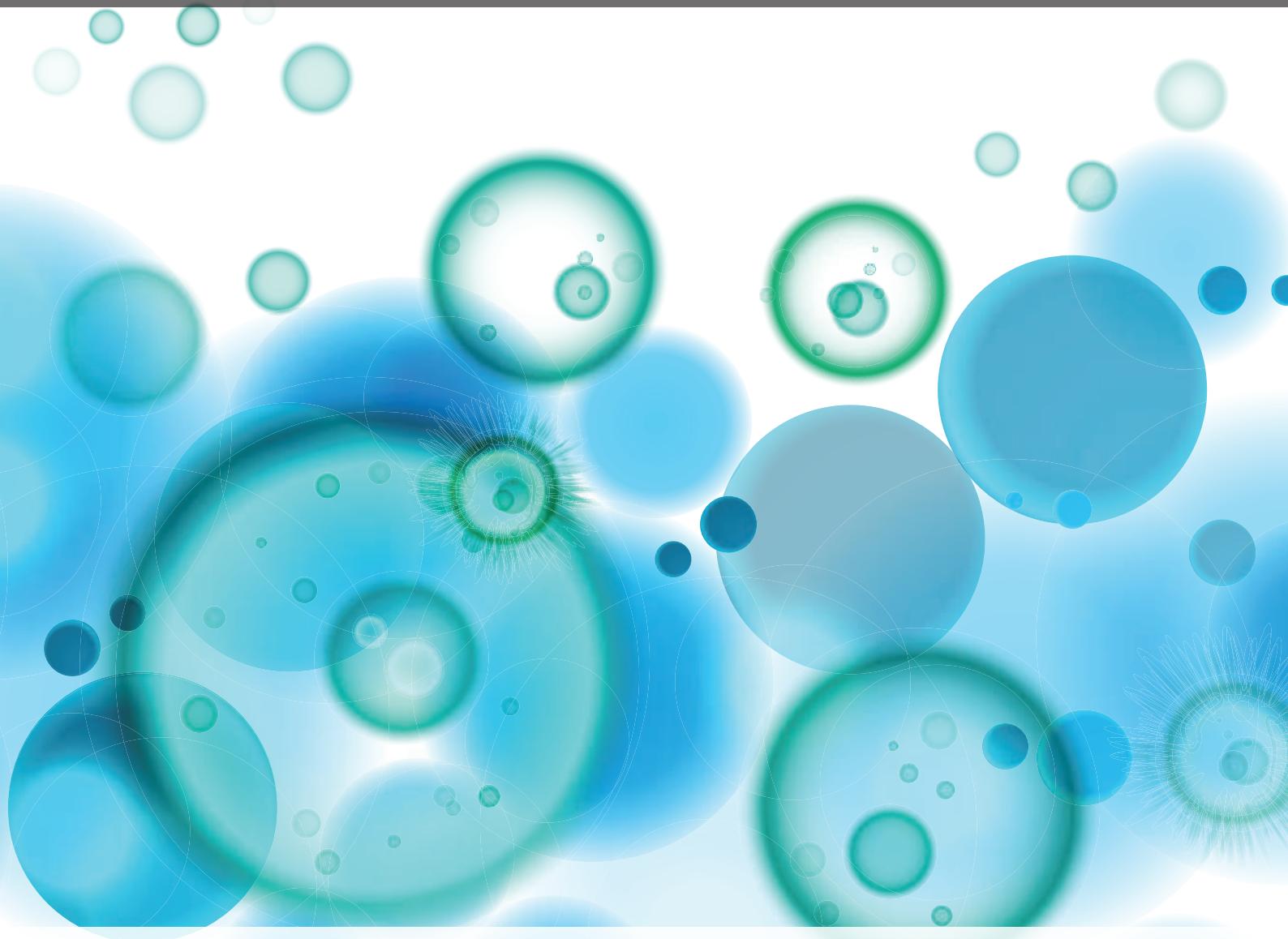
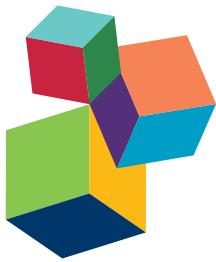


RECENT ADVANCES IN $\gamma\delta$ T CELL BIOLOGY: NEW LIGANDS, NEW FUNCTIONS, AND NEW TRANSLATIONAL PERSPECTIVES

EDITED BY: Dieter Kabelitz and Julie Déchanet-Merville

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RECENT ADVANCES IN $\gamma\delta$ T CELL BIOLOGY: NEW LIGANDS, NEW FUNCTIONS, AND NEW TRANSLATIONAL PERSPECTIVES

Topic Editors:

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Gamma/delta ($\gamma\delta$) T-cells are a small subset of T-lymphocytes in the peripheral circulation but constitute a major T-cell population at other anatomical localizations such as the epithelial tissues. In contrast to conventional α/β T-cells, the available number of germline genes coding for T-cell receptor (TCR) variable elements of $\gamma\delta$ T-cells is very small. Moreover, there is a preferential localization of $\gamma\delta$ T-cells expressing given $V\gamma$ and $V\delta$ genes in certain tissues. In humans, $\gamma\delta$ T-cells expressing the $V\gamma 9V\delta 2$ -encoded TCR account for anywhere between 50 and >95% of peripheral blood $\gamma\delta$ T-cells, whereas cells expressing non- $V\delta 2$ genes dominate in mucosal tissues. In mice, there is an ordered appearance of $\gamma\delta$ T-cell “waves” during embryonic development, resulting in preferential localization of $\gamma\delta$ T-cells expressing distinct $V\gamma V\delta$ genes in the skin, the reproductive organs, or gut epithelia. The major function of $\gamma\delta$ T-cells resides in local immunosurveillance and immune defense against infection and malignancy. This is supported by the identification of ligands that are selectively recognized by the $\gamma\delta$ TCR. As an example, human $V\gamma 9V\delta 2$ T-cells recognize phosphorylated metabolites (“phosphoantigens”) that are secreted by many pathogens but can also be overproduced by tumor cells, providing a basis for a role of these $\gamma\delta$ T-cells in both anti-infective and anti-tumor immunity. Similarly, the recognition of endothelial protein C receptor by human non- $V\delta 2$ $\gamma\delta$ T-cells has recently been identified to provide a link for the role for such $\gamma\delta$ T-cells in immunity against epithelial tumor cells and cytomegalovirus-infected endothelial cells. In addition to “classical” functions such as cytokine production and cytotoxicity, recent studies suggest that subsets of $\gamma\delta$ T-cells can exert additional functions such as regulatory activity and – quite surprisingly – “professional” antigen-presenting capacity. It is currently not well known how this tremendous extent of functional plasticity is regulated and what is the extent of $\gamma\delta$ TCR ligand diversity. Due to their non-MHC-restricted recognition of unusual stress-associated ligands, $\gamma\delta$ T-cells have raised great interest as to their potential translational application in cell-based immunotherapy. Topics of this Research Focus include: Molecular insights into the activation and differentiation requirements of $\gamma\delta$ T-cells, role of pyrophosphates and butyrophilin molecules for the activation of human $\gamma\delta$ T-cells, role of $\gamma\delta$ T-cells in tumor immunity and in other infectious and non-infectious diseases, and many others.

We are most grateful to all colleagues who agreed to write a manuscript. Thanks to their contributions, this E-book presents an up-to-date overview on many facets of the still exciting $\gamma\delta$ T-cells.

Dieter Kabelitz & Julie Déchanet-Merville

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Editorial: “Recent advances in gamma/delta T cell biology: new ligands, new functions, and new translational perspectives”

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Since their discovery in the mid-1980s, interest in the immunological significance of $\gamma\delta$ T cells has been subject to oscillations. The initial excitement over the unexpected discovery of a second T cell receptor (TCR) was followed by years of uncertainty as to the biological importance of these ambivalent T cells. Major breakthroughs led to the identification of specific and unique antigens for the $\gamma\delta$ TCR and accumulating evidence now shows that $\gamma\delta$ T cells play a major role in local immunosurveillance, thereby controlling tumorigenesis. Since 2004, biannual international $\gamma\delta$ T cell conferences are held to bring together experts in basic and clinical $\gamma\delta$ T cell research. To make accessible and synthesize the body of knowledge that has been put together, to date, we have organized a “Research Topic” on $\gamma\delta$ T cells consisting of a collection of original articles and focused reviews written by leading experts in the field. The idea of this Research Focus was to present the current status and “hot topics” as well as clinical perspectives on $\gamma\delta$ T cell research.

$\gamma\delta$ T Cells: Differentiation, Activation, and Signaling

The signaling pathways governing $\gamma\delta$ T cell differentiation and activation have been discussed in contributions from Carl Wares's (1) and Bruno Silva-Santos's (2) groups. Ribeiro et al. discuss the control of $\gamma\delta$ T cell activation and differentiation by distinct classes of cell surface receptors, namely (i) the TCR, (ii) costimulatory receptors (with a focus on CD27), (iii) cytokine receptors, (iv) NK receptors, and (v) inhibitory receptors. They further summarize how activation of $\gamma\delta$ T cells can be controlled by the TCR as well as by activating NK receptors. To terminate $\gamma\delta$ T cell responses, several inhibitory receptors can deliver negative signals, notably PD-1 and B- and T-lymphocyte attenuator (BTLA) (2). Bekiaris et al. focus on the cytokine control of innate $\gamma\delta$ T cells, and discuss the role of IL-7 in being critical for thymic development of $\gamma\delta$ T cells by regulating the survival of progenitor cells and inducing V(D)J recombination within the TCR γ gene locus. IL-7 also supports homeostatic proliferation of $\gamma\delta$ T cells and regulates surface expression of BTLA in a STAT5-dependent manner (1).

In recent years, IL-17 has been identified as an essential cytokine that regulates the recruitment of neutrophils during an inflammatory response. IL-17 must be rapidly available in an acute infection. $\alpha\beta$ T cells producing IL-17 (Th17 cells) require specific antigenic stimulation and an appropriate cytokine milieu for differentiation. $\gamma\delta$ T cells have been identified as an important “innate” source of rapid initial IL-17 production, which is thought to occur without specific TCR triggering (3). Several years ago, the group of Immo Prinz generated a unique mouse model to monitor early steps of $\gamma\delta$ T cell development (4). Using these reporter mice, they demonstrated that IL-17 producing $\gamma\delta$ T cells develop during the embryonic period and persist in adult mice as self-renewing, long-lived cells (5). Interestingly, Wei and colleagues have provided evidence that the TCR repertoire of such naturally occurring IL-17-producing murine $\gamma\delta$ T cells is highly restricted, with little

or no junctional diversity, regardless of their anatomical origin. These findings strongly suggest that antigen recognition is involved in the establishment and/or function of such “innate” IL-17 producing $\gamma\delta$ T cells (6). Further aspects of IL-17 producing $\gamma\delta$ T cells, also addressing their role in various pathophysiological conditions, are discussed in the review article by Patil et al. (7).

In addition to production of cytokines, such as IL-17, $\gamma\delta$ T cells can induce maturation of dendritic cells (DCs) and B cells. Petrasca and Doherty report on the upregulation of the expression of CD86, HLA-DR, IL-6, and TNF α in both DC and B cells upon coculture with human V γ 9V δ 2 T cells, whereas other cytokines, such as IFN γ or IL-4, were differentially induced in DC versus B cells (8). Collectively, their data indicate that $\gamma\delta$ T cells can drive the expression of antigen presenting cell (APC)-associated markers in both DC and B cells (8). Interaction with neighboring cells is a key feature of $\gamma\delta$ T cells. This is particularly true for tissue-resident $\gamma\delta$ T cells. Witherden et al. have reviewed the multiple molecular interactions that have been characterized between skin-resident dendritic epidermal $\gamma\delta$ T cells and keratinocytes. Among their many effector functions, epidermal $\gamma\delta$ T cells are involved in wound repair, maintenance of epithelial homeostasis, and protection from malignant transformation (9).

A final aspect of $\gamma\delta$ T cell differentiation and plasticity is presented in the report of Ziegler et al. (10). These authors made the surprising observation that the small population of human peripheral blood V δ 1 $\gamma\delta$ T cells that simultaneously express CD4, can differentiate into *bona fide* $\alpha\beta$ T cells in a process called transdifferentiation. The authors took all measures to avoid the potential contribution of possible artifacts that may influence their results. Their characterization of human CD4 $^+$ V δ 1 $\gamma\delta$ T cells as a source of extrathymic T cell development challenges current dogmas and opens interesting avenues for future research (10).

Recognition of Pyrophosphate Antigens by Human $\gamma\delta$ T Cells

A particularly exciting new area of $\gamma\delta$ T cell research is the role of butyrophilin and butyrophilin-like molecules (which consist of all members of the B7 family of co-stimulators) in $\gamma\delta$ T cell activation. A member of this family, Skint-1, has been shown as critical for positive selection of mouse V γ 5V δ 5 dendritic epidermal T cells (11). In humans, Butyrophilin 3A1 (BTN3A1) was recently found to play a critical role in the activation of V γ 9V δ 2 T cells by pyrophosphate molecules – collectively termed as “phosphoantigens” (pAgS). These intermediates of the eukaryotic mevalonate and the prokaryotic non-mevalonate pathway for isoprenoid synthesis have been identified as specific ligands for the V γ 9V δ 2 TCR that activate this subset of T cells at pico- to nanomolar (microbial pAgS) or micromolar (eukaryotic pAgS) concentrations. The recognition of metabolites that are produced by microbes [e.g., (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)] or that are overproduced by transformed eukaryotic cells [e.g., isopentenyl pyrophosphate (IPP)] provides a formal basis for the role of V γ 9V δ 2 T cells in both anti-infective and anti-tumor immunity (12). Interestingly, the recognition of non-peptidic small molecules is not restricted to human $\gamma\delta$ T cells. As an example, Zeng et al. recently reported

that small molecules, such as haptens, are recognized by specific murine $\gamma\delta$ TCRs (13).

For many years, it has been enigmatic how pAgS are actually recognized by the human $\gamma\delta$ TCR, and whether any “presenting” molecules are involved. A major breakthrough was the identification of an agonistic monoclonal antibody (called 20.1) that specifically triggers V γ 9V δ 2 T cell activation among peripheral blood mononuclear cells in a manner that is very similar to pAgS. This antibody is directed against CD277, a member of the B7 superfamily of butyrophilin (BTN) molecules. Detailed studies by Harly et al. identified an indispensable role of the BTN3A1 isoform in the activation of V γ 9V δ 2 T cells by pAgS (14). BTN3A1 is a transmembrane molecule with two Ig-like extracellular domains and an intracellular B30.2 domain. This discovery of the essential function of BTN3A1 in V γ 9V δ 2 T cell activation stimulated further research, particularly addressing the question whether BTN3A1 is directly involved in “presenting” pAgS to the $\gamma\delta$ TCR.

In continuation of the above studies, Gennaro de Libero’s group has come up with new evidence that suggests the Ig-like extracellular domain of BTN3A1 can directly bind pAgS and that there is specific and direct interaction of soluble V γ 9V δ 2 TCR with the BTN3A1-pAg complex (15). Quite surprisingly, however, such a “presenting” function of the extracellular BTN3A1 domain could not be verified by Erin Adam’s group. Instead, Adam and colleagues corroborated there being a crucial role of the intracellular B30.2 domain, which may actually directly interact with pAgS (16). In their model, the intracellular B30.2 domain might sense increased levels of intracellular pAgS (due to infection or cellular transformation), possibly inducing a conformational change in the BTN3A1 molecule, which could then be recognized by the V γ 9V δ 2 TCR (16). However, the issue appears to be even more complicated. Using CHO cells reconstituted with chromosome 6 as the only source of human genes, Riaño et al. demonstrate a potential role of other genes on chromosome 6 in addition to BTN3A1 that work to activate human V γ 9V δ 2 T cells (17). The various proponents of the seemingly conflicting models on the capacity of extra- versus intracellular BTN3A1 domains to directly interact with pAgS provide a balanced overview on this controversial issue in this special Research Topic (18–20).

Beyond this, “the intracellular pAg sensing model” recently received additional support from John Trowsdale’s group. These authors also reported direct binding of HMBPP to the intracellular B30.2 domain (21). Furthermore, using a yeast-two hybrid screen, these authors determined that the cytoskeletal protein plakin interacts with the cytoplasmic tail of BTN3A1 in a region proximal to the B30.2 domain. Such protein-protein interactions might be crucial in transmitting signals (e.g., conformational alterations of BTN3A1) from the $\gamma\delta$ TCR upon pAg binding to the intracellular B30.2 domain (21). It is thus evident that many of the mechanistic details of BTN3A1 are still unclear, beyond the undisputed fact that it is indispensable for $\gamma\delta$ T cell activation by pAgS.

Intriguingly, the mouse lacks BTN3A1 genes and homologous $\gamma\delta$ TCR that would be able to recognize similar pAgS. Karunakaran and Herrmann (22) have studied the ontogenetic evolution of V γ 9, V δ 2, and BTN3 genes. Their detailed database analysis suggests that these three genes have co-evolved in placental mammals, while they are lost in rodents and lagomorphs (22). Taken

together, the controversial issue of the ménage à trois of V γ 9V δ 2 TCR, pAgS, and BTN3A1 awaits additional elucidation. We are grateful that the major players in this particular field have shared their results and thoughtful reflections for this Research Topic.

$\gamma\delta$ T Cells in Tumor Immunity

$\gamma\delta$ T cells can kill a broad range of tumors of epithelial origin as well as many leukemias and lymphomas, and are further able to produce high levels of the anti-tumor cytokine, IFN γ . Given their HLA non-restricted method of ligand recognition, the role of $\gamma\delta$ T cells in anti-tumor immunity has stimulated great interest to explore their potential for cancer immunotherapy (23). Interestingly, the production of endogenous pAgS, such as IPP, can be pharmacologically manipulated by nitrogen-containing bisphosphonates (N-BP), which inhibit the downstream processing of IPP, leading to increased levels of endogenous IPP that is sensed by V γ 9V δ 2 T cells (24). Based on this knowledge, intravenous application of the N-BP zoledronate together with low-dose IL-2 has been evaluated as a means of *in vivo* activation of $\gamma\delta$ T cells in cancer patients (25). A recent survey has reviewed the available published studies on the *in vivo* activation and adoptive transfer of $\gamma\delta$ T cells in cancer patients (26). Most likely, strategies aiming to activate $\gamma\delta$ T cells *in vivo* will have to be combined with other treatment regimens to obtain optimal anti-tumor activity (27).

In addition to IPP and related pAgS, which serve as antigens for tumor-reactive V γ 9V δ 2 T cells, several cell surface-expressed antigens have been identified for tumor-reactive human non-V δ 2 $\gamma\delta$ T cells. One example illustrating the versatility of $\gamma\delta$ T cells is the endothelial protein C receptor (EPCR), which is an HLA-related molecule. We (Déchanet-Merville group) identified EPCR as a target for non-V δ 2 $\gamma\delta$ T cells that is expressed on endothelial cells infected by cytomegalovirus (CMV) and is also similarly expressed on epithelial tumor cells (28). It is thus conceivable that non-V δ 2 $\gamma\delta$ T cells have a similar potential for cancer immunotherapy as pAg-reactive V δ 2 T cells (23, 29). The recent discovery that CD1d, the closest structural homolog of EPCR, can present self lipids to the human V δ 1 TCR, also deserves further investigations in the domain of $\gamma\delta$ T cell-mediated anti-tumor protection and function (30, 31). It should be borne in mind that the anti-tumor efficacy of $\gamma\delta$ T cell subsets (V γ 9V δ 2, non-V δ 2) may, of course, vary between different tumor entities (32).

One of the basic observations supporting a role of immune surveillance in tumor development and progression is the sticking correlation between the proportion of tumor-infiltrating CD3 $^+$ T cells (TIL) and tumor progression and patient survival (33). $\gamma\delta$ T cells can represent a significant proportion of CD3 $^+$ TIL in different tumors (34, 35). Hidalgo et al. analyzed the presence of $\gamma\delta$ T cells among TIL in different types of breast carcinoma. Their results suggest a correlation between higher numbers of $\gamma\delta$ TIL and a better prognosis in medullary breast carcinoma as compared to invasive ductal carcinomas, two subtypes of the Her2- and hormone receptor-negative (“triple-negative”) breast carcinoma (35).

It is clear, however, that an increased presence of $\gamma\delta$ T cells within a tumor *per se* is not necessarily associated with a beneficial

effect. As discussed by Lo Presti et al., there are multiple interactions of tumor-infiltrating $\gamma\delta$ T cells within the local tumor microenvironment that strongly influence the functional outcome (36). Relevant factors include (but are not restricted to) tumor-derived immunosuppressive cytokines, such as TGF- β , locally expressed inhibitory molecules, such as PD-1, and myeloid-derived suppressor cells (MDSCs) located within the tumor stroma (36). Moreover, tumor-infiltrating $\gamma\delta$ T cells themselves may exert suppressive activity (37) or promote the accumulation of MDSCs (38). Pro-tumoral activity of $\gamma\delta$ T cells has also been demonstrated in several mouse models, and this is mainly mediated through their production of IL-17, which, in these instances, results in the attraction of immunosuppressive myeloid cells and promotion of angiogenesis. Depending on the priming signals in the tumor microenvironment, in addition to other $\gamma\delta$ T cell subtypes, even pAg-reactive V γ 9V δ 2 T cells may acquire suppressive activity (39). In the context of tumor immunity, multiple scenarios may thus convert $\gamma\delta$ T cells into suppressive cells, which can have counter-productive consequences for tumor immunity (40). Taken together, $\gamma\delta$ T cells can exert both anti- and pro-tumorigenic activities, and it is a major challenge for future studies to determine how to specifically boost the anti-tumor effects of $\gamma\delta$ T cells while simultaneously shunting their suppressive activity (41). This functional plasticity of mouse and human $\gamma\delta$ T cells in the anti-tumor immune response has been extensively discussed by Lafont et al. (42).

To enhance the anti-tumor activity of $\gamma\delta$ T cells, several strategies are under consideration. These include the use of antibodies to trigger Fc receptor-dependent ADCC, or the use of bispecific antibody constructs to cross-link the $\gamma\delta$ TCR with tumor cell surface antigens. Seidel et al. have explored Fc-optimized anti-CD19 antibodies as well as CD19/CD16 bispecific antibodies to enhance $\gamma\delta$ T cell-mediated killing of CD19 $^+$ B cell malignancies (43). For this purpose, they employed a label-free real-time cytotoxicity assay, which allows monitoring the tumor cell- $\gamma\delta$ T cell interactions over prolonged periods of time (43). This system was also used by us (Kabelitz group) to demonstrate the efficacy of a Her2-V γ 9 “tribody” construct in enhancing lysis of Her2-expressing pancreatic adenocarcinoma cells by V γ 9V δ 2 effector T cells (34). A different approach to explore the potential use of $\gamma\delta$ T cells for cancer immunotherapy would be to develop $\gamma\delta$ T cell-based cancer vaccines. This strategy is based on the seminal discovery of Bernhard Moser’s group that found activated human V γ 9V δ 2 T cells may actually serve as “professional” APCs that pick up and process exogenous antigens and load them onto both MHC class II and MHC class I presentation pathways for recognition by CD4 $^+$ and CD8 $^+$ $\alpha\beta$ T cells, respectively (44). Toward this end, the capacity of activated human $\gamma\delta$ T cells to take up tumor-derived antigens and to present processed peptides to tumor antigen-specific CD8 $^+$ $\alpha\beta$ T cells has been demonstrated (45). As discussed by Khan et al., the ease with which V γ 9V δ 2 T cells are expanded into large numbers *in vitro* offers an innovative strategy for the development of $\gamma\delta$ T cell-based tumor vaccines (46).

For the adoptive transfer of *in vitro* expanded $\gamma\delta$ T cells, a multitude of experimental protocols have been developed. One important aspect to bear in mind for this procedure is to design means to fully polarize $\gamma\delta$ T cells toward an efficient anti-tumor

functionality. Promotion of their IFN γ expression instead of IL-17 will certainly be an important step toward this aim (41). Along this line, Deniger and coworkers have summarized various strategies of *in vitro* expansion and further discuss additional perspectives involving genetic approaches to increase the efficacy of $\gamma\delta$ T cell-based immunotherapy (47). A crucial point in this context is the efficient monitoring of $\gamma\delta$ T cell subpopulations in the blood of cancer patients. We (Kabelitz group) have established a system to accurately determine absolute numbers of circulating V δ 1 and V δ 2 $\gamma\delta$ T cells from a small sample of whole blood. This method enabled us to determine a threshold number of V δ 2 T cells per microliter blood below which no cytotoxic activity toward pancreatic adenocarcinoma tumor targets could be triggered by pAg or Her2-V γ 9 tribody (48). Another important step for the application of $\gamma\delta$ T cell-based immunotherapy involves identifying the antigenic ligands recognized by $\gamma\delta$ T cells on tumor cells as they may potentially be used as agonistic drugs either *ex vivo* for $\gamma\delta$ T cell expansion or *in vivo* for active vaccination trials. Together, the collection of papers published in this Research Topic discuss critical issues pivotal for understanding the precise role of $\gamma\delta$ T cells in tumor immunosurveillance or tumor immune evasion as well as highlighting the future potential of $\gamma\delta$ T cell-based immunotherapies.

$\gamma\delta$ T Cells in Infection and Other Diseases

In view of the strong activation of human V γ 9V δ 2 T cells by microbial pAgs (notably HMBPP), it is not surprising to see significant expansion of peripheral blood $\gamma\delta$ T cells during the acute phase of many infections (49). However, not all microbes produce such $\gamma\delta$ T cell-stimulating molecules. The differential production of $\gamma\delta$ T cell ligands by microbes can be used for diagnostic purposes to identify the presence of (mostly Gram-negative) bacteria and thus allow for a pathogen-specific immune fingerprinting. This inventive diagnostic application of $\gamma\delta$ T cell research in the context of infectious diseases is highlighted in the Opinion Article by Eberl et al. (50).

While V γ 9V δ 2 T cells increase in numbers in many bacterial and parasitic infections, these cells are characteristically reduced in HIV infected individuals. Even upon efficient anti-retroviral therapy and CD4 T cell reconstitution, V γ 9V δ 2 T cell numbers usually remain low. Pauza and colleagues discuss the potential consequences of persistent low V γ 9V δ 2 T cell numbers in HIV infected individuals and argue in favor of designing innovative clinical trials to reconstitute normal levels of V γ 9V δ 2 T cells (51). Another example of viral infection with high relevance for $\gamma\delta$ T cells is CMV. Our (Déchanet-Merville group) early studies have

uncovered a remarkable expansion of V δ 2-negative $\gamma\delta$ T cells in CMV-positive but not -negative kidney transplant recipients (52). Further studies have revealed multiple possible functions of CMV-induced $\gamma\delta$ T cells, including direct anti-viral activity. Concomitant studies in mouse models point to a protective role of murine $\gamma\delta$ T cells against CMV infection (53, 54). All these aspects including the cross-reactivity of CMV-reactive non-V δ 2 $\gamma\delta$ T cells with certain epithelial tumor entities are discussed in a review article by Couzi et al. (55).

This Research Topic also addresses the role of $\gamma\delta$ T cells in non-infectious diseases. In addition to infection and cancer, autoimmune diseases, and wound healing and burn injuries have been addressed by Latha et al. (56). This review summarizes the extensive evidence showing the involvement of $\gamma\delta$ T cells in many pathophysiological conditions. An interesting specific example is reported by Marcu-Malina and coworkers (57) who observed a TNF-dependent induction of procoagulant tissue factor-1 (TF-1) in monocytes by zoledronate-stimulated $\gamma\delta$ T cells. This seemed to occur specifically in $\gamma\delta$ T cells from patients with systemic sclerosis (57). Last but not least, this Research Topic additionally covers the role of $\gamma\delta$ T cells in the diagnosis of immunodeficiencies. TCR immunodeficiencies can affect both $\alpha\beta$ and $\gamma\delta$ T cells. While $\alpha\beta$ T cells have been extensively studied, $\gamma\delta$ T cells are frequently ignored, partly due to their numerical scarcity in circulation. Garcillán et al. discuss these issues in detail and present a useful diagnostic flowchart (58).

Concluding Remarks

After 30 years of $\gamma\delta$ T cell research, it is clear that these cells are intimately involved in the control of tissue homeostasis, infection, and malignancy. The identification of specific ligands for the $\gamma\delta$ TCR provides strong support for the idea that $\gamma\delta$ T cells are non-redundant to $\alpha\beta$ T cells. Apart from the detailed knowledge of their physiological and pathophysiological significance, we are currently experiencing new exciting developments aimed at bringing $\gamma\delta$ T cells into clinical medicine.

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Mixing signals: molecular turn ons and turn offs for innate $\gamma\delta$ T-cells

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INTRODUCTION

The ability to generate antigen receptor diversity by somatic recombination evolved approximately 500 million years ago (1) and became the founding biological property of what we now know as adaptive immunity. This evolutionary milestone provided our immune system with an innate and an adaptive arm that synergized for the fight against infection and the recognition of oncogenesis. Lymphocytes of the gamma delta ($\gamma\delta$) T-cell lineage are evolutionary conserved among species (2) and although they express rearranged antigen-specific receptors, a large proportion display innate properties. In the mouse, where innate $\gamma\delta$ T-cells have been mostly studied, approximately 25% of lymph node $\gamma\delta$ T-cells respond rapidly to cytokine stimuli similar to innate lymphoid cells (ILCs) and appear to have reduced T-cell receptor (TCR) signaling capacity (3). Innate $\gamma\delta$ T-cells are characterized by the spontaneous and high expression of interleukin (IL)-17 ($\gamma\delta^{17}$) as well as IL-22 and express functional Toll-like receptors (TLR) (4, 5). Importantly, IL-17 and IL-23 receptor (IL-23R) expression, which is critical for IL-22 induction, are turned on during embryonic development in the thymus strongly pointing toward a *bona fide* innate nature (6–8). Although a new interferon gamma (IFN γ)-producing innate $\gamma\delta$ T-cell subset with no IL-17 potential has recently been described (3), this review will discuss briefly some of the key cytokines, cytokine receptors, and transcription factors (TFs) that regulate the development, activation, and inhibition of mouse innate $\gamma\delta^{17}$ cells (Figure 1).

Lymphocytes of the gamma delta ($\gamma\delta$) T-cell lineage are evolutionary conserved and although they express rearranged antigen-specific receptors, a large proportion respond as innate effectors. $\gamma\delta$ T-cells are poised to combat infection by responding rapidly to cytokine stimuli similar to innate lymphoid cells. This potential to initiate strong inflammatory responses necessitates that inhibitory signals are balanced with activation signals. Here, we discuss some of the key mechanisms that regulate the development, activation, and inhibition of innate $\gamma\delta$ T-cells in light of recent evidence that the inhibitory immunoglobulin-superfamily member B and T lymphocyte attenuator restricts their differentiation and effector function.

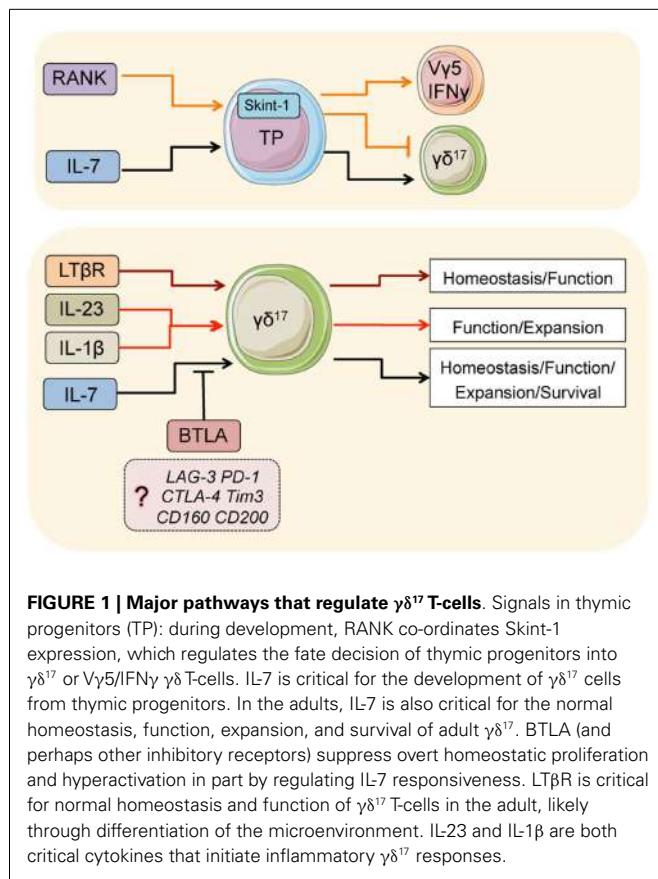
Keywords: BT LA, dermatitis, $\gamma\delta$ T-cell, IL-7, lymphotoxin, RORyt

IL-23 AND IL-1 β : KEY PROINFLAMMATORY AND ANTI-BACTERIAL MEDIATORS

Innate $\gamma\delta^{17}$ cells localize mainly at barrier and mucosal surfaces such as the skin, gut, and lung (9) and within the lymph nodes, they position themselves in close proximity to the subcapsular sinus and interfollicular regions both of which specialize in the capture of antigen (10). Therefore, infectious and inflammatory stimuli can readily activate $\gamma\delta^{17}$ cells either directly through TLR ligation or through cytokines such as IL-23 and IL-1 β that are produced by local innate sensors.

