Non-invasive methods represent a much safer and convenient way for the delivery of therapeutics (6).

This review will focus on antibody tools for the treatment of malignant gliomas with different mechanisms of passage through the BBB. Several approaches, including cell-based approaches, will be discussed with their future potential, and the currently active clinical trials will be overviewed.

CROSSING THE BBB

Transcellular mechanisms of transport such as adsorption-mediated transcytosis (AMT), and particularly receptor-mediated transcytosis (RMT), have gained most interest and have shown the highest potential for the non-invasive delivery of therapeutics through the BBB into the brain (5). In AMT, positively charged molecules can interact with the negatively charged membrane of endothelial cells, upon which endocytosis and crossing of the BBB can occur. The entire process is receptor independent and non-specific (5). Several mechanisms of AMT are being explored with potential therapeutic Abs (7–9). Cationized F(ab')₂ fragment against A β plaques have shown increased permeability across the BBB (10). Other cationized proteins that could serve as carrier proteins were also investigated—for example, cationized protein G for the delivery of IgG antibodies (11). AMT is also being investigated as a mechanism for the passage of nanoparticles where targeting brain tumors with cationized liposomes has shown great promise. Cationization has not only provided an efficient passage through BBB but has also served to enhance the binding of nanoparticles to the tumor endothelium (12–14).

Binding to specific receptors has promoted the transcytosis of a bound ligand where dissociation of the bound complex occurs after being transported across the cytoplasm. Certain peptides or proteins such as insulin and transferrin enter the brain tissue by RMT where they bind to a specific receptor expressed on the luminal side of the BBB. Some of the most studied receptors

for targeting brain tissue and promoting passage through the BBB are the insulin receptor (InsR), LDL-related protein type 1 (LRP1) Receptor and transferrin receptor (Tfr) (15, 16). Another way to mediate RMT is to target specific receptors using Abs that recognize and bind to them, a strategy known as the “Trojan horse” method. Therapeutics can be designed as bispecific Abs (bsAbs) where one Ab has specificity toward a receptor expressed on the luminal side of the BBB and the other has specificity toward a therapeutic target (17). Therapeutics can also act as a cargo where they are conjugated to a receptor targeting Abs. Another interesting strategy is to use cell-penetrating peptides (CPP) as a Trojan horse for the delivery of therapeutics to brain tissue (18). All these strategies will be discussed further on in later sections.

RECEPTORS MEDIATING RMT

The most common receptors for mediating RMT (Tfr, InsR, LRP1 receptor) have been successfully used for passing the BBB (19). However, they have all shown potential drawbacks. Their expression profile is not specific for brain tissue (20–22), causing side effects (acute clinical signs and decreased reticulocyte count) (23). The drawbacks of existing model receptors for passing the BBB (19, 23, 24) have led scientists to identify new potential target receptors in the BBB (24). Since abnormalities occur in the BBB in brain tumors, the expression of potential receptors that mediate RMT must be investigated specifically for the blood–brain tumor barrier (BBTB). For instance, some membrane transporters have been found to be over-expressed in the BBTB [e.g., P-glycoprotein (P-gP), multidrug resistance-associated protein 1 (MRP 1) and 3 (MRP3)] (6).

AB PROPERTIES NECESSARY TO PASS THE BBB-ABS THAT SERVE AS A TROJAN HORSE

Nearly 50% of Abs used in malignant glioma clinical trials are intact IgG Abs (6). These conventional Abs can remain in the peripheral circulation for days to weeks. Although their persistence in the peripheral system offers a therapeutic advantage, they can exhibit poor tissue penetration due to their large size. This is especially true in the case of targeting brain tissue and crossing the BBB (25). In mouse models, it has been reported that less than 0.1% of peripherally administered Abs can reach the brain tissue, with evidence indicating that only approximately $0.009 \pm 0.001\%$ of the injected dose of systematically administered intravenous immunoglobulins reached the cortex (26). The concentration of IgG Abs in the brain is additionally rapidly decreased through the activity of a neonatal Fc receptor (FcRn) which promotes reverse transcytosis. This could also be an advantage if the mechanism of accelerated circulation of IgG with the repeated transition of IgG is favorable. However, if prolonged exposure to higher concentrations of IgG is favorable, then FcRn-mediated efflux represents a disadvantage. Several solutions have been provided to escape FcRn-mediated efflux (27). Fc inhibition (28), and the use of low-affinity FcRn activity Abs (29) have successfully reduced the efflux of Abs from brain tissue. Alternatively, the use of Ab fragments lacking the Fc region avoids this problem.

Abs must possess certain properties to play a role as a Trojan horse by mediating RMT and crossing the BBB. The binding of Abs should not interfere with the binding of endogenous proteins and should promote receptor-mediated endocytosis. Manipulation of Abs that bind TfR, by decreasing their affinity (30, 31) and shifting their valency from bivalent to monovalent (32) has been shown to increase the successful delivery of Abs. Bivalent (32) and monovalent high-affinity (31) anti-TfR Abs have been associated with lysosomal degradation due to potential dimerization of the TfR receptor (32) or have predicted poor dissociation from the Ab–receptor complex (30). It can also be speculated that different epitopes on the extracellular part of TfR play an important role, but this has yet to be evaluated.

ALTERNATIVE FORMS OF Ab

Other Ab formats have been investigated for the treatment of brain tumors. Smaller Ab formats such as Fab or scFv possess several advantages over the use of conventional Ab formats. scFvs have been the most studied Ab fragment format for targeting brain diseases. Their small size improves tissue penetration. They are also easier to produce and genetically modify. The lack of an Fc region offers the advantage of circumventing FcRn-dependent efflux from brain tissue and eliminates Ab effector functions, such as complement-dependent cytotoxicity (CDC) and Ab-dependent cellular cytotoxicity (ADCC) where further inflammatory stimuli are prevented (33). These two characteristics offer a special advantage regarding targeting brain tissue. However, the absence of an Fc region also shortens the Ab half-life. Several techniques, e.g., the addition of PEG and conjugation of scFv to other proteins or molecules prolong their half-life. scFvs have been used to target brain tissue in the form of bispecific T-cell engagers (BiTE) (34), conjugated to liposomes (35), and linked with CPP (18, 36). They have also served as a Trojan horse where they target TfR and successfully mediate the passage of a conventional anti- $\text{A}\beta$ Ab (37). To our knowledge, passive passage of scFv across the BBB has not been directly compared with conventional Abs and remains to be evaluated. Several scFvs have shown therapeutic potential when targeting glioblastoma *in vivo*; however, most of them target non-orthotopic xenografts (38), or circumvented the BBB by direct distribution using convection-enhanced delivery (CED) (39, 40) and intracerebral injection (41). scFv D2C7 linked to immunotoxin targeting glioblastoma is in the phase I clinical trial stage and is being tested by intratumoral CED (42). Although CED represents a promising drug delivery method (43), it still is an invasive method.

CPPs AS ANOTHER KEY STRATEGY TO CROSS THE BBB

Cell-penetrating peptides are a group of short peptides, consisting of amphipathic and/or cationic sequences that enable crossing the cell membranes (Figure 1C). From a therapeutic point of view, they can be conjugated to therapeutics (e.g., Ab-based) and can be used to mediate their passage through the BBB. Generally, their uptake is non-specific without the need of a transporter. Although the mechanisms

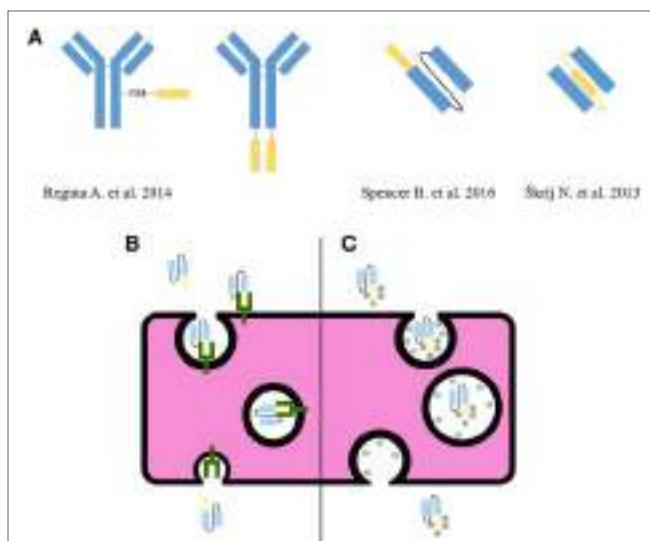


FIGURE 1 | (A) Flexibility of cell-penetrating peptide (CPP) incorporation into the Ab scaffold. Some examples efficiently passed through the BBB (18, 36, 44). The yellow color indicates CPP and the blue color indicates Abs. **(B)** CPPs can mediate RMT by binding receptor at the BBB and transporting the Ab across the cytosol to the other side of the BBB. **(C)** CPPs consisting of amphipathic and/or cationic sequences can mediate AMT and allow crossing the BBB.

of passage are still under investigation for some CPPs, AMT is the main mechanism. The non-specific uptake of peptides can be solved by incorporating a receptor targeting scaffold, as it has been shown for bi-functional liposomes conjugated to CPPs and transferrin, with improved BBB penetration compared with liposomes without included CPPs. Improved penetration most likely occurs because the incorporation of CPP overcomes receptor saturation (16). Some CPPs have been shown to target transporters at the BBB and mediate RMT (Figure 1B) (15). Their conjugation to therapeutic Abs allows efficient delivery of Abs into the brain tissue. Anti-human epidermal growth factor receptor 2 (HER2) monoclonal Ab conjugated to Angiopep-2 peptide, which binds to the LRP1 receptor in the BBB, efficiently passed through the BBB and prolonged the survival of mice with BT-474 brain tumor xenografts after systematic treatment (44).

Besides cell-penetrating properties, some peptides such as iRGD-amino-acid sequence: C(RGDKGPD) have also a more specific feature of tumor penetration. iRGD possesses affinity toward the tumor vasculature-specific αv integrins. After proteolytic cleavage, iRGD gains affinity toward neuropilin-1 (NRP-1) where it mediates further tumor tissue penetration (45). Nanoparticles conjugated to iRGD or co-administered with iRGD peptides have shown increased crossing of the BBB and enhanced intratumoral accumulation levels in glioma mouse models (46). Doxorubicin liposomes conjugated to a NRP-1-specific tumor-penetrating peptide prolonged the survival in mice and effectively crossed the BBB (47). Tumor-penetrating peptides represent a promising strategy for their ability to cross the BBB and specifically penetrate the tumor tissue. Inclusion of therapeutic Abs in the liposomes conjugated to tumor-penetrating peptides may provide further success in this field. Tumor-penetrating peptides

have also been used successfully when linked to Abs or administered together. TPP11, an NRP-1-specific peptide that also blocks the interaction of NRP-1 with vascular endothelial growth factor (VEGF), was linked to the Fc region of an anti-epidermal growth factor receptor (EGFR) monoclonal Ab. The design has allowed good tumor penetration and accumulation and has further presented anti-angiogenesis activity (48). The anti-HER2 Ab trastuzumab, when co-administered with iRGD, completely eradicates all tumors in orthotopic BT474 human breast tumor cell xenograft mouse models, whereas treatment with trastuzumab alone slows down tumor growth (45). The conjugation of tumor-penetrating peptides to Abs or their co-administration may provide enhanced therapeutic targeting of brain tumors.

Our research group has also provided a proof of flexibility in the design of Ab fragments coupled to CPP. Normally CPPs are conjugated to the C- or N-terminal end of an Ab molecule or other chemical groups on Ab (Figure 1). Our group has successfully prepared single-chain fragment variable (scFv) against the truncated form of prion protein with a penetratin used as a linker between the two variable domains. This design has allowed the passage of scFv through BBB (18). Altogether, CPPs present a prospective method to increase the brain uptake of different therapeutic Abs. New peptides against potential receptors in the BBB can be selected by phage display biopanning (49).