IL-23 induces the expression of IL-17 and IL-22 as well as the transcription factor retinoid-related orphan receptor gamma-t (RORyt) in T-helper 17 (T_H17) cells while at the same time promoting survival and cell proliferation (11). $\gamma\delta^{17}$ cells express functional IL-23R as early as embryonic day E18 in the thymus (7), in contrast to CD4 $^+$ T-cells that upregulate the IL-23R upon T_H17 differentiation (12). Although IL-23 or IL-23R has not been reported to be important for $\gamma\delta$ T-cell development, they enhance the production of IL-17 and IL-22 and can promote cellular proliferation (3, 13). *In vivo* infectious and inflammatory models have shown that IL-23 can be important for the activation of the $\gamma\delta$ T-cell response.

During imiquimod (IMQ)-induced psoriasis, genetic ablation of IL-23 or IL-23R results in a significant reduction of IL-17 production by $\gamma\delta^{17}$ cells, diminished accumulation of these cells in the skin, and a subsequent decrease in inflammatory symptoms (14–16). In this model, IL-23 is produced locally in the skin by resident



macrophage and dendritic cell (DC) populations that receive a combination of TLR and neuronal signals (15, 17, 18). The onset of experimental autoimmune encephalomyelitis (EAE), which is often used to model human multiple sclerosis, also depends to a certain extent on IL-23-driven IL-17 production by $\gamma\delta$ T-cells (5, 19). More specifically, it has been shown that IL-23-activated $\gamma\delta^{17}$ cells are important for optimal T_H17 polarization (5) and the suppression of regulatory T-cell responses (19). In a mouse model of brain ischemic injury, absence of IL-23 also abrogated $\gamma\delta^{17}$ -induced inflammation (20). In addition to regulating inflammatory reactions, $\gamma\delta^{17}$ cells and IL-23 have been linked with protection from a number of bacterial infections. Thus, cutaneous infection with *Staphylococcus aureus* triggers a $\gamma\delta$ T-cell orchestrated IL-17 response that depends on the combined effects of IL-23 and IL-1 β (21). Furthermore, infection with *Listeria monocytogenes* elicits an IL-23-driven $\gamma\delta^{17}$ response that is important for bacterial clearance (22, 23), and the IL-23 pathway appears also to operate during $\gamma\delta^{17}$ activation by *Mycobacterium tuberculosis* (24). Together, these data highlight the role of IL-23 in activating $\gamma\delta^{17}$ cell-induced inflammatory responses, both to pathogens and in driving autoimmune disease.

Similar to IL-23, IL-1 β has also been linked with IL-17-related immunity both in CD4 $^+$ T as well as in innate $\gamma\delta$ T-cells. $\gamma\delta^{17}$ cells constitutively express the IL-1 receptor and respond to *in vitro* IL-1 β stimulation by rapid proliferation and upregulation of IL-17 (3, 5, 13). Interestingly, IL-1 β appears to be important

for IL-23-mediated $\gamma\delta$ T-cell expansion and IL-17 production although the molecular mechanism is not yet understood (5, 13). Effective IL-1 β signaling was critical for $\gamma\delta$ T-cell activation and disease progression in the EAE model (5). However, during IMQ-induced psoriasis, usage of *Ilr1* $^{-/-}$ mice has resulted in conflicting conclusions. Whereas an earlier report presented no impact of IL-1 β on either dermatitis or $\gamma\delta^{17}$ activation (25), a more recent study showed that *Ilr1* $^{-/-}$ mice were consistently protected with severely compromised $\gamma\delta$ T-cell responses (13). A key difference in the two studies was the site of inflammation: ear (no IL-1 β effect) (25) versus dorsal epidermis (strong IL-1 β effect) (13), suggesting that IL-1 β may have site-specific regulatory roles, such as differential effects on resident stromal and epithelial cells or due to differences in lymphatic drainage.

IL-7: KEEPING THE BALANCE BETWEEN HOMEOSTASIS AND INFLAMMATION

IL-7 is one of the best-studied T-cell homeostatic cytokines. IL-7 deficiency is associated with lymphopenia and dysfunction of naïve and memory T-cell subsets (26). IL-7 is essential for the development of $\gamma\delta$ T-cells (27, 28) by regulating the survival of early thymic progenitors and by inducing V(D)J recombination within the TCR- γ locus (29, 30). Further experiments have shown that in addition to its developmental role, IL-7 supports the homeostatic proliferation of $\gamma\delta$ T-cells (31). Although IL-7 is strongly associated with signaling via the signal transducer and activator of transcription 5 (STAT5) (32), it has been shown to induce STAT3 phosphorylation in diverse lymphocyte populations such as thymocytes (33), B-cell progenitors (34), and $\gamma\delta$ T-cells (35). STAT3 is a critical component of the IL-23 and IL-6 signaling pathways, which are important for the differentiation of CD4 $^+$ T-cells into the T_H17 lineage (11, 36), in part by antagonizing STAT5 (37). Of the $\gamma\delta$ T subsets, IL-7 was found to preferentially expand and activate innate $\gamma\delta^{17}$ cells in a STAT3-dependent manner (35), although it sustained survival of all $\gamma\delta$ T-cells (38).

We have recently demonstrated that in $\gamma\delta^{17}$ cells, STAT5-mediated IL-7 signaling induces surface expression of the checkpoint receptor B and T lymphocyte attenuator (BTLA), which is necessary for their normal homeostasis and activation during skin inflammation (38). Blockade of IL-7 signaling itself has been shown to acutely diminish $\gamma\delta^{17}$ -driven dermatitis (35) while during viral hepatitis IL-7 co-operates with IL-23 to rapidly activate intrahepatic $\gamma\delta^{17}$ cells and initiate inflammation (39). Whether IL-7-induced STAT5 and STAT3 phosphorylation operate in parallel, sequentially, or as mutually exclusive processes within the $\gamma\delta^{17}$ population is unknown. However, $\gamma\delta$ T-cells deficient in STAT3 display normal homeostatic responses (40) suggesting that at steady state STAT5 may have a dominant role.

In addition to its direct effects on $\gamma\delta$ T-cells, IL-7 indirectly influences innate $\gamma\delta$ T-cell development by promoting the generation of lymphoid tissues in part by inducing the expression of tumor necrosis factor (TNF) superfamily members. IL-7 is produced homeostatically in the developing thymus and lymph node anlagen (41) and has been shown to induce the expression of surface lymphotoxin- $\alpha\beta$ (LT $\alpha\beta$) on resident embryonic lymphoid tissue inducer (LTi) cells (42). LT $\alpha\beta$ expressed by LTi interacts with the LT β receptor (LT β R) in order to initiate lymph node

development and organization (43, 44). Genetic ablation of LT β R results in the absence of all secondary lymphoid tissues in addition to disorganized splenic and thymic architecture (45, 46). Several members of the TNF superfamily have been shown to directly regulate $\gamma\delta$ T-cell development, homeostasis, and function, as outlined below.

LYMPHOTOXIN AND THE TNF NETWORK: CRITICAL REGULATORS OF INNATE $\gamma\delta$ T-CELLS

Innate IL-17 producing $\gamma\delta$ T-cells as well as V γ 5 (V γ 3 in German nomenclature) expressing cells that colonize the skin as resident dendritic epidermal T-cells (DETCs) are strictly dependent on the embryonic microenvironment (8, 47). Thus, adult progenitors cannot reconstitute either of the aforementioned populations even if they are provided with a fetal thymus suggesting the need for embryonic-only progenitors (8). Thus, the fetal thymus contains fully functional $\gamma\delta^{17}$ cells that develop between E15–18 (8). The development of these cells is intimately associated with the TNF superfamily since as early as E15 V γ 5 $^+$ progenitors express the TNF ligand RANKL (receptor activator of NF- κ B ligand) and condition the thymic medulla to upregulate Skint-1 (48), an immunoglobulin (Ig) superfamily protein that is necessary for the development of V γ 5 cells (49–51). Interestingly, in Skint-1 deficient animals, V γ 5 cells are reprogrammed into a $\gamma\delta^{17}$ -like phenotype with severely reduced IFN γ production (52). This suggests that innate $\gamma\delta^{17}$ T-cells are likely to represent the default differentiation pathway of most $\gamma\delta$ T-cell progenitors pre-Skint-1 selection. This is in line with the evolutionary evidence that IL-17-producing $\gamma\delta$ T-cells are conserved between non-jawed vertebrates and human beings (2) while Skint-1 and related genes (e.g., Btn1a1) are highly restricted to mammals (www.ensembl.org).

In addition to RANK, LT β R has also been linked with the development and functional maturation of $\gamma\delta$ T-cells. Early reports showed that $\gamma\delta$ T-cells can acquire LT β R expression in the thymus, and that activation of these receptors by LT $\alpha\beta$ - and LIGHT-expressing double-positive (DP) thymocytes drives maturation of $\gamma\delta$ T-cells assessed by the production of IFN γ (53). However, the expression of IL-17 or other $\gamma\delta^{17}$ -related properties was not evaluated. The authors suggested that LT β R-induced maturation likely occurred at the late stages of thymic development when DP cells predominate. Given that $\gamma\delta^{17}$ T-cells develop during early embryonic life (8), one scenario to explain these findings is that during thymic development the LT β R pathway in part regulates the IFN γ potential of $\gamma\delta$ T-cells, presumably following Skint-1 selection. In agreement with this argument, the TNF receptor CD27 is required by thymic progenitors to induce the innate IFN γ -related differentiation program and to sustain expression of LT β R (7). Thus, while CD27 deficient animals retain an intact $\gamma\delta^{17}$ compartment, they showed a marked reduction in IFN γ and LT β R expression (54). These results predict that LT β R signaling is not absolutely necessary for $\gamma\delta^{17}$ development and function, although mice deficient in LT β R or its ligands had very few IL-17-producing $\gamma\delta$ T-cells in the spleen and thymus (55). Mice lacking the NF- κ B TFs RelA and RelB also showed reduced IL-17-producing $\gamma\delta$ T-cells (55). Since the NF- κ B pathway is central to TCR signaling and T-cell development (56), low

IL-17 production might be reflective of impaired TCR stimulation rather than loss of LT β R signals. Furthermore, lack of lymph nodes in LT β R deficient mice (45) may relocate $\gamma\delta^{17}$ cells to the skin or intestine and thus explain their reduced numbers in the spleens. Importantly, loss of LT β R results in abnormal thymic organization and maturation of the medullary epithelium (46, 57), which may negatively affect $\gamma\delta^{17}$ T-cell development. Alternatively, organized secondary lymphoid tissues may be important for the survival and steady-state turnover of $\gamma\delta^{17}$ cells. Of note, LT β R has been shown to participate in the production of IL-7 by fibroreticular stromal cells in the lymph node (58), which might explain why deficiency in LT β R can result in reduced $\gamma\delta^{17}$ responses.

In addition to its involvement in stromal cell development, LT β R is expressed on tissue resident DCs and macrophages (59) both of which have been linked with the IL-23-mediated activation of $\gamma\delta^{17}$ T-cells, whether this is in the context of skin (15, 17) or brain inflammation (5). Notably, LT β R regulates the homeostasis of DCs (60, 61) and can directly induce their production of IL-23 (62). Interestingly, an LT β R-LT $\alpha\beta$ interaction has been linked with the production of IL-22 by intestinal ILCs (63, 64) raising the possibility that a similar mechanism may be in place at sites where $\gamma\delta^{17}$ cells preferentially localize, such as the skin.

BTLA AND INHIBITORY RECEPTORS: PUTTING THE BRAKES ON

In human beings, herpesvirus entry mediator (HVEM) interacts with the two TNF ligands LIGHT (shared with LT β R) and soluble LT α , and the Ig superfamily members CD160 and BTLA. BTLA is an inhibitory receptor with an immunoreceptor tyrosine inhibitory motif (ITIM) that has been shown to interact with the Src homology 2 (SH2)-domain containing protein tyrosine phosphatase 1 (SHP1) and SHP2 and to inhibit T-cell activation (65–67) upon interacting with HVEM, its only identified ligand thus far (66, 68, 69). In addition to its inhibitory role in T-cell responses, BTLA was shown to prevent overt TLR stimulation in DCs (70) and to diminish cytokine production by natural killer T (NKT) (71) and follicular T-cells (72) suggesting a regulatory role both in adaptive and innate immunity.

BTLA and HVEM signal bi-directionally providing inhibitory signals in T-cells and survival signals in cells expressing HVEM (68). BTLA expression varies $\sim 10^3$ fold among hematopoietic lineages, and co-expressed with HVEM forming a complex *in cis* that may contribute to homeostatic signaling (73). Constitutive surface expression of BTLA (74) implicates a unique ability among inhibitory receptors to sustain the homeostatic balance of T-cells (75) and DCs (61). Similarly, our recent data showed that BTLA is necessary to inhibit homeostatic expansion and activation of lymph node and skin resident $\gamma\delta^{17}$ T-cells (38). $\gamma\delta^{17}$ but not other $\gamma\delta$ T-cell subsets deficient in *Btla* were hyperresponsive to IL-7 stimulation suggesting that BTLA diminishes IL-7 receptor (IL-7R) signaling. Interestingly, IL-7 increased surface BTLA on $\gamma\delta^{17}$ cells in a STAT5-dependent way revealing the presence of a negative feedback loop between IL-7 and BTLA (38) (Figure 2). Given the broad range of SHP1 and SHP2 targets (76), it is likely that these phosphatases can inactivate both STAT3 and STAT5 in response to IL-7. However, the exact molecular details of BTLA-mediated

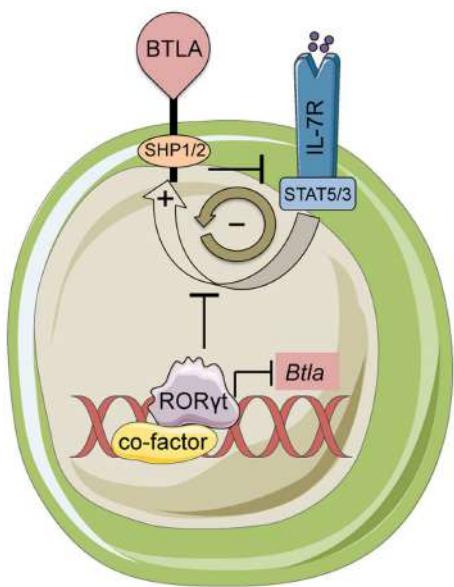


FIGURE 2 | BTLA and ROR γ t mediated control of $\gamma\delta^{17}$ T-cells. BTLA expression limits inflammatory responses and homeostasis of $\gamma\delta^{17}$ cells by antagonizing IL-7 signaling. In turn, IL-7 induces BTLA expression creating a negative feedback loop. The transcription factor ROR γ t represses the *Btla* promoter limiting the expression level of BTLA. This regulatory loop maintains BTLA expression at very low levels on the cell surface in resting $\gamma\delta$ T-cells.

suppression of IL-7R or other $\gamma\delta^{17}$ -expressed cytokine receptors are currently not known.

Although there are numerous functional inhibitory receptors that have been reported on the surface of lymphocyte subsets either at steady state or after activation, there is little information regarding their role on innate or non-innate $\gamma\delta$ T-cells. Several reports have mapped the expression of inhibitory molecules like programmed death-1 (PD-1) (77, 78), lymphocyte activation gene-3 (LAG-3) (79), CD200 (80), Tim-3 (81), CD160 (82), and cytotoxic T lymphocyte antigen-4 (CTLA-4) (83) on human or murine $\gamma\delta$ T-cells but the capacity to target these receptors using agonistic or antagonistic manipulation has in general not been addressed. Notably, we found that activating BTLA receptors using an agonistic antibody limited pathology in mice (38). Additionally, blockade of BTLA signaling enhanced activation of lymphoma-specific human V γ 9V δ 2 T-cells (84). Thorough investigation of the expression patterns and function of the different inhibitory receptors on innate $\gamma\delta$ T-cells may provide promising targets for intervening when these lymphocytes need to be turned on or off. Currently, and in combination with its suppressive activity, BTLA appears to be a key targetable pathway for regulating innate $\gamma\delta$ T-cells.

TRANSCRIPTIONAL CONTROL: IS EVERYTHING PRE-PROGRAMMED?

It is now well-appreciated that there is an extensive network of TFs that are expressed early in pre-committed progenitors and are necessary for the development, functional differentiation, and survival

of all innate cells including $\gamma\delta^{17}$ T-cells. A subset of these TFs control lineage specification, either through activating or repressing gene transcription. A number of TF mouse knockout lines result in the complete abolition or severe reduction in the numbers of the $\gamma\delta^{17}$ subset in the periphery and in the thymus. Thus, mice deficient for the high-mobility group (HMG) box TFs Sox13 and Sox4 show severe reduction of IL-17-producing $\gamma\delta$ T-cells due to a differentiation block early on during development (85, 86), which correlates with high expression levels of Sox13 and Sox4 in $\gamma\delta^{17}$ -committed T-cell progenitors (86–88). Interestingly, the function of Sox13 can be counteracted embryonically by Egr3, which drives the DETC differentiation program and IFN γ expression (52), while TCF1, another HMG box TF, suppresses $\gamma\delta^{17}$ differentiation (86). Notch signaling turns on TCF1 (89), which can also induce expression of Hes1, another TF critical for the generation of $\gamma\delta^{17}$ cells during embryonic differentiation (40). Interestingly, a subset of innate $\gamma\delta$ T-cells has been shown to depend on the expression of promyelocytic zinc finger (PLZF), which is also required for the development of ILCs (90, 91). It remains to be seen whether PLZF is specifically required for the development of $\gamma\delta^{17}$ cells.

Although, ROR γ t is necessary for the differentiation of T H 17 cells (36), it is not essential for the development of $\gamma\delta^{17}$ progenitors in the fetal thymus (40). However, consistent with its ability to bind to and transactivate the *Il17* promoter (92), ROR γ t is important for optimal IL-17 production (40). Interestingly, despite being developed, ROR γ t deficient $\gamma\delta$ T-cells cannot persist in the periphery (40), suggesting a potentially critical role for ROR γ t in the homeostasis of adult $\gamma\delta^{17}$ T-cells. This could be either cell-extrinsic or cell-intrinsic. ROR γ t is necessary for the development of all secondary lymphoid tissues (93). Thus, upon export in the periphery, $\gamma\delta^{17}$ T-cells may not have the appropriate microenvironment in order to sustain homeostasis (cell-extrinsic). In the cell-intrinsic scenario, ROR γ t may be important for the survival of $\gamma\delta^{17}$ cells by regulating the levels of the anti-apoptotic protein Bcl-xL (93). Our data have demonstrated that via its interaction with LxxLL containing nuclear co-factors ROR γ t can function as a transcriptional repressor and suppress expression of BTLA (38) (Figure 2). Therefore, an alternative cell-intrinsic hypothesis is that loss of ROR γ t results in aberrant expression of BTLA and perhaps other co-inhibitory receptors (such as LAG-3; Bekiaris/Ware, unpublished observations) leading to a sustained inhibition of homeostatic expansion.

CONCLUSION

$\gamma\delta^{17}$ and other $\gamma\delta$ T-cell subsets comprise a unique family of lymphocytes that provides an innate powerhouse to the immune system. The innate nature of $\gamma\delta^{17}$ cells is demonstrable by a number of key biological properties including rapid response to cytokines, functional maturation during embryogenesis, largely TCR-independent responses, and TF-dependent lineage commitment. Resolving the complex and fascinating biology of these cells has been breaking the Frontiers of Immunology for a number of years and has taught us a great deal about how lymphocytes develop and function. The continued knowledge of how all innate $\gamma\delta$ T-cells work will certainly push forward these frontiers and perhaps allow us to develop tools in order to manipulate them for the treatment of human disease.

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Five layers of receptor signaling in $\gamma\delta$ T-cell differentiation and activation

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The contributions of $\gamma\delta$ T-cells to immunity to infection or tumors critically depend on their activation and differentiation into effectors capable of secreting cytokines and killing infected or transformed cells. These processes are molecularly controlled by surface receptors that capture key extracellular cues and convey downstream intracellular signals that regulate $\gamma\delta$ T-cell physiology. The understanding of how environmental signals are integrated by $\gamma\delta$ T-cells is critical for their manipulation in clinical settings. Here, we discuss how different classes of surface receptors impact on human and murine $\gamma\delta$ T-cell differentiation, activation, and expansion. In particular, we review the role of five receptor types: the T-cell receptor (TCR), costimulatory receptors, cytokine receptors, NK receptors, and inhibitory receptors. Some of the key players are the costimulatory receptors CD27 and CD28, which differentially impact on pro-inflammatory subsets of $\gamma\delta$ T-cells; the cytokine receptors IL-2R, IL-7R, and IL-15R, which drive functional differentiation and expansion of $\gamma\delta$ T-cells; the NK receptor NKG2D and its contribution to $\gamma\delta$ T-cell cytotoxicity; and the inhibitory receptors PD-1 and BTLA that control $\gamma\delta$ T-cell homeostasis. We discuss these and other receptors in the context of a five-step model of receptor signaling in $\gamma\delta$ T-cell differentiation and activation, and discuss its implications for the manipulation of $\gamma\delta$ T-cells in immunotherapy.

Keywords: $\gamma\delta$ T-cells, T-cell receptor, T-cell costimulation, cytokines, natural killer receptors

INTRODUCTION

$\gamma\delta$ cells endow the T-cell compartment with a rapid, innate-like reaction to insults, which places them in the afferent phase of the immune response. Namely, $\gamma\delta$ T-cells are responsible for “lymphoid stress surveillance,” i.e., sensing and responding immediately to infections or non-microbial stress without the need of clonal expansion or *de novo* differentiation, in synchrony with prototypic innate immune responses (1). Critically, this implicates $\gamma\delta$ T-cells in inflammation (2), autoimmunity (3), infectious diseases (4, 5), and tumor surveillance (6–8).

Many of the studies elucidating the physiological roles of $\gamma\delta$ T-cells have been performed in murine models, where a major breakthrough has been the identification of pro-inflammatory subsets naturally producing either IFN γ or IL-17 (9–11). Moreover, these studies have been greatly facilitated by the identification of cell surface markers that segregate the two functional $\gamma\delta$ T-cell subsets: CD27, CD122, and NK1.1 mark IFN γ -producing $\gamma\delta$ cells, whereas their IL-17-expressing counterparts display a CD27⁺CCR6⁺ phenotype (9–11). Moreover, the two subsets show distinct V γ chain usage in their TCR repertoires, with a bias toward V γ 1 among IFN γ -producing $\gamma\delta$ cells, and an enrichment in V γ 4 and V γ 6 in IL-17-producing $\gamma\delta$ cells (12).

In humans, $\gamma\delta$ T-cells are primarily identified by their V δ chain usage, with V δ 1⁺ cells predominating in the thymus and in peripheral tissues, while V δ 2⁺ cells (mostly co-expressing a V γ 9 chain) constitute the majority of blood-circulating $\gamma\delta$ T-cells. Both human $\gamma\delta$ T-cell subsets are highly prone to secrete IFN γ , but IL-17 can be induced in highly

inflammatory conditions triggered by infections (13) or tumors (14, 15).

In both murine and human $\gamma\delta$ T-cells, functional responses are initiated upon recognition of antigens that are likely induced by stress signals and sensed by either T-cell or natural killer receptors. Some $\gamma\delta$ T-cell populations are also particularly responsive to cytokines or innate toll-like receptor (TLR) agonists (16, 17). Following proliferation and effector responses, the return to homeostasis is controlled by inhibitory receptors. Here, we discuss the various layers of contributions of T (TCR and costimulatory/inhibitory receptors), NK, and cytokine receptors to the activation and differentiation of effector $\gamma\delta$ T-cell populations in mice and humans.

SIGNAL 1: T-CELL RECEPTOR

The $\gamma\delta$ TCR complex is composed by the $\gamma\delta$ TCR itself and various CD3 chains following the stoichiometry: TCR $\gamma\delta$ CD3 $\epsilon_2\gamma\delta\zeta_2$ in humans and TCR $\gamma\delta$ CD3 $\epsilon_2\gamma_2\zeta_2$ in mice (18). The assembly of a $\gamma\delta$ TCR complex in thymic progenitors has immediate consequences for $\gamma\delta$ T-cell development. The “strong” signals stemming from the $\gamma\delta$ TCR (when compared to the “weaker” pre-TCR signaling) drive $\gamma\delta/\alpha\beta$ common precursors into the $\gamma\delta$ lineage (19, 20). These “stronger” $\gamma\delta$ TCR signals associate with increased phosphorylation of ERK1/2, abundant calcium release and induction of early growth response (Egr) transcription factors (21, 22).

The TCR complex does not present intrinsic kinase activity but the intracellular signaling is initiated after phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the

CD3 cytoplasmic domains by the Src-family kinases (SFKs) Lck and Fyn (23). The recruitment of these SFKs to the TCR complex in $\gamma\delta$ T-cells remains obscure since these cells do not express the CD4 or CD8 co-receptors, which have been shown, in $\alpha\beta$ T-cells, to be responsible for recruiting SFKs upon $\alpha\beta$ TCR ligation (23). Nonetheless, the importance of SFKs in $\gamma\delta$ T-cells is underscored by the substantial phosphorylation of ERK upon inhibition of Csk, a potent inhibitor of SFKs (24).

SFK-mediated phosphorylation of the ITAMs on CD3 chains allows the recruitment, phosphorylation, and activation of Zap70 that facilitates phosphorylation of the scaffolding proteins SLP-76 and LAT. This lead to the formation of a supramolecular signalosome that recruits the phospholipase PLC γ 1, resulting in propagation of downstream signaling events (22). Here again, $\gamma\delta$ T-cell signaling is different from $\alpha\beta$ T-cells, since mutations on the binding site of PLC γ 1 on LAT resulted in a severe block in murine $\alpha\beta$ thymocyte development while $\gamma\delta$ T-cell numbers were only modestly reduced in the thymus, intestine, and liver, and remained normal in the skin. Unexpectedly, a population of $\gamma\delta$ T-cells in the secondary lymphoid organs in these mice underwent uncontrolled expansion and caused autoimmune pathology, suggesting distinct functions for LAT/PLC γ 1-mediated signaling in subpopulations of $\gamma\delta$ T-cells (21, 25).

In humans, the major $\gamma\delta$ T-cell subset in the peripheral blood, V γ 9V δ 2 T-cells, are uniquely and specifically reactive to self- and foreign non-peptidic phosphorylated intermediates of isoprenoid synthesis – “phosphoantigens” or “phosphoagonists” (P-Ags) (26–28). These P-Ags were shown to trigger bona fide V γ 9V δ 2 TCR signaling in various studies. Cipriani and colleagues showed that the activation of V γ 9V δ 2 T-cells with the P-Ag isopentenyl pyrophosphate (IPP), induced rapid and persistent PKC-dependent phosphorylation of ERK1/2, p38 MAPK, and JNK, resulting in NF- κ B and AP-1 activation as well as the release of MIP-1 α , MIP-1 β , IFN- γ , and TNF- α (29). Moreover, P-Ag stimulation and CD3-crosslinking produced identical phosphorylation of the signaling proteins Zap70, PI3K, LAT, ERK1/2, and p38 MAPK (30, 31); and induced highly sustained calcium signaling in V γ 9V δ 2 T-cells (32). Importantly, activation by P-Ags is the basis of current cancer immunotherapy strategies involving V γ 9V δ 2 T-cells (33).

Recent work has produced some puzzling results on the role of the $\gamma\delta$ TCR in the development of effector subsets of murine $\gamma\delta$ T-cells (34–36), namely, CD27 $^+$ CD122 $^+$ $\gamma\delta$ T-cells producing IFN- γ or CD27 $^-$ CCR6 $^+$ $\gamma\delta$ T-cells making IL-17 (9, 10). First, Chien and co-workers showed that T10/T22-specific $\gamma\delta$ T-cells required thymic expression of their TCR ligand to differentiate into IFN- γ producers, in contrast with “ligand naïve” IL-17 producers (9). Consistent with this, TCR-dependent thymic selection was also shown to set the functional potential of dendritic epidermal T-cells (DETC) progenitors away from IL-17 production (37). Furthermore, peripheral IL-17-producing CD27 $^-$ CCR6 $^+$ $\gamma\delta$ T-cells were shown to expand and produce IL-17 independently of TCR activation (38). However, a subsequent study by Chien and collaborators demonstrated that a subset of phycoerythrin (PE)-specific $\gamma\delta$ T-cells produced IL-17 specifically upon TCR ligation (39). Moreover, a recent study by Hayday and colleagues suggested that an impairment in Zap70 signaling (in SKG mice) mostly affected the development of IL-17 $^+$ rather than IFN- γ $^+$

$\gamma\delta$ T-cells (40). The authors further proposed that “innate-like” $\gamma\delta$ T-cell populations, including IL-17 producers and some subsets of IFN- γ producers, receive strong TCR signals during thymic development to become hyporesponsive to TCR stimulation in the periphery (40). Future research should aim to resolve the apparent contradictions of the available data, namely, by clarifying the requirement on TCR ligand engagement, as well as the developmental effects of manipulating distinct $\gamma\delta$ TCR signaling pathways and their downstream (transcriptional and post-transcriptional) mechanisms on $\gamma\delta$ T-cell subsets.

SIGNAL 2: COSTIMULATORY RECEPTORS

A series of T-cell costimulatory receptors are known to induce qualitative and quantitative changes that lower activation thresholds, prevent “anergy” and enhance T-cell functions. Typical costimulatory receptors are type I transmembrane proteins that can be divided into two groups, based on their structural characteristics: immunoglobulin (Ig) or tumor necrosis factor receptor (TNFR) superfamilies. Ig superfamily members have a variable Ig-like extracellular domain and a short cytoplasmic tail, whereas TNFR family members present extracellular domains rich in six cysteine repeats (which form disulfide bridges) and a more complex cytoplasmic tail [reviewed in Ref. (41)]. These two main types of costimulatory receptors display different modes of intracellular signaling: whereas the CD28 family members associate directly with protein kinases (like PI3K or ITK), TNFR superfamily co-receptors require the adaptor proteins TRAF (TNFR-associated factor), namely TRAF2 and TRAF5, to link to downstream signaling mediators (Table 1). Here, based on their specific roles in $\gamma\delta$ T-cells, we shall discuss CD28 (of the Ig superfamily) and the TNFR superfamily members, CD27, CD30, and CD137 (4-1BB).

The best studied costimulatory receptor, CD28, has historically yielded paradoxical results on $\gamma\delta$ T-cells (46). We have recently readdressed this issue for both human and mouse $\gamma\delta$ T-cells. We described that CD28 is constitutively expressed on lymphoid $\gamma\delta$ T-cells and promotes survival and proliferation via IL-2 production. CD28 receptor agonists enhanced $\gamma\delta$ T-cell expansion, which was conversely inhibited by blocking antibodies against its B7 ligands (42). Importantly, CD28-deficient mice displayed lower (relative to controls) numbers of total or activated $\gamma\delta$ T-cells upon *Plasmodium berghei* infection, and failed to expand both their IFN- γ $^+$ and IL-17 $^+$ subsets (42). In contrast, Hayes and colleagues reported that both functional $\gamma\delta$ T-cell subsets differentiated and expanded normally in a *Listeria* model (80). It would be interesting to determine how variable is the dependence on CD28 costimulation for $\gamma\delta$ T-cell responses to distinct infectious agents.

In naïve mice, while CD28 is not required for the development of either IFN- γ $^+$ or IL-17 $^+$ $\gamma\delta$ T-cell subsets (80), the TNFR superfamily member CD27 is selectively implicated in the generation of IFN- γ $^+$ $\gamma\delta$ T-cells (10). In fact, we showed that CD27 expression segregates IFN- γ $^+$ (CD27 $^+$) and IL-17 $^+$ (CD27 $^-$) $\gamma\delta$ T-cells. Most interestingly, these phenotypes are established in the thymus, and since embryonic stages. Based on the results from our (10) and Chien’s (9) teams, the development of IFN- γ -producing $\gamma\delta$ T-cells seemingly requires strong TCR signaling and CD27 costimulation in the thymus.