NANOPARTICLES AND LIPOSOMES— A VEHICLE FOR THE DELIVERY OF THERAPEUTICS INTO THE BRAIN

Nanoparticles and liposomes have proven to be efficient tools for the delivery of Ab-based therapeutics into the brain; particularly, liposomes have been extensively used to target glioma. The passage of liposomes through the BBB has already been shown to be increased by cationization (12–14), conjugation to Abs (50, 51), CPPs (47, 52–54), protein ligands of receptors at the BBB (55) or conjugation to two of them, namely protein ligands of receptors at the BBB and CPPs (16, 56). Another strategy to increase the passage through the BBB is the use of magnetoliposomes, where magnetic nanoparticles are incorporated into liposomes and external magnetic fields are used for guidance across the BBB (57). Liposomes conjugated to Abs to cross the BBB or specifically target tumor tissue are called immunoliposomes, and they are being studied extensively for the targeted drug delivery to tumor tissue. Cationic liposomes, encapsulating temozolomide and conjugated to anti-TfR scFvs, show prolonged survival and inhibition of tumor growth in an intracranial glioblastoma xenograft (U87-luc2) model (58). Liposomes can incorporate hydrophilic, hydrophobic, and lipophilic substances due to their composition where one or more lipid bilayers surround an aqueous compartment. Therefore, they are suitable for the delivery of various drugs, including Abs (59). The administration of some therapeutic Abs may lead to off-target effects and cytotoxicity. Encapsulating those in liposomes and providing specific delivery may help to circumvent this problem (60). In addition to controlled drug release and specific delivery, liposomes also present good biocompatibility, biodegradability, and low toxicity.

Although only passive targeting strategies using liposomes are currently in clinical trials, their drawbacks include poor penetration through the BBB, non-specific uptake and a low enhanced permeability and retention effect (EPR) (61). However, active targeting strategies using (tumor) penetrating peptides and Abs against receptors present in the BBB have improved their therapeutic potential. Therefore, immunoliposomes and liposomes conjugated to (tumor) penetrating peptides present an interesting, more specific targeting strategy, with controlled release of therapeutics and provide a promising strategy for targeting brain tumors. Apart from liposomes, nanoparticles can successfully cross the BBB. This can be accomplished by conjugation of nanoparticles to protein ligands of receptors at the BBB (62), CPPs (63), and Abs (64, 65). Nanoparticles also serve as carriers of various drugs, where they can be adsorbed, covalently bound or encapsulated.

BISPECIFIC Abs (bsAbs)—A PROMISING TECHNOLOGY

Bispecific Abs recognize two different epitopes. Many different technologies to produce bsAbs have been described (66, 67). The passage through the BBB can be mediated using a bsAb where one Ab's specificity recognizes a receptor at the BBB, which then promotes transcytosis. The other Ab's specificity recognizes a potential therapeutic target. Within this scaffold, a therapeutic potential and ability to promote crossing over the BBB are combined in one molecule. Until now, only bsAbs targeting TfR and beta-secretase 1 (BACE1) have been described (30, 68). The same mechanism of action could be used to target brain tumors, where one specificity would target a receptor suitable for RMT, while the other would target a tumor-specific or tumor tissue-overexpressed antigen. Affinities for specific epitopes may change when designing bsAbs; therefore, affinities for both epitopes should be adjusted to allow efficient delivery and therapeutic response.

Bispecific Abs have also been proven to mediate a more efficient therapeutic response when targeting two epitopes simultaneously. Treating (non-brain) tumors with VEGF inhibitors alone promotes tumor metastasis, VEGF-independent angiogenesis and increased hypoxia (69). Angiopoietin-2 (Ang-2), an angiogenic growth factor, was overexpressed in bevacizumab-treated glioblastomas, while translocator protein (TSPO) was upregulated in bevacizumab-treated glioblastomas and promoted apoptosis resistance. Targeting both epitopes with bsAb in bevacizumab-treated rats resulted in significantly prolonged survival and showed promise for the treatment of the aggressive and apoptotic-resistant nature of bevacizumab-treated glioblastomas (70). Another bsAb targeting Ang-2 and VEGF prolonged survival and provided other clinical benefits in a mouse brain tumor model with glioblastoma xenografts (71). However, how these bsAbs passed through the BBB has not been evaluated. One possible speculation is that small concentrations of these therapeutic Abs are sufficient for the therapeutic effect. On the other hand, Ang-2 upregulation is associated with BBB disruption and enhanced paracellular and transcellular passage (72). It could be considered that, in glioblastoma, where Ang-2 is overexpressed, the passage of Abs across BBB is enhanced through a passive mechanism.

Redirection of immune cells to target tumor cells using bsAbs offers another promising mechanism of treatment. By linking a tumor-specific epitope to a T-cell activated ligand, an immune synapse is formed. Particularly, a successful group of bsAbs in this field turned out to be a group of BiTEs, where two scFvs, each targeting its own antigen, are linked together in tandem. A BiTE, targeting a specific T-cell activated ligand, CD3, and tumor-specific mutated EGFR receptor (EGFR vIII) that is constitutively activated and is often found in glioblastoma, had promising therapeutic effects in mice using a human glioblastoma xenograft model U87MG. Δ EGFR. Treating affected mice resulted in prolonged survival, and, in the case of higher dosages, the mice were completely cured without apparent cytotoxicity (73). However, the mechanism of passage across the BBB remains unknown and was not investigated in many cases of targeted brain tumor models (**Table 1**). Enhanced passage was predicted due to the reduced size of BiTEs compared with conventional Abs, and it was assumed that they elicited their effect even when present in considerably low concentrations (74).

STEM CELLS (SCs) AS DELIVERY VEHICLES FOR Ab TO TUMORS

Stem cells are a promising strategy for *in vivo* Ab production and delivery, mainly because of their pathotropism properties and ability to cross the BBB (88, 89). Mesenchymal stem/stromal cells (MSCs) are multipotent and can differentiate into many adult cell types of mesenchymal origin (90, 91). Neural stem cells (NSCs) have self-renewal capacity and multipotent potential to differentiate into neurons, astrocytes, and oligodendrocytes (92–94).

The major problem of treating malignant gliomas is that they infiltrate the surrounding normal brain tissue and are elusive to standard therapies. MSCs and NSCs from different sources have significant tropism to tumors and are usually used in studies of therapeutic protein delivery. It was shown that both NSCs and MSCs have tumor tropism properties and can migrate toward malignant glioma, distribute across the tumor bed and continue expressing a foreign gene (95–97). NSCs were observed while migrating from the transplantation site to the tumor. They were clearly tumor tropic, but some migrated to other areas such as the hippocampus and auditory cortex (98).

Understanding the mechanisms regulating SC migration is necessary to optimize the use of SCs as therapeutic delivery vehicles (99). Glioma cells produce their own extracellular matrix (ECM) and invade the surrounding brain parenchyma by expression of additional ECM molecules, including tenascin, fibronectin, laminin, vitronectin, and different types of collagen (100). The ECM of malignant glioma facilitates NSC migration *in vitro*. When different ECM molecules were tested for NSC migration, laminin was the most permissive, whereas tenascin, fibronectin, and vitronectin also supported NSC motility (101).

It was shown that NSCs preferentially target hypoxic glioma regions *in vivo*. Knockdown of HIF-1 α , which is a master regulator of many genes involved in tumors, resulted in the inhibition of hypoxia-induced NSC tropism. Hypoxia is a key factor for NSC tropism and the process is mediated by stromal derived factor

1/chemokine receptor type 4 (SDF-1/CXCR4), urokinase-type plasminogen activator/its receptor (uPA/uPAR), VEGF/VEGFR2, and hepatocyte growth factor (HGF)/c-Met signaling pathways (102). HGF and other growth factors [VEGF, epidermal growth factor (EGF) and transforming growth factor α (TGF α)] can also induce the migration of NSCs. This is similar to the migration of cancer cells in glioma invasion, only that it is deregulated and constitutive (103). IL-8 appears to be another chemoattractant promoting SC migration. The migration of MSCs toward a glioma cell line was enhanced also by the overexpression of its receptor chemokine receptor 1 (CXCR1) in MSCs. This implies that the overexpression of CXCR1 could be a way of improving MSC tropism in glioma therapy (104). It was also shown that both MSCs and NSCs show significantly greater migration toward cancer cell lines of solid tumors that express high levels of uPA and uPAR compared with those with low uPA/uPAR expression. Therefore, MSCs and NSCs can use multiple cytokines for tropism to tumors, but a common feature is the expression of uPA and uPAR (105).

The migratory capacities of MSCs and NSCs to brainstem glioma were compared *in vitro* and *in vivo*, and it was shown that MSCs from various sources have similar migratory capacities to NSCs. It was also reported that not all but only approximately 30% of all SCs migrated to the target glioma from the injection site (forebrain). It is possible that only astrocytic precursors migrate to the tumor (106). Understanding the mechanism of NSC glioma targeting can help in designing genetically engineered NSCs with optimal cytokines and receptor combination for effective NSC migration and drug delivery to solid tumors.

The tumor tropism of SCs can be exploited to deliver therapeutic agents selectively to tumors. MSCs were first tested for the delivery of therapeutic proteins to tumors in pulmonary metastases (97) and later on gliomas using an intracranial glioma model and hMSCs engineered to release interferon beta (IFN- β) (99). For NSCs, it was reported that, using an immortalized NSC cell line expressing an anti-cancer prodrug (rCE; activates CPT-11), a tumor-free survival of 100% of mice (model of pediatric neuroblastoma) for longer than 6 months was achieved. MSCs continue to replicate *in vivo* and incorporate into tumor stroma and could possibly support tumor growth. They also engraft in the bone marrow of recipients, whereas NSCs are only detectable in the bone marrow if tumor cells are present. Thus, it was proposed that NSCs may be preferable to MSCs when a relatively short-term survival of SCs is desirable, such as in cancer therapy (107). These pioneer studies serve as a foundation for other SC therapies combined with Abs against glioma or other cancers. Studies where SCs expressing Abs were used are summarized in **Table 2** and are described below.

Neural stem cells were genetically engineered to secrete properly assembled anti-HER2 Ab (trastuzumab equivalent), which can inhibit the proliferation of HER2-positive breast cancer *in vitro*. GM NSCs could deliver these Abs to human breast cancer xenografts in mice. The anti-HER2 Ab was detected only at the tumor site but not in the blood of NSC-treated mice, showing the potential for a robust localized anti-tumor effect with minimal systemic toxicity (111). In a later study, the anti-HER2 Ab SC therapy was tested for its efficacy against brain tumors

TABLE 1 | Ab-based therapies targeting glioma models *in vivo*, their proposed mechanism of passage and their therapeutic outcomes (2013–present).