Table 1 | Co-receptors of $\gamma\delta$ T-cells – extracellular ligands and intracellular signaling pathways.

| Receptor | Ligands | Intracellular signaling initiators/adaptors | Downstream signaling pathway | Target molecules | Reference |
|----------------|--|---|--|---|-----------------|
| CD28 | B7.1 (CD80) B7.2 (CD86) | PI3K ITK Grb2 | PI3K/AKT Grb2/MEK/ERK | IL-2, NF- κ B, AP-1, Bcl-x _L , NFAT | (42–45) |
| CD27 | CD70 | TRAF2 TRAF5 Siva | IKK/NF- κ B JNK | NF- κ B, Ca ²⁺ , <i>cyclinD2</i> , <i>Bcl2a1</i> , Bcl-x _L | (46–49) |
| CD30 | CD30L | TRAF2 TRAF5 | TRAF/IKK/IkB Ca ²⁺ | NF- κ B, IL-4, IFN γ , IL-8, CC chemokines | (46, 50, 51) |
| 4-1BB (CD137) | CD137L | TRAF2 | | NF- κ B, IFN γ | (52–54) |
| IL-2R | IL-2 | Jak1 | PI3K/AKT | IFN γ , TNF- α , T-bet, | (55–58) |
| IL-15R | IL-15 | Jak3 | Jak/STAT4/STAT5 MEK/ERK STAT1 | eomesodermin | |
| IL-7R | IL-7 | Jak1 Jak3 | STAT3 | IL-17, SOCS3 | (59) |
| IL-21R | IL-21 | Jak1 Jak3 | STAT3 | CXCL13, CXCR5 | (60) |
| NKG2D | MIC(A–B) ULBP (1–6) H60 MULT1 RAE1 | DAP10 | PI3K/AKT Grb2/VAV1/SOS1 PKC θ /Ca ²⁺ | NF- κ B, RelB, Bcl-x _L , Bcl-2 | (32, 46, 61–63) |
| NKp30 | B7-H6 BAT3 | CD3 ζ | cAMP/PKA | CC chemokines: CCL3, CCL4, CCL5 | (64–67) |
| NKp44 | NKp44L | DAP12 | Zap70/Syk | | (64, 68–70) |
| DNAM-1 (CD226) | Nectin-like-5 Nectin-2 | PKC LFA-1 Fyn | SLP-76/VAV1/ERK | | (71, 72) |
| PD-1 | PD-L1 (B7-H1) PD-L2 (B7-DC) | SHP-1 SHP-2 | CK2/PTEN/PI3K/AKT MEK/ERK | GSK-3, Bcl-x _L Smad3, Cdc25A, IFN γ , IL-2 | (73–76) |
| BTLA | HVEM | SHP-1 SHP-2 | Zap70/ERK | IL-17, TNF, IL-2 | (77–79) |

Beyond its role in thymic differentiation, CD27 is critical for the expansion of peripheral IFN- γ -producing $\gamma\delta$ T-cells upon infection with herpes viruses or malaria parasites in mice (81). We showed that, in the context of TCR stimulation and upon ligation to CD70, CD27 signaling activates the non-canonical NF- κ B pathway and enhances the expression of anti-apoptotic and cell cycle-related genes, thus promoting murine $\gamma\delta$ T-cell survival and proliferation (81).

We have also addressed the impact of CD27 costimulation on the activation of human $\gamma\delta$ T-cells. Administration of soluble recombinant CD70 enhanced, whereas anti-CD27 (or anti-CD70) antibodies reduced, V γ 9V δ 2 T-cell expansion *in vitro* (82).

Moreover, CD27 signals induced calcium fluxes and upregulated the expression of *Cyclin D2* and the anti-apoptotic gene *Bcl2a1*. Given the typical IFN- γ secretion and cytotoxicity of activated V γ 9V δ 2 T-cells (30), our work suggests that the modulation of CD70–CD27 signals may be beneficial in the context of $\gamma\delta$ T-cell-based cancer immunotherapy.

Upon activation, human $\gamma\delta$ T-cells can also express another TNFR superfamily member, CD30 (83). CD30 signaling, which potentiated calcium fluxes induced by TCR activation, also enhanced pro-inflammatory cytokine production (50). Recently, Yoshikai and colleagues compared $\gamma\delta$ T-cell homeostasis and response to *Listeria monocytogenes* in CD30-sufficient versus

deficient mice. They demonstrated a selective depletion of IL-17-producing V γ 6 $^+$ T-cells in mucosal tissues in the steady-state and upon infection (84). This associated with reduced bacterial clearance, which could be rescued, alongside the IL-17 $^+$ V γ 6 $^+$ T-cell pool, by agonistic anti-CD30 antibody administration. In contrast, Lee et al. reported that agonistic anti-CD137 (4-1BB) antibodies promoted the expansion of IFN- γ $^+$ V γ 1 $^+$ T-cells, which protected (in an IFN- γ -dependent manner) also from *Listeria* infection (52). This study also showed that 4-1BB was expressed and functional on activated human $\gamma\delta$ T-cells, and its ligation upon cell transfer protected NOD/SCID mice against *Listeria* infection.

Interestingly, activated V γ 9V δ 2 T-cells also express high levels of 4-1BBL (CD137L) (85), which besides acting as a ligand for 4-1BB on T and NK-cells, may also participate in V γ 9V δ 2 T-cell activation due to its known reverse signaling ability (86). This may, in fact, also apply to CD70 (CD27-ligand), which is highly induced upon phosphoantigen-mediated stimulation of V γ 9V δ 2 T-cells (82, 87). These possibilities deserve further investigation.

SIGNAL 3: CYTOKINE RECEPTORS

Interleukins are key determinants of T-cell survival, proliferation, and differentiation. IL-7, IL-15, and IL-2 are essential for lymphocyte development and homeostasis; upon inflammation, other cytokines, namely, IL-1 β , IL-12, IL-18, IL-21, and IL-23, take a central role in determining T-cell functions. Here, we review the main contributions of homeostatic and inflammatory cytokines specifically to $\gamma\delta$ T-cell physiology.

IL-7 and IL-15 are seemingly the key determinants of murine $\gamma\delta$ T-cell development (88–90) and homeostasis (91). A recent study that depleted IL-7 specifically from (Foxn1 $^+$) thymic epithelial cells showed that $\gamma\delta$ T-cells were significantly reduced in the adult thymus and in the gut, whereas they were completely absent in the fetal thymus and epidermis (89). In the dermis, it was also IL-7, but not IL-15, that supported the development and survival of the resident $\gamma\delta$ T-cell population (92). Conversely, in the gut, IL-15 seems to play the primordial role in sustaining the local intraepithelial $\gamma\delta$ T-cell compartment (93).

Unexpectedly, IL-7 was recently reported to promote the selective expansion of murine IL-17-producing $\gamma\delta$ T-cells (59). STAT3-dependent IL-7 signals allowed CD27 $^-$ $\gamma\delta$ T-cells to resist activation-induced cell death (AICD) and undergo proliferative responses to TCR agonists. Such an IL-7/IL-17 axis was also reported to be required for the $\gamma\delta$ T-cell response to viral hepatitis infection *in vivo* (94). Moreover, IL-7 also seems to support the expansion of human IL-17-producing $\gamma\delta$ T-cells (59).

We recently assessed the functional differentiation of human $\gamma\delta$ thymocytes, which are >80% of the V81 subtype. We observed that IL-15 and IL-2, but not IL-7, induced the cytotoxic type 1 (IFN- γ -producing) program in functionally immature $\gamma\delta$ thymocytes (55). This was consistent with previous data on peripheral $\gamma\delta$ T-cells isolated from cancer patients (95). However, additional reports on peripheral V γ 9V δ 2 T-cell cultures showed that IL-15 or IL-2 stimulation, despite efficient ERK and AKT activation, were not sufficient to induce effector responses; these required phosphoantigen-dependent TCR activation and downstream calcium mobilization (56, 96). Unexpectedly, in our cultures of $\gamma\delta$ (mostly V81) thymocytes, TCR stimulation was not required for

neither ERK activation nor T-bet and eomesodermin induction and the acquisition of effector functions (55).

IL-2 and IL-15 play key roles in the peripheral expansion of V γ 9V δ 2 T-cells in response to microbial phosphoantigens or synthetic drugs like bisphosphonates (56, 97). This notwithstanding, it is important to note, toward the therapeutic application of V γ 9V δ 2 T-cells, that optimal effector responses seemingly require the combination of these cytokines with TCR agonists. Thus, recent work from Chen and colleagues demonstrated that the differentiation of cytotoxic type 1 V γ 9V δ 2 T-cells capable of controlling *Mycobacterium tuberculosis* infection in macaques required a phosphoantigen/IL-2 combination (98).

Effector $\gamma\delta$ T-cell differentiation is also greatly impacted by inflammatory cytokines, particularly IL-12 and IL-18 that typically promote IFN- γ production; and IL-1 β and IL-23 that mostly drive IL-17 production.

High expression of IL-12R β expression on activated murine $\gamma\delta$ T-cells guarantees a dominance of type 1 (IFN- γ $^+$) over type 2 (IL-4 $^+$) effector fates (99). Type 1 differentiation is also predominant in human $\gamma\delta$ T-cells, and can be further enhanced by IL-18 (100, 101) or IL-21 (102). The induction of a type 17 program in human $\gamma\delta$ T-cells requires persistent stimulation with IL-23 for neonatal V γ 9V δ 2 T-cells (15); and IL-23 and IL-1 β in the presence of TGF- β for adult V γ 9V δ 2 T-cells (13, 103). In mice, IL-1 β and IL-23 are also the main drivers of abundant IL-17 production by peripheral $\gamma\delta$ T-cells (3, 5, 81, 104–106), although recent data surprisingly suggest that IL-18 can replace IL-1 β in combining with IL-23 to induce IL-17 expression (107). In contrast, IL-1 β upstream of IL-1R seems essential for GM-CSF production by $\gamma\delta$ T-cells (108).

Finally, IL-21 was recently suggested to endow human V γ 9V δ 2 T-cells with B-cell helper activity associated with a T follicular helper cell-like phenotype (60, 109), which may impact on the generation of high affinity antibodies against microbial infections.

SIGNAL 4: NATURAL KILLER RECEPTORS

An important key characteristic that allows the recognition of transformed cells by $\gamma\delta$ T-cells is the expression of a wide set of germline-encoded receptors that were initially described in NK-cells and hence are collectively known as NK receptors (NKR), including natural cytotoxicity receptors (NCRs).

The C-type lectin-like NK receptor group 2 member D (NKG2D) is the best studied NKR in $\gamma\delta$ T-cells. NKG2D binds extracellularly to multiple ligands of the MIC(A–B) and ULBP (1–6) families in humans; and to H60, MULT1, and various RAE1 molecules in mice (110). NKG2D ligands are induced upon cellular stress, for example, downstream of the DNA-damage response pathway in tumor cells (111, 112). The biological significance of this recognition system is underlined by the increased susceptibility of NKG2D-deficient mice to tumor development (113).

Intracellularly, NKG2D binds to DNAX-activating protein of 10 kDa (DAP10), which carries an YXNM motif that after tyrosine phosphorylation recruits PI3K or a Grb2–Vav1–SOS1 signaling complex (Table 1). This motif is similar to that in CD28, and thus, NKG2D/DAP10 may provide T-cells with costimulatory signals that synergize with the ITAM-based TCR/CD3 complex (61). However, unlike $\alpha\beta$ T-cells but similarly to NK-cells, $\gamma\delta$ T-cells can

express both DAP10 and DAP12 (62). The latter contains an ITAM motif, which after tyrosine phosphorylation recruits and activates Syk and ZAP70. Interestingly, only murine but not human NKG2D is able to associate with DAP12 (in addition to DAP10).

The controversy on a primary stimulatory versus costimulatory role of NKG2D in $\gamma\delta$ T-cells has been discussed elsewhere (46, 114). Briefly, the costimulatory function of NKG2D in human V γ 9V δ 2 T-cells was supported by additive effects on TCR-mediated activation: an upregulation of cytokine production upon MICA-NKG2D interactions (115); and an increase in intracellular calcium mobilization and cytotoxic activity (32). However, other lines of evidence have suggested that NKG2D signals can activate $\gamma\delta$ T-cells in the absence of TCR engagement: NKG2D ligation can upregulate CD69 expression in V γ 9V δ 2 T-cells to similar extent as TCR stimulation (116); NKG2D but not TCR blockade can inhibit V γ 9V δ 2 T-cell cytotoxicity against various hematological tumors (117); and murine DETC can target tumors upon recognition of NKG2D ligands (6, 118).

Another NKR implicated in tumor cell recognition by V γ 9V δ 2 T-cells is DNAX accessory molecule-1 (DNAM-1). DNAM-1 is an Ig-like family glycoprotein composed of a cytoplasmic domain containing three putative sites of phosphorylation by intracellular kinases. The phosphorylation of the Ser329 by protein kinase C (PKC) was shown to be critical for the association between DNAM-1 and LFA-1, which recruits the Fyn Src kinase to phosphorylate the Tyr322 of DNAM-1, thus initiating downstream signaling leading to SLP-76 and Vav1 phosphorylation (Table 1) (119). Antibody-mediated DNAM-1 blockade impaired V γ 9V δ 2 T-cell cytotoxicity and IFN- γ production against hepatocellular carcinoma lines expressing Nectin-like-5 (71).

Recently, we characterized a V δ 1 $^+$ T-cell population capable of targeting hematological tumors resistant to fully activated V γ 9V δ 2 T-cells (120). Unexpectedly, the enhanced killer function resulted from induced NCR expression, namely NKp30 and NKp44, which had been previously regarded as NK-specific markers. Although neither V δ 1 $^+$ nor V δ 2 $^+$ cells express NCRs constitutively, these can be upregulated selectively in V δ 1 $^+$ cells by PI3K/AKT-dependent signals provided by γ c cytokines (IL-2 or IL-15) and TCR stimulation. Once expressed on the cell surface, NKp30 and NKp44 can signal via CD3 ζ and DAP12, respectively (64). We further showed that NKp30 and NKp44 are both functional in NCR $^+$ V δ 1 $^+$ T-cells and synergize with NKG2D to target lymphocytic leukemia cells (120).

In sum, NKRs seem critical for tumor recognition and deployment of the cytotoxic program that is endowed by TCR/ γ c cytokine-dependent differentiation, thus defining distinct mechanisms to be integrated in $\gamma\delta$ T-cell-mediated cancer immunotherapy.

SIGNAL 5: INHIBITORY RECEPTORS

Beyond efficient activation and deployment of effector functions, it is necessary to negatively regulate the T-cell response in order to return to the homeostatic baseline. Inhibitory receptors like PD-1 or CTLA-4 are known to be critical for this contracting phase of the T-cell response and have become major clinical targets in cancer immunotherapy. Although $\gamma\delta$ T-cells rarely express CTLA-4, they can upregulate PD-1 upon activation, while they constitutively

express BTLA, and thus these two receptors may be the key to control $\gamma\delta$ T-cell responses.

Programmed death-1 (PD-1) is absent or low expressed on circulating V γ 9V δ 2 T-cells but is rapidly induced upon activation (121). The cytoplasmic tail of PD-1 contains conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) and switch motif (ITSM), both of which are phosphorylated to recruit negative regulators that block Lck activity downstream of the TCR complex (122). Moreover, PD-1 ligation can augment the activity of the protein phosphatase and tensin homolog (PTEN), a cellular phosphatase that inhibits PI3K/AKT signaling and thus leads to impaired survival, proliferation, and IL-2 release (123). The expression of the ligand PD-L1 on tumor cells inhibited V γ 9V δ 2 T-cell cytotoxicity and IFN- γ production (121). However, zoledronate-induced accumulation of P-Ags in tumor cells and consequent V γ 9V δ 2 TCR activation seemed to overcome the inhibitory effect of PD-1/PD-L1 interactions. More research is required to understand the full extent to what PD-1 may control $\gamma\delta$ T-cell functions and homeostasis.

B- and T-lymphocyte attenuator (BTLA) is another inhibitory receptor, member of the CD28 family and structurally related to PD-1 and CTLA-4. Binding to its ligand, herpesvirus entry mediator (HVEM), induces phosphorylation of the ITIM domain and association with SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) and SHP-2, which leads to attenuation of cellular activation and growth (124). Recent data showed that BTLA engagement with HVEM reduced P-Ag/TCR-mediated signaling and inhibited V γ 9V δ 2 T-cell proliferation, including in response to lymphoma cells (77). Conversely, BTLA-HVEM blockade using monoclonal antibodies enhanced V γ 9V δ 2 TCR signaling and may thus have therapeutic potential for the positive manipulation of $\gamma\delta$ T-cells.

A detailed study on BTLA function in murine $\gamma\delta$ T-cells has revealed a selective involvement in the homeostasis of the IL-17-producing CD27 $^-$ $\gamma\delta$ T-cell subset (78). Although these cells constitutively express low levels of BTLA, it is upregulated by IL-7 stimulation and thereby limits $\gamma\delta$ T-cell numbers. Consequently, BTLA-deficient mice accumulated IL-17 $^+$ CD27 $^-$ $\gamma\delta$ T-cells and were more susceptible (than wild-type controls) to dermatitis, which could be reversed by agonist BTLA antibodies. Thus, BTLA may be an important target for controlling pathogenic $\gamma\delta$ T-cells in inflammatory and autoimmune diseases.

CONCLUDING REMARKS

A multitude of surface receptors has been shown to participate in $\gamma\delta$ T-cell differentiation and activation. However, some crucial aspects remain to be elucidated, such as the identity of most $\gamma\delta$ TCR ligands. Most importantly, we must improve the transfer of past and current basic research into future protocols for $\gamma\delta$ T-cell-based immunotherapy. In this context, some key questions are: how to balance $\gamma\delta$ TCR activation with “exhaustion” due to chronic stimulation? What can be achieved by manipulating the NK-like activation mode of $\gamma\delta$ T-cells? Which costimulatory receptors should be modulated, and at what stages, to boost the desired $\gamma\delta$ T-cell responses? Which combinations of cytokines enable the best effector $\gamma\delta$ T-cells for each therapeutic application? Which receptors are most useful to tune down or switch off pathogenic effector

$\gamma\delta$ T-cells? The answers to these questions must be obtained in appropriate *in vivo* pre-clinical models and hopefully next in the clinic.

For now, we would like to propose that the five types of receptor signals reviewed here define five distinct layers of regulation of $\gamma\delta$ T-cell differentiation, activation, and function. The $\gamma\delta$ TCR is critical for the initial stages of differentiation and for proliferative responses; both processes further require cytokine signals that promote cell survival, proliferation, and terminal effector function. Costimulatory and inhibitory receptors control the extent of $\gamma\delta$ T-cell expansion, with interesting biases toward specific effector subsets. Finally, NK receptors play a decisive role in tumor cell targeting by $\gamma\delta$ T-cells. Thus, we believe that the recognition of “stressed self” can be mediated by the $\gamma\delta$ TCR but also chiefly by NK receptors like NKG2D. As such, the characterization of both type of ligands on tumors may be critical to design protocols, select and monitor patients, and increase the chances of efficacious $\gamma\delta$ T-cell-based cancer immunotherapies.

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A highly focused antigen receptor repertoire characterizes $\gamma\delta$ T cells that are poised to make IL-17 rapidly in naïve animals

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Interleukin (IL)-17 plays a key role in immunity. In acute infections, a rapid IL-17 response must be induced without prior antigen exposure, and $\gamma\delta$ T cells are the major initial IL-17 producers. In fact, some $\gamma\delta$ T cells make IL-17 within hours after an immune challenge. These cells appear to acquire the ability to respond to IL-1 and IL-23 and to make IL-17 naturally in naïve animals. They are known as the natural Ty δ 17 (nTy δ 17) cells. The rapidity of the nTy δ 17 response, and the apparent lack of explicit T cell receptor (TCR) engagement for its induction have led to the view that this is a cytokine (IL-1, IL-23)-mediated response. However, pharmacological inhibition or genetic defects in TCR signaling drastically reduce the nTy δ 17 response and/or their presence. To better understand antigen recognition in this rapid IL-17 response, we analyzed the antigen receptor repertoire of IL-1R $^+$ /IL-23R $^+$ $\gamma\delta$ T cells, a proxy for nTy δ 17 cells in naïve animals directly *ex vivo*, using a barcode-enabled high throughput single-cell TCR sequence analysis. We found that regardless of their anatomical origin, these cells have a highly focused TCR repertoire. In particular, the TCR sequences have limited V gene combinations, little or no junctional diversity and much reduced or no N region diversity. In contrast, IL-23R $^-$ cells at mucosal sites similar to most of the splenic $\gamma\delta$ T cells and small intestine epithelial $\gamma\delta$ lymphocytes expressed diverse TCRs. This remarkable commonality and restricted repertoire of IL-1R $^+$ /IL-23R $^+$ $\gamma\delta$ T cells underscores the importance of antigen recognition in their establishment/function.

Keywords: $\gamma\delta$ T cells, TCR repertoire, high throughput TCR sequencing, IL-1R $^+$ $\gamma\delta$ T cells, IL-23R $^+$ $\gamma\delta$ T cells, IL-17 $^+$ $\gamma\delta$ T cells

INTRODUCTION

Interleukin (IL)-17 is an important cytokine in the inflammatory response. It induces chemokines and cytokines that mediate the maturation and release of neutrophils from the bone marrow. Neutrophil recruitment focuses the immune response at the site of infection to reduce pathogen load, and induces the subsequent phases of the inflammatory response, which primes antigen-specific $\alpha\beta$ T cell and B cell activation and initiates the resolution program. Although both $\alpha\beta$ T cells and $\gamma\delta$ T cells can make IL-17, $\alpha\beta$ T cells producing IL-17 (Th17 cells) require antigen-specific priming and a specific cytokine environment to develop. In acute infections, a rapid IL-17 response must be initiated without prior antigen exposure, and $\gamma\delta$ T cells have been identified as the major initial IL-17 producers in infections and after immunization [reviewed in Ref. (1)].

Some naïve $\gamma\delta$ T cells in secondary lymphoid organs undergo antigen-driven activation and differentiation to become IL-17 producers: within 24 h after immunization, antigen-specific $\gamma\delta$ T cells in the draining lymph node increase in numbers and show activated phenotypes (e.g., becoming CD44 hi and CD62L lo).

Forty-eight hours after immunization, activated $\gamma\delta$ T cells express ROR γ t and after another 12 h, these cells make IL-17A and IL-17F (2, 3), these are the inducible Ty δ 17 cells. Importantly, encountering antigen in an immune response induces the expression of inflammatory cytokine receptors such as IL-1R and IL-23R on $\gamma\delta$ T cells. Signaling through the T cell receptor (TCR) and the cytokine receptors can then induce sustained, high magnitude IL-17 production (2, 4). These observations provide a mechanistic basis for the induction of a sustained antigen-specific $\gamma\delta$ T cell IL-17 response, which is much more rapid than that of Th17 $\alpha\beta$ T cells.

In addition to the inducible Ty δ 17 cells discussed above, some $\gamma\delta$ T cells in naïve mice, such as those in the skin dermis, the peritoneum, intestinal lamina propria, the lung, and the spleen have an activated phenotype (CD44 hi CD62L lo) and express IL-1R and IL-23R. These cells make IL-17 a few hours after immune challenge—these are the natural Ty δ 17 (nTy δ 17) cells (1). The observation that IL-17 can be induced with IL-1 and IL-23 alone without deliberate TCR triggering has led to the supposition that the antigen recognition by these cells is irrelevant to their response (5).

Nonetheless, this response is inhibited by cyclosporine A (CsA) or by FK506 (2). Both compounds reduce nuclear factor of activated T cells (NFAT) activity and disrupt the calcineurin-NFAT signaling circuit activated by signaling through the antigen receptor (6). Furthermore, the amount of IL-17 induced by the inflammatory cytokines alone is much lower in magnitude when compared with that induced by cytokines together with TCR stimulation (2, 4), suggesting that robust IL-17 production requires combined signaling through the TCR and cytokine receptors. Moreover, the number of rapid IL-17 responding IL-1R $^+$ $\gamma\delta$ T cells in the intestinal lamina propria and peritoneum is markedly reduced in germ free mice, and in SPF mice treated with the antibiotic neomycin sulfate, vancomycin but not in mice treated with metronidazole when compared with SPF mice and the numbers can be restored by SPF microbiota reconstitution. However, the presence of these IL-1R $^+$ $\gamma\delta$ T cells requires signaling through VAV1, a guanine nucleotide exchange factor required for the activation of $\gamma\delta$ T cells via $\gamma\delta$ TCR ligation (7), but not the myeloid differentiation primary response protein 88 (MyD88) or toll-like receptor 3 signaling pathways (8). In addition, the number of n $\gamma\delta$ 17 cells is drastically reduced in the SKG mouse (9), which carries a mutation that reduces the function of the kinase domain of the TCR-proximal signaling kinase Zap70. These observations demonstrate the importance of TCR signaling in n $\gamma\delta$ 17 induction and function. To evaluate the contribution of antigen recognition to their function, we seek to determine the antigen receptor repertoire of n $\gamma\delta$ 17 cells. To this end, we use a bar-code-enabled high throughput single-cell TCR sequencing strategy, which allows us to identify the TCR γ and δ gene pair from each cell directly *ex vivo*, without the bias introduced through generating T cell clones or hybridomas. This method determines the entire sequence of both the TCR γ and δ chains, including the V gene segment and CDR3 region, such that we can properly define the antigen receptor specific repertoire, rather than describing these cells solely based on their V γ or V δ usage. The results are discussed below.

MATERIALS AND METHODS

MICE

C57BL/6 mice were purchased from Jackson Laboratories and housed in the Stanford Animal Facility for at least 1 week before use. IL-17F $^{Thy1.1/Thy1.1}$ mice (10) were bred and housed in the pathogen-free Stanford Animal Facility. IL-23R EGFP mice (11) were bred and housed in the pathogen-free Merck Research Laboratories, Palo Alto Animal Facility. All experiments were performed in accordance with the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee.

ANTIBODIES AND CELL ISOLATION

Antibodies were purchased from either eBioscience or BD Biosciences unless otherwise stated. All analyses and sorting were performed on a BD Aria or Falstaff sorter. $\gamma\delta$ T cells were enriched from mouse splenocytes or peritoneal cells by negative depletion as described (2).

To isolate Thy1.1 positive spleen $\gamma\delta$ T cells from IL-17F $^{Thy1.1/Thy1.1}$ reporter mice, enriched $\gamma\delta$ T cells were stained with PE-GL3, Pacific Blue-CD3e, PerCP-Cy5.5-Thy1.1, PerCP/Cy5.5 Mouse IgG1, κ Isotype Ctrl (OX-7 and its isotype control;

BioLegend), LIVE/DEAD Aqua, APC-Cy7 conjugated anti-TCR β , CD19, CD11b, CD11c, F4/80, TER-119. APC-Cy7 and Aqua positive cells are excluded from analysis. Peritoneal IL-1R positive $\gamma\delta$ T cells were isolated from C57BL/6J mice i.p. infected with 1000 tachyzoites of Type II Me49 strain of *Toxoplasma gondii* 5 h prior. To isolate IL-1R (CD121a) positive cells, enriched $\gamma\delta$ T cells were stained with PE-GL3 (pan anti- $\gamma\delta$ TCR), PE-Cy7-CD3e (145-2C11), APC-CD121a (JAMA-147; BioLegend), LIVE/DEAD Aqua, and APC-Cy7 conjugated anti-TCR β H57-597, CD19 (1D3), CD11b (M1/70), CD11c (N418), F4/80 (BM8), TER-119 (TER-119). APC-Cy7 and Aqua positive cells are excluded from analysis. Dermal split-thickness skin was obtained from C57BL/6J mice ears. Dermal sheets were prepared by incubation of split-thickness skin with 0.25% trypsin for 16 h at 4°C, and subsequent removal of the epidermis. Dermal sheets were digested with 2.5 mg/ml collagenase and 0.3 mg/ml hyaluronidase for 45 min at 37°C to release dermal cells. Dermal cells were stained with PE-GL3, APC-Cy7-CD3e antibodies and Live/Dead Aqua. GL3 and CD3e positive dermis $\gamma\delta$ T cells were isolated with FACS.

Two- to four-month-old female IL-23R EGFP $^{+/-}$ mice were used for the isolation of IL-23R $^+$ and IL23R $^-$ $\gamma\delta$ T cells. Five mice were combined for each type of tissue preparation. Visceral fat was directly minced in 4 mg/ml collagenase II (Worthington), 5% FBS in RPMI followed by shaking for 45 min at 37°C. Cells were further purified with 36% Percoll gradient (GE Healthcare) in PBS and spun at 2000 rpm for 5 min at room temperature. The floating layer and Percoll layer were aspirated and the resulting cell pellet was suspended in PBS, counted, and stained for flow cytometry. Colons were cleaned and washed in PBS and minced into 1 cm segments and placed into 0.5 mM EDTA in PBS. After shaking for 20 min at 37°C, the intraepithelial cell rich supernatant was discarded. Colon fragments were washed with PBS, then further minced to pieces <0.25 cm 3 in size in digestion buffer [PBS + 10% FCS + 1 mg/ml collagenase D (Sigma) + 2000 U/ml DNase I (Sigma) + Dispase (Corning, dilute 1:100)], and incubated with shaking for 20 min at 37°C. Cells were further purified with percoll gradient as described for isolating cells from fat. Isolated cells were stained with FcBlock, CD3 Percp-Cy5.5, TCR δ APC (Clone GL3), TCR β APC-Cy7, CD4-PE, CD8 α PE-Cy7, Live/Dead Aqua. IL-23R GFP $^+$ and IL-23R GFP $^-$ $\gamma\delta$ T cells were single sorted into the wells of a 96-well plate using a FACsAria II (BD Biosciences).

BARCODE-ENABLED HIGH THROUGHPUT SINGLE-CELL TCR DETERMINATION

Single T cells are sorted into 96-well PCR plates and sequencing is performed as described (12), except murine $\gamma\delta$ TCR specific primers are used for this study. $\gamma\delta$ TCR primer sequences and the sequencing reaction are described in detail in Supplemental Methods in Supplementary Material. Briefly, an RT-PCR reaction is carried out with TCR primers. The products are then used in a second PCR reaction, with nested primers for TCR genes. A third reaction is then performed that incorporates individual barcodes into each well. The products are combined, purified, and sequenced using the Illumina MiSeq platform. The resulting paired-end sequencing reads are assembled and de-convoluted using barcode identifiers at both ends of each sequence by a custom software pipeline to separate reads from every well in every

plate. The resulting sequences are analyzed using VDJFasta (13), which we have adapted to resolve barcodes and analyze sequences with a customized gene-segment database. The CDR3 nucleotide sequences are then extracted and translated. Barcode design is shown in Figure S1 in Supplementary Material and TCR sequencing primer sequences are shown in Table S2 in Supplementary Material.

RESULTS

A defining feature of nT γ 17 cells is their surface expression of IL-1R and IL-23R in naïve animals. To determine the antigen receptor repertoire of γ T cells that are “poised” to mount a rapid IL-17 response, we analyzed skin dermal cells, and IL-23R $^{+}$ γ T cells in the colon lamina propria, fat, and spleen of naïve IL-23R reporter mice (IL-23R EGFP). Peritoneal nT γ 17 cells are characterized by their IL-1R expression in rapid response situations (8); therefore, we analyzed IL-1R $^{+}$ peritoneal γ T cells from C57/BL6 mice that were intra-peritoneally (i.p.) infected with *T. gondii* 5 h prior. Representative FACS analysis and gates used to isolate these cells are shown in Figure 1. The TCR sequences were determined from a single FACS sorted γ T cell using a bar-code-enabled high throughput single-cell TCR sequencing strategy. We found that

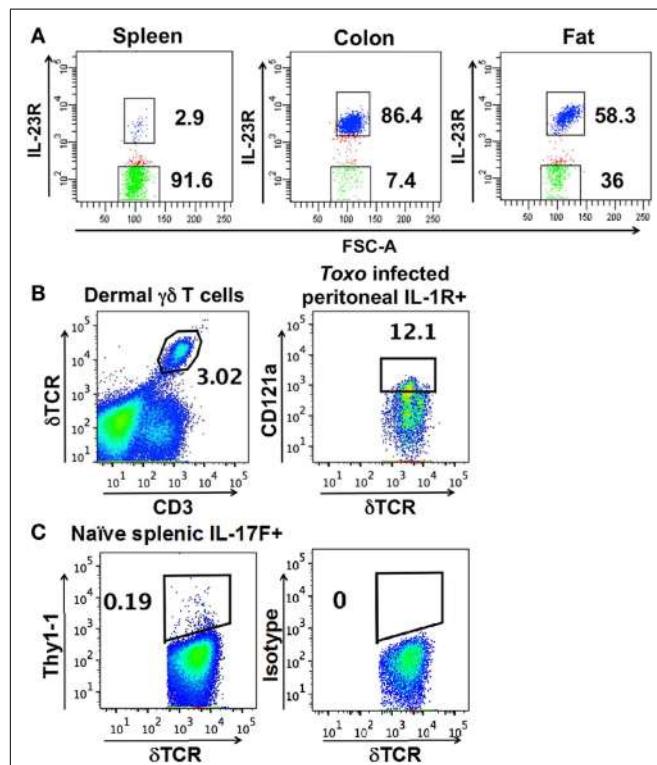


FIGURE 1 | Representative FACS analysis and gates used to isolate (A) IL-23R $^{+}$ (in blue) and IL-23R $^{-}$ $\gamma\delta$ T cells (in green) (using FACSDiva) from IL-23R reporter mice. (B) Dermal cells, IL-1R $^{+}$ $\gamma\delta$ T cells from the peritoneum of C57BL/6 mice infected with *T. gondii* 5 h prior. (C) Thy1.1 $^{+}$ cells from the spleen of naïve IL-17F reporter mice. (B,C) are plotted using FlowJo. The number within each graph indicates the percentage of the designated population of cells out of the total $\gamma\delta$ T cells.