Therapeutic agent	Mechanism of passage	Brain tumor model	Therapeutic outcome	Reference
1 ANG-4043: anti-HER2 Ab conjugated to CPP Angiopep-2	RMT	Intracranial breast ductal carcinoma ^a xenograft (BT-474) in mice	Increase in median survival (for 80 days)	(44)
2 Anti-Ang-2/TSPO bispecific Ab	Unknown	Intracranial glioblastoma xenograft (GL261) in mice; glioblastoma bearing rats treated with bevacizumab prior to treatment	Reduced tumor size and increased survival in mice; increased overall survival and reduced macrophage infiltration in rats	(70)
3 Anti-Ang-2/VEGF bispecific Ab	Unknown	Intracranial glioblastoma xenografts (GL261, MGG8) in mice	Decreased vessel density, delayed tumor growth, prolonged survival, reprogramming of macrophages in GL261 mice; prolonged survival and reprogramming of macrophages in MGG8 mice	(71)
4 Anti-EGFRvIII/CD3 BiTE	Unknown	Intracranial glioblastoma xenograft (U87MG.ΔEGFR) in mice	Prolonged survival and complete cure rates up to 75%	(75)
5 NZ-1-(scdsFv)-PE38KDEL: anti-podoplanin immunotoxin	n/a—CED	Intracranial medulloblastoma ^a (D425MED) xenograft in mice	Increase in survival (41%)	(39)
6 D2C7-(scdsFv)-PE38KDEL: anti-EGFR/EGFRvIII immunotoxin	n/a—CED	Intracranial glioblastoma xenografts (43MG, NR6M and D270MG) in mice	Increased survival (43MG by 310%, NR6M by 28%, D270MG by 160%)	(76)
7 IP10-EGFRvIII scfV	n/a—i.c.	Intracranial glioblastoma xenograft (U87MG.ΔEGFR) in mice	Reduced tumor growth and prolonged survival	(77)
8 Anti-PD-1 Ab (\pm radiation therapy)	Route of administration is unknown	Intracranial glioblastoma xenograft (GL261-Luc) in mice	Long-term survival (180 + days) for 15–40% of animals	(78)
9 Ficlatuzumab (\pm temozolomide)	Unknown	Intracranial glioblastoma xenograft (U87MG) in mice	Prolonged survival in monotherapy. More prolonged survival in combination therapy where 80% of animals remained free of clinical signs of the disease after treatment	(79)
10 mAb9.2.27: anti-NG2 Ab (\pm NK cells)	n/a— intra-lesional treatment	Intracranial glioblastoma xenografts (U251-NG2, U87MG) in rats	Prolonged median survival time (combination therapy: U251-NG2 for 5,5 days and U87MG for 52 days)	(80)
11 AMG 595: Ab drug conjugate anti-EGFRvIII conjugated to DM1	Unknown	Intracranial glioblastoma xenograft [D317(EGFRvIII positive)] in mice	Inhibition of tumor growth	(81)
12 TTAC-0001: anti-VEGFR-2/KDR Ab	Unknown	Intracranial glioblastoma xenograft (U87MG) in mice	Inhibition of tumor growth	(35)
13 Nanocomplex scL-TMZ: cationic liposomes encapsulating temozolomide and conjugated to anti-TfR scFv	RMT	Intracranial glioblastoma xenograft (U87-luc2) in mice	Inhibition of tumor growth, prolonged survival	(58)
14 Anti-EGFRvIII Ab + rapamycin	Unknown	Intracranial glioblastoma xenograft (U251-EGFRvIII) in mice	Prolonged median survival time (combination therapy by 31,5 days)	(82)
15 Anti-Ang2 Ab + cediranib	Unknown	Intracranial glioblastoma xenografts (U87, GL261) in mice	Prolonged median survival time (combination therapy U87 by 21 days and GL261 by 18 days), slower tumor growth rate in the GL261 model, development of early necrosis in the U87 model, structural vessel normalization in both models, alteration of tumor-associated macrophages	(83)
16 Anti-CD47 Ab	Unknown	Intracranial glioblastoma xenografts (GBM4, GBM5) in mice	Reduced tumor burden, survival benefit, alteration of tumor-associated macrophages	(84)
17 Anti-GITR Ab + radiation therapy	Unknown	Intracranial glioblastoma xenograft (GL261-luc) in mice	Combination therapy: improved survival, delayed tumor progression, a subset of cured long-term survivors	(85)
18 Anti-CD40 Ab	n/a—CED	Intracranial glioblastoma xenografts (GL261, NSCL61, bRITs-G3) in mice	Prolonged survival	(86)
19 Bevacizumab	n/a—transcranial focused ultrasound	Intracranial glioblastoma xenograft (U87) in mice	Increase in median survival time (135%)	(87)

^aNot glioma models.

TABLE 2 | Therapies with SCs expressing Abs and Ab fragments against brain tumor antigens and their outcome in preclinical studies.

Stem cell	Therapeutic protein	Brain tumor model	Outcome	Reference
1 NSC HB1.F3	Full length anti-HER2 Ab (trastuzumab equivalent)	Breast cancer brain metastases (BT474Br cells)	Significant improvement of survival in mice (approximately 30 days)	(108)
2 NSC	EGFR-specific nanobodies (ENbs) and ENb2-TRAIL immunoconjugate	Intracranial glioblastoma model (U87)	Significant inhibition of tumor growth with NSC-ENb2 and complete prevention of outgrowth with NSC-ENb2-TRAIL; increased survival; inhibition of tumor invasiveness	(109)
3 hMSC	Anti-EGFRvIII scFv	Intracranial glioma xenografts (U87-EGFRvIII)	Survival prolonged for 1 week in mice; an additional injection further prolonged survival	(110)

(**Table 2**). In a breast cancer brain metastases mouse model, the intracranial injection of NSCs secreting anti-HER2 Ab showed a significant improvement in survival. It was reported that anti-HER2 Ab secreted by NSCs binds to HER2-overexpressing human breast cancer cells and inhibits PI3K-Akt signaling and inhibits growth *in vitro*. PI3K-Akt signaling is activated by HER2 dimerization and leads to increased invasion responsible for metastatic breast cancer. These benefits are not efficient against brain metastases if the Ab fails to penetrate the BBB (108).

Neural stem cells were also tested for the delivery of EGFR-targeting nanobodies (ENbs) or ENb-derived immunoconjugates (**Table 2**). They maintained transgene expression *in vivo* and *in vitro* over a period while maintaining stem properties. ENbs secreted by NSCs inhibited EGFR signaling *in vitro* and reduced glioblastoma growth in mice but did not result in significant regression of the tumor size. To increase the efficacy, an ENb2—tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) immunoconjugate was designed. This induced caspase-3/7-mediated apoptosis in GBM cell lines with various degrees of TRAIL resistance. With some cell lines, it was indicated that simultaneous EGFR inhibition might sensitize the cells to TRAIL-induced apoptosis. It was also reported that continuous exposure of tumor cells to ENbs is more effective than a single high dose (109).

hMSCs were engineered to express an scFv Ab against EGFRvIII on the cell surface (**Table 2**). Engineered MSCs showed enhanced binding to U87-EGFRvIII cells *in vitro* and an increased retention in U87-EGFRvIII expressing tumors *in vivo* (110). Down regulation of pAkt was also observed. The growth of U87-EGFRvIII xenografts was inhibited, and survival was significantly improved after *in vivo* treatment with scFvEGFRvIII hMSCs. An additional injection of engineered hMSCs further prolonged the survival. Adding an additional therapeutic gene to these SCs may boost their therapeutic potential even more. The use of GM MSCs with scFv to target tumor-specific antigens, such as EGFRvIII, might achieve stem cell accumulation at the tumor site and prolong therapeutic effect (112).

The presented potential therapies were all performed using intracranial or intravenous injection of SCs, but the first method is invasive and not optimal for repeated administrations. The second method does not deliver the largest number of cells to the brain and can lead to off-target effects although intravenously injected SCs have the potential to cross the BBB and localize to tumors. Intranasal delivery is showing promise in overcoming this challenge. Studies have shown that the intranasal delivery

of MSCs or NSCs modified for drug delivery can prolong the survival of glioma animal models (113, 114).

T-CELL THERAPY

In recent years, adoptive T-cell transfer therapy was developed, where tumor-specific T cells are rapidly expanded *ex vivo* and transferred to patients. T cells used in therapy can also be modified to increase their specificity and survival or become resistant to immune evasion mechanisms. Activated T cells (ATC) can cross the BBB irrespective of their antigen specificity, so they are suitable for glioma therapies (115, 116). A chimeric antigen receptor (CAR) can be inserted that encodes Ab fragments specific for tumor-associated antigens. CARs provide T-cell activation regardless of MHC-restricted presentation (117). Potential glioma-specific antigens currently targeted by CAR-T are HER2 (118), EGFRvIII (119–121), EphA2 (122), and IL13Ra2 (123, 124).

A promising use of this technique in glioma therapy is arming anti-CD3-activated T cells with bsAbs that target the T-cell receptor and the tumor-associated antigen and can redirect the non-MHC-restricted cytotoxicity to ATC to lyse tumors. Good targets for this treatment are antigens expressed on glioma stem cells (GSCs). It was reported that arming ATC with either HER2 or EGFR bsAb converts ATC into a specific cytotoxic T cell (125). A recombinant bsAb against the epitopes CD133 and CD3 was developed and locally applied together with autologous CD8⁺ cells. The bsAb redirected polyclonal T cells to CD133⁺ GSCs, where it induced their targeted lysis and prevented the outgrowth of glioblastoma xenografts (126).

CLINICAL TRIALS OVERVIEW

In May 2017, over 70 active clinical trials (including pilot studies) addressing the use of Abs in gliomas were registered at clinicaltrials.gov (Table S1 in Supplementary Material). The roles of Abs in these studies are various and include Abs used as agonistic or antagonistic drugs individually or in combination with other Abs, other biologicals, chemotherapeutics, radiotherapeutics, or surgery. Moreover, the combinatorial use of Abs makes them an invaluable tool (e.g., vehicle) in Ab-drug conjugates, Ab-radiodrug conjugates or (with tremendous gain of popularity; **Table 3**) a part of a molecular construct expressed on the cell surface (CAR on T cells) to bring the drug/toxin,

TABLE 3 | Overview of the current phase III clinical trials in Ab-based drugs.

Drug	Target antigen	Ab Type	Phase	Cancer type	Sponsor
1 Bevacizumab (with or w/o Vorinostat, Temozolomide, radiation)	VEGF-A	humanized monoclonal Ab	Ph II, Ph III	High-Grade Glioma	National Cancer Institute (NCI), USA
2 Bevacizumab (with or w/o Lomustine)	VEGF-A	humanized monoclonal Ab	Ph III	Recurrent glioblastoma	European Organisation for Research and Treatment of Cancer—EORTC
3 Bevacizumab (combined with or w/o Temozolomide and radiation)	VEGF-A	Humanized monoclonal Ab	Ph III	Glioblastoma	National Cancer Institute (NCI), USA
4 Nivolumab (with or w/o Bevacizumab and Ipilimumab)	1. PD-1 2. VEGF-A 3. CTLA-4	1. Human monoclonal Ab 2. Humanized monoclonal Ab 3. Human monoclonal Ab	Ph III	Recurrent Glioblastoma	Bristol-Myers Squibb
5 Nivolumab (with or w/o Temozolomide, Radiation)	PD-1	Human monoclonal Ab	Ph III	Glioblastoma	Bristol-Myers Squibb

Currently, the most commonly targeted antigen in glioma by Ab-based drugs is VEGF-A, followed by programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). All biological treatments include chemo- and/or radio-therapy or the use of other biologicals.

TABLE 4 | Recent chimeric antigen receptor-T cell (CAR-T)-based clinical trials in glioma.

Biological/drug	Target	Ab type	Clinical Trial Phase	Cancer Type	Sponsor
1 HER2-specific T cells	HER2	scFv	Ph I	Glioblastoma	Nabil Ahmed, Baylor College of Medicine, USA
2 Genetically modified HER.CAR CMV-specific CTLs	HER2	scFv	Ph I	Glioblastoma	Nabil Ahmed, Baylor College of Medicine, USA
3 Anti-EphA2 CAR-T	EphA2	scFv	Ph I, Ph II	Malignant glioma	Fuda Cancer Hospital, Guangzhou, China
4 Anti-EGFRvIII CAR-T (with Aldesleukin, Fludarabine, Cyclophosphamide)	EGFRvIII	scFv	Ph I, Ph II	Malignant glioma	National Cancer Institute (NCI), USA
5 Anti-MUC1 CAR-T cells	MUC-1	scFv	Ph I, Ph II	MUC-1 positive solid tumors, glioma	PersonGen BioTherapeutics (Suzhou) Co., Ltd., China
6 IL13Ra2-specific, hinge-optimized, 41BB-costimulatory CAR/truncated CD19-expressing Autologous T lymphocytes	Interleukin-13 receptor alpha 2 (IL13Ra2)	scFv	Ph I	Malignant glioma	City of Hope Medical Center, USA
7 Anti-MUC1 CAR-pNK cells ^a	MUC-1	scFv	Ph I, Ph II	MUC-1 positive solid relapsed or refractory tumor, glioma	PersonGen BioTherapeutics (Suzhou) Co., Ltd., China
8 Anti-HER2 CAR-T	HER-2	scFv	Ph I, Ph II	HER2 Positive Cancer, glioma	Zhi Yang, Southwest Hospital, China
9 EGFRvIII CAR T cells	EGFRvIII	scFv	Ph I	Glioblastoma	Gary Archer Ph.D., Duke University Medical Center, USA
10 CMV-specific cytotoxic T lymphocytes expressing CAR targeting HER2 (HERT-GBM)	HER-2	scFv	Ph I	Glioblastoma	Nabil Ahmed, Baylor College of Medicine, USA
11 HER2-specific T cells (iCAR)	HER-2	scFv	Ph I	Glioblastoma	Nabil Ahmed, Baylor College of Medicine, USA

Currently, the most commonly targeted antigens in glioma by CAR-T based cell therapy is HER-2, followed by EGFRvIII, MUC1, EphA2, and IL13Ra2.

^aThese studies also include anti-MUC1 CAR-pNK cells, where NK cells are used in place of T cells. Most of the trials use a single type of therapy, without preconditioning.

radiodrug or a therapeutic cell (a payload) to its specific antigen target in glioma.