IL-17F $^{+}$ spleen $\gamma\delta$ T cells from naïve IL-17F reporter mice (IL17 thy1.1/thy1.1) and IL-23R $^{+}$ spleen $\gamma\delta$ T cells from naïve IL-23R reporter mice have similar TCR repertoires (Figure 2). This observation is consistent with the supposition that IL-23R $^{+}$ $\gamma\delta$ T cells in naïve animals can be used as a proxy for nT γ 17 cells in TCR repertoire analysis.

A striking characteristic of the TCR repertoire of IL-1R $^{+}$ /IL-23R $^{+}$ $\gamma\delta$ T cells is the lack of diversity. They express TCRs with limited V gene combinations, little or no junctional diversity and much reduced or no N region diversity. In particular, a single pair of TCR sequences encoded by V81D82J81 and V γ 6J γ 1 (Group 1 sequences, Figure 2) dominates the repertoire of dermal cells, IL-23R $^{+}$ $\gamma\delta$ T cells from the lung, colon, and IL-1R $^{+}$ $\gamma\delta$ T cells from the peritoneum. These cells also utilize two sets of closely related TCR sequences, which consist of similar V γ 4J γ 1 rearrangements, paired with very similar V85D82J82 (designated as Group 2, 3 sequences, Figure 2). Naïve spleen IL-23R $^{+}$ and IL-17F $^{+}$ T cells did not have a dominant population that expressed Group 1 sequences. Instead, cells with the Group 3 sequences were more represented. Some of these $\gamma\delta$ T cells also expressed TCRs consisting of Group 3 TCR γ chains paired with a very similar V84D82J82 TCR δ chains (designated as the Group 4 sequences, Figure 2).

In contrast, reported TCR sequences identified from spleen $\gamma\delta$ T cells and small intestine epithelial $\gamma\delta$ lymphocytes (IELs) (14–16) and IL-23R $^{-}$ $\gamma\delta$ T cell populations in the spleen, lung, and colon lamina propria analyzed here (Table S1 in Supplementary Material) are highly diverse, using different V γ 's and V8's, with CDR3 regions consisting of both D81 and D82 gene segments in all three reading frames, and N regions in each of the gene-segment junctions. An analysis of CDR3 paratope convergence within IL-23R $^{-}$, IL-23R $^{+}$, and IL-17F $^{+}$ $\gamma\delta$ T cell populations is shown in Figure 3. Along this line, it should be noted that the antigen-specific $\gamma\delta$ T cells, including the inducible T γ 17 cells, also utilize diverse TCRs (2, 3, 16). In this context, ~1/3 of the IL-23R $^{+}$ or IL-17F $^{+}$ spleen $\gamma\delta$ T cells, and ~1/5 of IL-23R $^{+}$ lung $\gamma\delta$ T cells express TCRs with different V γ V δ genes and diverse CDR3 regions. The spleen and lungs are continuously exposed to blood-borne or air-borne environmental antigens. It is likely that the TCR repertoire of IL-1R $^{+}$ /IL-23R $^{+}$ $\gamma\delta$ T cells reflects both the natural and the inducible T γ 17 cells.

Despite the fact that a substantial number of IL-1R $^{+}$ /IL-23R $^{+}$ $\gamma\delta$ T cells and dermal $\gamma\delta$ T cells express TCRs with similar V γ 4J γ 1 rearrangement (CSYG-(X)Y-SSGFHK), V γ 4 $^{+}$ TCR γ chain sequences are not utilized exclusively by this set of T cells. In fact, ~50% of the IL-23R $^{-}$ cells also expressed TCRs with V γ 4, and more than half of these V γ 4 sequences were also expressed in IL-23R $^{+}$ cell populations (Figure 4).

DISCUSSION

Our analysis showed that regardless of their anatomical location, IL-1R $^{+}$ /IL-23R $^{+}$ $\gamma\delta$ T cells express a highly focused antigen receptor repertoire. While all major groups of TCR sequences expressed by these cells result from rearrangements with exonuclease digestion and P nucleotide addition (17), only Group 3 and 4 TCR sequences have N nucleotides at the CDR3 γ and δ junctions. The N nucleotides are generated at the terminal of the combining gene segments by terminal transferase (TdT) in a template-independent

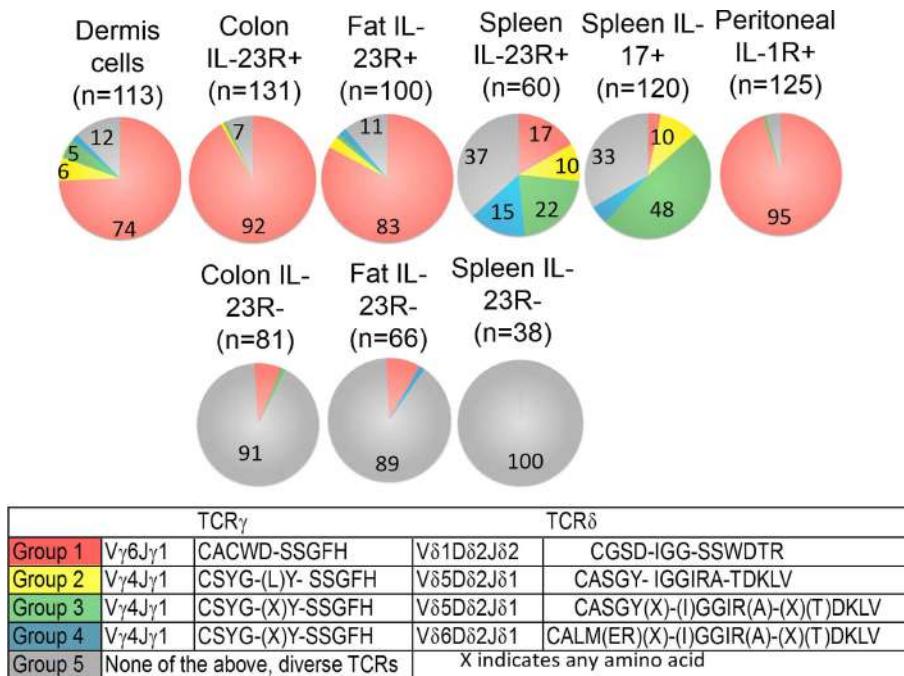


FIGURE 2 | Frequency of each major group of TCR sequences in IL-1R⁺/IL23R⁺ $\gamma\delta$ T cell populations. Spleen IL-17⁺, IL-23R⁺, IL-23R⁻ $\gamma\delta$ T cells, lung, fat, and colon lamina propria IL-23R⁺ and IL-23R⁻ $\gamma\delta$ T cells, peritoneum IL-1R⁺ $\gamma\delta$ T cells 5 h after intraperitoneum *Toxoplasma gondii* infection and skin dermal $\gamma\delta$ T cells were analyzed. Each cell population is represented by one pie chart. Each section of the pie chart represents one group of TCR sequences, color-coded as described. *n*, total number of analyzed sequences. The number within each section of the pie chart indicates the percentage of a given group of TCR sequences in the total

number of analyzed sequences of that cell population (Table S1 in Supplementary Material). All experiments were performed two independent times, except the analysis of spleen IL-23R⁺ and IL-23R⁻ $\gamma\delta$ T cells, which were isolated and analyzed once. TCR sequences from two independent isolations and analyses are very similar and the combined results are shown. In two independent experiments, 58% and 82% of the total colon $\gamma\delta$ T cells are IL23R⁺; 74% and 86% of total fat $\gamma\delta$ T cells are IL-23R⁺; 0.1% and 0.2% of spleen cells are IL-17F⁺; 2.9% of spleen $\gamma\delta$ T cells are IL23R⁺. In the peritoneum 5 h after infection, 12 and 30% of the $\gamma\delta$ T cells are IL-1R⁺.

manner. In mice, TdT is not expressed in developing thymocytes until 4–5 days after birth (18). Thus, $\gamma\delta$ T cells that express Group 1 and 2 sequences are most likely generated during the fetal and/or neonatal stages. Indeed, Group 1 TCR has also been described for hybridomas derived from fetal and newborn $\gamma\delta$ thymocytes (19) and is also present at the mucosal sites (20–22). Our observation that Group 3, 4 TCR expressing IL-1R⁺/IL-23R⁺ $\gamma\delta$ T cells are prevalent in the spleen and present in the lung and skin is consistent with the observation that adult precursor cells contribute to the n γ δ 17 cell pool and that these cells express V γ 4⁺ TCR γ chains (23–25).

Group 1 TCR sequences have been described for $\gamma\delta$ T cell hybridomas generated from lung epithelium (26), from expanded $\gamma\delta$ T cells after *Listeria monocytogenes* and *Bacillus subtilis* infection and in models of autoimmune inflammation (27–29). In addition, the rapid appearance of V γ 6 and/or V δ 1 T γ δ 17 cells has been reported in various infection systems: *E. coli* (i.p.) (30, 31), *L. monocytogenes* (i.p. oral) (32, 33) and *Staphylococcus aureus* (i.p.) (34). V γ 6⁺ and V γ 4⁺ dermal $\gamma\delta$ T cells making IL-17 in response to imiquimod applied topically to induce skin inflammation has also been reported (24, 25). Separated TCR γ and δ chains of Group 4 sequences were identified from CFA-induced IL-17 making $\gamma\delta$ T cells (35, 36). Taken together, our repertoire analysis confirms

and advances previous studies of TCR usage of n γ δ 17 cells by defining the precise TCR sequences of these cells and observing how constrained they are. These observations suggest that antigen encountering is important for establishing their functional attributes, a finding consistent with observations that signaling through the TCR is essential for this process (2, 8, 9).

It is unclear what n γ δ 17 cells recognize. However, the identification of their TCR sequences is an important step forward in characterizing the antigens of these cells. In this context, O'Brien, Born and their colleagues demonstrated that a multimeric staining reagent of soluble TCR expressing the Group 1 sequences can bind L cells, NIH 3T3 cells, a keratinocyte cell line XB-2, as well as freshly isolated macrophages from naïve mice and from mice infected with *Listeria* (37, 38).

While n γ δ 17 responses are well documented in the mouse, it is unclear whether or not a human counterpart exists. In this regard, human and murine $\gamma\delta$ TCR gene sequences are very different. Thus, it is unlikely that one would find human $\gamma\delta$ TCRs that show the sequence equivalent of the TCRs described for the murine n γ δ 17 cells. However, one of the defining characteristics of adaptive immune recognition is that the antigen specificity, but not the particular antigen-specific receptor sequences, is conserved through evolution. The recognition of lysozyme by specific

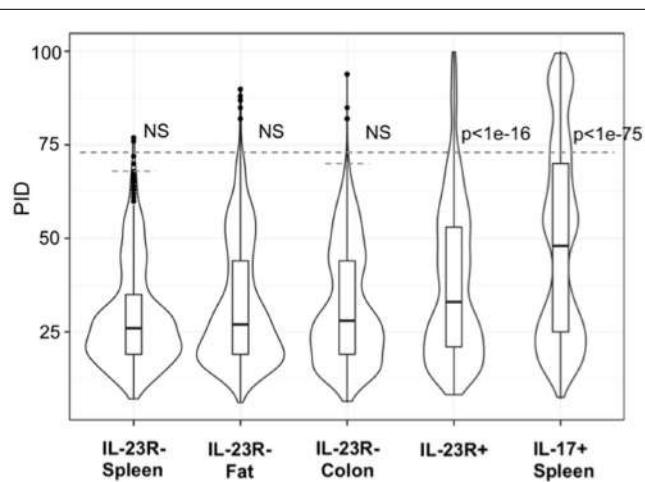


FIGURE 3 | Analysis of CDR3 paratope convergence of all unique sequences of IL-23R $^{-}$ $\gamma\delta$ T cells from the spleen, fat, colon samples, IL-17F $^{+}$ $\gamma\delta$ T cells from spleen and IL-23R $^{+}$ $\gamma\delta$ T cells (combined from all anatomical sites). Percent identity among aligned γ and δ CDR3 amino acid sequences for all pairwise comparisons within each group are represented in violin/Box plot. Significance assessed by the Mann-Whitney-Wilcoxon test with Bonferroni's correction for multiple testing given $a = 0.01$ set to $p < 0.001$ to be considered significance. Dotted line indicates average 99th percentile percent identity for the IL-23R $^{-}$ T cell populations (68% ID for spleen, 73% ID for fat, 70% ID for colon).

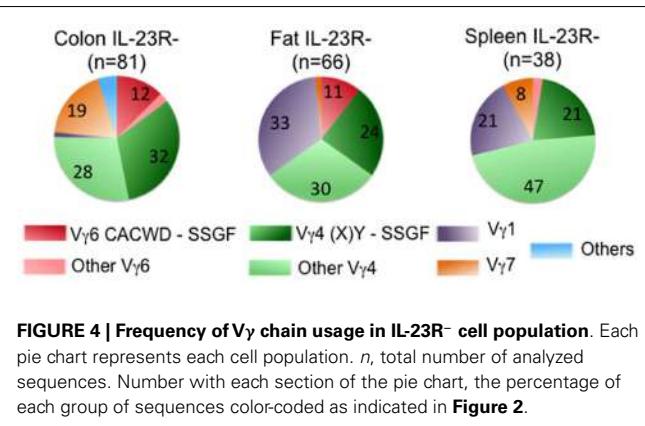


FIGURE 4 | Frequency of V γ chain usage in IL-23R $^{-}$ cell population. Each pie chart represents each cell population. n , total number of analyzed sequences. Number with each section of the pie chart, the percentage of each group of sequences color-coded as indicated in Figure 2.

murine, human, and camel antibodies as well as by the adaptive immune receptors of sea lamprey (39), and the recognition of the algae protein phycoerythrin (PE) by specific human and murine $\gamma\delta$ TCRs (2) are such examples. Thus, differences in the TCR gene sequences among different species should not preclude the presence of nT γ 17 cells.

It should be noted that the focused antigen receptor repertoire described here is based on the analysis of pairs of TCR γ and δ chains, consisting of V gene segments as well as CDR3 regions. While the majority of these $\gamma\delta$ T cells expressed V γ 6 or V γ 4, not all V γ 6 and V γ 4 expressing cells belong to this group of nT γ 17 cells. These observations underscore the need for caution in categorizing $\gamma\delta$ T cell function solely according to V gene

usage. The approach of determining TCR sequences from a single cell directly *ex vivo*, as outlined here, should facilitate future analysis of the contributions of $\gamma\delta$ T cells to a range of immune responses.

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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.2015.00118>

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The Jekyll and Hyde story of IL17-producing $\gamma\delta$ T cells

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In comparison to conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells are considered as specialized T cells based on their contributions in regulating immune response. $\gamma\delta$ T cells sense early environmental signals and initiate local immune-surveillance. The development of functional subtypes of $\gamma\delta$ T cells takes place in the thymus but they also exhibit plasticity in response to the activating signals and cytokines encountered in the extrathymic region. Thymic development of $T\gamma\delta 1$ requires strong TCR, CD27, and Skint-1 signals. However, differentiation of IL17-producing $\gamma\delta$ T cells ($T\gamma\delta 17$) is independent of Skint-1 or CD27 but requires notch signaling along with IL6 and TGF β cytokines in the presence of weak TCR signal. In response to cytokines like IL23, IL6, and IL1 β , $T\gamma\delta 17$ outshine Th17 cells for early activation and IL17 secretion. Despite expressing similar repertoire of lineage transcriptional factors, cytokines, and chemokine receptors, $T\gamma\delta 17$ cells differ from Th17 in spatial and temporal fashion. There are compelling reasons to consider significant role of $T\gamma\delta 17$ cells in regulating inflammation and thereby disease outcome. $T\gamma\delta 17$ cells regulate mobilization of innate immune cells and induce keratinocytes to secrete anti-microbial peptides thus exhibiting protective functions in anti-microbial immunity. In contrast, dysregulated $T\gamma\delta 17$ cells inhibit Treg cells, exacerbate autoimmunity, and are also known to support carcinogenesis by enhancing angiogenesis. The mechanism associated with this dual behavior of $T\gamma\delta 17$ is not clear. To exploit, $T\gamma\delta 17$ cells for beneficial use requires comprehensive analysis of their biology. Here, we summarize the current understanding on the characteristics, development, and functions of $T\gamma\delta 17$ cells in various pathological scenarios.

Keywords: $\gamma\delta$ T cell, IL17, $T\gamma\delta 17$, infection, inflammation, cancer

INTRODUCTION

Decades have passed since the accidental discovery of T cells expressing γ and δ chains (1), yet it is hard to define $\gamma\delta$ T cells like $\alpha\beta$ T cells. Ambiguity in understanding the functions of $\gamma\delta$ T cells is attributed to their unparalleled characteristics as compared to $\alpha\beta$ T cells. Current understanding of T cell biology has emerged extensively from studies on $\alpha\beta$ T cells; however, recent findings have underlined the crucial role of $\gamma\delta$ T cells in shaping the immune response in infections, inflammatory diseases, and cancer. They are involved in early immune response like innate cells, produce proinflammatory cytokines (IFN γ , IL17, and TNF α), and activate adaptive immune cells. The cytokines secreted by $\gamma\delta$ T cells determine their effector functions. In humans, the major cytokine produced by $\gamma\delta$ T cells is IFN γ , contributing to its role in anti-viral, anti-bacterial, and anti-tumor immunity (2–4). However, upon activation $\gamma\delta$ T cells can be skewed toward IL17, IL4, or TGF β producing phenotype governed by the polarizing cytokines present in the surrounding milieu (5). Recent investigations in mice and human have highlighted the role of IL17-producing $\gamma\delta$ T cells (hereafter referred as $T\gamma\delta 17$) in bacterial infection, inflammatory disease, and cancer (6–8). They are the primary source of IL17 in early disease condition and are pivotal in progression and disease outcome (9, 10). To understand the functional significance of $T\gamma\delta 17$ in pathological conditions, many efforts have made in mouse models but there is scanty literature available on human $T\gamma\delta 17$ cells. In this review, we will discuss the recent findings of

$T\gamma\delta 17$ differentiation, mechanisms regulating IL17 production, and their relevance in pathological conditions.

$\gamma\delta$ T CELLS: UNIQUE BUT VERSATILE

Survival of $\gamma\delta$ T cells over strong evolutionary selection pressure highlights their exclusive importance and disparate properties from conventional $\alpha\beta$ T cells. Initially, $\gamma\delta$ T cells were considered as cells of innate immunity owing to their ability to recognize conserved non-peptide antigens expressed by stressed cells. In addition to this, they recognize pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP) through pattern recognition receptors (PRR) expressed by them (11). Like adaptive immune cells, human $\gamma\delta$ T cells undergo clonal expansion and exhibit antigen-specific memory (12). Thus, $\gamma\delta$ T cells link innate and adaptive immunity thereby enhancing the immune response against invading pathogen or danger signal posed by “self” cells. Antigen recognition by murine or human $\gamma\delta$ T cells does not require antigen presentation by major histocompatibility complex (MHC) class I or class II (13) and the crystal structure of $\gamma\delta$ TCR has revealed its close homology with immunoglobulins suggesting that antigen recognition by $\gamma\delta$ T cells is similar to antigen–antibody interaction (14). However, diversity of antigens recognized by $\gamma\delta$ T cells brands it different from B cells. The antigens exclusively recognized by $\gamma\delta$ T cells are not peptides of protein antigens rather are small mono- and pyrophosphates of linear C5 isoprenoids called as phosphoantigens (13). These

prenyl pyrophosphates are metabolites of cholesterol biosynthesis and are recognized through complementarity determining regions (CDRs) of T γ T cells (15). In humans, during cholesterol biosynthesis, phosphorylated precursors such as isopentenyl pyrophosphate (IPP) and DMAPP (dimethylallyl pyrophosphate) are synthesized by mevalonate pathway (16). However, microbial pathogens use non-mevalonate pathway to produce these phosphorylated precursors (17). T γ T cells respond to these natural or synthetic stimulators with varying degree. Based on this, stimulators are classified either as weak or potent stimulators. HMBPP [(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate], a metabolite of non-mevalonate pathway of bacteria *Mycobacterium tuberculosis* is 10⁴ times more potent stimulator of human T γ T cells than IPP (18). The exclusive response of T γ T cells to these phospho-antigens has a potential therapeutic significance and synthetic pyrophosphates can be used to harness the cytotoxic potential of T γ T cells.

Murine and human T γ T cells also recognize phycoerythrin (PE) – fluorescent molecule of cyanobacteria and red algae. PE is directly recognized by T γ T cells but there is no sequence similarity between PE-specific murine and human T γ T TCR (19). Naturally occurring primary alkyl amines activate human V γ 2V δ 2 T cells and enhance immunity against certain microbes and plant-derived antigens (20, 21). Similar to natural killer (NK) cells, human T γ T cells also recognize the stress-induced MHC class I-related molecules MICA, MICB, and the UL16-binding proteins that are upregulated on malignant or stressed cells (22, 23). The stress-related molecules are ligands for NKG2D expressed by T γ T cells and this engagement also enhances T γ T cells' response to non-peptide antigens (24). Human and murine T γ T cells recognize lipid antigens presented by CD1 molecules, a classical ligand for NK T cell suggesting the phenomenon similar to MHC-restricted antigen recognition by $\alpha\beta$ T cells (25–27). The murine T γ T cells also recognize non-classical MHC class I molecules like T10 and T22 (β 2 microglobulin-associated molecules lacking peptide binding groove) (28, 29). In addition to non-protein and MHC related antigens, murine and human T γ T cells also recognize small peptides such as heat shock proteins (HSPs) (30–32). However, they do not require antigen-presenting cells (APCs) and recognition of antigen is MHC unrestricted, resembling B cells (33). Thus, the broad spectrum antigen responsiveness of T γ T cells helps them to mount faster immune response.

Like $\alpha\beta$ T cells, T γ T cells develop in the thymus from CD4 $^-$ CD8 $^-$ (double negative, DN) thymocytes (34); however, they precede $\alpha\beta$ T cells in T cells ontogeny. T γ T TCR rearrangements can be traced in early embryonic stages in mice as well as in humans (35, 36). This highlights their role in neonatal protection as conventional T cells are functionally impaired and APCs are immature in newborns (37). During thymic development, the decision of T γ versus $\alpha\beta$ T cell commitment is determined by TCR signal strength or notch signaling (38). In mice, the strong TCR signaling in absence of notch signal induces T γ T cells lineage commitment whereas low TCR signal strength in presence of strong notch signaling promotes $\alpha\beta$ T cell lineage (39–41). However, notch signaling alone is insufficient to decide T γ /T $\alpha\beta$ T cell commitment. The intrinsic signals from T cell receptor complex and trans-conditioning by different subsets of thymocytes also

determine thymic development of T γ T cells (42). In humans, notch has opposite role in $\alpha\beta$ versus T γ T cell lineage decision, sustained notch signaling is required for the development of T γ T cells (43) which is determined by differential notch receptor-ligand interaction importantly Jagged2/Notch3 signaling (44). In human, T γ T cells differentiate along two pathways, a notch-independent DN pathway, generating mature DN and CD8 $\alpha\alpha^+$ SP (single positive) TCR $\gamma\delta^+$ cells. In the notch-dependent DP (double positive) pathway, immature CD4 $^+$ SP, and subsequently DP TCR $\gamma\delta^+$ cells are generated. Human postnatal thymus thus exhibits a scenario of DN, DP, and SP TCR $\gamma\delta^+$ population, which highlights heterogeneity in human T γ T cell development (45). The activated extrathymic T γ T cells, in humans, express notch receptors, which regulate their effector functions. Inhibiting notch signaling in T γ T cells dampened their anti-tumor cytotoxic potential (46). Thus, validates the requirement of notch signaling in both thymic development and functions of human T γ T cells. The diversity of human T γ T cell repertoire at birth (majorly contributed by V81 $^+$ subset of T γ T cells in cord blood) is restricted in adulthood especially to V γ 9V δ 2, a circulating subset of T γ T cells. The absolute numbers of V γ 9V δ 2 T cells increase from minor population at birth to more than 75% of T γ T cells pool in peripheral blood (35), which constitute around 1–10% of total T cells in humans. The T γ T cells exit the thymus as mature T cells and express markers that are associated with antigen-experienced T cells (47).

The other important feature of T γ T cells apart from antigen recognition is their tissue tropism. In humans, the first T γ T cells to arise from thymus are V81 $^+$ (paired with various V γ chains), which preferentially populate in epithelial tissue and constitute larger proportion of intraepithelial lymphocytes (IELs) (48). They rapidly and innately recognize stressed cells found to be enriched in various tumor tissues (4). The V γ 9V δ 2 is a lymphoid homing subset of T γ T cells, which continually expand in response to microbial antigen in circulation and exhibit characteristics of adaptive immune system (49). These cells recognize, expand, and secrete cytokines in response to non-peptide antigens associated with microbes in circulation. In mouse, a substantial proportion of T γ T cells reside as the IEL in the skin, intestine, and genitourinary tract. In response to the chemokine signals, V γ 5V δ 1 $^+$ T cells leave the fetal thymus, reside in the epidermis, and form dendritic-like network similar to Langerhans cells. These cells are called as dendritic epidermal T cells (DETcs) and constitute more than 90% of epidermal T cells (50). V γ 6 $^+$ T cells home to tongue and reproductive tract whereas V γ 7 $^+$ T cells home to intestinal tract suggesting that distinct TCR repertoire are present at different anatomical site and respond to antigens unique to their resident tissues (51–53). However, the functions of IELs are determined by the environment at the anatomical site (54) and hence specific T γ T cell subset could be used in tissue repair and generation of effective immune response at different epithelial sites.

T γ T cells perform diverse effector functions determined by the TCR expressed, tissue localization, and activation status. Apart from these, MHC-independent recognition of antigens, production of IFN γ , and expression of cytotoxic granules classify T γ T cells as potential cytotoxic cells (55). They can kill activated, infected, stressed, and transformed cells using various strategies such as engagement of death-inducing receptors, such as FAS

and TNF-related apoptosis-inducing ligand receptors (TRAILR) and the release of cytotoxic effector molecules such as perforin and granzyme (56, 57). Human $\gamma\delta$ T cells also recognize HSP (HSP60/70) expressed on tumor cells and enhance its cytolytic activity against the tumors (31, 58). $\gamma\delta$ T cells support the maturation and activation of other lymphocytes, NK cells, and macrophages with the help of secreted chemokines (CCL3, CCL4, CXCL10) (55). Another chemokine CXC-chemokine ligand 13 (CXCL13) produced by V γ 9V δ 2 cells can regulate B cell organization within lymphoid tissues and help B cells to produce antibodies (59). Human $\gamma\delta$ T cells can also crosstalk with dendritic cells (DCs) influencing each other functions like the antigen presentation by DCs, activation, and secretion of IL12 and IFN γ by $\gamma\delta$ T cells, which result in DC maturation (11, 60). These properties of $\gamma\delta$ T cells aid in generation of the effective immune response in the appropriate condition. Not only this, activated V γ 9V δ 2 cells can take up and process the soluble antigens, opsonize target cells, and can migrate to lymph nodes through CC-chemokine receptor 7 (CCR7) where they upregulate expression of MHCs and co-stimulatory receptors CD80 and CD86 (61, 62). Activated V γ 9V δ 2 cells has also been licensed to act as APC and activate CD4 and CD8 T cells (63). Collectively, these observations highlight the multi-talented role of $\gamma\delta$ T cells, having both Th- and Tc-like properties along with acting as APC. The special trait of $\gamma\delta$ T cells is their ability to recognize phosphorylated non-protein antigens and mediate its effector function in spatial and temporal manner making them a robust cell type, which can be manipulated to develop a promising tool for novel immunotherapies against certain types of diseases. However, care should be adapted while designing such immunotherapies because these cells have capacity to secrete various cytokines under different conditions.

T γ δ17: A SUBTYPE OF $\gamma\delta$ T CELLS

Unlike $\alpha\beta$ T cells, in mice, which leave thymus as naïve cells and are primed in the peripheral compartment, $\gamma\delta$ T cells undergo subset commitment in the thymus itself. However, in humans, upon activation with different cytokines, V γ 9V δ 2 cells can be polarized toward different effector subtypes like γ δ1, γ δ2 (64), γ δ17 (65, 66), and γ δTreg (67, 68). This functional plasticity of $\gamma\delta$ T cells assists them to tackle the distinct disease conditions and play important role in the early responses to invasive pathogens. The recent findings have stated that $\gamma\delta$ T cells are major IL17 producers and have shown their involvement in early onset of immune activation (69). Similar to Th17 cells, T γ δ17 cell express ROR γ t as a lineage determination transcriptional factor (70). Healthy adult human peripheral blood V γ 9V δ 2 T cells distinctively express Th1 signature and 50–80% produce IFN γ but <5% produce IL17 (6). However, T γ δ17 cells have been demonstrated to be involved in the pathogenesis of transplantation rejection (71), autoimmune disease (72), allergy (73), and cancer (74) in humans. The biology of T γ δ17 is so naive that it compels us to cross-examine its genesis, functions, and clinical relevance to understand its therapeutic potential.

MOLECULAR EVIDENCES OF T γ δ17 GENESIS

The molecular mechanism of IL17-producing $\gamma\delta$ T cells remains an enigma. Most of the studies carried out to understand the

differentiation mechanisms of T γ δ17 cells are based on the murine models. $\gamma\delta$ T cells preferentially localized to barrier tissues are the initial source of IL17 and are likely to originate from the fetal thymus. These are called as the natural IL17-secreting $\gamma\delta$ T cells. $\gamma\delta$ T cells that make IL17 within 24 h fall in this category (75). $\gamma\delta$ T cells acquire IL17-secreting phenotype in secondary lymphoid tissues after antigen exposure, which is referred to as induced T γ δ17 cells (76, 77).

During development of T cells in thymus, murine $\gamma\delta$ T cells branch off at the transition of thymocytes from DN3 stage to DN4 stage (34). It is also reported that $\gamma\delta$ T cells develop from DN2 stage and specifically produce IL17 whereas IFN γ -producing $\gamma\delta$ T cells can develop from both DN2 and DN3 precursors (78) (Figure 1). This suggests that $\gamma\delta$ T cells do not develop like $\alpha\beta$ T cells and follow evolutionary ancient path of T cell development. However, the precise DN stage from which $\gamma\delta$ T cells develop is elusive (79). Fetal thymic $\gamma\delta$ T-cell development occurs in successive waves by using the different V γ and V δ segments during the embryonic development (34, 80). Successful gene rearrangement of $\gamma\delta$ T cells from early thymic precursors (CD44^{hi}) lead to the development of naïve $\gamma\delta$ T cell characterized by CD44^{lo} CD27⁺CD62L⁺ phenotype. This phenotype can either leave the thymus to populate in secondary lymphoid organs or it can undergo further intrathymic differentiation that results in the development of multiple $\gamma\delta$ T cell subtypes such as dendritic epidermal $\gamma\delta$ T cell (DETCs), T γ δ17, or NK 1.1⁺ $\gamma\delta$ cell (γ NKT cells) (80, 81). Recently, it was described that when thymic lobes of mice at E14 were colonized with DN1a cells from mice at E13 and E18, respectively. It was observed that although both populations (E13 DN1a cells and E18 DN1a cells) generated similar number of $\gamma\delta$ T cells, only E13 DN1a cells generated V γ 3⁺ DETCs. These observations indicate that precursor lineage of DETCs may be different and needs further investigation (82). DETCs develop at embryonic day 13 (E13) to approximately E17 and readily secrete IFN γ when activated. After the development of DETCs, the next functional developmental wave consists of T γ δ17 cells. T γ δ17 cells are heterogeneous in using TCR chains that mainly include V γ 6⁺ and V γ 4⁺ but also use V γ 1⁺ chain. V γ 6⁺ cells develop by E14 to around birth and finally V γ 1 and V γ 4 cells develop E16 onward (81). The other subtypes of $\gamma\delta$ T cells, which develop in thymus, are γ NKT cells, which are similar to invariant TCR $\alpha\beta$ ⁺ NKT cells (83, 84).

There are different thymic signaling processes, which determine functional phenotype of $\gamma\delta$ T cells in thymus before migration to periphery and contribute to the balance between IFN γ committed versus IL17-committed subtypes (85). This biasness toward IL17 or IFN γ depends on the antigen experience in thymus. The $\gamma\delta$ T cells that have encountered the cognate antigen interaction in thymus, gain the potential to differentiate into the IFN γ -producing functional phenotype while antigen naïve $\gamma\delta$ T cells develop into IL17-producing $\gamma\delta$ T cells (86). This skewedness also reflects in their distribution outside the thymus. Most of T γ δ17 cells reside in lymph nodes whereas IFN γ -producing $\gamma\delta$ T cells are mainly found in the spleen and the mechanism for this distribution is not clear (86). Similar distribution is also found in $\alpha\beta$ T cells and it seems to be logical as the lymph nodes serve as the site of initial exposure to foreign antigens and propagate the wave of inflammation, thus are suited for the earliest source of the IL17 secretion (87).