The predominant therapeutic Ab-based drugs in these trials are the humanized blocking Abs anti-VEGF-A (Bevacizumab) and human anti-PD1 Ab (Nivolumab). As part of more complex therapy regimens, humanized anti-VEGF-A Abs and human anti-PD1 Abs are also among the five current phase III clinical trials (**Table 3**). Both bevacizumab (127–129) and nivolumab

(130–133) have been a part of clinical trials of glioma for some time alone or combined with other treatment types. Bevacizumab is currently FDA approved for the treatment of glioblastoma that recur after treatment. However, it may be used in the off-label setting if the treating physician prescribes this treatment. “Off-label” indicates the use of an approved treatment for any purpose other than that described in the treatment’s FDA-approved labeling (American Brain Tumor Association).

Fragment Ab-based drugs tested in current clinical trials are used as a vehicle in ADC (D2C7-immunotoxin) (42, 134); or, in most clinical trials, as a part of therapeutic CAR-T cells described in the previous sections, or CAR-pNK cells in one case. The CARs in current studies are led by scFv against five antigens (HER-2, EGFRvIII, MUC-1, IL13R α 2, and EphA2), specific for antigens expressed on glioma cells and/or other solid tumors. As opposed to the biological or combined drugs used in **Table 3** and Table S1 in Supplementary Material, in the case of CAR-T (**Table 4**), we are dealing with cellular therapeutics and, thus, potentially an additional problem in reaching the target by breaching the BBB. However, only one of the current clinical trials on CAR-T cells uses an intratumoral or intracavitary or intraventricular administration of CAR-T cells (**Table 4**). This implies that the BBB (impaired in brain malignancies) can be adequately breached and that therapeutic cells migrate toward and act against a specific antigen-labeled tumor cell (135, 136).

DISCUSSION

Targeting brain tumors and other brain diseases represents a major issue because of the inaccessibility of brain tissue for therapeutics, especially biologics. The aim of therapy is to achieve specific targeting to brain tissue and further on to tumor tissue. Although potential glioma-specific antigens have been identified (118–124, 137–142), the major obstacle still resides in the (in)ability for the specific passage of therapeutics through the BBB to reach tumor tissue in adequate concentrations. In the past few years, many different mechanisms for reaching brain pathologies have been investigated. A Trojan horse method seems especially attractive where Abs and CPPs represent the key players. In the role of a Trojan horse, Abs have already successfully mediated the passage of liposomes containing chemotherapeutics (58), other therapeutic Abs (37), and nanoparticles carrying therapeutic peptides (65). Based on targeting the TfR receptor, it has been shown that Ab valency (32) and affinity (30, 31) are crucial for efficient RMT, and caution must be taken when designing new Abs to mediate RMT. Anti-TfR Abs provide an important insight into how important tuning the interaction and mechanism of interaction can be for the efficient passage through the BBB. When we find an appropriate target and raise an Ab against it, we must evaluate the most appropriate avidity of the therapeutic Ab that would allow the most efficient transcytosis, without redirecting it to the lysosomal pathway. This process is most likely dependent on the target receptor and epitope. To maximize uptake and exposure of a therapeutic Ab, a therapeutic dose must be selected. The saturation concentration of the receptor and decrease in the Ab concentration over time must be considered. Another key player to mediate RMT are CPPs, and they have already successfully mediated the delivery of therapeutic Abs (44), liposomes containing chemotherapeutics (47) and nanoparticles (46). The design of Abs conjugated to CPPs is relatively simple and flexible due to their small size as we have shown previously (18). CPPs present a prospective method to increase the brain uptake of therapeutic Abs. Regarding therapeutic strategies, liposomes and nanoparticles have gained interest and have shown promise as carriers for therapeutics. bsAbs,

combining the role of a Trojan horse and a therapeutic agent, have been investigated only for targeting Alzheimer's disease (30, 68), and their promise as a therapeutic agent for glioblastoma remains to be seen. bsAbs have already been investigated for targeting glioblastoma in the role of mediating a T-cell response (143) and targeting two antigens simultaneously (71, 144). We have not discussed the role of nanobodies for targeting brain diseases. They appear promising since they possess an advantage of high stability, solubility, and small size, providing better tissue penetration, as well as low immunogenicity. A small molecular size and high isoelectric point (pI) have been shown to influence their passive passage through the BBB, possibly *via* ATM (145). However, their concentration in brain tissue remains low (146), and further investigation regarding their passage through the BBB and modifications is needed to evaluate their full therapeutic potential for targeting brain tumors. Although there have been some advances in the discovery of mechanisms for the passage through the BBB, most *in vivo* experiments on brain tumor models still do not investigate the passage and needed concentration for the efficiency of Ab-based therapeutics in orthotopic brain tumor models (**Table 1**). Many evaluations of potential therapeutics targeting brain tumors circumvent this obstacle by using CED, i.e. administration or non-orthotopic models. Many different Ab-based therapeutic strategies are currently known that present promising future therapies against glioblastoma. However, deeper knowledge regarding the passage through the BBB, identification of new target receptors, Trojan horse agents, and more research in the field of novel therapeutics design and combinational therapy will provide the tools needed for more efficient and safer treatment of brain tumors. The major pitfall resides in the evaluation of the bioavailability of Abs needed to exert their therapeutic potential in the brain. Only few studies have quantitatively assessed the Abs' capacity to pass the BBB and remain in the brain (26, 147, 148). Also the same methods must be used for evaluation of the capacity to allow comparison among them. Different Abs have different biochemical characteristics, such as amino-acid sequence, isoelectric point, and degree of hydrophobicity. These characteristics can affect the Abs' physiological properties, including capability to cross the BBB and remain in the brain parenchyma (26). Therefore, we must be careful when comparing capacities of different Abs (e.g., polyclonal Abs to monoclonal Abs) to cross the BBB. Another issue is to extrapolate the findings found in mouse models to humans. Therefore, more studies quantitatively evaluating the capacity of different Ab-based therapies must be performed with the same methods for the evaluation of these properties and allowing comparison between them.

Using SCs for the delivery of therapeutic proteins, including Abs to tumors, seems to be a promising mode of anti-glioma therapy. The main advantages are the ability to cross the BBB and tumor tropic properties, while the largest disadvantages presently are the lack of experience with this sort of therapy and its potential side effects. The results of the first in-human study [NCT01172964 (149)] provided the base for future SC-based clinical trials for patients with brain tumors (primary or metastatic). The NSC cell line used in the study could be further used for the delivery of other anti-tumor drugs, such as Abs. The principle can also be used for other SCs in clinical trials. Main

issues that still need to be resolved are the SC lineage and source, immunogenicity, and route of administration. The mechanisms underlying tumor tropism, crossing the BBB and other therapeutic advantages of SCs need to be studied further. Currently, only a few preclinical studies use stem cells as delivery vehicles for Abs or Ab fragments against brain tumors, but they show the potential for the use of Ab-expressing SCs in future clinical studies. It is important that an Ab specific for glioma cells is used that has an adequate therapeutic effect. This calls for meta studies to identify and functionalize reliable glioma-specific markers that could be used as targets to identify and remove these cells.

Adoptive T-cell transfer represents a promising technique in future anti-glioma therapy, especially the use of CARs, which encode scFv Abs specific for tumor-associated antigens fused with endo- and transdomains. However, there are still many challenges to overcome before routine clinical use. Some of these include the loss of antigen in recurring tumors and safety concerns if the antigen is also recognized at low levels in healthy cells.

The number and variety of current clinical trials (**Tables 3** and **4**; Table S1 in Supplementary Material) show a strong interest in Abs as therapeutic tools. As therapeutic tools Abs can be used either as an active component, vehicle or else. The form of Abs in pharmaceutical formulation can include Abs either as a whole molecule or fragments and can be used either individually or combined with another type of treatment. The frequency of certain therapeutics being used in clinical trials individually or in combination narrows down the current antigens of interest for the future development of Ab-based drugs. Certain drugs being used in other tumors are also being tested in gliomas. The experience with one of the most common Ab-based drugs being used in glioma in clinical trials in the past years, an anti-angiogenic drug, showed that its application changed the tumor phenotype by increasing hypoxia and leading to a metabolic switch toward glycolysis (128, 142). This metabolic switch, in turn, led to increased cell invasion in glioblastoma (150). The metabolic adaptability of GBM cells highlights the difficulty of targeting one specific

metabolic pathway for effective therapeutic intervention (151). Thus, by suppressing one specific metabolic pathway, other fronts emerge that we may not be able to anticipate. Currently, the way this is being handled is by combining anti-angiogenic treatment with others (**Table 3**). Also, a reliable tumor cell marker must be most thoroughly investigated and functionalized preclinically prior to defining it as an adequate drug target.

Therefore, the strongest issues noted here that need to be addressed in the future remain (i) the ability of the Ab-based drug to pass the BBB and reach therapeutic concentrations *in situ*, (ii) functional, fully characterized tumor-specific antigens that would limit the delivery or action of the Ab to tumor cells only and minimize the (cytotoxic, invasive, or else) side effects, and (iii) the immunogenicity of biological and cell-based therapies.

AUTHOR CONTRIBUTIONS

RR, NN, VČŠ, and UR contributed to the conception and design of this work, drafted the work, approved of the final version to be published and agreed 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.

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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.2017.01181/full#supplementary-material>.

TABLE S1 | Clinical Trials Ph I, II.

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Antitumor Activity of a Mesenchymal Stem Cell Line Stably Secreting a Tumor-Targeted TNF-Related Apoptosis-Inducing Ligand Fusion Protein

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Mesenchymal stem cells (MSCs) are currently exploited as gene delivery systems for transient *in situ* expression of cancer therapeutics. As an alternative to the prevailing viral expression, we here describe a murine MSC line stably expressing a therapeutic protein for up to 42 passages, yet fully maintaining MSC features. Because of superior antitumoral activity of hexavalent TNF-related apoptosis-inducing ligand (TRAIL) formats and the advantage of a tumor-targeted action, we choose expression of a dimeric EGFR-specific diabody single-chain TRAIL (Db-scTRAIL) as a model. The bioactivity of Db-scTRAIL produced from an isolated clone (MSC.TRAIL) was revealed from cell death induction in Colo205 cells treated with either culture supernatants from or cocultured with MSC.TRAIL. *In vivo*, therapeutic activity of MSC.TRAIL was shown upon peritumoral injection in a Colo205 xenograft tumor model. Best antitumor activity *in vitro* and *in vivo* was observed upon combined treatment of MSC.TRAIL with bortezomib. Importantly, *in vivo* combination treatment did not cause apparent hepatotoxicity, weight loss, or behavioral changes. The development of well characterized stocks of stable drug-producing human MSC lines has the potential to establish standardized protocols of cell-based therapy broadly applicable in cancer treatment.

Keywords: mesenchymal stem cells, apoptosis, non-viral transfection, TNF-related apoptosis-inducing ligand, diabody, cell-based therapy, mouse xenograft

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stem cells that have generated a great deal of interest since their first identification in 1960s by Friedenstein (1, 2) due to their exceptional capabilities, foremost multilineage differentiation potential (3, 4), and hypoimmunogenic properties (5, 6). Because of these features, MSCs were early on applied in the field of regenerative medicine (7) and subsequently in a variety of other diseases, including autoimmune disorders, cardiovascular malignancies, and liver diseases [reviewed by Squillaro et al. (8)].

In addition, because of their tumor-homing capability [reviewed by Hagenhoff et al. (9)], MSCs are currently exploited as cell-based delivery systems for cancer protein therapeutics (10, 11).