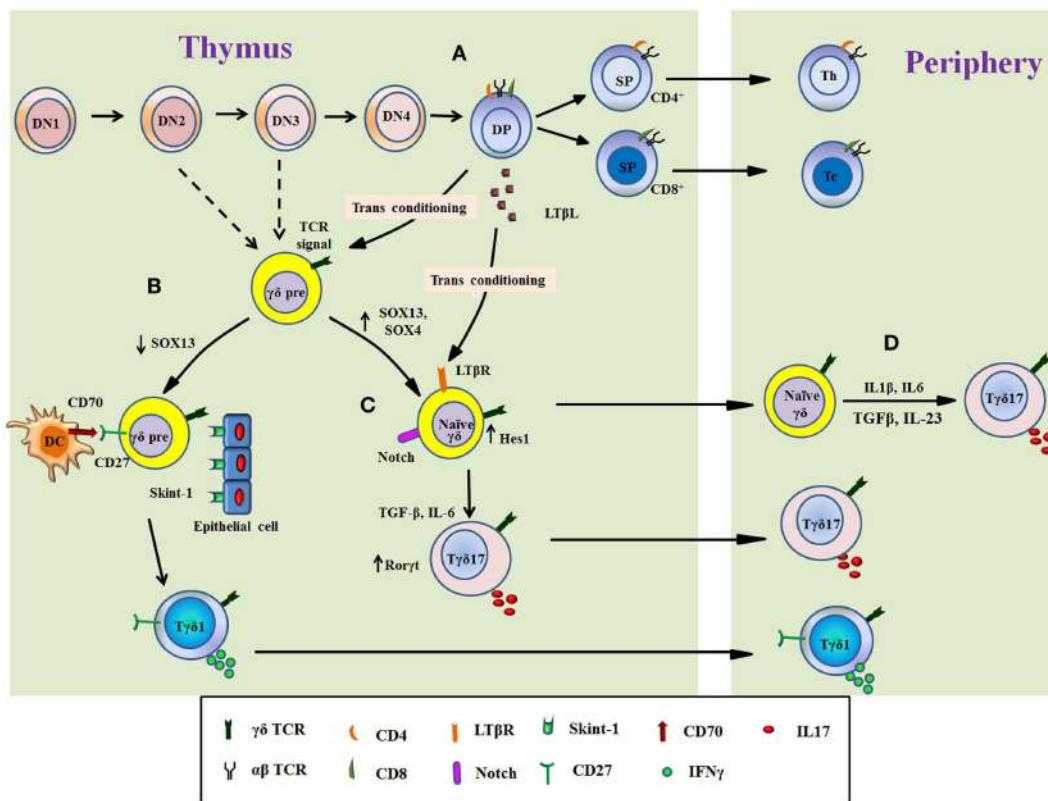


FIGURE 1 | Overview of $\text{Ty}\delta 17$ cells development. The figure illustrates the differentiation of $\text{Ty}\delta 17$ cells from T cell progenitors in the murine thymus (**A–C**) and from naïve $\gamma\delta$ T cells in periphery in human (**D**). Progenitor T cells differentiate through double negative stage 1 (DN1) to DN stage 4 (**A**). The decision of $\alpha\beta$ or $\gamma\delta$ TCR expression takes place at early T cells precursor (from DN2 or DN3 stage) as showed by dashed line. The thymocytes expressing $\alpha\beta$ TCR develop into double-positive thymocytes, which support differentiation of functional subtypes of $\gamma\delta$ T cells called as transconditioning. DP thymocytes secrete LT β L, which support differentiation of $\text{Ty}\delta 17$. The DP $\alpha\beta$ thymocytes then exit the thymus as mature single positive T cells (either CD4⁺ or CD8⁺ T cells) (**A**). The functional programming of $\gamma\delta$ T cells is

determined by TCR signal and/or other related signals. TCR signal, interaction with Skint-1 from epithelial cells, downregulation of SOX13, and signaling through CD27/CD70 divert $\gamma\delta$ thymocytes toward IFN γ -producing phenotype ($\text{Ty}\delta 1$), which migrate to periphery (**B**). Conversely, signaling through Notch receptor maintain Sox13 levels with increase in Hes1 and ROR γ t expression induce $\gamma\delta$ thymocytes to produce IL17. Progression of $\gamma\delta$ thymocytes to $\text{Ty}\delta 17$ cells is independent of signaling through Skint-1 and/or CD27 but require inputs from IL6 and TGF β . The natural $\text{Ty}\delta 17$ cells developed in thymus migrate to tissue or periphery (**C**). In human, naïve $\gamma\delta$ T cells, which exit thymus, can also differentiate into $\text{Ty}\delta 17$ cells in presence of TCR signal and cytokines such as IL6, IL1 β , IL23, and TGF β (**D**).

Besides the $\gamma\delta$ TCR signaling (86), expression of tumor necrosis factor receptor family member, CD27, determines the IL17 versus IFN γ production by $\gamma\delta$ T cells (88). CD27⁺ $\gamma\delta$ T cells differentiate into IFN γ producing cells whereas IL17 production was restricted to CD27⁻ T cells (89) (Figure 1). Thus thymic “imprinting” of the $\gamma\delta$ T cells as CD27⁺ or CD27⁻ regulates effector functions of $\gamma\delta$ T cells and is preserved in the periphery (89). CD27 is not only associated with IFN γ production but also aids $\gamma\delta$ T cells to interact with its ligand CD70 expressed on DCs, thymic epithelial cells, and double-positive thymocytes thus acting as a costimulatory receptor (89). Therefore, CD27 conveys an intrathymic message that licenses the CD27⁺ $\gamma\delta$ T cells for the production of IFN γ (47). Another signaling pathway that influences the differentiation of $\text{Ty}\delta 17$ is the signaling through lymphotoxin- β receptor (LT β R), a member of the tumor necrosis factor receptor family (90). Signaling through LT β R leads to the activation of the alternative nuclear factor (NF)- κ B pathway via RelB. Ligands for LT β R regulating this developmental process are produced by CD4⁺CD8⁺ thymocytes

(91). The homeostasis of this functional phenotypic differentiation, influenced by other thymic progenitors is known as transconditioning (91), which highlights coordination between different signaling pathways in thymus that occur in physically separate thymic niche (92). LT β R signaling pathway controls $\text{Ty}\delta 17$ development by regulating transcription factors ROR γ t and ROR α 4, required for IL17 expression in $\gamma\delta$ thymocytes (93). The role of LT β R signaling, however, remains controversial as LT β R is present downstream to CD27 signaling, which is associated with the IFN- γ production (89).

The maturation of $\text{Ty}\delta 17$ cells from its precursors requires TCR signaling as mice with reduced ZAP70 show decreased number of $\text{Ty}\delta 17$ cells (94). However, TCR signaling alone is not sufficient as it also requires other signals (95). An src family kinase, Blk (B lymphoid kinase), is required for $\text{Ty}\delta 17$ cells development in thymus as Blk-deficient mice was reported to have less number of IL17-producing $\gamma\delta$ T cells (96). Similarly, high-mobility group (HMG) box transcription factors, SOX4 and SOX13 are

positive regulators of Ty δ 17 development (95, 97). These transcription factors expressed in immature T cells (98) highlight that the development of Ty δ 17 is from early precursors (DN2) (78, 95). Other thymic determinant, which is responsible for the functional dichotomy in Ty δ 17 and Ty δ 1, is Skint-1, a thymic epithelial cell determinant. The interaction between Skint-1 $^+$ cells and $\gamma\delta$ thymocytes (V γ 5 $^+$ V δ 1 $^+$) induce an Egr3-mediated pathway, leading to differentiation toward IFN γ -producing $\gamma\delta$ T cells. Further, it suppresses Sox13 and an ROR γ t transcription factor-associated Ty δ 17 cells lineage differentiation suggesting that the functions of the earliest T cells are substantially preprogrammed in the thymus (99). Notch signaling is known to be involved in thymic determination and development of Ty δ 17 cells. Hes1, one of the basic helix-loop-helix (bHLH) proteins induced by Notch signaling is critical for the IL17 expression by $\gamma\delta$ T cells and its thymic development (100–102). Further, the specific expression of Hes1 in CD25 $^+$ and CD27 $^-$ $\gamma\delta$ T cells and decreased levels of Ty δ 17 in Hes1-deficient mice highlights the critical role of Notch–Hes1 pathway in Ty δ 17 development in thymus as well as in periphery (101). The thymic development of Ty δ 17 is independent of STAT3 but partly dependent on ROR γ t (101) and most peripheral IL17-producing $\gamma\delta$ cells express ROR γ t and respond rapidly to IL23 (103).

Developmental process of Ty δ 17 also requires signaling through different cytokines. TGF β signaling is necessary for Ty δ 17 development (104). It has been shown that in absence of TGF β 1 or Smad3 (a component of the TGF β signaling), the number of Ty δ 17 thymocytes reduced drastically relative to that of wild-type mice (104). As compared to TGF β , requirement of IL6 for Ty δ 17 development is not well understood as there are contrasting reports on its role (72, 105). It is also reported that IL6 does not act directly on uncommitted $\gamma\delta$ thymocytes but instead it acts indirectly by regulating the expression of Delta-like ligand 4, a ligand for notch receptor, expressed by thymic epithelial cells that promote the differentiation of Ty δ 17 (101, 106). Moreover, IL23 and IL1 produced by DCs are crucial for IL17 production by $\gamma\delta$ T cells. IL23 $^{-/-}$ and IL23R $^{-/-}$ mice showed the significant reduction in Ty δ 17 cells after *L. monocytogenes* infection supporting earlier observation (107–110).

Thymic development of human Ty δ 17 cells is poorly investigated. Around 80% circulating human V γ 9V δ 2 T cells are IFN- γ producers and express CD27 whereas CD27 negative cells are IL17-producing $\gamma\delta$ T cells are <5% (65). Interaction of CD70 with CD27 promotes the expansion of Th1-biased V γ 9V δ 2 T cells in periphery (111). However, such role in their thymic development is unknown. Human V γ 9V δ 2 T cells can be polarized to Ty δ 17 cells in periphery upon IPP activation and in the presence of cytokines like TGF β , IL1 β , IL6, and IL23, followed by a week of culture in differentiation medium supplemented with IL2 can induce IL17 in these cells (65, 66). In humans, there are contrasting reports on role of IL6 and IL23 in differentiation of Ty δ 17. It has been shown that IL6 is required for differentiation of neonatal Ty δ 17, and IL23 is required for the generation of adult IL17-producing $\gamma\delta$ T cells (65). In another study, it is reported that in the presence of TCR signaling, IL23 promotes the induction of IL17 in neonatal (but not adult) $\gamma\delta$ T cells (112). However, it appears that IL23 induces $\gamma\delta$ T cells to coproduce IL17 and IFN γ in adults but

support development of Ty δ 17 cells in neonates. In addition to the above-mentioned cytokines, IL7 selectively promotes the mouse and human IL17-producing $\gamma\delta$ T cells. IL7 activates STAT3 preferentially in $\gamma\delta$ T cells competent to produce IL17 (113). However, the increased IL17 production by $\gamma\delta$ T cells upon TCR stimulation in presence of IL7 is observed only in case of cord blood cells but not with peripheral lymphocytes. Thus, it is important to note that the antigen naive $\gamma\delta$ T cells only can be reprogramed *in vitro* toward Ty δ 17 phenotype (66, 113).

The kinetic study of IL17 production by $\gamma\delta$ T cells has shown that murine $\gamma\delta$ T cells secrete IL17 within few hours after stimulation (70). This phenomenon can be reasoned by the thymic development of murine Ty δ 17 cells and constitutive presence of transcriptional regulators for IL17 production. However, human $\gamma\delta$ T cells in thymus are functionally immature and can attain their functional differentiation in periphery in presence of cytokines (114). This supports the kinetics of IL17 production by human $\gamma\delta$ T cells that mRNA expression of IL17 and ROR γ t peaks by day 3–6 and decrease by day 9 onward, after stimulation. The expression of cytokine receptors (IL1 β R, IL6R, TGF β R, and IL23R) on V γ 9V δ 2 T cells peaks on day 3 and decrease by day 6 (66). Thus, coordinated combination of TCR and cytokine stimulation could be necessary for the sustained secretion of IL17 by $\gamma\delta$ T cells, which highlights the difference in kinetics of IL17 secretion by murine and human Ty δ 17 cells. This underscores that human $\gamma\delta$ T cells can be “reprogramed” in the periphery into different functional lineages.

Upon antigenic challenge, T cells differentiate to memory phenotype; either central memory (TCM) or effector memory (TEM) (115). Human Ty δ 17 cells present in non-lymphoid environment belong to CD27 $^-$ CD45RA \pm effector (74) or terminally differentiated (TEMRA) (66) memory phenotype. Similarly, murine Ty δ 17 cells also show effector memory phenotype with CD44 high , CD45RB low , and CD62L low (116). Thus, Ty δ 17 cells differentiated either in thymus or in periphery, belong to memory phenotype, and licensed to patrol the blood, lymphoid organs, and peripheral tissues.

Ty δ 17 IN MICROBIAL INFECTIONS

Ty δ 17 cells can rapidly produce IL17 upon Toll-like receptors (TLR) or cytokine stimulation alone even in absence of antigen presentation. The general proinflammatory functions of IL17 [reviewed in Ref. (117, 118)] could be associated with $\gamma\delta$ T cells as they are major producers of IL17. Studies carried out in various infection models showed that Ty δ 17 cells are protective against infection. During mycobacterial infection, IL17 produced by V γ 4 $^+$ and V γ 6 $^+$ cells induce pulmonary granuloma formation by recruitment of granulocytes and monocytes. The IL17 participates in maturation of granuloma by promoting tight cell to cell binding via ICAM1 and LFA1 induction (119). Mycobacteria-infected DCs secrete IL23, which regulate IL17 production by $\gamma\delta$ T cells emphasizing that the early activation of Ty δ 17 cells is important for initiating inflammation and recruiting innate immune cells to the site of infection thereby enhancing bacterial clearance from host (120, 121). Ty δ 17 cells also support cell-mediated immunity by inducing Th1 cells against pulmonary mycobacterial infection (122).

In *Escherichia coli* infection model also, $\gamma\delta$ T cells were reported to be the major producers of IL17, which enhanced neutrophil infiltration to the peritoneum. The infiltration of cells diminished after antibody depletion of resident V δ 1 $^{+}$ subtype of $\gamma\delta$ T cells highlighting its involvement in IL17 secretion in response to IL23 (9). Thus, IL23 and Ty δ 17 cells play a dominant role as first line of defense in infection before CD4 T cell activation. In case of *L. monocytogenes* infection, a large number of $\gamma\delta$ T cells accumulate in the lymph organs shortly after infection and begin to produce IL17A, signifying the role of Ty δ 17 cells in the *Listeria* infection (123). IL17 was also shown to promote proliferation of CD8 $^{+}$ cytotoxic T lymphocytes by enhancing DC cross-presentation *in vitro*. DCs stimulated with IL17 showed upregulation of MHC-I molecule H2Kb and enhanced secretion of cytokines (IL12, IL6, and IL1 β). CD8 $^{+}$ DCs from *Il17a* $^{-/-}$ mice also produced less IL12 and are less potent in activating naive CD8 $^{+}$ T cells (123). This indicate that Ty δ 17 cells not only induce innate response but also critical for optimal adaptive cytotoxic response against intracellular bacterial infection. The alliance of IL23 and Ty δ 17 is also demonstrated to have a protective role during infections such as *Klebsiella pneumonia* (124), *Citrobacter rodentium* (125, 126), *Salmonella enterica* (127, 128), and *Toxoplasma gondii* (129). The Ty δ 17 cells also play a vital role in clearing fungal infections. The rapid production of IL17A was reported in the lungs at a very early stage after intravenous infection with *C. albicans*. Lung resident $\gamma\delta$ T cells were the major source of early IL17A production regulated by IL23 and TLR2/MyD88-dependent pathway (130). Presence of Ty δ 17 cells were also reported in the lungs of neutropenic mice during *C. neoformans* infection. These Ty δ 17 cells played an important role in the chemotaxis of leukocytes and induction of protective immune response (131). Ty δ 17 cells thus orchestrate the protective immunity by acting at the early onset in infection models (108).

Relatively few studies have evaluated the role of Ty δ 17 cells in human microbial immunity. In patients with tuberculosis (TB), elevated levels of Ty δ 17 cells were found in peripheral blood and were major producers of IL17 (6). As a protective role, in response to bacterial antigens, IL17-producing V γ 9V δ 2 T cells induce neutrophil migration through secretion of CXCL8 and promote their phagocytic activity (66). Ty δ 17 cells also induce epithelial cells to secrete anti-microbial peptides like β -defensins in response to bacterial antigens (66). This signifies the modulatory effects of Ty δ 17 cells on keratinocytes and other immune cells in anti-microbial defense. In children with bacterial meningitis, the population of IL17 $^{+}$ V γ 9V δ 2 T cells significantly increase in peripheral blood and at the site of infection (cerebrospinal fluid). The reversal of this pattern after successful anti-bacterial therapy clearly suggests the anti-microbial role of Ty δ 17 cells (66). Collectively, these studies provide new insight into the functions of $\gamma\delta$ T cells as the first line of host defense against bacterial and fungal infection in human and may pave a path in designing newer treatment modalities.

TOLL-LIKE RECEPTORS REGULATE IL17 PRODUCTION IN Ty δ 17 CELLS

$\gamma\delta$ T cells express various chemokine receptors, cytokine receptors, and PRRs, which regulate IL17 production. TLRs are the well-studied PRRs expressed by DCs, macrophages, and $\gamma\delta$ T cells. The

unique microbial molecules called as PAMP are recognized by TLRs, which orchestrate the anti-microbial response in $\gamma\delta$ T cells (11). In malarial infection, MyD88 deficiency results in severe impairment of IL17A producing $\gamma\delta$ T cells levels, but not IFN γ producing $\gamma\delta$ T cells highlighting differential control by innate signaling through TLRs in infections (132). Murine Ty δ 17 cells specifically express TLR1 and TLR2 but not TLR4. High number of Ty δ 17 cells were induced upon *in vivo* stimulation with Pam3CSK4 (ligand for TLR2) but not with LPS (TLR4 ligand) or CpG (TLR9 ligand) (70). Interestingly, it has been shown that TLR4 indirectly controls IL17 generation by $\gamma\delta$ T cells through IL23 secreted by TLR4 expressing macrophages in response to HMG Box 1 (HMGB1, a damage-associated protein and TLR4 ligand) (133). Moreover, Ty δ 17 cells promote experimental intraocular neovascularization (134) as well as early acute allograft rejection (135) in response to HMGB1. Signaling through TLR2 is indispensable for Ty δ 17 in anti-microbial functions. Absence of TLR2 or MyD88 in cutaneous *Staphylococcus aureus* infection, or in *Candida albicans* infection, caused an impaired IL17 production and poor microbial clearance in the skin infiltrated with V γ 5 $^{+}$ $\gamma\delta$ T cells (130, 136). Ty δ 17 cells also express DC-associated C-type lectin 1 (dectin 1) and intraperitoneal injection of curdlan (dectin 1 ligand), induced IL17 production by $\gamma\delta$ T cells (70). In imiquimod (IMQ)-induced psoriasis-like model, dermal $\gamma\delta$ T cells spontaneously secreted a large amount of IL17 in IMQ-treated skin cells. Thus, it appears that TLR7/8 (receptor of IMQ) may regulate the IL17 production by $\gamma\delta$ T cells. It is important to note that the modulatory effects of TLRs on $\gamma\delta$ T cells as showed in *in vivo* murine models are mediated through IL23 and/or IL1 β cytokines. The direct stimulation of CD27 $^{-}$ $\gamma\delta$ T cells by TLR ligands (LPS or PAM) show no effect on IL17 production (132). This suggests that TLR signaling indirectly modulates Ty δ 17 function.

RECEPTOR REPERTOIRE EXPRESSED BY Ty δ 17 CELLS

The receptor profile of Ty δ 17 cells is similar to Th17 cells. In mice, the majority of IL17-producing CD4 cells belong to CCR6 $^{+}$ compartment compared to CCR6 $^{-}$ (137). Sorted CCR6 $^{+}$ $\gamma\delta$ T cells showed increased mRNA expression of IL17, IL22, IL23R, Ror γ t, and aryl hydrocarbon receptor (AhR) compared to CCR6 $^{-}$ $\gamma\delta$ T cells (70, 138). This suggests that CCR6 can be a phenotypic surface marker of Ty δ 17 cells. Besides CCR6, Ty δ 17 cells express various chemokine receptors including CCR1, CCR2, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR3, CXCR4, CXCR5, and CXCR6 (7). The early onset recruitment of Ty δ 17 to the site of inflammation is determined by the type of chemokine receptor on them. Ty δ 17 cells expressing CCR6 and CCR9 show selective migration toward allergic inflamed tissue in response to CCL25 (ligand for CCR9). α 4 β 7 integrin expression is indispensable for this migration and transendothelial crossing of Ty δ 17 cells. (139). Since migration through CCL2/CCR2 axis is determinant for total $\gamma\delta$ T cells, CCL25/CCR9-mediated migration seems to be specific for Ty δ 17 subtype (140, 141).

In humans, Ty δ 17 cells express CCR6 but not CXCR3, CXCR5, CCR3, CCR4, or CCR5. However, they express granzyme B, FASL, and TRAIL but not perforin (66). The lack of granzyme B and perforin coexpression may be responsible for absence of cytolytic activity of Ty δ 17 cells. On the contrary, it has been shown that the

human colorectal tumor-infiltrating Ty δ 17 cells do not express FASL or TRAIL but express CD161 and CCR6 (74). The inconsistency in expression of cytolytic markers and their relevance on Ty δ 17 cells needs to be understood in detail. The AhR is indispensable for Ty δ 17 cells as it promotes differentiation of naïve V γ 9V δ 2 T cells toward Ty δ 17 phenotype (66).

In mouse model, it has been shown that Ahr $^{-/-}$ Ty δ 17 cells express IL17 but fail to produce IL22 (70). Moreover, in mouse model of *Bacillus subtilis* induced pneumonitis, deficiency of Ahr resulted into low IL22 production but IL17 levels were maintained (142). Thus, although Ahr promotes IL17, it is indispensable for IL22 production by Ty δ 17 cells.

INFLAMMATORY DISORDERS AND MANIA OF Ty δ 17

Th17 cells and Ty δ 17 cells are essential in disease progression and are pathogenic in autoimmune disease. Dysregulated levels and sustained secretion of proinflammatory cytokines by $\gamma\delta$ and/or CD4 T cells have devastating effects on autoimmune disease progression. In a collagen-induced arthritis (CIA) model (resembling human rheumatoid arthritis), IL17-producing V γ 4/V δ 4 $^{+}$ T cells selectively increase in joints and lymph nodes. Depletion of $\gamma\delta$ T cells by anti V γ 4 antibody, markedly reduced the disease severity score revealing its pathogenic nature (143). Interestingly, both Th17 and Ty δ 17 are present in the joints but Th17 cells localize proximal to the bone, which facilitates its interaction with osteoclast. Selective depletion of Th17 cells abrogated the bone resorption suggesting that Th17 but not Ty δ 17 cells are responsible for bone destruction. Thus, Ty δ 17 cells may be responsible for enhancing joint inflammation and exacerbate CIA (144). In contrast, absence of Ty δ 17 was reported in patients with rheumatoid arthritis and in murine model of autoimmune arthritis (SKG model) (145). The SKG mouse model has defects in the differentiation of Ty δ 17 cells (94), which might result into low Ty δ 17 cells in the inflamed joints. Thus, the role of Ty δ 17 cells in autoimmune arthritis need to be evaluated comprehensively.

Ty δ 17 also enhanced experimental autoimmune encephalomyelitis (EAE) (mouse model for human multiple sclerosis). Upon immunization of mice with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA), V γ 4 $^{+}$ CCR6 $^{+}$ IL23 $^{+}$ $\gamma\delta$ T cells accumulate in the central nervous system (CNS), which expand by 20-fold in absolute number during development of clinical signs of the disease (72). In contrast, IFN γ -producing $\gamma\delta$ T cells are low in CNS and marginally increase during course of EAE (103). The mechanism behind aggravation of EAE could be attributed to restraining the development of Foxp3 $^{+}$ regulatory T cells (Tregs) functions by Ty δ 17 cells. Supernatants from IL23-activated $\gamma\delta$ T cells inhibited the TGF β driven conversion of naïve Foxp3 $^{-}$ $\alpha\beta$ T cells into Foxp3 expressing T cells and also reversed the suppressive effect of Treg cells (72). Similar function of Ty δ 17 was reported in cardiac transplantation in mice. IL17, majorly produced by $\gamma\delta$ T cells, accelerates acute rejection of transplanted heart but IL17 deficiency enhanced Treg expansion and prolonged allograft survival (71). In ischemic brain injury, Ty δ 17 were reported to be present at the infarct areas (146). Ty δ 17 rather than Th17 was the major source of IL17 whereas IFN γ was majorly produced by Th1 cells. In mice, genetically deficient for IL17 or IL23, the infarct areas were reduced suggesting a role of

Ty δ 17 as a key contributor of neuroinflammation (146). Overall, this suggests that in chronic inflammatory condition, innate cytokines IL23 and IL1 β promote infiltration and generation of IL17-producing $\gamma\delta$ T cells, which aggravate the disease.

Experimental silicosis is a useful model for depicting chronic lung inflammation, tissue damage, and fibrosis. Ty δ 17 along with Th17 accumulated in the lung in response to IL23 expressing macrophages by third day after silica treatment but interestingly did not induce lung fibrosis (73). On the contrary, in allergic lung inflammation, Ty δ 17 cells are known to be protective (147, 148). Functional blockage of both IL17 and $\gamma\delta$ T cells impaired the resolution of airway lung inflammation (148). It is claimed that this protective role is mediated by prostaglandins (PGs), which are abundant at the site of inflammation. PGI2 analog iloprost enhanced IL17 production by $\gamma\delta$ T cells in the thymus, spleen, and lungs, reducing airway inflammation (147). This highlights the role of PGI2 analogs that can be exploited in the development of immune response in immunotherapeutic approaches. Age-related macular degeneration (AMD) is another chronic inflammation associated disease, characterized by choroidal neovascularization (CNV). In an experimental model, Ty δ 17 cells along with Thy-1 $^{+}$ ILCs (innate lymphoid cells) infiltrate the eye after laser treatment and promote neovascularization. This recruitment is in response to IL1 β but not IL23 produced by macrophages (134).

Ty δ 17 CELLS AS HEROES OR VILLAINS IN CANCER

The unmatched characteristics of human $\gamma\delta$ T cells to have MHC unrestricted tumor directed cytotoxicity, release of copious amounts of IFN γ , and recognition of cancer cells through variety of mechanisms render them as potential candidate for cancer immunotherapy (4, 149). Upon activation, $\gamma\delta$ T cells show cytotoxicity against myeloma (150), lymphoma (151), leukemia (152, 153), and other epithelial carcinomas (57, 154, 155) *in vitro*. Several clinical trials have been launched using $\gamma\delta$ T cells based therapies in cancer patients. The hallmark characteristic of $\gamma\delta$ T cells to be used for therapy is their ability to infiltrate tumors (156). *In vivo* activation by phosphoantigens or adaptive transfer of preactivated autologous $\gamma\delta$ T cells have proved successful in cancer treatment (157). However, the role of Ty δ 17 cells as anticancer effector cells is not well defined.

In a chemotherapeutic approach, Ty δ 17 cells are reported to play decisive role in several transplantable tumor models (EG7 thymoma, MCA205 sarcoma, CT26 colon cancer, and TS/A mammary carcinomas). Ty δ 17 (V γ 4 $^{+}$ /V δ 6 $^{+}$) cells were shown to invade the tumor bed early in response after drug treatment. This was followed by infiltration and induction of IFN γ -producing CD8 (Tc1) cells to the tumor bed. This infiltration of Ty δ 17 and Tc1 cells was correlated and associated with tumor regression post radio or chemotherapy (158). Thus, IL17-producing V γ 4 $^{+}$ /V δ 6 $^{+}$ cells are critical for the induction of Tc1 response in tumor tissue in response to drug treatment or radiation. Another study in bladder cancer supports the helper function of Ty δ 17 cells in cancer treatment. Ty δ 17 cells induce neutrophil infiltration to the tumor site and show anti-tumor effect upon *Mycobacterium bovis* BCG treatment (159).

In contrast to anti-tumor role of Ty δ 17 cells, they also promote tumor development. With the notion that IL17 is a proangiogenic

cytokine (160), Ty δ 17 cells promote angiogenesis in tumor model. In IL17 $^{-/-}$ tumor bearing mice, the blood vessel density was markedly decreased compared to wild type. In addition, IL17 induced the expression of Ang-2 (angiopoietin) and VEGF (vascular endothelial growth factor) in tumor cells (8). In ovarian cancer model, it has been reported that CD27 $^{-}$ V γ 6 $^{+}$ cells produced higher IL17 and induce VEGF and Ang-2 in peritoneal exudates of tumor bearing mice after 6 weeks of post-tumor inoculation (161). Additionally, Ty δ 17 cells induce mobilization of protumor small peritoneal macrophages (SPM) to the tumor bed, which express IL17-dependent proangiogenic profile (*Il1b*, *Il6*, *vegfa*, *tgfb*, *mif*, *cxcl1*, *cxcl8*, and *tie2*). SPMs also enhance ovarian cancer growth by stimulating tumor cell proliferation (161). In hepatocellular carcinoma mouse model, it was reported that IL17, majorly produced by V γ 4 $^{+}$ γ 8T cells, induced CXCL5 production by tumor cells, which enhance migration of MDSCs (myeloid-derived suppressor cells) expressing CXCR2 to the tumor site. In addition, IL17 also enhanced suppressive functions of MDSCs by inhibition of T cells proliferation and cytokine (IFN γ and TNF α) production (162). In return, MDSCs induced γ 8T cells to produce IL17 through IL23 and IL1 β secretion forming positive feedback loop for Ty δ 17 activation (162). Thus, Ty δ 17 cells interact with myeloid cells and counteract tumor immune-surveillance.

In human colorectal cancer, IL8 and GM-CSF secreted by Ty δ 17 promote migration of MDSCs while IL17 and GM-CSF enhanced their proliferation. Ty δ 17 cells also support survival of MDSCs through IL17, IL8, and TNF α (74). Thus, it is possible to speculate that Ty δ 17 cells might be responsible for gradual shift from initial inflammatory to immunosuppressive tumor environment in advanced stage cancer (163). In human colorectal carcinoma, Ty δ 17 cells were positively correlated with advancing tumor stages as well as with clinicopathological features including tumor size, tumor invasion, lymphatic and vascular invasion, lymph node metastasis, and serum CEA (Carcinoembryonic antigen) levels suggesting their pathogenic role (74).

Collectively, these findings highlight the apparently opposite roles of Ty δ 17 cells in cancer immunity. It seems that during tumor development, inflammatory environment (IL1 β and IL23) modulate the cytokine profile of γ 8T cells from primary IFN γ toward proinflammatory IL17, which support tumor progression.

CONCLUDING REMARKS

Despite the small percentage in total T cell population, γ 8T cells have emerged as an important modulator of early immune responses. The development of functional subtypes of γ 8T cells require polarizing cues including molecular and cellular

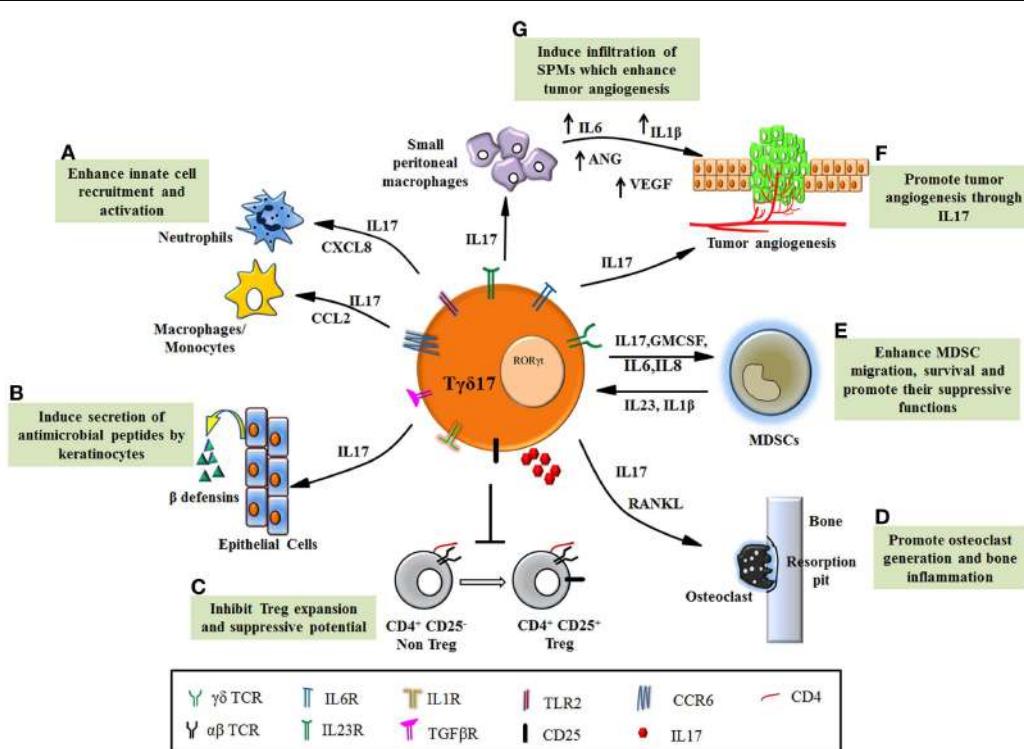


FIGURE 2 | Functions of Ty δ 17 cells in pathological conditions.