Conceptionally, it is anticipated that through tumor homing of MSC the localized production of a given therapeutic protein is advantageous over systemic application of a recombinant protein considering not only effective *in situ* concentrations of the drug and thus favorable pharmacokinetic parameters but also minimizing unwanted systemic actions, often being the dose-limiting factor in clinical application. The TNF-related apoptosis-inducing ligand (TRAIL), also known as Apo2L (12), has raised great hopes for a novel, broadly applicable treatment of cancers due to its apparently selective induction of tumor cell apoptosis. However, the clinical trials with a recombinant soluble form of TRAIL, consisting of a non-covalently assembled homotrimer, by and large, failed to show therapeutic activity (13, 14), whereas inadvertently existing agglomerates in preparations of soluble TRAIL displayed toxic activity toward non-malignant tissue, in particular hepatocytes (15). Over the past decades, many recombinant versions of TRAIL have been generated to enhance its pharmacokinetics and/or antitumoral activity (16–18). By now, it is evident that the failure of a soluble, strictly trimeric TRAIL in clinical trials is not only due to very short serum half-life but even more related to the fact that proper death receptor activation requires stable receptor crosslinking, which can be achieved by at least a hexavalent organization of the TRAIL molecule (19). Nevertheless, to cope with insufficient pharmacokinetic properties, several studies have addressed the use of *in situ* production of a standard soluble TRAIL molecule by different adult stem cells (20–22). Further, two studies have reported antitumoral activity of human MSC expressing antibodies in a diabody format (23, 24).

So far, use of viral vectors prevails to introduce therapeutic genes into stem cells, despite still existing safety concerns [reviewed by Stuckey and Shah (25)] because, conceptionally, viral transduction allows the use of autologous, patient-derived stem cells for gene delivery. However, due to the apparently low immunogenicity of MSCs, allogeneic transplantation is effectively used in regenerative medicine (26, 27) and, thus allows an alternative concept for *in situ* cell-based production of protein therapeutics. Based on these considerations and on knowledge about the requirements of effective apoptosis induction by TRAIL ligands, we investigated whether it is possible to generate a MSC line stably producing a highly bioactive, tumor-targeted single-chain TRAIL fusion protein under retention of its full MSC properties. Here, we report on the establishment of such a cell line (MSC.TRAIL) and its therapeutic activity in a xenotransplantation tumor model.

MATERIALS AND METHODS

Cell Lines

Mouse bone marrow-derived MSC have been previously described (28) and were kindly provided by Dr. Angelika Hausser (IZI, University of Stuttgart, Germany). These cells were cultivated under sterile conditions, at 37°C in a 5% CO₂ humidified atmosphere, in alpha-MEM supplemented with 10% FBS (HyClone) plus 1% penicillin/streptomycin. MSCs were passaged at a confluence of 70% every 3–4 days if not mentioned otherwise. Colo205 and HCT116 cells were obtained from ATCC

(Manassas, VA, USA) and cultured, at 37°C and 5% CO₂, in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS (Thermo Fisher Scientific).

MSC Transfection

Mesenchymal stem cells were transfected with polyethylenimine (PEI) using a ratio 1:3 for DNA and PEI. Briefly, 150 × 10³ cells/well in a six-well plate were grown in 2-ml culture medium for 18 h. Next, cell culture medium was removed, and 1.5 ml of serum-free alpha-MEM was added. Three hundred microliters of Opti-MEM were incubated with 12 µg of PEI for 5 minutes (min) at room temperature (RT). Next, 4 µg of plasmid DNA was added to the mixture, and after 20 min incubation, the mix was carefully added drop-wise to the cells. After 18 h incubation at 37°C cells were transferred into a flask and allowed to grow in cell culture medium for 24 h. Next, in order to select the transfected cells, 250 µg/ml of geneticin (G418) was added to the medium. Subsequently, a single clone selection, making limiting dilutions with a statistical density of 1 cell/well was performed. The best clone, named MSC.TRAIL, was used for further studies. The coding sequence of Db-scTRAIL (EGFR-specific pCR3-Db-scTRAIL) construct was reported by Siegemund et al. (19).

Purification of Recombinant Proteins

The EGFR targeting Db-scTRAIL fusion protein (see Figure S1 in Supplementary Material) was produced from transfected MSCs and purified from cell culture supernatant by anti-FLAG affinity chromatography as described previously (19). In brief, cell-free supernatant was incubated with anti-FLAG M2 Affinity Gel (0.3 ml bead volume/100 ml supernatant, Sigma-Aldrich) for at least 2 h or alternatively overnight at 4°C on a roller mixer, prior to collecting of beads in an empty column, washing with TBS, and eluting with 100 µg/ml FLAG peptide in TBS. After dialysis in PBS, eluates were concentrated with Vivaspin 20 devices (50 kDa, Sartorius), and the purified protein was analyzed by western blotting.

Cell Death Assays

Colo205 (4 × 10⁴ cells/well), HCT116 (3 × 10⁴ cells/well), or MSCs (2 × 10⁴ cells/well) were grown in 100-µl culture medium in 96-well plates for 18 h, followed by treatment either with serial dilutions of Db-scTRAIL proteins or supernatant from transfected MSCs, in triplicates. For positive control, cells were killed with 0.25% Triton X-100. Cell death assays were performed in the absence or presence of bortezomib (BZB) (250 ng/ml; UBPBio). TRAIL blocking antibody (1 µg/ml; Enzo Life Sciences) was used in the combined treatments. BZB was added 30 min before incubation with the proapoptotic proteins to sensitize cancer cells for cell death induction. After 16 h of incubation, cell viability was determined by crystal violet staining (19).

Coculture of MSCs and Cancer Cells

Colo205 (1 × 10⁴ cells/well) or HCT116 (8 × 10³ cells/well) were seeded in 24-well plates, in 600 µl of MSC medium (alpha-MEM), and allowed to grow at 37°C. After overnight incubation, MSCs were added using different ratios of MSCs:colorectal cancer (CRC) cells, in a final volume of 1 ml/well. Different treatments

with BZB (250 ng/ml) and TRAIL blocking antibody (1 µg/ml) were performed and finally analyzed by crystal violet staining as described above.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay assays were performed using the kit OptEIA™ human TRAIL ELISA Set (BD), according to the manufacturer's instruction. Briefly, the ELISA plate was coated with 100 µl/well of capture antibody and incubated overnight at 4°C, and the remaining binding sites were blocked with 2% (w/v) dry milk/PBS. Next, a titration (1:3) of standard TRAIL protein and either serial dilutions of MSC supernatant or serum blood (dilution 1:20) were added and incubated for 2 h at RT, in duplicate. After five washing steps, working detector solution was incubated for 1 h at RT. Bound proteins were detected using ELISA developing solution (0.1 mg/ml TMB, 100 mmol/l sodium acetate buffer, pH 6.0, 0.006% H₂O₂). The reaction was stopped with H₂SO₄ (1 mol/l). Absorbance was measured at 450 nm in an ELISA reader.

Western Blotting

TNF-related apoptosis-inducing ligand secreted by transfected MSCs in culture medium was purified by an anti-FLAG affinity chromatography as described above. The purified proteins were separated on SDS-PAGE (12%) and then blotted on PVDF membrane. After incubation with primary antibody (anti-TRAIL MAB687, R&D), the secondary anti-mouse HRP-conjugated antibody (Sigma-Aldrich) was added. Finally, the membrane was treated with a peroxidase substrate (enhanced chemiluminescence detection system from Pierce) according to the manufacturer's instructions to visualize the signals and exposed to an X-ray film.

Flow Cytometry

To analyze the expression of surface markers, MSCs (10×10^4 cells/well) were seeded in a 96-well plate and incubated with the directly conjugated antibody (CD9, CD44, CD71, CD105, CD14, and CD34). Signals from respective isotype control antibodies were subtracted from all samples to compensate unspecific antibody binding.

Propidium iodide (PI, Sigma-Aldrich) staining of cells was done after 18 h of treatment. The cells were collected, incubated with 10 µg/ml of PI, and immediately analyzed by flow cytometry.

In order to test cleaved caspase-3 activation, Colo205 (8×10^4 cells/well) were seeded in the bottom of a transwell plate (Costar) and incubated at 37°C overnight. Then, BZB (250 ng/ml) was added into the culture medium and incubated for 30 min. Next, into the upper chamber of the transwell, 16×10^3 cells for each MSC line were seeded. After 18 h of coculture, Colo205 cells from the lower chamber were collected, fixed in PFA (4%), and permeabilized with 0.1% Triton X-100. Then, primary antibody (Asp 175, Cell Signaling Technology) was incubated for 1 h at RT. After two washing steps and secondary antibody incubation, the cells were resuspended and analyzed.

Immunofluorescence and Microscopy

Mesenchymal stem cells were seeded on glass coverslips and incubated for 3 h at 37°C. Then, coverslips were washed with PBS and cells were fixed with 4% PFA and permeabilized with 0.1% Triton X-100. The blocking step was performed by incubating the cells with 5% FBS in PBS for 30 min at RT. Next, cells were washed and incubated with primary antibody and DAPI (1 µg/ml, Sigma) diluted in blocking buffer for 2 h. When required, incubation with secondary antibody, diluted in blocking buffer, was performed for 1 h. Coverslips were mounted in Fluoromount G and analyzed with the Spinning Disc (Zeiss) using 488, 543, and 633 nm excitation and a 20×/0.8 DIC objective lens. Images were processed with ZEN software.

Adipogenic and Osteogenic Differentiation

For the adipogenic differentiation, MSCs were grown to confluence on Permanox 4-well chamber slides (Thermo Scientific). Then, the culture medium was replaced with adipogenic medium (α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1 µM dexamethasone, 500 µM IBMX, 10 µg/ml human insulin, and 100 µM indomethacin) and changed every 2–3 days. Twelve days after initial adipogenic induction, cells were washed with PBS and fixed for 10 min in 4% Histofix (Roth). Next, cells were rinsed once with H₂O, incubated in 60% isopropanol for 5 min, and then with Oil Red O for 10 min. Finally, the cells were washed once with 60% isopropanol followed by H₂O. Nuclei were counterstained with hemalaun. As a negative control, cells grown in culture medium for 12 days were used. In order to analyze the osteogenic differentiation, MSCs were grown to 90–100% confluence in 24-well plates and then cultured in osteogenic medium (α-MEM supplemented with 15% FBS, 1% penicillin/streptomycin, 100 nM dexamethasone, 50 µg/ml ascorbate-2-phosphate, and 10 mM beta-glycerol phosphate) for 21 days. At this time point, the differentiation was assessed by Alizarin Red staining. In brief, cells were washed with PBS and allowed to dry for 5–10 min. Next, cells were fixed with 50% ethanol for 20 min. The fixed cells were then stained with 1% Alizarin red (Roth) at pH 6.4 for 30 min under continuous shaking. Finally, cells were rinsed three times with H₂O, and transmitted light pictures were taken. As a negative control, cells grown in culture medium for 21 days were used.

Xenograft Mouse Tumor Model

Animal care and all experiments performed were in accordance with federal guidelines and had been approved by university and state authorities. Female NMRI nu/nu mice (Janvier), 8 weeks old, were injected subcutaneously (s.c.) with 3×10^6 Colo205 cells in 100 µl PBS at left and right dorsal sides. Treatment started 10 days after tumor cell inoculation when tumors reached ~100 mm³. In particular, 4×10^6 MSCs were resuspended in 100 µl PBS mixed with 100 IU/ml of heparin (29) and then peritumorally injected (p.t.). During the injections of all cell lines, mice were anesthetized with isoflurane. The Colo205-bearing mice received three p.t. injections of MSCs at day 10, 17, and 27. In addition, 5 µg of BZB in 100 µl PBS were injected i.p. every second day, starting from day 11 until day 31. Mice in the control groups received either 100 µl PBS i.p. injected or MSCs.Mock. Tumor growth was monitored as described (30). Blood samples were taken from the

tail at day 31, centrifuged ($10,000 \times g$, 10 min, 4°C) and then stored at -80°C . Activity of ALT was determined by an enzymatic assay accordingly to the manufacturer's instructions (BIOO Scientific, Austin, TX, USA).

Statistics

All values are expressed as means \pm SD, while for the analysis of the *in vivo* studies the 95% confidence interval (95% CI) was used. Significances, for each experiment, were calculated with GraphPad prism one-way ANOVA with Tukey's post-test. In particular: * represents a p -value < 0.05 , ** represents a p -value < 0.01 , and *** represents a p -value < 0.001 .

RESULTS

Engineered MSCs Express Bioactive Soluble Db-scTRAIL

As a prerequisite to study the application of MSCs as cell-based therapy for Db-scTRAIL fusion protein expression, we first investigated the TRAIL sensitivity of these cells in comparison to a CRC cell line Colo205. In accordance with our previous study (19, 30), combinatorial activity of the Db-scTRAIL fusion protein with the apoptosis sensitizer BZB exerts a potential superior apoptotic effect on CRC cells. In fact, we observed a strong enhancement of cell death induction on Colo205 cells upon combined treatment resulting in a ~9-fold increase of TRAIL-mediated apoptosis induction with BZB (EC₅₀ values:

Db-scTRAIL 19 pM; Db-scTRAIL + BZB 2.2 pM) (Figure 1A). Conversely, under the same conditions, MSCs were fully resistant to Db-scTRAIL activity even in the presence of the sensitizer (Figure 1B), confirming that MSCs are a well-suited cell delivery system of highly active TRAIL variants. Next, we tested Db-scTRAIL expression after transient transfection by ELISA and immunoblotting as well as *in vitro* bioactivity. MSCs were able to secrete Db-scTRAIL, revealing an accumulation of intact product over the observed time period of 5 days (Figures 1C,D). With the applied transient transfection protocol, Db-scTRAIL production, as revealed by specific ELISA and bioassay (induction of Colo205 cell death) was highest 1 day after transfection (~0.44 pg/cell) and declined thereafter, with still significant protein and bioactivity detectable after 5 days of culture and four subsequent media changes (Figure 2). Additionally, we tested cell death induction with PI staining after coculture of MSCs and Colo205 at ratios of 1:5 and 1:50, showing a significant increase of PI levels in a cell-dose and BZB-dependent manner (Figure S2 in Supplementary Material). These data collectively demonstrate that MSCs are a suitable system for the expression of bioactive Db-scTRAIL proteins.

Stable MSCs TRAIL Cell Line Induce Tumor Apoptosis by Caspase 3 Activation *In Vitro*

Based on the positive results with transient transfection of MSCs, we aimed at generation of stable producer clones by standard

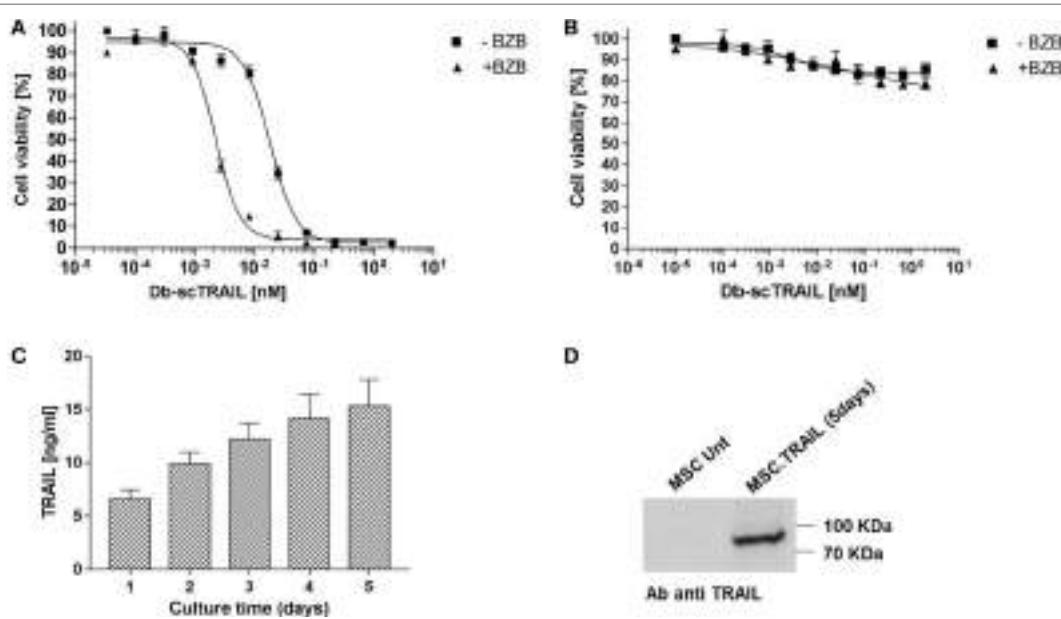


FIGURE 1 | Sensitivity of Colo205 and mesenchymal stem cells (MSCs) to diabody single-chain TNF-related apoptosis-inducing ligand (Db-scTRAIL) activity. (A) Colo205 cells and (B) MSCs were treated with serial dilutions (titration 1:3) of Db-scTRAIL in the absence (filled squares) or in the presence (filled triangles) of 250 ng/ml of bortezomib (BZB). After 18 h, cell viability was determined using crystal violet staining. Data were normalized using BZB-treated cells or cells treated with normal medium for Db-scTRAIL + BZB or Db-scTRAIL alone, respectively (mean \pm SD, $n = 3$). (C) MSCs were transiently transfected (PEI), and the amount of soluble Db-scTRAIL released in cell culture medium was measured by enzyme-Linked Immunosorbent Assay, every 24 h (mean \pm SD, $n = 3$). (D) After 5 days of transient transfection, Db-scTRAIL secreted in cell medium was purified and analyzed by western blotting using a specific antibody against TRAIL (MSC.Unt: MSC untransfected, MSC.TRAIL: MSC transfected with TRAIL).

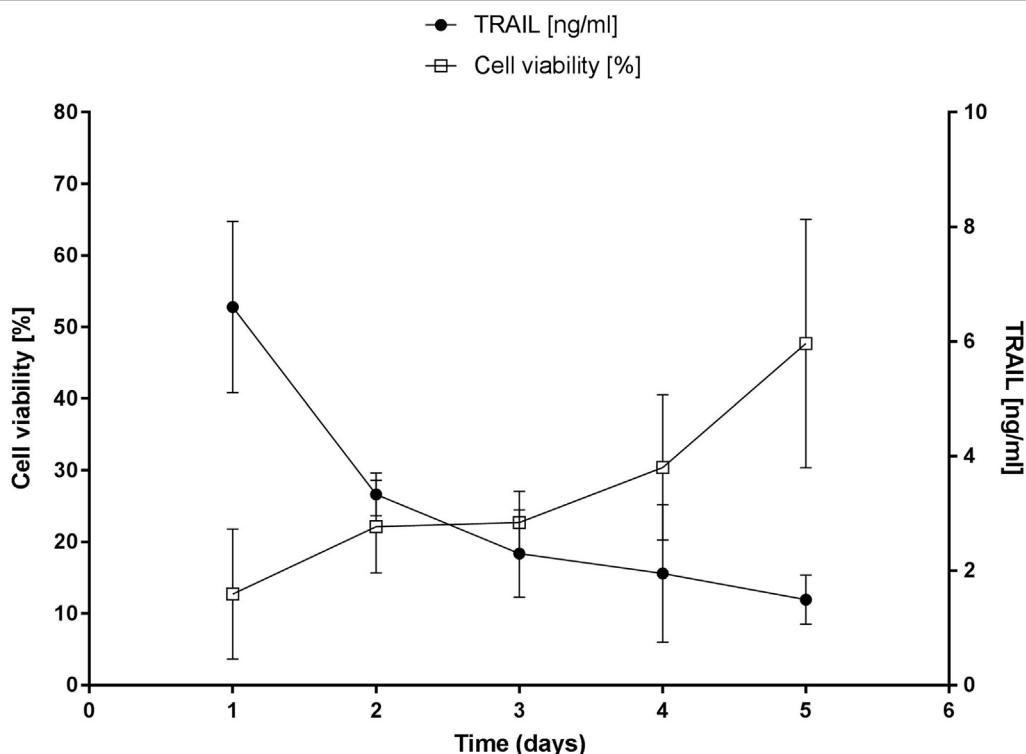


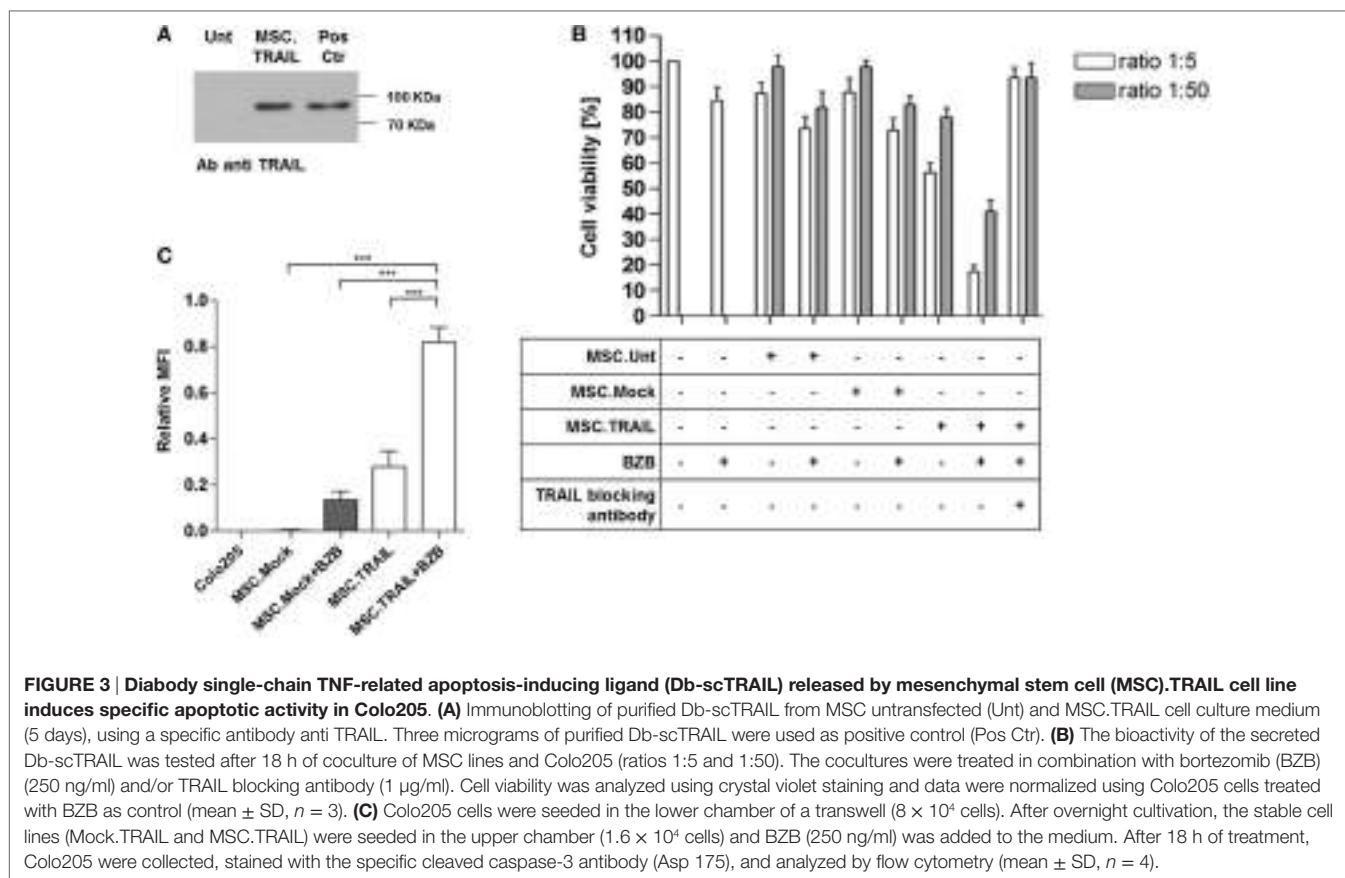
FIGURE 2 | Cell death induction assay of secreted diabody single-chain TNF-related apoptosis-inducing ligand (Db-scTRAIL) on Colo205. Left axis: Colo205 cells were sensitized with bortezomib (BZB, 250 ng/ml) and treated with an aliquot of daily collected and renewed medium (dilution 1:3) from transiently transfected mesenchymal stem cells (MSCs). After 18 h of treatment, cell viability of Colo205 was determined using crystal violet staining and data were normalized using BZB-treated cells as control (mean \pm SD, $n = 4$). Right axis: the daily amount of soluble Db-scTRAIL released in cell culture medium of MSCs was measured by enzyme-linked immunosorbent assay (mean \pm SD, $n = 3$).

selection methods and isolation of individual clones by limiting dilution. The two highest expressing clones out of 13 identified positive clones after the first screening round proofed to be stable expressors *in vitro* and one clone, named MSC.TRAIL was used for further analyses of long-term expression and *in vivo* activity (Figure S3 in Supplementary Material). MSC.TRAIL showed cumulative secretion of the protein during culture for 5 days and stable expression of the product *in vitro* for 44 passages (data not shown). Western blot analysis of the purified TRAIL verified secretion of full-length protein (Figure 3A). Bioactivity of the secreted Db-scTRAIL was tested in coculture assays with Colo205 as target cells in the presence or absence of BZB (Figure 3B). A strong reduction of the cell viability in combination with BZB and complete neutralization of cell death in the coculture by a TRAIL blocking antibody proofed secretion of bioactive protein and strictly TRAIL-dependent cell death. The same results were observed after coculture of MSC.TRAIL with a different CRC cell line (HCT116) sensitive to TRAIL (Figures S4A,B in Supplementary Material). In order to confirm that the observed reduction of cell number is due to an apoptotic process, we analyzed cleaved caspase-3 levels as a hallmark of apoptosis induction. For this, we performed coculture using a double chamber system with a membrane allowing free exchange of soluble mediators. Colo205 cells were seeded in the bottom chamber and the MSC.TRAIL or the Mock cells were seeded in the upper

chamber. Cleaved caspase-3 levels in Colo205 cells were determined by flow cytometry after 18 h of coculture (Figure 3C). As expected, a strong increase of cleaved caspase-3 levels was found when Colo205 cells were exposed to Db-scTRAIL-producing cells in combination with the sensitizer BZB. MSC.Mock served as negative control and a weak signal was noted upon incubation with sensitizer BZB only. Collectively, the *in vitro* studies show that stable MSC producer clone can be established exerting long-term expression of a highly bioactive Db-scTRAIL fusion protein.