(A) Ty δ 17 cells promote infiltration of neutrophils and monocytes/macrophages to the site of inflammation through chemokines. (B) IL17 secreted by Ty δ 17 cells induces keratinocytes to produce anti-microbial peptides such as β defensins and protect host in infections. (C) Dysregulated Ty δ 17 cells in autoimmune diseases inhibit Treg expansion and its ability to suppress autoreactive cell, thereby exacerbating the disease. (D) The inflammatory condition in arthritis is

worsened by IL17, which foster osteoclast formation through induction of RANKL. Ty δ 17 cells are involved in bone resorption and enhance joint inflammation. (E) Human Ty δ 17 cells support MDSC migration, survival, and promote their suppressive functions through IL17, GMCSF, and IL8. MDSCs also form feedback loop and promote Ty δ 17 differentiation through IL23 and IL1 β . (F) Ty δ 17 cells secrete IL17 and induce tumorigenesis by their proangiogenic activity. (G) Murine Ty δ 17 cells recruit small peritoneal macrophages to the tumor bed, which induce angiogenesis.

interaction and combination of multiple cytokines and chemokine receptors that regulate their distribution. This suggests that the functional determination of T γ δ17 cell subtypes is dictated by the local environment (thymus or peripheral blood or the inflamed tissue) in which they are present. T γ δ17 is a special T γ δ17 cell subset, distinctly present at early immune response in the tissue and can modulate the functions of other immune and epithelial cells but their relevance in disease outcome remains controversial. In response to microbial antigens, T γ δ17 cells promote infiltration of neutrophils and macrophages and induce production of antimicrobial peptides resulting in clearance of microbial load. Such protective behavior of T γ δ17 cells in infections can be exploited to develop newer approaches to tackle the microbial pathology (**Figure 2**).

The opposite side of T γ δ17 functions has revealed its detrimental role in enhancing inflammation in autoimmunity and cancer (**Figure 2**). The mechanism, which regulates such dual personality of T γ δ17 cells is unknown. It appears that the obvious common role executed by these cells is enhancement of inflammation but due to functional heterogeneity and their complex interdependency on other cells (innate and adaptive); the emerging scenario of their biology is far from complete. This provokes us to consider contextual behavior of T γ δ17 cells in disease pathology. Current progress in understanding the significance of T γ δ17 cells in inflammatory diseases has revealed their novel but debilitating functions such as suppression of Tregs in autoimmunity, induction of angiogenesis, and recruitment and activation of MDSCs in various malignancies. Thus, in inflammatory disorders, T γ δ17 cells can be targeted using various immunotherapeutic approaches. However, need of hour is to expand the understandings of T γ δ17 in humans and develop a protocol for their propagation and activation. The future therapies will rely on regulating the key transcription factor ROR γ t by designing suitable antagonists that will help in fine tuning T γ δ17 differentiation and eventually their function in chronic inflammation and infection.

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Human V δ 2 $^{+}$ $\gamma\delta$ T cells differentially induce maturation, cytokine production, and alloreactive T cell stimulation by dendritic cells and B cells

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Human $\gamma\delta$ T cells expressing the V γ 9V δ 2 T cell receptor can induce maturation of dendritic cells (DC) into antigen-presenting cells (APC) and B cells into antibody-secreting plasma cells. Since B cells are capable of presenting antigens to T cells, we investigated if V γ 9V δ 2 T cells can influence antigen-presentation by these cells. We report that V γ 9V δ 2 T cells induced expression of CD86, HLA-DR, and CD40 by B cells and stimulated the release of IL-4, IL-6, TNF- α , and IgG, IgA, and IgM. V γ 9V δ 2 T cells also augmented the ability of B cells to stimulate proliferation but not IFN- γ or IL-4 release by alloreactive T cells. In contrast, V γ 9V δ 2 T cells induced expression of CD86 and HLA-DR and the release of IFN- γ , IL-6, and TNF- α by DC and these DC stimulated proliferation and IFN- γ production by conventional T cells. Furthermore, CD86, TNF- α , IFN- γ , and cell contact were found to be important in DC activation by V γ 9V δ 2 T cells but not in the activation of B cells. These data suggest that V γ 9V δ 2 T cells can induce maturation of B cells and DC into APC, but while they prime DC to stimulate T helper 1 ($T_{H}1$) responses, they drive maturation of B cells into APC that can stimulate different T cell responses. Thus, V γ 9V δ 2 T cells can control different arms of the immune system through selective activation of B cells and DC *in vitro*, which may have important applications in immunotherapy and for vaccine adjuvants.

Keywords: human $\gamma\delta$ T cells, dendritic cells, B cells, cytokines, antibody production, APC, T cell proliferation

INTRODUCTION

T cells expressing the V γ 9V δ 2 T cell receptor (TCR) comprise the most abundant $\gamma\delta$ T cell subset in human blood, where they typically account for 1–5% of T cells in healthy adults (1–4). In many microbial infections, V γ 9V δ 2 T cells dramatically expand, reaching >50% of all T cells at infected sites (5), thus indicating their importance in antimicrobial immunity and their potential for diagnostic and therapeutic use. The V γ 9V δ 2 TCR recognizes a variety of low molecular weight pyrophosphate intermediates of isoprenoid biosynthesis (phosphoantigens), but the most potent phosphoantigen known is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the non-mevalonate pathway that is found in the majority of Gram-negative bacteria, some Gram-positive species and some parasites, such as *Plasmodium falciparum* and *Toxoplasma gondii* (1, 6). Recently, butyrophilin 3A (BTN3A/CD277) was shown to bind to phosphoantigens within cells, resulting in activation of V γ 9V δ 2 T cells (7, 8). HMB-PP can be used to induce *in vitro* expansion and activation of V γ 9V δ 2 T cells (9, 10). Activated V γ 9V δ 2 T cells exhibit a range of effector functions including direct cytotoxicity of infected and tumor cells, the induction of inflammatory and immunoregulatory processes and promotion of the survival, differentiation and activation of monocytes, neutrophils, dendritic cells (DC), $\alpha\beta$ T cells, and B cells (1–4).

Recent studies have provided evidence that V γ 9V δ 2 T cells can bridge innate and adaptive immune responses by promoting the differentiation of a number of cell types into antigen-presenting

cells (APC). DC are the most potent professional APC. They exist in peripheral tissues as specialized cells for pathogen recognition and uptake by phagocytosis, endocytosis, and pinocytosis, which results in their upregulated expression of antigen-presenting and co-stimulatory molecules, secretion of cytokines, and migration to lymphoid organs where they present antigen to naïve T cells (11, 12). V γ 9V δ 2 T cells, alone and in synergy with pathogen products, can induce differentiation of DC into immunogenic APC that express co-stimulatory markers, produce cytokines and stimulate T cells (10, 13–17). Furthermore, HMB-PP-stimulated V γ 9V δ 2 T cells are also capable of promoting survival and differentiation of monocytes into inflammatory DC (18, 19). V γ 9V δ 2 T cells are also capable of inducing recruitment, activation, and survival of neutrophils (20, 21) and a recent study has shown that neutrophils exposed to V γ 9V δ 2 T cells acquire the ability to present microbial antigens to CD4 $^{+}$ T cells and to cross-present endogenous antigens to CD8 $^{+}$ T cells (22).

B cells are also capable of presenting antigens to T cells (23) and secreting cytokines that activate and regulate adaptive immune responses (24). A number of studies have demonstrated that V γ 9V δ 2 T cells can induce differentiation of B cells into antibody-producing plasma cells (25–28). They can be found in germinal centers, can acquire features of follicular helper T cells and can induce the production and affinity maturation of class-switched antibodies. However, it is not known if V γ 9V δ 2 T cells contribute to antigen-presentation and cytokine secretion by B cells. The aim of the present study was to investigate the ability of V γ 9V δ 2 T cells

to induce differentiation, cytokine secretion, antibody production, and T cell allostimulation by B cells and how this compares to the adjuvant effect of V γ 9V δ 2 T cells for DC. We also examined the requirements for cell contact, co-stimulatory molecule, and cytokine receptor engagement between V γ 9V δ 2 T cells and B cells or DC for their reciprocal stimulatory activities. Our results show that V γ 9V δ 2 T cells induce maturation of both DC and B cells into APC that express co-stimulatory molecules and produce cytokines, and that these mature DC and B cells are capable of inducing alloreactive T cell proliferation. In addition, V γ 9V δ 2 T cell-stimulated B cells secrete antibodies. However, we show that V γ 9V δ 2 T cell-matured DC and B cells have different cytokine profiles and distinct stimulatory capacities for T cells and are mediated by different molecular interactions. Thus, V γ 9V δ 2 T cells can control different effector arms of the immune system through interactions with DC and B cells *in vitro*.

MATERIALS AND METHODS

DONORS

Peripheral blood mononuclear cells were prepared from healthy human buffy coat packs obtained from the Irish Blood Transfusion Service (IBTS, St. James's Hospital, Dublin, Ireland) by standard density gradient centrifugation over Lymphoprep™ (Nycomed Pharma, Oslo, Norway). The IBTS provides *pro bono* blood components to Irish third level educational facilities or health care facilities for the purposes of research and education. This blood is from voluntary, anonymous, non-remunerated donors donated primarily for therapeutic application to patients.

IN VITRO V δ 2 T CELL EXPANSION

$\gamma\delta$ T cells were enriched from peripheral blood mononuclear cells (PBMC) by positively selecting $\gamma\delta$ TCR $^+$ cells using a magnetic Microbead cell sorting kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). V γ 9V δ 2 T cells were expanded in 24-well plates by stimulating with 10 nM HMB-PP (kindly provided by Dr. Hassan Jomaa and Dr. Armin Reichenberg) and culturing them in complete RPMI (cRPMI) medium (RPMI 1640 with Glutamax containing 10% heat inactivated fetal calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 μ g/ml fungizone, and 25mM HEPES buffer, Gibco-BRL, Paisley, UK) supplemented with 50 IU/ml IL-2 (Peprotech, New Jersey, USA or Miltenyi Biotec). The medium was changed every 3–4 days by replacing with fresh IL-2-supplemented cRPMI. The cells were harvested on days 14–28 and used for co-culture with DC or B cells. We previously found that virtually all V δ 2 $^+$ T cells express the V γ 9 chain. Therefore, V γ 9V δ 2 T cells were subsequently identified by a V δ 2 monoclonal Ab (mAb) and are referred to as V δ 2 T cells hereafter (10). Cell purities were determined by staining with mAb against CD3 and V δ 2 and analyzing by flow cytometry.

B CELL ISOLATION

B cells were obtained from human PBMC by positive selection magnetic bead cell sorting (Miltenyi Biotec) of CD19 $^+$ lymphocytes or by negative selection magnetic bead cell sorting of CD19 $^-$ lymphocytes (Stemcell Technologies, Canada). The B cells were suspended in cRPMI and used fresh for co-culture with V δ 2 T cells. Purity was determined by staining the cells with anti-CD19 and anti-CD20 mAb and analysis by flow cytometry.

DENDRITIC CELL PREPARATION

Monocyte-derived DC were obtained from human PBMC by positively selecting CD14 $^+$ cells (Miltenyi Biotec). The monocytes were induced to differentiate into immature DC by culturing them in DC medium (RPMI 1640 supplemented with 10% heat inactivated, filtered low-endotoxin HyClone fetal calf serum, 1% penicillin-streptomycin, 1% fungizone, 1% L-glutamine, 0.1% β -mercaptoethanol, 1% sodium pyruvate, 1% non-essential amino acid mixture, 1% essential amino acid mixture, and 2% HEPES; Gibco-BRL; Logan, UT, USA) containing IL-4 (70 ng/ml) and GM-CSF (50 ng/ml) (Immunotools, Friesoythe, Germany). After 3 days, medium was replaced with fresh DC medium containing IL-4 and GM-CSF. On day 6, immature DC were harvested and used for co-culture with V δ 2 T cells.

ANTIBODIES AND FLOW CYTOMETRY

Fluorochrome-conjugated human mAb specific for CD3, CD11c, CD14, CD19, CD20, CD40, CD80, CD86, HLA-DR, IFN- γ , IL-4, IL-6, IL-10, IL-12p40, IL-13, TNF- α , and V δ 2 were obtained from Biolegend (San Diego, CA, USA), Immunotools or eBioscience (Hatfield, UK). Fixable viability dye eFluor 506 (eBioscience) was used to determine cell viability. Staining was carried out in PBA buffer (phosphate-buffered saline containing 1% bovine serum albumin and 0.02% sodium azide; Gibco-BRL; Sigma-Aldrich, Ireland) and analyzed using CyAn ADP (Beckman Coulter, High Wycombe, UK) or FACS Canto-II (Becton Dickinson, USA) flow cytometers and FlowJo software (Treestar, Ashland, OR, USA) using fluorescence-minus-one controls. Flow cytometry was used to look at cell surface phenotypes, intracellular cytokines, antibody production, co-stimulatory marker expression, and alloreactive T cell proliferation.

ANALYSIS OF CO-STIMULATORY MARKER EXPRESSION BY DC AND B CELLS

V δ 2 T cells were cultured with either B cells or DC in equal numbers in the presence or absence of HMB-PP (10 nM) for 72 or 24 h in cRPMI, respectively. The cells were stained for expression of CD11c (DC) or CD19 (B cells) and markers of antigen-presentation CD40, CD80, CD86, and HLA-DR. Surface expression of these markers was compared by mean fluorescence intensity (MFI) readings obtained using flow cytometry.

ANALYSIS OF CYTOKINE RELEASE FROM CO-CULTURES

V δ 2 T cells were cultured with either B cells or DC in equal numbers in the presence or absence of HMB-PP for 72 or 24 h, respectively. The supernatants were then harvested and assayed for levels of IFN- γ , IL-4, IL-6, IL-10, IL-12p70, and TNF- α by enzyme-linked immunosorbent assay (ELISA) using R&D Systems DuoSet kits (Abingdon, UK).

ANALYSIS OF INTRACELLULAR CYTOKINE PRODUCTION

V δ 2 T cells were cultured with either B cells or DC at 1:1 ratios in the presence or absence of HMB-PP for 24 h and then treated with monensin (10 μ l/ml, Biolegend) overnight. The cells were then stained for cell surface expression of CD3 and V δ 2 (V δ 2 T cells), CD19 (B cells), or CD11c (DC). The cells were then fixed and permeabilized and stained for intracellular expression of IFN- γ ,

IL-4, IL-6, IL-10, IL-12p40, IL-13, and TNF- α for analysis by flow cytometry.

MEASUREMENT OF ANTIBODY PRODUCTION BY B CELLS

V δ 2 T cells were cultured with B cells at 1:1 ratios in the presence or absence of HMB-PP (10 nM) for 7 days. The supernatants were harvested and analyzed using immunoglobulin cytometric bead array kits (Becton Dickinson) for IgA, IgM, IgE, and total IgG levels.

BLOCKING EXPERIMENTS

V δ 2 T cells were cultured with either DC or B cells in equal numbers in the presence or absence of HMB-PP and low-endotoxin, azide-free functional grade blocking antibodies against CD86, CD40L, IFN- γ and IFN- γ R, IL-4 and IL-4R, or TNF- α or isotype control mAbs for 24 h. Similar cultures were set up in transwell plates to prevent cell contact. The effects of blocking on DC and B cell phenotypes, cytokine expression and release and antibody production were determined as described above.

ALLOREACTIVE T CELL PROLIFERATION

V δ 2 T cells were cultured with either B cells or DC in equal numbers in the presence or absence of HMB-PP for 24 h. $\gamma\delta^-$ PBMC were enriched for CD3 $^+$ cells using a magnetic bead cell sorting kit (Miltenyi Biotec) and stained using a CellTrace™ kit (Invitrogen, CA, USA). The CellTrace-labeled resting alloreactive $\alpha\beta$ T cells were added to the overnight culture at ratios of 10:1 or 1:1 and cultured for 6 days before analysis of CellTrace dye dilution of CD3 $^+$ T cells by flow cytometry. Phytohemagglutinin-P (Sigma-Aldrich)-stimulated $\alpha\beta$ T cells cultured with IL-2 and irradiated PBMCs were used as positive controls. Similar co-cultures, except using unlabeled alloreactive $\alpha\beta$ T cells, were incubated for 3 days to look for expression of intracellular cytokines by alloreactive T cells. The supernatants harvested on day 3 were assayed for IL-2, IL-4, IL-10, and IFN- γ secretion by ELISA.

STATISTICAL ANALYSIS

GraphPad Prism 5.0 (San Diego, CA, USA) was used to carry out paired and unpaired *t*-tests to compare the means between groups. *P* values of <0.05 were considered statistically significant.

RESULTS

V δ 2 T CELLS INDUCE APC MARKER EXPRESSION BY DC AND B CELLS

We initially investigated if V δ 2 T cells can induce differentiation of B cells into cells with phenotypes of APC. Therefore, we examined the expression of CD40, CD86, and HLA-DR by B cells or DC after co-culture with non-stimulated or HMB-PP-activated V δ 2 T cells. V δ 2 T cells induced an increase in CD86 (Figure 1A) and HLA-DR (Figure 1C), but not CD40 (Figure S1A in Supplementary Material) expression by DC after 24 h and CD86 (Figure 1B), HLA-DR (Figure 1D) and CD40 (Figure S1B in Supplementary Material) expression by B cells after 72 h. CD86 expression was also upregulated on B cells after 24 h. To investigate which molecules are involved in DC and B cell activation by V δ 2 T cells or whether it is cell contact dependent, the same co-cultures were set up in the presence of HMB-PP-activated V δ 2 T cells and one of several blocking antibodies or transwell inserts which prevent

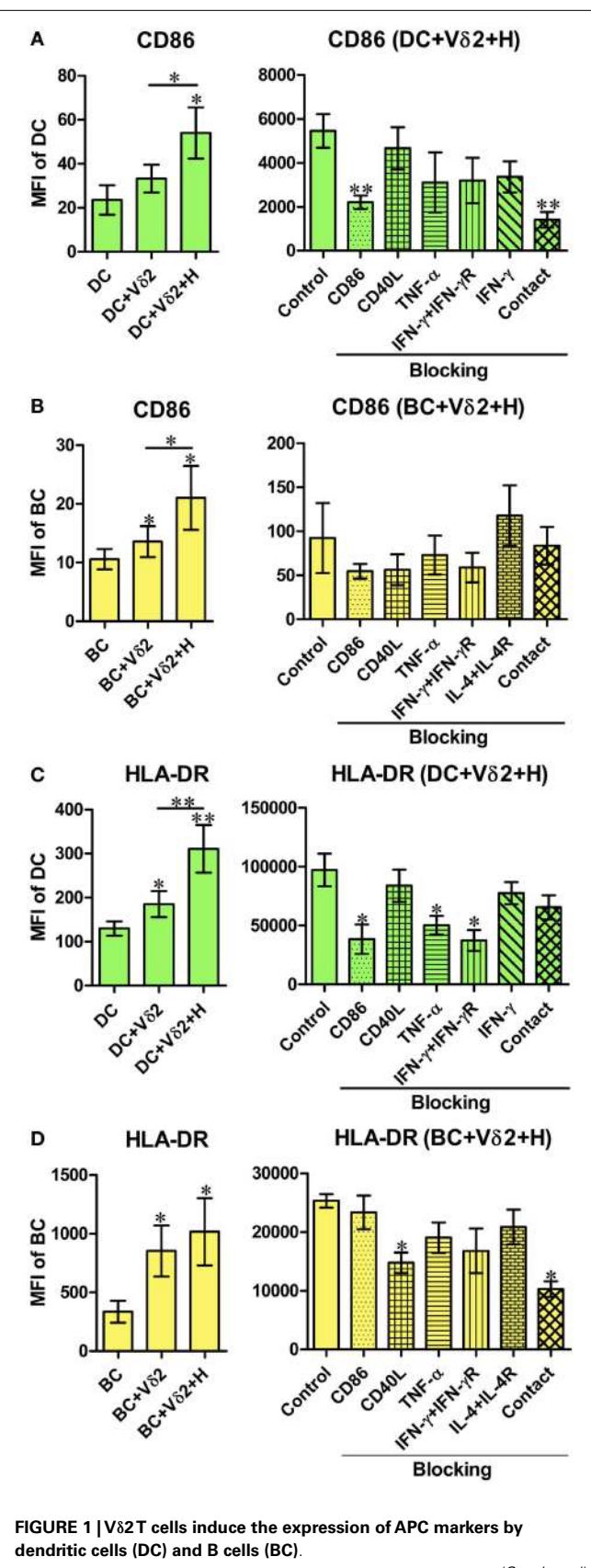


FIGURE 1 |V δ 2 T cells induce the expression of APC markers by dendritic cells (DC) and B cells (BC).

(Continued)

FIGURE 1 | Continued

Monocyte-derived DC or enriched peripheral blood B cells were co-cultured for 24 or 72 h with HMB-PP-expanded human V δ 2 T cells in the absence or presence of HMB-PP (denoted H). Cells were then stained using mAb specific for CD11c or CD19 and CD86 and HLA-DR and analyzed by flow cytometry. Left panels show average (\pm SEM) mean fluorescence intensities (MFI) of staining for CD86 expression by (A) DC ($n=11$) and (B) B cells ($n=12$) and HLA-DR expression by (C) DC ($n=9$) and (D) B cells ($n=7$). Right panels show average (\pm SEM) MFI of staining for CD86 or HLA-DR by DC or B cells after co-culturing them with V δ 2 T cells in the presence of HMB-PP in the absence (control) or presence of blocking mAbs specific for CD86, CD40L, TNF α , IFN- γ + IFN- γ R, IL4 + IL4R or with the DC or B cells separated from V δ 2 T cells using transwell inserts ($n=5$ for DC treatments and $n=3$ for BC treatments). * $p < 0.05$, ** $p < 0.01$ using a paired t -test, compared to DC or BC alone (left panels) or compared to BC control (right panels) and unpaired t -test compared to DC control (right panels) except where indicated by horizontal lines.

cell contact between the different cell types in the co-cultures. The results show that cell contact is important for CD86 expression by DC (Figure 1A), while CD86, TNF- α , and IFN- γ are important for HLA-DR expression by DC (Figure 1C). In contrast, CD40L and cell contact are important for HLA-DR expression (Figure 1D) but not CD40 expression (Figure S1B in Supplementary Material) by V δ 2-stimulated B cells.

V δ 2 T CELLS INDUCE DISTINCT CYTOKINE EXPRESSION BY DC AND B CELLS

To further characterize the influence of V δ 2 T cells on DC and B cell activation, we examined the same co-cultures for intracellular cytokine expression. The co-cultures, as described above, were treated with monensin for 16 h and the DC or B cells were analyzed for intracellular IFN- γ , IL-4 (Figures 2A,B), and TNF- α (Figure S2 in Supplementary Material) expression by flow cytometry. V δ 2 T cells induced IFN- γ expression by DC (Figure 2C) but not B cells and IL-4 expression by B cells (Figure 2D) but not DC. In contrast, V δ 2 T cells induced TNF- α expression by both DC and B cells (Figure S2 in Supplementary Material). The blocking studies revealed that CD86 and IFN- γ are important for IFN- γ expression by DC (Figure 2C), but not for cytokine production by B cells (Figure 2D).

V δ 2 T CELLS INDUCE PRO- AND ANTI-INFLAMMATORY CYTOKINE SECRETION FROM DC AND B CELL CO-CULTURES

While the flow cytometric cytokine assay revealed the percentage of cells expressing cytokines, we wanted to quantify the levels of cytokine production from the co-cultures. After 24 h co-culture of V δ 2 T cells and DC or B cells, supernatants were analyzed for levels of IFN- γ , TNF- α , IL-4, IL-6, IL-10, and IL-12 by ELISA. Since the cellular source of the cytokines produced cannot be identified, we also examined cytokine production by V δ 2 T cells alone. We found that V δ 2-DC co-cultures produced IFN- γ (Figure 3A), TNF- α (not shown), and IL-6 (Figure S3A in Supplementary Material) but not IL-4 (Figure 3C), IL-10, or IL-12 (Figure S3A in Supplementary Material) after 24 h. In contrast, V δ 2-B cell co-cultures produced TNF- α and IL-6 but did not augment IFN- γ (Figure 3B), IL-4 (Figure 3D), IL-10, or IL-12 (Figure S3B in Supplementary Material) production compared with V δ 2 T cells cultured alone. IFN- γ production by HMB-PP-activated V δ 2 T

cells was also observed by flow cytometry (data not shown). None of the molecules tested in the blocking studies, nor cell contact were found to be important for cytokine secretion by these co-cultures. However, surprisingly, blocking of CD86 resulted in augmented IFN- γ secretion after co-culture with V δ 2 T cells.

V δ 2 T CELLS INDUCE ANTIBODY PRODUCTION BY B CELLS

Previous studies have shown that a subset of V δ 2 T cells can provide help for antibody production by B cells and that it was mediated by CD40L, ICOS, and IL-10 (28). To investigate whether V δ 2 T cells can induce immunoglobulin production by fresh peripheral B cells *in vitro*, V δ 2 T cells were cultured with B cells for 7 days, and the supernatants were analyzed for total IgG, IgA, IgM, and IgE by a flow cytometric bead array. V δ 2 T cells induced IgG (Figure 4A), IgA (Figure 4B), IgM (Figure 4C) but not IgE (Figure 4D) production by B cells, while HMB-PP-activated V δ 2 T cells prevented IgA (Figure 4B) and IgM (Figure 4C) production. The blocking studies revealed that the cytokines and co-stimulatory markers examined and cell contact, do not play a part in antibody production by B cells.

V δ 2-MATURED DC AND B CELLS STIMULATE PROLIFERATION OF RESTING ALLOGENEIC T CELLS

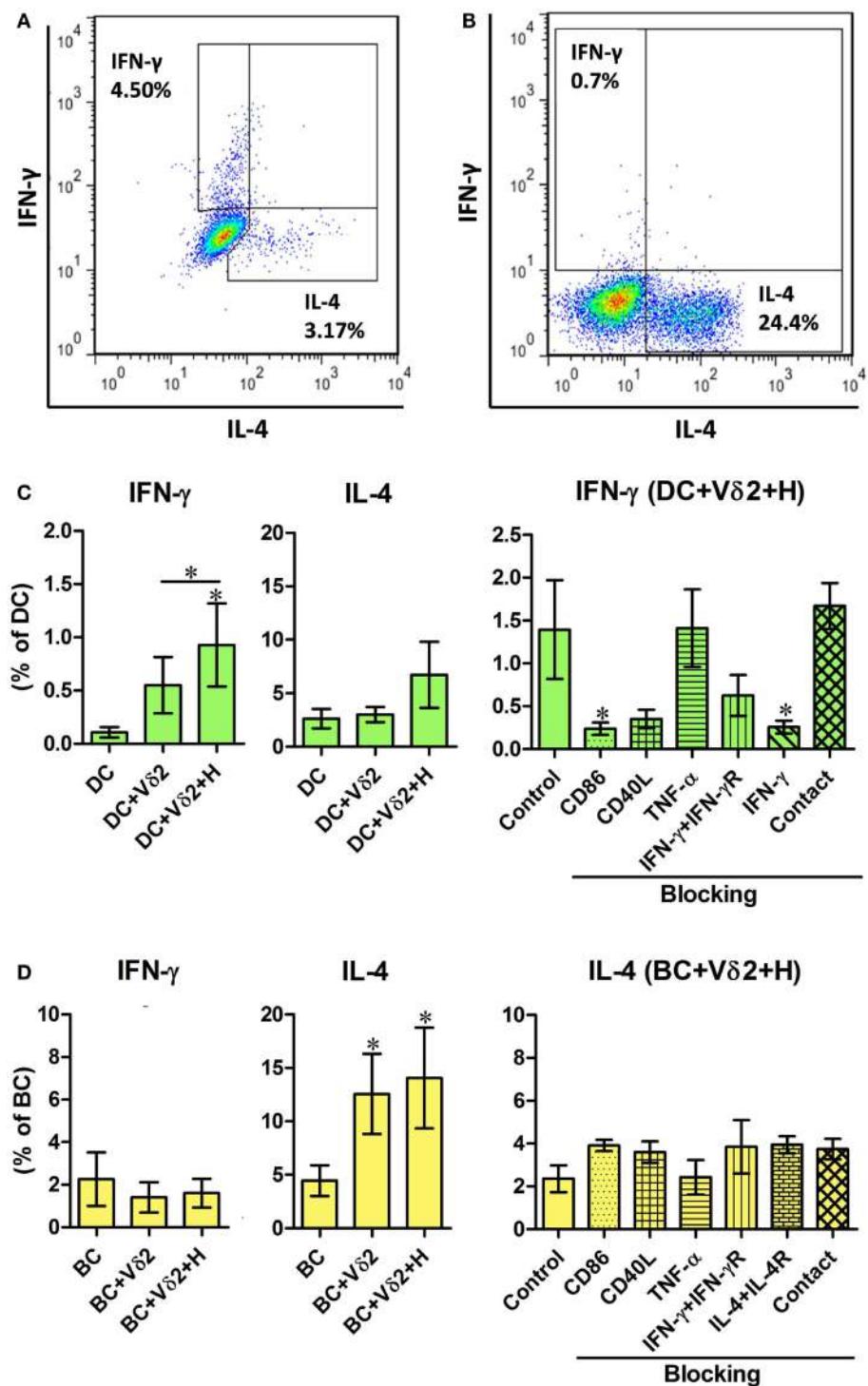
We investigated whether V δ 2 T cell-matured DC and B cells can induce activation and proliferation of resting $\alpha\beta$ T cells. V δ 2 T cell-matured DC or B cells were cultured with 10 times as many CellTrace-labeled resting allogeneic $\alpha\beta$ T cells for 6 days and dye dilution due to cell proliferation was examined by flow cytometry (Figures 5A,B). The co-cultures showed that both DC (Figure 5C) and B cells (Figure 5D) induced activation and proliferation of resting T cells after co-culture with V δ 2 T cells. Similar 3 day co-cultures were set up for analysis of cytokine secretion. ELISA showed that V δ 2 T cell-matured DC induced IFN- γ but not IL-4 production by T cells, whereas V δ 2 T cell-matured B cells did not stimulate cytokine production by T cells (Figures 5C,D; Figure S5 in Supplementary Material).

ALLOGENEIC AND AUTOLOGOUS V δ 2 T CELLS EQUALLY ACTIVATE DC AND B CELLS

The experiments described above indicate that V δ 2 T cells can differentially induce MHC and co-stimulatory molecule expression, cytokine production, and T cell allostimulation by allogeneic DC and B cells. We also investigated if the same outcomes could be observed when V δ 2 T cells were cultured with autologous DC or B cells. Figure S4 in Supplementary Material shows that V δ 2 T cells could equally induce CD86 expression (Figure S4A in Supplementary Material) and IL-12 secretion (Figure S4B in Supplementary Material) by autologous and allogeneic DC, and CD86 expression (Figure S4C in Supplementary Material) and IL-4 secretion (Figure S4D in Supplementary Material) by autologous and allogeneic B cells. Thus it appears that allogeneic V δ 2 T cells can be substituted for autologous V δ 2 cells as adjuvants for DC or B cells.

DISCUSSION

V γ 9V δ 2 T cells exhibit a myriad of effector functions in innate and adaptive immunity. They can kill infected, tumor, and stressed

**FIGURE 2 |**V δ 2T cells induce distinct cytokine profiles by DC and B cells.

DC or B cells were co-cultured with HMB-PP-expanded human V δ 2T cells in the absence or presence of HMB-PP (denoted H) for 24 h. The cultures were then treated with monensin for a further 16 h and stained for cell surface expression of CD11c or CD19 and CD3 and V δ 2 and intracellular expression of IFN- γ or IL-4 and analyzed by flow cytometry. **(A,B)** Representative flow cytometric dot plots showing IFN- γ and IL-4 expression by gated CD11c $^+$ cells (DC) and CD19 $^+$ cells (BC), respectively. **(C,D)** Left and center panels show mean (\pm SEM) percentages of **(C)** DC ($n=10$) and **(D)** BC ($n=10$) that express

IFN- γ and IL4, respectively. Right panels show mean (\pm SEM) percentages of **(C)** DC and **(D)** BC expressing IFN- γ and IL-4, respectively, after co-culturing them with V δ 2T cells in the presence of HMB-PP in the absence (control) or presence of blocking mAbs specific for CD86, CD40L, TNF- α , IFN- γ + IFN- γ R, IL-4 + IL-4R or with the DC ($n=5$), or BC ($n=3$) separated from V δ 2T cells using transwell inserts. * $p < 0.05$ using a paired t -test, compared to DC or BC alone (left panels) or compared to BC control (right panels) and unpaired t -test compared to DC control (right panels) except where indicated by horizontal lines.

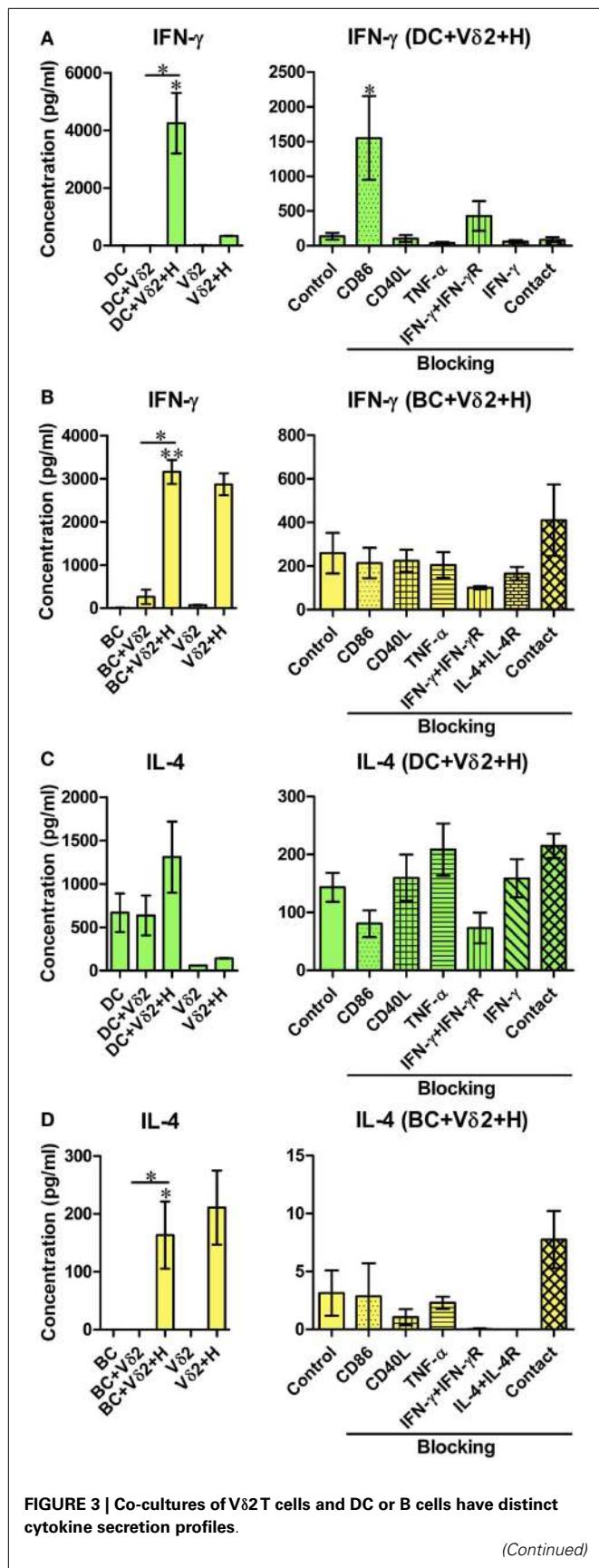


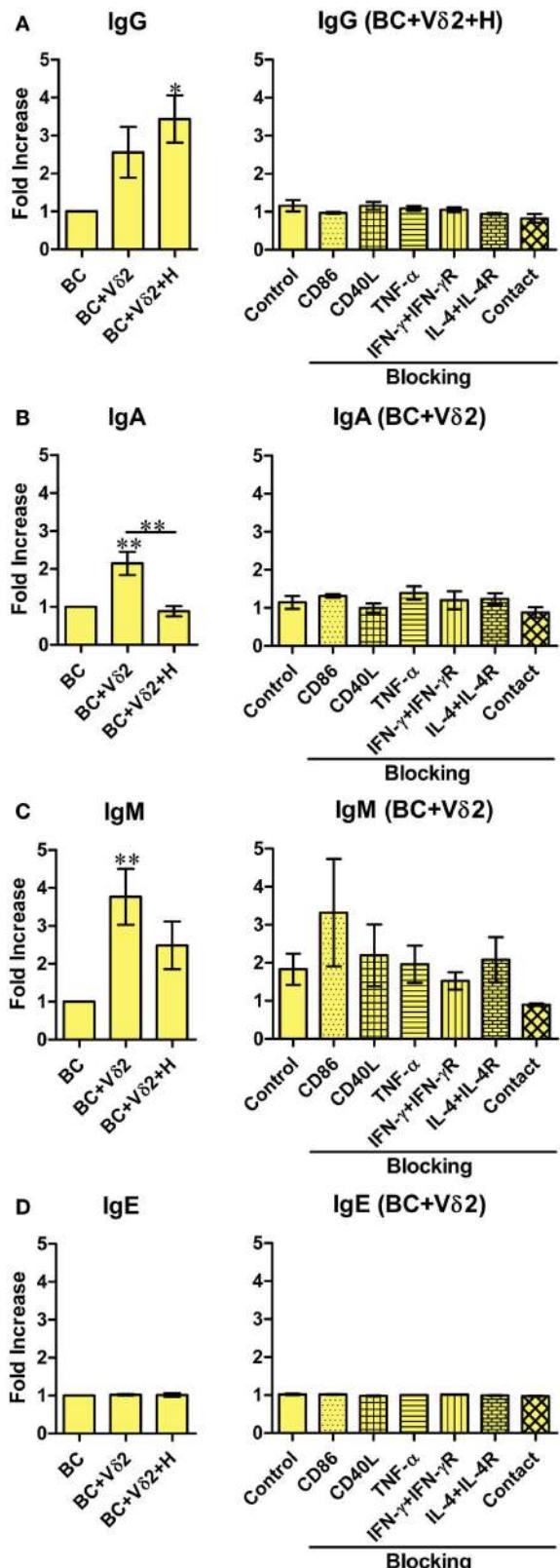
FIGURE 3 | Co-cultures of V δ 2 T cells and DC or B cells have distinct cytokine secretion profiles.

(Continued)

target cells, promote inflammation and wound healing, promote the survival, differentiation and activation of monocytes, neutrophils, and DC, provide B cell help for antibody production and prime CD4 $^{+}$ and CD8 $^{+}$ T cells (1–4). V γ 9V δ 2 T cells can also link innate and adaptive immune responses by promoting differentiation of different types of cells into APC that are capable of initiating antigen-specific T cell responses and long-term immunological memory (10, 13–19, 22, 29). These findings implicate V γ 9V δ 2 T cells as candidate targets for development of novel therapies and vaccines.

The findings in the present study confirm previous reports that V γ 9V δ 2 T cells can induce maturation, MHC and co-stimulatory receptor expression, and T H 1 cytokine production by DC (10, 13–17) and further show that these matured DC can stimulate proliferation and T H 1 cytokine production by alloreactive $\alpha\beta$ T cells. We found that V δ 2-DC co-cultures secreted IFN- γ , TNF- α , and IL-6 but not IL-4 and IL-10 after 24 h. While V δ 2 T cells were not potent inducers of IL-12 production by DC, they exhibited a strong synergistic effect with TLR ligands, such as LPS in inducing IL-12 release. Importantly, DC matured with V δ 2 T cells could stimulate proliferation and IFN- γ production by resting alloreactive T cells *in vitro*, suggesting that these APC also prime antigen-specific T H 1 responses. Although we did not test if V δ 2 T cell-matured DC could present specific antigen to T cells, their ability to stimulate alloreactive T cells to a greater degree than DC that had not been cultured with V δ 2 T cells, suggests that V δ 2 T cells are promoting differentiation of DC into APC.

Previous studies have demonstrated that V γ 9V δ 2 T cells can induce maturation of B cells into antibody-secreting plasma cells (25–28), suggesting that they can promote humoral immune responses *in vivo*. We showed that HMB-PP-stimulated V δ 2 T cells can stimulate the production of IgG, IgM, and IgA but not IgE by B cells *in vitro* and that HMB-PP prevents IgM and IgA production. We also examined the phenotypic changes to B cells that occur in response to co-culturing them with V δ 2 T cells and found that, like for DC, B cells upregulated HLA-DR, CD40, and CD86, suggesting that V δ 2 T cells can drive maturation of B cells into APC. However, analysis of cytokine production revealed that V δ 2-B cell co-cultures could produce TNF- α , IL-6, and IL-4 but not IFN- γ or IL-12. Thus V δ 2-matured DC and B cells have distinct cytokine profiles, with B cells lacking the T H 1-promoting cytokine bias seen for DC. Analysis of the capacity of V δ 2 T cell-matured B cells to stimulate alloreactive T cells indicated that they could induce

**FIGURE 4 |** V δ 2 T cells induce antibody production by B cells.

(Continued)

FIGURE 4 | Continued

B cells were co-cultured with HMB-PP-expanded human V δ 2 T cells in the absence or presence of HMB-PP (denoted H). After 7 days the supernatants were harvested and analyzed for IgA, IgM, IgE, and total IgG levels by cytometric bead array and flow cytometry. Left panels show average mean (\pm SEM) MFI of staining for (A) IgG ($n=5$), (B) IgA ($n=8$), (C) IgM ($n=7$), and (D) IgE ($n=2$). Right panels show average (\pm SEM) MFI intensities of IgG, IgA, IgM, and IgE of B cells after co-culturing them with V δ 2 T cells in the presence of HMB-PP in the absence (control) or presence of blocking mAbs specific for CD86, CD40L, TNF- α , IFN- γ +IFN- γ R, IL-4+IL-4R, or with the B cells separated from V δ 2 T cells using transwell inserts ($n=3$).

* $p < 0.05$, ** $p < 0.01$ using a paired *t*-test, compared to BC alone (left panels) or compared to B cell control (right panels) except where indicated by horizontal lines.

proliferation but not IFN- γ , IL-2, IL-4, or IL-10 production. These findings suggest that V δ 2 T cells can drive the differentiation of DC into T H 1-promoting APC and B cells into APC that can stimulate different T cell responses.

Several studies have demonstrated a flexibility of DC maturation and their ability to differentiate into APC that selectively promote T H 1, T H 2, or tolerogenic T cell responses (30–33). The factors that determine the fate of DC differentiation include the nature of antigen and the presence of TLR ligands and cytokines and it appears that V γ 9V δ 2 T cells contribute by driving T H 1-promoting APC generation. Tolerogenic APC are characterized by the expression of MHC class II and co-stimulatory molecules in the absence of pro-inflammatory cytokine production and they can present antigen to T cells resulting in the induction of anergy or the expansion of regulatory T cells (30–33). Our data suggest that V δ 2 T cell-matured B cells may function as tolerogenic APC, since they display phenotypes of APC but they do not produce pro-inflammatory cytokines and they stimulate proliferation but not cytokine production by alloreactive T cells. Furthermore, the ability of V δ 2-matured B cells to produce the anti-inflammatory cytokine IL-4 further supports a tolerogenic phenotype and we speculate that the IL-4 may function in promoting antibody responses. This is supported by the study by Caccamo (26), which showed that a subset of V δ 2 T cells that produce IL-4 and IL-10 provide help to B cells for antibody production. B cells have previously been shown to present antigen, resulting in tolerogenic T cell responses (34, 35), but future work is required to determine if the T cells stimulated by V δ 2-matured B cells have tolerogenic or immunosuppressive activities.

Since the mechanisms underlying DC and B cell activation by V δ 2 T cells are poorly understood, we aimed to identify the molecules required to mediate these functional changes. We found that while co-stimulatory molecules, pro-inflammatory cytokines and physical contact with V δ 2 T cells were important for DC maturation, co-stimulatory markers, and contact played only a minor role in B cell maturation and were not important for antibody production. Blocking CD40L and separating the B cells from V δ 2 T cells resulted in less upregulation of HLA-DR by B cells, but did not significantly affect the other readouts. Our results are in contrast to the study by Caccamo (26), which showed that IL-10, IL-4, CD40L, and ICOS abrogated antibody production. However, they did not investigate the role of these factors on co-stimulatory

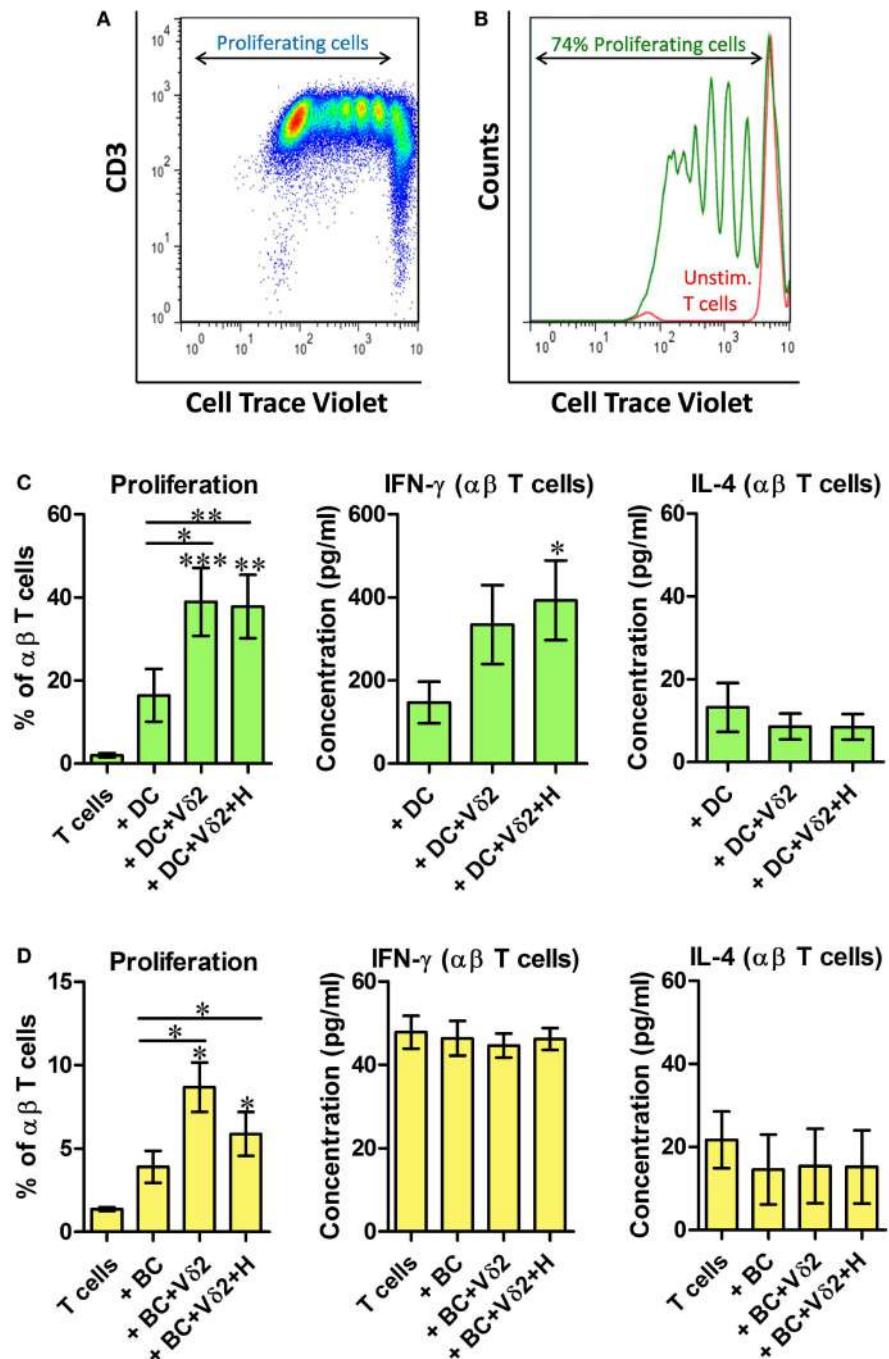


FIGURE 5 | V δ 2-matured DC and B cells stimulate proliferation of resting allogeneic T cells. DC or B cells were co-cultured with HMB-PP-expanded human V δ 2 T cells in the absence or presence of HMB-PP (denoted H). After 24 h CellTrace-labeled allogeneic resting $\alpha\beta$ T cells were added at a ratio of 10:1 and cultured for 6 days. **(A)** Representative dot plot showing proliferating T cells. **(B)** Histogram showing proliferating T cells (green peaks) versus unstimulated T cells (red peak) by flow cytometric analysis of cell trace

dilution. **(C)** Average (\pm SEM) percentage of proliferating T cells when cultured with V δ 2-matured DC ($n = 10$; left) and levels of IFN- γ and IL4 secreted by cultures of V δ 2 T cell matured DC with $\alpha\beta$ T cells ($n = 6–10$). **(D)** Average (\pm SEM) percentage of proliferating T cells when cultured with V δ 2-matured B cells ($n = 4$) and levels of IFN- γ and IL4 secreted by cultures of V δ 2 T cell matured DC with $\alpha\beta$ T cells ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired t -test versus T cells except where indicated by horizontal lines.

marker expression and cytokine production by B cells. Thus, the mechanisms responsible for B cell activation need to be further elucidated.

The adjuvant effects of V δ 2 T cells display similarities to those of other innate T cells. Invariant natural killer T (iNKT) cells, so called because of their conserved TCR α -chains that recognize

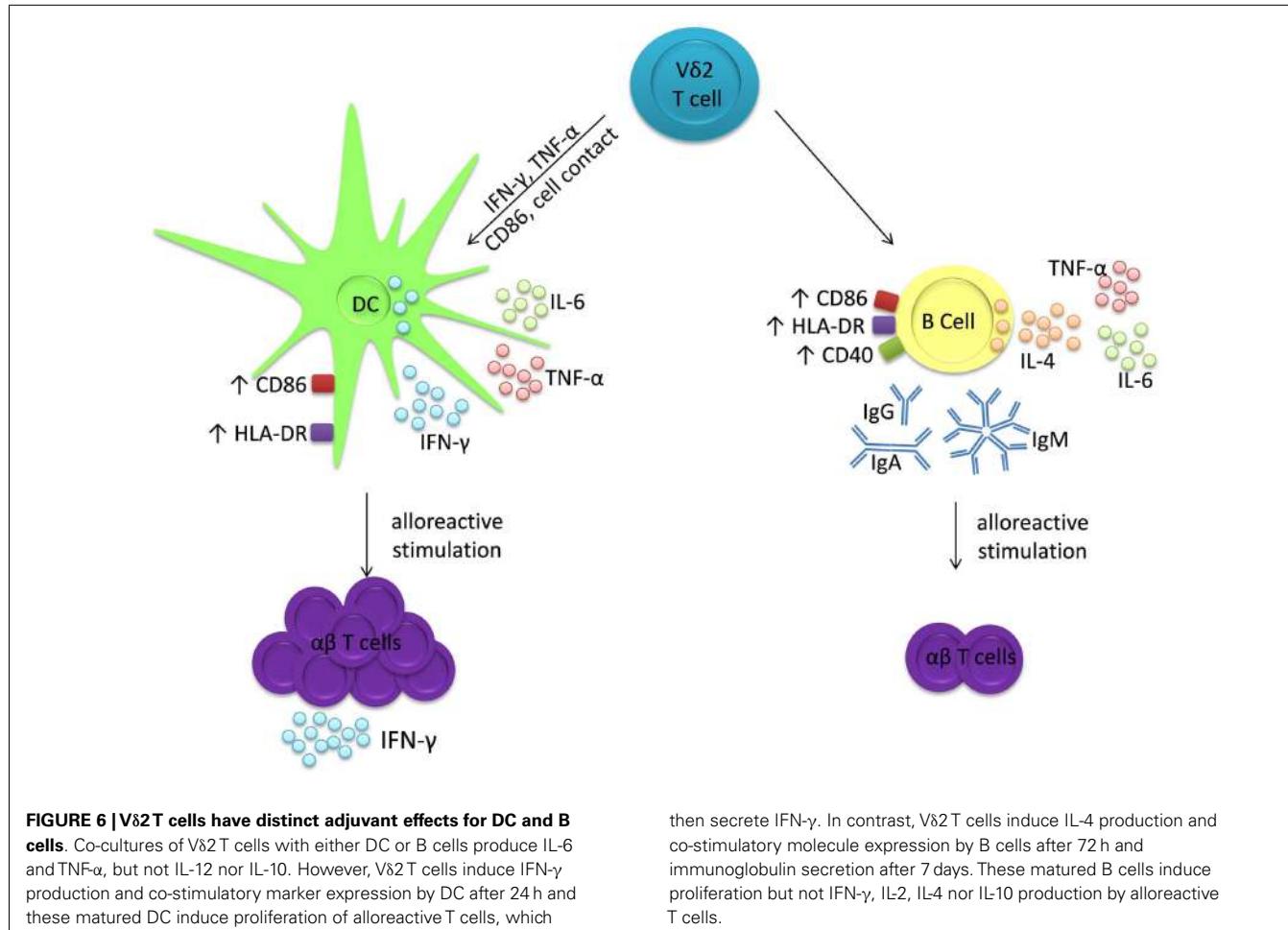


FIGURE 6 |V δ 2 T cells have distinct adjuvant effects for DC and B cells. Co-cultures of V δ 2 T cells with either DC or B cells produce IL-6 and TNF- α , but not IL-12 nor IL-10. However, V δ 2 T cells induce IFN- γ production and co-stimulatory marker expression by DC after 24 h and these matured DC induce proliferation of alloreactive T cells, which

then secrete IFN- γ . In contrast, V δ 2 T cells induce IL-4 production and co-stimulatory molecule expression by B cells after 72 h and immunoglobulin secretion after 7 days. These matured B cells induce proliferation but not IFN- γ , IL-2, IL-4 nor IL-10 production by alloreactive T cells.

glycolipid antigens presented by CD1d, can also induce maturation of DC into APC (36–38) and B cells into antibody-secreting plasma cells (39–42). Similar to V δ 2 T cells, iNKT cells induce MHC and co-stimulatory molecule expression by both DC and B cells, but they predominantly induce IL-12 production by DC (36, 37, 43) and IL-4 and IL-10 production by B cells (44). Furthermore, DC cultured with iNKT cells acquire phenotypes and functions of immunogenic APCs, whereas B cells cultured with iNKT cells differentiate into antibody-producing plasma cells but they are inhibited in their ability to stimulate alloreactive T cell proliferation (44). Other subsets of human $\gamma\delta$ T cells also possess adjuvant activities. T cells expressing the V δ 1 and V δ 3 TCRs can promote maturation of DC into APCs capable of driving T cell proliferation (45–47) and one study has shown that a population of V δ 1 $^+$ T cells specific for pollen-derived antigens can drive IgE production by B cells *in vitro* (48). Therefore, V δ 2 T cells belong to a family of innate T cells that can differentially promote or regulate T cell and antibody responses through selective interactions with DC and B cells. Whereas V δ 2 T cells promote immunogenic Th1 responses by inducing maturation of DC into APCs, they appear to promote T cell tolerance via their adjuvant activities on B cells, while at the same time promoting antibody production (Figure 6). While V δ 2 T cells are already under investigation as adjuvants for

immunotherapies in clinical trials for cancer (49–51), their distinct effects on DC and B cells must be considered in order to prevent unwanted immunosuppression or autoimmunity.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00650/abstract>

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Multiple receptor-ligand interactions direct tissue-resident $\gamma\delta$ T cell activation

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$\gamma\delta$ T cells represent a major T cell population in epithelial tissues, such as skin, intestine, and lung, where they function in maintenance of the epithelium and provide a crucial first line defense against environmental and pathogenic insults. Despite their importance, the molecular mechanisms directing their activation and function have remained elusive. Epithelial-resident $\gamma\delta$ T cells function through constant communication with neighboring cells, either via direct cell-to-cell contact or cell-to-matrix interactions. These intimate relationships allow $\gamma\delta$ T cells to facilitate the maintenance of epithelial homeostasis, tissue repair following injury, inflammation, and protection from malignancy. Recent studies have identified a number of molecules involved in these complex interactions, under both homeostatic conditions, as well as following perturbation of these barrier tissues. These interactions are crucial to the timely production of cytokines, chemokines, growth factors, and extracellular matrix proteins for restoration of homeostasis. In this review, we discuss recent advances in understanding the mechanisms directing epithelial-T cell crosstalk and the distinct roles played by individual receptor-ligand pairs of cell surface molecules in this process.

Keywords: epithelial, $\gamma\delta$ T cell, activation, costimulation, epidermis, skin

INTRODUCTION

Epithelial tissues represent barriers between the body and the outside world. These barrier tissues contain resident populations of T cells that help maintain homeostasis and provide a defense against disruption to the epithelium. One such T cell population is the $\gamma\delta$ T cell. Subsets of $\gamma\delta$ T cells are present in virtually all epithelial tissues of all species and, in many cases represent the major, or even exclusive, T cell population in the tissue (1). A variety of roles have been ascribed to these tissue-resident $\gamma\delta$ T cells, including maintenance of epithelial homeostasis, tissue repair, inflammation, response to infection, and protection from malignancy (2–5). Thirty years have already passed since the discovery of $\gamma\delta$ T cells and, although a considerable amount of progress has been made in the understanding of the varied functions of these cells, much remains unknown about the mechanisms by which these functions are elicited.

Like $\alpha\beta$ T cells, $\gamma\delta$ T cells express a rearranged T cell receptor (TCR), although with far more limited diversity than $\alpha\beta$ T cells (1, 6). In contrast to $\alpha\beta$ T cells, most epithelial-resident $\gamma\delta$ T cells do not express the CD4 or CD8 coreceptors or the well characterized $\alpha\beta$ T cell costimulatory molecule, CD28 (7, 8). This gave rise to the hypothesis that alternate molecules on $\gamma\delta$ T cells may serve analogous functions to those well characterized as essential for $\alpha\beta$ activation and that additional novel interactions may be responsible for some of the functions unique to epithelial $\gamma\delta$ T cells.

Indeed, the intimate contact between $\gamma\delta$ T cells and the neighboring epithelial cells they surveil, suggests that multiple receptor-ligand interactions likely maintain $\gamma\delta$ T cells in their homeostatic state as well as participate in their activation and effector functions.

This review will focus on recent advances in the identification and characterization of such molecules and the unique roles they play in epithelial $\gamma\delta$ T cell function.

ANTIGEN RECOGNITION

Stress-induced self-antigens have been postulated for many years to represent ligands for $\gamma\delta$ T cells (7, 9). Although $\gamma\delta$ T cell ligands are not the focus of this review, the $\gamma\delta$ TCR forms an essential component of the cell's ability to survive and function and the importance of TCR-ligand interactions for $\gamma\delta$ T cell activation are undisputed. In some cases, bone fide ligands have been identified [reviewed in Ref. (10)]. Despite the restricted use of the $\gamma\delta$ TCR, ligands appear to be varied and diverse in nature and the majority of those identified to date are ligands for circulating $\gamma\delta$ T cells as opposed to the tissue-resident epithelial $\gamma\delta$ T cells.

One of the populations of epithelial-resident $\gamma\delta$ T cells that has received much attention, yet TCR-ligands remain unidentified, is the dendritic epidermal $\gamma\delta$ T cell (DETC) of the murine epidermis (11, 12). These cells express an invariant V γ 3V δ 1 TCR [nomenclature according to Garman (13)]; alternative nomenclature V γ 5V δ 1 (14), that is expressed exclusively by DETC in skin and DETC precursors in fetal thymus (15). Recent work has demonstrated rapid and transient expression of the unknown TCR ligand following wounding, as well as a restricted distribution of expression to sites immediately adjacent to the wounds (16). In this study, no ligand was detectable under steady-state conditions in non-wounded tissue. In contrast, another study using intravital microscopy found constitutive V γ 3V δ 1 TCR signaling from interaction with neighboring epithelial cells, with wounding eliciting a reorganization of TCR molecules rather than an increase

in signal strength (17). This suggested constitutive TCR-ligand interactions under homeostatic conditions. As neither study identified a TCR ligand, both lack definite proof of constitutive ligand absence or presence, respectively. The Skint1 molecule does represent an attractive candidate for a steady-state V γ 3V δ 1 TCR ligand, as it is constitutively expressed by keratinocytes (18). However, as yet, no direct binding of Skint1 to the V γ 3V δ 1 TCR has been demonstrated. Until the identity of the V γ 3V δ 1 TCR ligand is firmly established, it cannot be concluded that this constitutive signaling in DETC in the steady state is indeed ligand-induced. Nevertheless, Skint1 deficiency has a profound effect on DETC development (19, 20) and studies in Skint1-deficient animals have added to the body of evidence demonstrating the importance of the V γ 3V δ 1 TCR to DETC function.

Studies of animals with disruption of the V γ 3 gene provided the first evidence that TCR conformation was essential for localization to, and residence in, the skin (21). The epidermis of mice lacking the V γ 3 gene product is populated by $\gamma\delta$ T cells expressing alternate V γ chains, yet these T cells are still recognized by a V γ 3V δ 1 clonotype-specific monoclonal antibody (21). This demonstrates the requirement of TCR conformation for localization of $\gamma\delta$ T cells to the epidermal layer of the skin.

Subsequent studies, disrupting the entire TCR δ locus, demonstrated the functional importance of the $\gamma\delta$ TCR to both epidermal homeostasis and wound repair. In these TCR δ -deficient animals, the epidermis is populated by replacement T cells bearing diverse $\alpha\beta$ TCRs (22). The lack of true DETC in these animals results in keratinocyte apoptosis due to IGF-1 deficiency (23) and gradual decline in epidermal T cell numbers over time as the atypical $\alpha\beta$ T cell population is not maintained in the epidermis (22). Upon damage to the epidermal layer, the $\alpha\beta$ T cell population found in the epidermis of TCR $\delta^{-/-}$ animals is unable to mount an efficient response to repair the epidermal damage and facilitate the return to homeostasis. One major defect in these animals is a lack of KGF-1 production (24) by the replacement $\alpha\beta$ T cells. This results in reduced keratinocyte proliferation and delayed wound closure. In addition, hyaluronan production is defective, resulting in reduced or delayed recruitment of additional immune cells, such as macrophages, required to facilitate the repair process (25).

Wound repair functions of $\gamma\delta$ T cells are not restricted to the epidermis. In the DSS-induced mouse model of colitis, it is possible to analyze both tissue damage and repair in the intestine, and thus the role of $\gamma\delta$ T cells in these processes. In this model, the importance of $\gamma\delta$ T cells in the intraepithelial compartment of the intestine ($\gamma\delta$ IEL) to the repair process is clear, yet once again the ligand for the $\gamma\delta$ TCR is unknown. Following DSS treatment, $\gamma\delta$ IEL localize to sites of epithelial cell damage and express KGF-1, resulting in vigorous epithelial cell proliferation to repair the damage (26). In the absence of $\gamma\delta$ T cells, there is increased severity of DSS-induced damage and a delay in tissue repair due, at least in part, to defective KGF-1 production resulting in severely impaired epithelial cell proliferation (26). Together, studies in skin and intestine highlight the importance of the communication between $\gamma\delta$ TCR bearing cells and epithelial cells for homeostatic tissue maintenance as well as repair from epithelial damage. What is becoming increasing clear is that TCR-ligand interactions are not the sole

communicators for epithelial $\gamma\delta$ T cell interactions with their neighboring epithelial cells.

COSTIMULATION

Costimulation, integral to effective $\alpha\beta$ T cell activation, has not been as clearly defined for $\gamma\delta$ T cells. However, recent studies have begun to identify novel molecules, and decipher their costimulatory mechanisms, for epithelial $\gamma\delta$ T cells.

Junctional adhesion molecule-like (JAML) is a type I transmembrane glycoprotein found on a variety of effector cells of both the innate and adaptive immune system. Most notably, JAML expression has been demonstrated on neutrophils, monocytes, and memory T cells (27, 28). More recently, JAML was found to be expressed at low levels on epithelial $\gamma\delta$ T cells under steady-state conditions and rapidly upregulated upon stimulation (29). *In vitro* assays with isolated epidermal $\gamma\delta$ T cells demonstrated a key role for JAML in $\gamma\delta$ T cell costimulation (29). Strikingly, this costimulatory function of JAML appears restricted to the epithelial subsets of $\gamma\delta$ T cells. Emerging evidence suggests that circulating $\gamma\delta$ T cells may too have their own unique set of costimulatory and accessory molecules (30–32).