MSC Properties Are Not Affected by Stable Transfection *In Vitro*

In order to investigate whether the transfection and isolation of a stable producer cell line affected MSC characteristics *in vitro*, we analyzed the properties of this cell line at different passages and compared it to mock-transfected and untransfected MSCs. We first tested the phenotype of MSCs by staining the cells with phalloidin to visualize the F-actin. All cell lines (MSCs untransfected, MSC.Mock, and MSC.TRAIL) displayed a typical spindle-shaped phenotype as described for MSCs (31). Remarkably, the phenotype did not change during *in vitro* cultivation up to passage 42 (Figure 4A). Next, we investigated the expression of stem cell markers. In accordance with the International Society of Cellular Therapy, all MSC lines analyzed were positive for CD9, CD44,

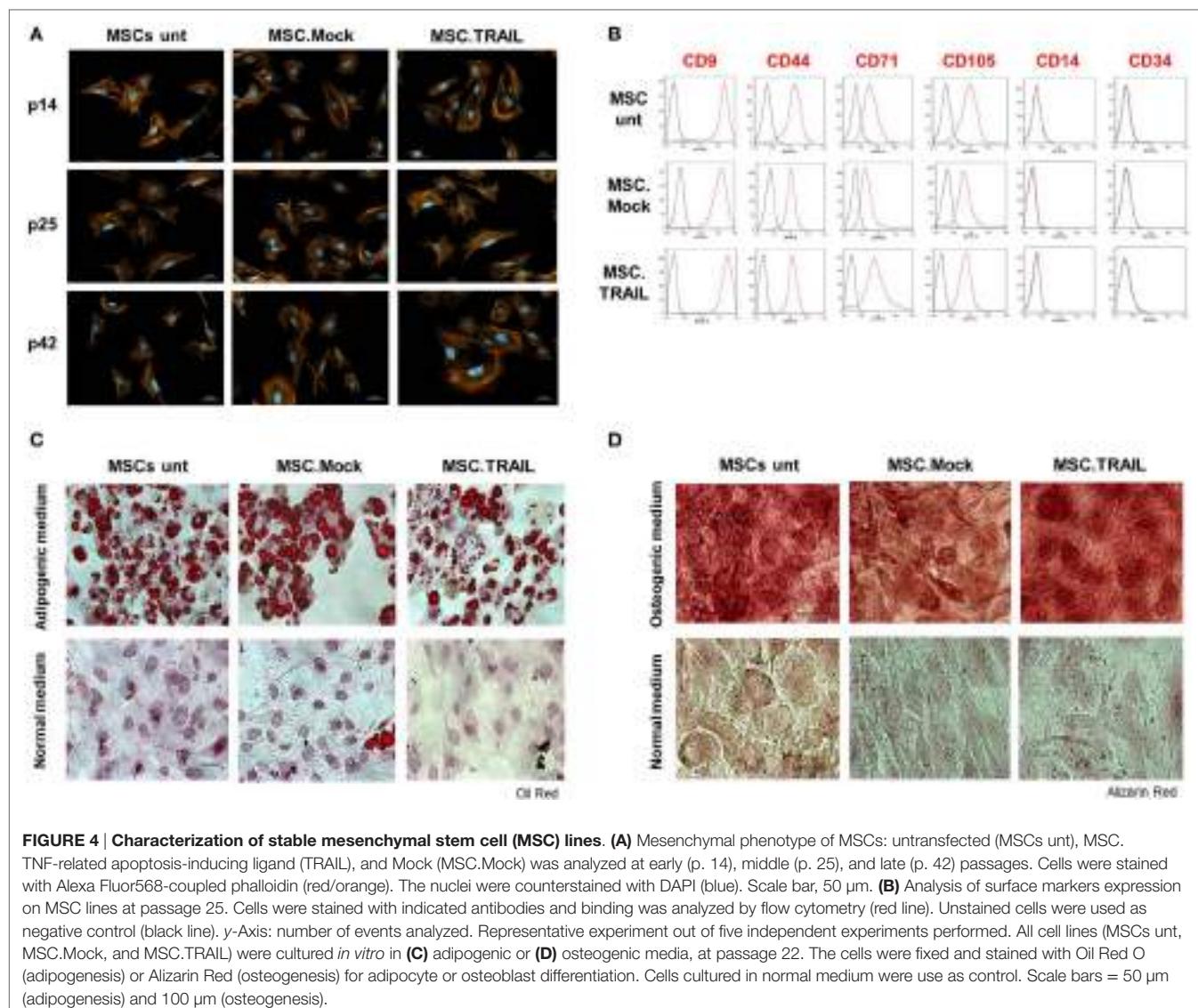


CD71, and CD105 and lacked the expression of CD14 and CD34, as shown in **Figure 4B**. No differences between untransfected MSCs and the stably transfected cell lines were observed. Interestingly, the pattern of marker expression was maintained from early passage (p9) up to passage 42 (Figure S5 in Supplementary Material) and is in accordance with murine MSCs lines described by others [reviewed by Boxall and Jones (32)]. Finally, we verified the multilineage differentiation capability of MSCs, which is the most characteristic feature. All MSC lines showed, on one hand, the capability to generate lipid droplets which indicate a successful adipogenic differentiation (**Figure 4C**). In rare cases, a spontaneous adipogenic differentiation was observed, probably due to a high cell density in the differentiation cultures, without a statistically significant frequency. Further, the cell lines were also able to display mineralization, observed by Alizarin red staining, confirming osteogenic differentiation ability (**Figure 4D**). The same results were observed at late passages for all cell lines (data not shown). These findings demonstrate that the stable non-viral transfection and the constitutive Db-scTRAIL secretion do not alter the typical properties of MSCs, even during long-term *in vitro* culture.

MSC.TRAIL Exert a Significant Antitumor Activity in Combination with BZB *In Vivo*

Based on our *in vitro* results, we assessed the potential therapeutic utility of MSC.TRAIL *in vivo*. First, we performed single

subcutaneous injection of MSCs (s.c.; 4×10^6 cells) in nude mice in order to verify the presence of Db-scTRAIL in the serum fraction after 1, 3, 7, 14, and 21 days by ELISA (Figure S6 in Supplementary Material). No TRAIL signals could be detected up to 3 days after injection, whereas after 7 and 14 days specific TRAIL signals were identified in the range of 1.5 ng/ml for MSC.TRAIL cells. As expected, all control cell injections gave no positive signal. Next, we investigated the antitumor activity of MSC.TRAIL in a Colo205 mouse xenograft tumor model, in which MSCs were peritumorally (p.t.) injected at three time points. The treatments started when tumors were palpable and vascularized, reaching a volume of ~ 100 mm 3 . At this time point, the first MSC (4×10^6 cells) injection was performed, with Mock cells and PBS used as controls. In the combination treatment groups (MSC.TRAIL + BZB and MSC.Mock + BZB), 5 µg of BZB was intraperitoneally (i.p.) injected every other day. Up to 10 days after the first MSC injection, we observed no differences in tumor growth for all groups. However, from day 17 on, coincident with the second MSC administration, a slight, but increasingly significant reduction of tumor size was observed for the combination treatment group MSC.TRAIL + BZB over the whole observation period (31 days) (**Figure 5A**). Importantly, at day 26, serum analysis of Db-scTRAIL in animals receiving MSC.TRAIL revealed a concentration of ~ 1.5 ng/ml (**Figure 5C**) supporting a direct correlation of TRAIL activity and tumor reduction. Tumor response was maintained with the third application of MSC.TRAIL, although



complete remission was achieved only in one case (**Figure 5C**). The observed MSC.TRAIL-dependent tumor response required co-administration of a low dose of the sensitizer BZB, corroborating the *in vitro* data. Thus, under the applied protocol, the MSC.TRAIL cells alone showed only a small, non-significant reduction in tumor growth, similar to the Mock-transfected MSC in combination with BZB (**Figure 5B**).

Administration of MSC.TRAIL *In Vivo* Does Not Induce Acute Side Effects

In order to get insights into potential off-target, systemic side effects of continuous Db-scTRAIL expression in tumor-bearing animals, we analyzed serum levels of the liver enzyme alanine aminotransaminase (ALT) as an established marker of acute liver toxicity. ALT serum levels were determined in tumor-bearing mice at day 31, after three MSC p.t. injections. The analysis showed for all MSC groups only slightly elevated serum ALT levels compared to PBS group (<50 U/l), but low compared

to the Fas ligand treatment known to cause acute liver toxicity (**Figure 5D**). This result revealed that the applied treatment protocol (three MSC administrations with and without BZB) did not induce discernable acute hepatotoxic effects *in vivo*, in accordance with a recent study from Yan and colleagues (21). Additionally, all the other standard parameters, such as body weight, remained in the normal range for the entire period of treatment (data not shown).

DISCUSSION

In this study, we explored the possibility of generating stable MSC producer cell lines as delivery system for the expression of an antitumor protein drug, using a tumor-targeted variant of the proapoptotic protein TRAIL as a model substance. Since its first identification (33, 34), TRAIL was extensively studied due to its characteristics of inducing apoptosis in human cancer cell lines while sparing normal cells (35, 36). However, TRAIL-based

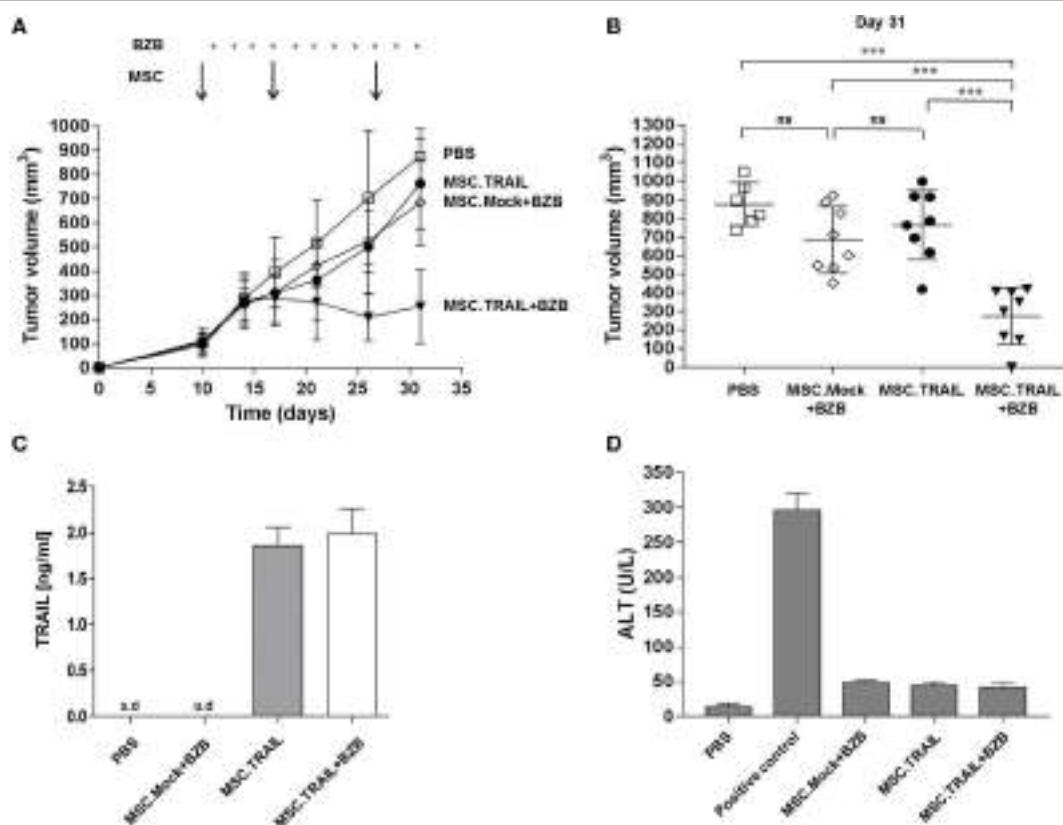


FIGURE 5 | Antitumor activity of mesenchymal stem cell (MSC).TNF-related apoptosis-inducing ligand (TRAIL) in a Colo205 xenograft tumor model.

(A) Tumor volume was analyzed as a function of time after p.t. injection of: PBS (squares), MSC.Mock + BZB (diamonds), MSC.TRAIL (filled circles), or MSC.TRAIL + BZB (filled triangles). Arrows, MSC p.t. administrations (4×10^6 cells/injection on day 10, 17, and 27); asterisks, BZB application (5 µg every second day); $n = 8$ tumors/treatment group. (B) Individual tumor volumes at day 31 ($n = 6$ tumors for PBS group and $n = 8$ tumors for MSC.Mock + BZB, MSC.TRAIL, and MSC.TRAIL + BZB). Bars, mean of tumor volumes \pm 95% confidence interval (CI) (n.s.: not significant; *** $p < 0.001$). (C) Diabody single-chain TRAIL mouse serum levels were analyzed at day 26 by enzyme-linked immunosorbent assay assay (mean \pm SD, $n = 3$; u.d., under detectable level). (D) Alanine aminotransaminase (ALT) activity was analyzed in mouse serum, at day 31, after three MSC p.t. injections (mean \pm SD, $n = 3$). Positive control, 0.1 nmol Fas ligand fusion protein; negative control, PBS.

therapies tested in several clinical trials, in a broad range of different tumors, yielded very disappointing results [reviewed by Lemke et al. (37)]. Three major features are considered to limit the therapeutic activity of conventional recombinant TRAIL proteins, low *in vivo* bioactivity and short plasma half-life (38), intrinsic or acquired resistance to TRAIL (39), and inefficient delivery of the proapoptotic protein to the tumor cells, altogether requiring multiple doses with potential increase in side effects (40). In attempts to overcome these intrinsic negative features of recombinant soluble TRAIL, several studies in different tumor models, including ovary-, lung-, colon-, and pancreas-derived tumors, exploited the possibility of a transient cell-based TRAIL expression making use of MSC as delivery system because of their presumed tumor-homing potential (41–43). Despite that the specific role of MSCs in the tumor microenvironment is not fully understood, different studies reported that TRAIL expressed by MSCs can overcome resistance in colorectal and breast cancer to treatment with recombinant TRAIL (44, 45). This suggests that the advantage of using MSC-based *in situ* production of soluble TRAIL is not only favorable to overcome the short plasma

half-lives of this antitumor drug but also may contribute to break TRAIL resistance of tumor cells.

Based on this knowledge, we aimed at an improvement of MSC-based drug delivery systems from two sides, the producer cell and the product itself. Thus, using a murine MSC line as a model which was shown to maintain its phenotype and differentiation potential *in vitro* and *in vivo* (28), we questioned whether instead of transient expression, stable producer clones can be obtained to lay the ground for similar approaches with human MSC and for establishment of defined drug producer cell banks suitable for allogeneic transplantation in cancer patients. Concerning the therapeutic protein, we reasoned that second-generation TRAIL molecules, with tumor targeting features and optimized apoptotic potential are functionally superior to conventional soluble TRAIL. A cell-based *in situ* expression system of the model drug, an EGFR-specific diabody single-chain TRAIL (Db-scTRAIL), comprising a hexavalent TRAIL, could be well advantageous over costly GMP expression and purification of such a complex molecule. MSCs isolated from three different sources proved to be insensitive to apoptosis induced by

human recombinant TRAIL, despite the expression of DR4 and DR5 (46). Because of a low intrinsic bioactivity of such soluble TRAIL preparations, and the several orders of magnitude higher bioactivity of the Db-scTRAIL used here, we first confirmed the insensitivity of MSCs to Db-scTRAIL, even in combination with the sensitizer BZB. Thus, the model cell system used qualified for establishing a producer cell.

Concerning the transfection method, despite that the majority of the studies so far used viral vectors to genetically modify stem cells, this technology is still debated. This is due to the fact that some of these viral vectors, like lentiviruses, are immunogenic and show instability of the transgene, which can cause severe immune responses when introduced into the patients [reviewed by Stuckey and Shah (25)]. Additionally, the specific integration site of the vector DNA into the genome of the cells is crucial, and disruptions of essential genes that may cause malignant transformation have to be avoided. Despite these potential safety issues of viral transduction methods, the prospects of higher efficiency so far have favored this over non-viral transfection methods for stem cells (47, 48). Therefore, with the aim to provide alternative approaches to generate MSC drug producer cell systems, in this study, we exploited a non-viral transfection method based on PEI. We achieved the isolation of a stable and long-term expressing MSC line producing highly bioactive Db-scTRAIL under retention of its full MSC typical differentiation capability. Moreover, a significant reduction of tumor volume could be achieved already after two peritumoral administrations of MSC.TRAIL, showing that the localized production achieves therapeutically effective doses of the drug when combined with BZB, and the systemically detectable levels of the Db-scTRAIL fusion protein were well tolerated by the treated animals.

A particular feature attributed to MSCs is their potential tumor-homing capacity (9). Because we detected circulating levels of the Db-scTRAIL upon local s.c. injection of MSCs in non-tumor bearing animals, we focused in this study on analysis of macroscopic tumor regression; therefore, we cannot tell to which extent peritumorally injected MSC might have migrated to the tumor tissue and whether or not this was instrumental for the observed tumor regression. Further, the *in vivo* fate of transferred MSCs remains to be defined in future studies. Specifically parameters affecting the duration of therapeutics production, such as long-term survival with or without potential tissue-specific differentiation need to be unraveled.

Currently, the clinical application of MSC-based therapy in the regenerative medicine is widely accepted with clear benefits (49–51) while its use in oncology is still in an early exploratory phase and a general treatment concept is not yet available. Provided the model described here can be translated into a clinical application, the data obtained here suggest that with local administration of a stable producer line, aside from achieving clear tumor responses, potential additional benefits for the patients could be expected, for example concerning the frequency of therapeutic injections. In fact, in the case of TRAIL therapeutics, the standard clinical treatments but also animal tumor models with second-generation TRAIL reagents require daily or biweekly injections (19, 52). While based on the obtained results using

stable producer MSCs, a regimen with a reduced frequency of administrations, weekly or even less frequent, seems achievable. Importantly, an efficient translation of cell-based therapy into clinical application often requires the ability to readily administer a safe and efficacious product at the optimal dosage. Toward this aim, an established MSC producer cell bank suited for allogeneic transplantation potentially offers enormous advantages over autologous transplantation concerning time constraints and the unclear chance of isolation of autologous MSCs from the patient suitable to use for transfection and successful protein expression. Specifically, in the context of autologous sources, patients are generally older and may present with multiple comorbidities, may impact MSC isolation and propagation both in quantitative and qualitative terms. Because of the hypo-immunogenic feature of MSCs in general, autologous transplantation appears not mandatory. Accordingly, we propose that a well-characterized stock of MSC producer lines, as the model cell line described here, is a robust alternative to cell-based expression systems relying on autologous patient material.

In conclusion, in the present study using a murine MSC line and xenografted tumors as model system, we revealed proof of concept that a stable MSC line expressing a therapeutic protein and maintaining MSC characteristics can be generated and can be applied *in vivo* to achieve a significant tumor response without apparent side effects. Our results support the exploitation of this approach for generation of stable well-characterized cell banks of human MSC producer lines for local expression of highly active, yet difficult or costly to produce, cancer therapeutics.

ETHICS STATEMENT

Animal care and treatment were carried out in accordance with the local Ethical Committee guidelines at University of Stuttgart on the use of experimental animals and experiments approved by State authorities (Regierungspräsidium Stuttgart) under no. 35-9185.81/0413.

AUTHOR CONTRIBUTIONS

IM contributed to the design of the work, performed the experimental work and data analysis, and contributed to data interpretation. MH, RK, and MS were responsible for the design and genetic engineering of Db-scTRAIL molecules and contributed to study data analyses. KP was responsible for the overall concept and design of the study, data interpretation, and the final manuscript. All the authors contributed to manuscript writing and approved the submitted 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.2017.00536/full#supplementary-material>.

FIGURE S1 | Scheme of the diabody single-chain TNF-related apoptosis-inducing ligand fusion protein. An EGFR-specific scFv antibody fragment, comprising V_H and V_L , was fused to the N-terminus of scTRAIL in which three extracellular TRAIL domains are genetically linked to one polypeptide chain. A peptide linker (GGGGS) between V_H and V_L was chosen to obtain a diabody configuration and, therefore, dimerization of the molecule. F, FLAG tag.

FIGURE S2 | Analysis of cell death induction after coculture of Colo205 with mesenchymal stem cells (MSCs). One day after transient transfection, MSCs (MSC.TNF-related apoptosis-inducing ligand) were cocultured with Colo205 cells (1×10^5 cells) in the presence or absence of BZB (250 ng/ml) for additional 24 h. Two different ratios of MSCs and Colo205 were tested, 1:5 and 1:50. After 18 h of coculture, cell viability was analyzed by PI staining. y-Axis: number of events analyzed.

FIGURE S3 | Single clone selection. Thirteen clones selected after Dot Blot analysis were seeded (1×10^6 cells) and cultured. After 3 days, the amount of

soluble diabody single-chain TNF-related apoptosis-inducing ligand released in culture media was measured by enzyme-linked immunosorbent assay. Mesenchymal stem cells untransfected (UNT) and the pool cell line were used as controls (mean \pm SD, $n = 3$; #, under detectable level).

FIGURE S4 | Diabody single-chain TNF-related apoptosis-inducing ligand (Db-scTRAIL) secreted by mesenchymal stem cell (MSC).TRAIL cell line induces apoptotic activity in HCT116. HCT116 were (**A**) treated with serial dilutions (titration 1:3) of purified Db-scTRAIL in the absence (circles) or in the presence (squares) of 250 ng/ml of BZB or (**B**) cocultured with MSC lines (MSC:HCT116 ratios 1:5 and 1:50) in combination with BZB (250 ng/ml) and/or TRAIL blocking antibody (1 μ g/ml). After 18 h, cell viability was analyzed using crystal violet staining, and data were normalized using HCT116 cells treated with BZB as control (mean \pm SD, $n = 3$).

FIGURE S5 | Surface mesenchymal stem cells (MSC) marker expression. Analysis of MSC marker expression at passages (**A**) 9 and (**B**) 42. Cells were stained with indicated antibodies and binding was analyzed by flow cytometry (red). Unstained cells were used as negative control (black). y-Axis: number of events analyzed.

FIGURE S6 | Analysis of diabody single-chain TNF-related apoptosis-inducing ligand (Db-scTRAIL) production *in vivo* from s.c.-injected mesenchymal stem cells (MSCs). MSC.TRAIL, MSCs untransfected (MSC unt), and MSC.Mock (4×10^6 cells) were subcutaneously injected in one flank of nude mice [$n = 4$ animals for each group, $\pm 95\%$ confidence interval (CI)] or 100 μ l of PBS (s.c.) as control. After 1, 3, 7, 14, and 21 days serum concentration of Db-scTRAIL was analyzed by enzyme-linked immunosorbent assay assay (#, under detectable level). The groups MSC Unt, MSC.Mock, and PBS did not reveal detectable protein levels.

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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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