JAML binds to coxsackie and adenovirus receptor (CAR) (28, 29) expressed on epithelial cells (29). CAR ligation of JAML recruits PI3K to JAML (33) and subsequently costimulates DETC proliferation and cytokine production (29). Of note is that PI3K is also able to mediate costimulatory signals through the prototypic $\alpha\beta$ T cell costimulatory molecule, CD28, through a binding motif similar to that found in JAML and another $\alpha\beta$ costimulatory receptor, ICOS (34). In the absence of JAML-CAR interactions *in vivo* in the skin, DETC activation in response to wounding is impaired, cytokine responses are diminished and subsequent wound closure is delayed (29). Thus, crosstalk between JAML and CAR is a key component of DETC activation and the wound repair process. The comparable expression of JAML and CAR in the mouse intestine (29), suggests that these molecules may play a parallel role in $\gamma\delta$ IEL activation in the intestine. Whether interactions between JAML and CAR are also vital for human skin and intestinal T cell activation and damage repair is still unknown.

In addition to JAML, the C-type lectin-like NKG2D receptor is also expressed on effector cells of both the innate and adaptive immune systems. NKG2D can be found on NK, NKT, $\gamma\delta$, and CD8 $^{+}$ T cells and is best characterized as providing activating signals upon ligation to one of its multiple ligands (35–37). In humans, NKG2D ligands include MICA and MICB and members of the ULBP family of molecules, while in the mouse, H60a-c, MULT1, and RAE1 serve as ligands (37–39). The expression of NKG2D ligands is generally low under homeostatic conditions, but can be upregulated by a variety of signals of cellular stress including infection, tumorigenesis, and tissue damage.

In the mouse, epidermal $\gamma\delta$ T cells express NKG2D. While ligand engagement of NKG2D activates these DETC (40), it is not yet clear whether this activation signal relies on concomitant TCR signaling or can alone activate DETC. H60c is an NKG2D ligand expressed in the epidermis upon skin damage and on cultured keratinocytes (41). H60c engagement of NKG2D, in the absence of TCR-mediated signals, is unable to activate DETC *in vitro*. Instead, H60c provides a costimulatory signal to DETC through

NKG2D (41). Blockade of interactions between H60c and NKG2D impairs KGF production and the wound repair response (42). In contrast, keratinocyte specific upregulation of another NKG2D ligand, RAE1, is able to activate DETC directly without an apparent requirement for simultaneous TCR engagement (43, 44). Whether this difference in TCR requirement could be due to the nature of the damage and thus the nature of the induced ligand, and elicited DETC response, is an intriguing question that remains unanswered.

In human beings, the NKG2D ligands MICA and MICB can be recognized by intestinal epithelial T cells expressing the V δ 1 $\gamma\delta$ TCR (45, 46). As expression of MIC in the intestinal epithelium is apparently stress induced, these NKG2D ligands have been proposed to be recognized by V δ 1 $\gamma\delta$ T cells in their surveillance for signs of damaged, infected, or transformed intestinal epithelial cells (47). Data suggest that MIC recognition can be either directly through the TCR or via NKG2D and that recognition may in fact be sequential, utilizing both molecules (48). This hypothesis, however, remains to be tested experimentally. In addition, both circulating and intestinal $\gamma\delta$ T cells have been shown to recognize lipid antigens bound to CD1d [reviewed in Ref. (49)]. Recently, a previously described MICA binding V δ 1 TCR was also found to interact with high affinity with CD1d-sulfatide (50), opening the possibility of multiple ligands for some $\gamma\delta$ TCRs.

MORPHOLOGY AND MIGRATION

Epidermal $\gamma\delta$ T cells develop in the thymus during fetal life and migrate to the epidermis, proliferate locally, and then remain in the epidermis for the life of the animal. These cells are sessile under homeostatic conditions (17, 51), and one of their most unique features is their striking dendritic morphology (11, 12), with basal dendrites being highly motile and immobile dendrites apically oriented, anchoring DETC at keratinocyte tight junctions (17). Adoption of these dendritic features seems to be dictated somewhat by environment, as recent work has shown a similar morphology of CD8 $^{+}$ T_{RM} in the epidermis (52, 53). Interestingly, T_{RM} cells form short dendrites and small projections that extend laterally within the epidermis (52, 53), in contrast to the long dendrites of DETC projecting upwards toward the stratum corneum (17), indicating that additional non-microenvironmental cues may control epidermal T cell morphology. At least for the $\gamma\delta$ T cells in the skin, this dendritic morphology is dramatically lost upon activation of DETC (24). Activated DETC assume a rounded shape and recent studies have identified the semaphorin, CD100, and one of its ligands, Plexin B2, in regulating this process (54). Mice deficient in the CD100 molecule were found to exhibit delayed DETC rounding upon wounding. A direct role for CD100 and plexin B2 in this morphology change was demonstrated by *in vitro* ligation of CD100 leading to ERK kinase and cofilin activation, concurrent with rapid DETC rounding. The importance of the CD100-plexin B2 mediated rounding in epithelial wound repair was demonstrated by the delayed wound closure observed in animals deficient for the CD100 molecule (54). Plexin B2 is broadly expressed on many epithelial tissues where CD100-expressing $\gamma\delta$ T cells reside, suggesting a more general role for CD100-plexin B2 in epithelial cell-T cell interactions. Indeed, a more severe colitis and a similar delay in repair, is

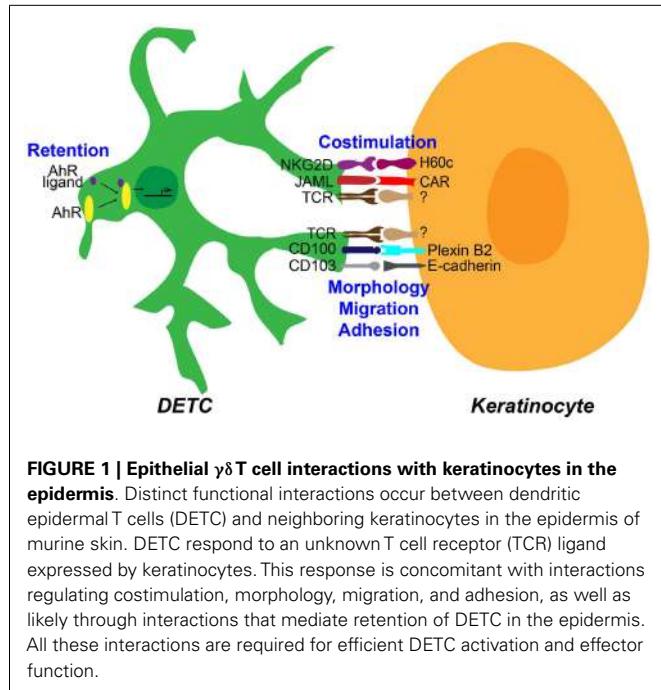
seen in the absence of CD100 in a mouse model of DSS-induced colitis (55).

Despite an increased understanding of the mechanisms controlling the characteristic DETC rounding upon activation, the function of this morphology change remains to be determined. One idea that has been put forth is that rounding is required for motility of DETC, either to the site of damage following a wound (54), or to draining lymph nodes in response to contact hypersensitivity reactions (56). Interestingly, CD103 has been demonstrated to play a role in DETC dendrite attachment to keratinocytes (17) and has recently been shown to be important for retention of T_{RM} in the skin (57). By analogy with this, CD103 is down-regulated upon DETC activation (22), which may thus allow detachment from keratinocytes and movement of the normally sessile DETC. Consistent with this hypothesis is the reduced number of DETC in the epidermis of CD103-deficient animals (58), although it has not been shown definitively that this is due to a lack of retention of DETC in the skin. Other possible explanations for the reduced DETC numbers in these animals are a defect in DETC development or reduced homing of DETC to the epidermis.

Persistence in the epidermis is also reliant on the aryl hydrocarbon receptor (AhR). AhR is expressed by keratinocytes, Langerhans cells, melanocytes, and DETC (59). In the absence of AhR, DETC undergo apparently normal intrathymic development and are able to home to the epidermis (59, 60). However, DETC in AhR $^{-/-}$ animals do not exhibit their normal dendritic morphology (59). They do not extend dendrites to neighboring epithelial cells, instead remaining round. Furthermore, DETC do not take up residence in the epidermis, but steadily decline in number in the first weeks after their initial homing to the tissue (59, 60). Conditional knock-out animals have demonstrated that it is specifically a deficiency in AhR in the DETC themselves that is responsible for the lack of retention in the epidermis (60), possibly as a result of a defect in c-kit interaction with its ligand, stem cell factor caused by the AhR deficiency (59). AhR-deficient DETC may thus be unable to make the necessary contacts with keratinocytes, and possibly Langerhans cells, that are required for maintenance in the epidermal compartment.

A similar loss of intestinal epithelial T cells in the absence of AhR has been described (60). While normal numbers of $\gamma\delta$ T cells were found in lymph node, spleen, and thymus, AhR-deficient animals were virtually devoid of small intestinal TCR $\alpha\beta$ CD8 $\alpha\alpha$ and $\gamma\delta$ IEL. As in the epidermis, loss of AhR activity was found to be responsible for a lack of maintenance of these cells in the intestine. Additionally, a reduction in AhR ligands or AhR deficiency itself results in increased immunopathology in DSS-induced colitis (60). Although clearly important for epithelial homeostasis, just how AhR signals maintain DETC and IEL at epithelial sites is unknown. In addition, the role of AhR in the activation of these cells during the wound repair process still requires investigation, but likely requires coordinated interactions between resident $\gamma\delta$ T cells and their neighboring epithelial cells.

Interestingly, differences exist between epidermal-resident and intestinal-resident $\gamma\delta$ T cells. The epithelia in these two tissues are quite distinct with the epidermis containing a stratified epithelial layer and the intestine lined with a single layer epithelium which may account for some of the differences in the features of $\gamma\delta$ T



cells in these tissues. As mentioned above, DETC are sessile under homeostatic conditions using their multiple dendritic projections to survey multiple neighboring keratinocytes simultaneously (17). In contrast, $\gamma\delta$ T cells in the intestine migrate actively within the intraepithelial compartment in the normal steady state (61). In this way, the limited number of $\gamma\delta$ IEL are presumably able to surveil the entire intestinal epithelium for signs of damage or disease. Evidence points to occludin expression by IEL as vital to this process (61) but the contribution of other molecules thought to be involved in epithelial $\gamma\delta$ T cell migration, such as CD100 and CD103, is unknown at this time.

CONCLUDING REMARKS

Although sharing some characteristics with $\alpha\beta$ T cells, the identification of an increasing number of novel molecules functioning in various aspects of epithelial $\gamma\delta$ T cell activation (Figure 1), highlights the distinct nature of these cells. Numerous molecules, such as integrins, adhesion molecules, cytokine receptors, and known markers of activation are expressed by DETC and other $\gamma\delta$ IEL and are modulated *in vitro* and/or *in vivo* by activation signals (62–64). Future studies designed at elucidating the precise role of these various molecules in epithelial $\gamma\delta$ T cell activation, should shed further light on the unique functional properties of this enigmatic T cell population.

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Human peripheral CD4⁺ V δ 1⁺ $\gamma\delta$ T cells can develop into $\alpha\beta$ T cells

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The lifelong generation of $\alpha\beta$ T cells enables us to continuously build immunity against pathogens and malignancies despite the loss of thymic function with age. Homeostatic proliferation of post-thymic naïve and memory T cells and their transition into effector and long-lived memory cells balance the decreasing output of naïve T cells, and recent research suggests that also $\alpha\beta$ T-cell development independent from the thymus may occur. However, the sites and mechanisms of extrathymic T-cell development are not yet understood in detail. $\gamma\delta$ T cells represent a small fraction of the overall T-cell pool, and are endowed with tremendous phenotypic and functional plasticity. $\gamma\delta$ T cells that express the V δ 1 gene segment are a minor population in human peripheral blood but predominate in epithelial (and inflamed) tissues. Here, we characterize a CD4⁺ peripheral V δ 1⁺ $\gamma\delta$ T-cell subpopulation that expresses stem-cell and progenitor markers and is able to develop into functional $\alpha\beta$ T cells *ex vivo* in a simple culture system and *in vivo*. The route taken by this process resembles thymic T-cell development. However, it involves the re-organization of the V δ 1⁺ $\gamma\delta$ TCR into the $\alpha\beta$ TCR as a consequence of TCR- γ chain downregulation and the expression of surface V δ 1⁺V β ⁺ TCR components, which we believe function as surrogate pre-TCR. This transdifferentiation process is readily detectable *in vivo* in inflamed tissue. Our study provides a conceptual framework for extrathymic T-cell development and opens up a new vista in immunology that requires adaptive immune responses in infection, autoimmunity, and cancer to be reconsidered.

Keywords: extrathymic T-cell development, V δ 1⁺ $\gamma\delta$ T cells, T-cell development, $\delta\beta$ heterodimer, inflammation, hematopoietic progenitor cell, extrathymic T-cell progenitor

INTRODUCTION

Hematopoietic stem-cells (HSCs) are rare, phenotypically and functionally diverse cells that can give rise to all cell lineages of the immune system (1). T-cell development commences when bone-marrow-derived HSCs seed the thymus. They are the most immature progenitors and thus constitute the CD4⁻CD8⁻ double negative (DN) T-cell fraction. Stroma- and thymocyte-derived signals then induce their T-cell lineage commitment and the cells' differentiation into either $\alpha\beta$ or $\gamma\delta$ T cells through well-defined stages (DN1–DN4). In humans, these stages can be recognized by the expression of CD34, CD38, and CD1a surface proteins. The expression of functionally rearranged TCR- γ and TCR- δ chain genes in DN2/3 thymocytes leads to $\gamma\delta$ TCR complexes, which drive cellular proliferation and promote differentiation into $\gamma\delta$ T cells (2, 3). In order to become an $\alpha\beta$ T cell, developing DN3 thymocytes need to express functionally rearranged TCR- β chain genes that associate with pre-T α molecules to form pre-TCR complexes. The pre-TCR signal drives proliferation, induces transcriptional silencing of the TCR- γ chain (4) and initiates the transition of the T cells into CD4⁺ and CD8⁺ expressing double-positive (DP) stages. In humans, this transition involves immature single-positive (ISP) CD4⁺ intermediates (5). DP T cells initiate the rearrangement of TCR- α genes, which leads to the deletion and

thus "silencing" of the TCR- δ chain because the genes encoding the TCR- δ chain are embedded in the TCR- α locus (6–10). TCR- α and - β chains form $\alpha\beta$ TCRs, which are selected for their ability to recognize peptide-presenting self-MHC molecules (positive selection). In this repeated process, cells that carry non-functional TCRs undergo TCR- α rearrangement (11) until selected (2). DP T cells that recognize self-MHC class I or II molecules below an acceptable threshold of reactivity (negative selection) develop into single-positive (SP) CD4⁺ or CD8⁺ $\alpha\beta$ T cells, and are exported from the thymus into the periphery.

It is undisputed that the thymus provides the foremost source of naïve T cells and orchestrates normal T-cell lymphopoiesis to some degree throughout life (12, 13). However, thymic involution begins as early as 1 year after birth, resulting in an exponentially decreasing output of naïve T cells, which is almost completely extinguished post adolescence (14). The total size of the T-cell pool nevertheless remains relatively constant throughout life (14, 15), which suggests that the T-cell pool must be replenished in some other way. The decreasing number of naïve T cells is in part balanced by the proliferation of peripheral, post-thymic T cells, including naïve (16) and memory $\alpha\beta$ T cells (16–18), $\gamma\delta$ T cells (19, 20), and NKT cells (21), leading to effector or long-lived memory T cells (22–24). Moreover, there is a growing body of evidence

that suggests that T cells may develop at extrathymic sites in mice (25) and in humans, e.g., in tonsils (26), lymph nodes, spleen, and the bone marrow (27–30). However, detailed knowledge about the precursors, site, and routes of extrathymic T-cell development is still elusive.

Recent research indicates that HSC – generally present in a dormant state in a specialized niche in the bone marrow – can be induced to proliferate and differentiate under conditions of stress (31–33). It has also been shown that they respond to T-cell consumption by inducing the proliferation of common lymphoid progenitors (CLPs), which are the immediate progenitors of T cells (31–33). V δ 1 $^+$ $\gamma\delta$ T cells are key players in the lymphoid stress-surveillance response. They constitute a minor T-cell population in the peripheral blood, but a major subset among tissue-residing and intraepithelial lymphocytes (34–37).

In this study, we show that the rare and so far unappreciated entity of human CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells, isolated from the peripheral $\gamma\delta$ T-cell pool of healthy individuals, expresses markers that are characteristic of the earliest hematopoietic progenitor cells, i.e., multipotent (MPP) and CLPs. Like thymus-seeding, early T (ETP), and DN1 progenitors, CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells express CD34 and CD38 but not CD1a (CD34 $^+$ CD38 $^+$ CD1a neg) on their surface; they also carry full-length transcripts of in-frame δ , γ , and β TCR gene rearrangements and express recombination-activating gene (RAG) and terminal deoxynucleotidyl transferase (TdT), which are typically found in DN2 and DN3 thymocytes. We show that CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells that lack thymus-homing properties but carry chemokine receptors (CCR) that direct circulating T cells to sites of inflammation, can develop into functional, mature CD4 $^+$ or CD8 $^+$ $\alpha\beta$ T cells in an inflammatory environment. In this study, we pinpoint the individual steps of this development, a process that is very similar to thymic T-cell development, but proceeds via a V δ 1 $^+$ V β $^+$ intermediate instead of a pre-TCR. We also show that the progenitors' cellular intermediates are present *in vivo* in inflamed tissue and to a considerably lesser extent in peripheral blood of healthy individuals.

This fundamentally new role of $\gamma\delta$ T cells as an $\alpha\beta$ T-cell precursor contributes to the emerging concept of T-cell plasticity and recommends the reconsidering of adaptive immune responses in infection, autoimmunity, and cancer.

RESULTS

CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-CELL CLONES DISPLAY CHARACTERISTICS OF A T-CELL PROGENITOR

In this study, we aimed to characterize the scarce T-cell entity of CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells. We generated CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T clones from the peripheral blood of 12 healthy individuals, from leukapheresis products (LPH) of GM-CSF-mobilized healthy stem-cell donors ($n = 12$), and also from the bone marrow of potential stem-cell donors ($n = 10$). Clones of this phenotype were extremely long-lived – they could be cultivated uninterruptedly *ex vivo* for up to more than a year under standard culture conditions. Importantly, over time, some clones could change their $\gamma\delta$ TCR into $\alpha\beta$ TCR. The morphology of the CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones was similar to that of large granular lymphocytes (LGLs) (Figure S1A in Supplementary Material). In contrast to most other V δ 1 $^+$ cells, their TCR- $\gamma\delta$ chain (Figure 1A) contained a constant-region segment

1 (C γ 1) (Figure S1B in Supplementary Material) and was thus able to form disulfide bonds between TCR- δ and - γ chains (38–40).

To elucidate the nature of the clones' transdifferentiation from $\gamma\delta$ into $\alpha\beta$ T cells and to clarify whether the change in TCR constitutes a certain form of TCR revision or whether it is the result of progenitor differentiation, clones were examined for the expression of stem-cell and progenitor markers. Although already committed to T-cell lineage (CD3 $^+$) CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones nevertheless uniformly expressed CD34 lo (22/22), which is the common marker of most immature hematopoietic stem/progenitor cells. The clones also expressed C-X-C chemokine receptor type 4 (CXCR4), which maintains the quiescence of the HSC pool in bone-marrow niches (41), TGF- β , a regulator of hematopoietic stem/progenitor cell self-renewal (42–44), and its receptor CD105, which, to some extent, indicates a self-sustaining circuit (Figure 1B). CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones expressed a functional IL-7 receptor (CD127 $^+$ /CD132 $^+$) (Figure 1C), CD117lo(c-kit) and the FLT3 ligand receptor CD135 (Figure 1B). FLT3 and the CD117-activated signal transduction cascade promote cell survival and proliferation. The marker set identified on CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones characterizes different progenitors, namely lin $^-$ multipotent hematopoietic progenitors (MPP) as well as CLP in human bone marrow, as well as linlo ETPs, and canonical DN1 in the thymus (1). Like DN1-stage T-cell progenitors, CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones were CD34 $^+$ CD38 $^+$ CD1a $^-$ (Figure 1D).

Clones that were established directly from the bone marrow – the place where hematopoietic stem and progenitor cells reside – expressed significantly higher quantities of CD135 ($p = 0.0182$) ($69.5 \pm 3.6\%$ cells positive/clone, $n = 4$) than peripheral blood-derived clones did ($48.7 \pm 6.8\%$ cells positive/clone, $n = 4$) (Figure S1C in Supplementary Material), which is evidence for the presence of a more primitive precursor type in the bone marrow. Although CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones did not initially express CD2 on their surface, they did so rapidly in the course of cultivation. This is additional evidence of the CD4 $^+$ V δ 1 $^+$ T-cell clones' premature phenotype (Figure 1D). Moreover, CD4 $^+$ V δ 1 $^+$ clones transcribed RAG and TdT (Figure 2A), had fully rearranged TCR- β loci (Figure S1D in Supplementary Material), and the TCR V β protein was readily detectable in the cytoplasm (Figure 2B) and on the cell surface (Figure S2A in Supplementary Material). Thus established CD4 $^+$ V δ 1 $^+$ clones were V δ 1 $^+$ C β $^+$ but not TCR $\alpha\beta$ $^+$. CD4 $^+$ V δ 1 $^+$ clones were negative for pre-T α ($n = 9$) (Figure 2A). In newly established clones, fully rearranged V α segments were found in rare cases in periphery-derived clones, though never in LPH-derived CD4 $^+$ V δ 1 $^+$ clones (not shown). This suggests that the precursors found in the bone marrow are more primitive. GATA-3 was the major transcription factor while T-bet, RORc, and Foxp3 were only transcribed at very low levels (Figure 2C). CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones spontaneously produced low level regulatory, T H 1- and T H 2-related, and proinflammatory cytokines (TGF- β , IL-2, -4, -5, -6, -10, -13, -17A, IFN- γ , and TNF- α) in standard culture conditions after stimulating the cells with PMA/ionomycin (Figure 3A). The CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones did not express the CD45RA antigen, which clearly distinguishes them from recent thymic emigrants (Figure 3B). CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones were CD45RO $^+$, CD45RA $^-$, CD62L $^-$, CD27 $^-$, and CCR7 $^-$, and can thus be classified as effector-memory cells (Figure 3B).

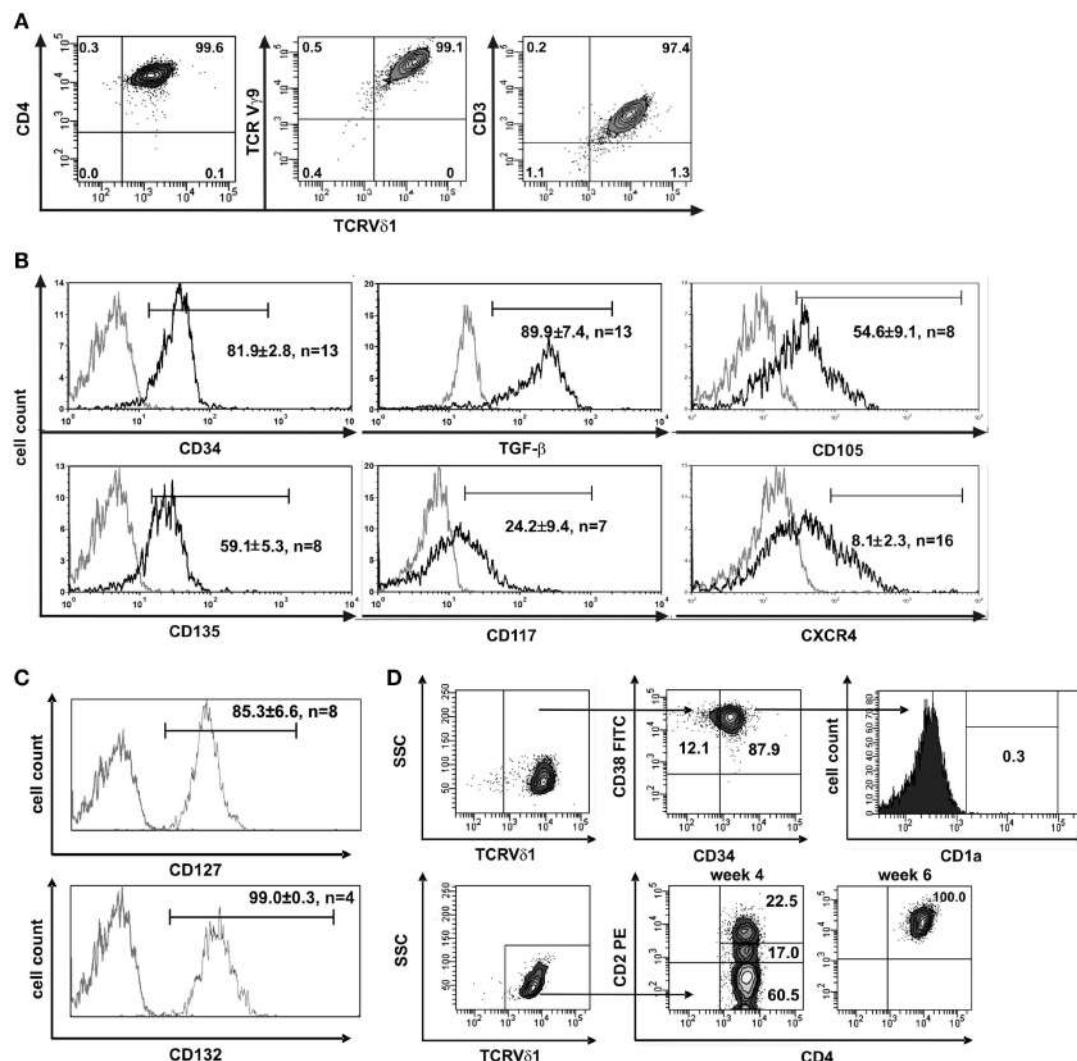


FIGURE 1 | CD4 $^{+}$ V δ 1 $^{+}$ cells express hematopoietic stem/progenitor cell markers. **(A)** CD4 $^{+}$ V δ 1 $^{+}$ T-cell clone TCRs contain a V γ 9 chain and the cells are CD3 $^{+}$. **(B)** CD4 $^{+}$ V δ 1 $^{+}$ T-cell clones express the stem-cell and progenitor markers CD34, CD135 (FLT3), CD117 (c-kit), CD105 (TGF- β R), and CXCR4 on their surface and express large amounts of TGF- β . Gray line: isotype control. Histogram marker shows cells that stained positive for antigen of interest. Numbers indicate mean \pm SEM of CD4 $^{+}$ V δ 1 $^{+}$ T cells

that stained positive for the respective marker (given in %). Each histogram shows one representative experiment of all clones tested. Numbers of clones tested are given in each histogram. **(C)** V δ 1 $^{+}$ CD4 $^{+}$ T-cell clones express IL-7 receptor composed of α subunit CD127 and the common γ chain CD132 of IL-2R. **(D)** FACS analysis showed that CD4 $^{+}$ V δ 1 $^{+}$ T-cell clones are CD34 $^{+}$ CD38 $^{+}$ CD1a neg , may lack CD2 expression, but become CD2 $^{+}$ during cultivation.

Thus, CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T-cell clones expressed marker molecules that are characteristic for a thymus-seeding progenitor (TSP), as well as DN1–DN4 stage thymocytes and an ISP thymocyte, which in humans is CD4 $^{+}$.

CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T-CELL CLONES EXPRESS CHEMOKINE RECEPTORS THAT DIRECT CIRCULATING T CELLS TO SITES OF INFLAMMATION

The function of T cells is strongly connected with their microenvironment. CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T cells did not express the chemokine receptors CCR9 (8/9) and CCR7 (9/9), which makes homing to the thymus less likely (Figure 4) (45). However, they expressed five chemokine receptors: CCR4 (46, 47), CXCR1/CXCR2 (48), CCR6 (49), and CXCR4 (50), which direct the movement of

circulating T cells to sites of tissue injury, infection, inflammation, and under physiological conditions to the skin (46, 47) (Figure 4).

CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T-CELL CLONES CAN CHANGE THEIR TCR INTO $\alpha\beta$ TCR AND MATURE INTO FUNCTIONAL $\alpha\beta$ T CELLS

CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T-cell clones turned out to be extremely long-lived. When grown under standard culture conditions, the CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T-cell clones successively downregulated the expression of CD34 on the cell surface (not shown) and some expressed $\alpha\beta$ instead of $\gamma\delta$ TCR (Figure 5A, top). TCR change occurred rarely under standard culture conditions – only 1 out of 50 clones changed their V δ 1 $^{+}$ TCR into $\alpha\beta$ TCR. TCR change occurred either within

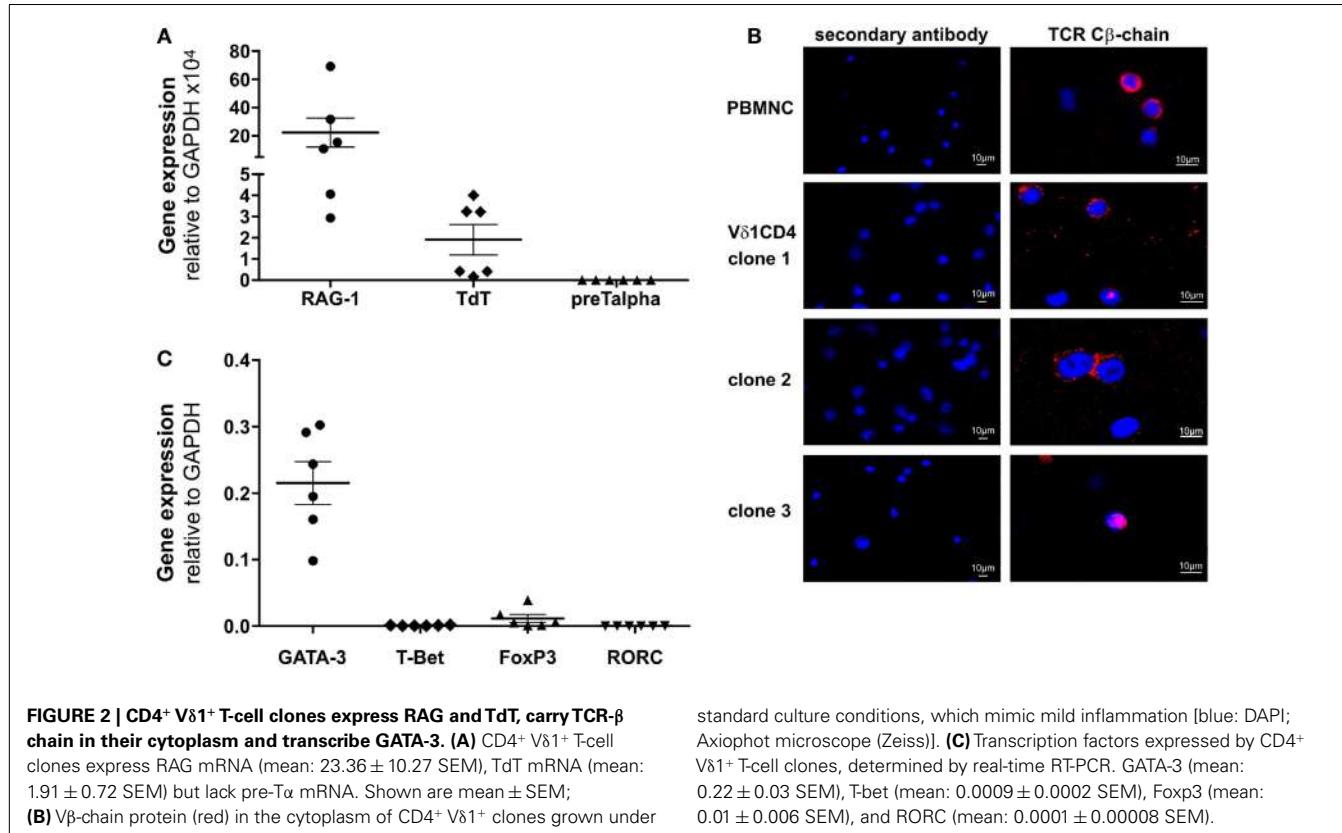


FIGURE 2 | CD4 $^+$ V δ 1 $^+$ T-cell clones express RAG and TdT, carry TCR- β chain in their cytoplasm and transcribe GATA-3. **(A)** CD4 $^+$ V δ 1 $^+$ T-cell clones express RAG mRNA (mean: 23.36 ± 10.27 SEM), TdT mRNA (mean: 1.91 ± 0.72 SEM) but lack pre-T α mRNA. Shown are mean \pm SEM; **(B)** V β -chain protein (red) in the cytoplasm of CD4 $^+$ V δ 1 $^+$ clones grown under

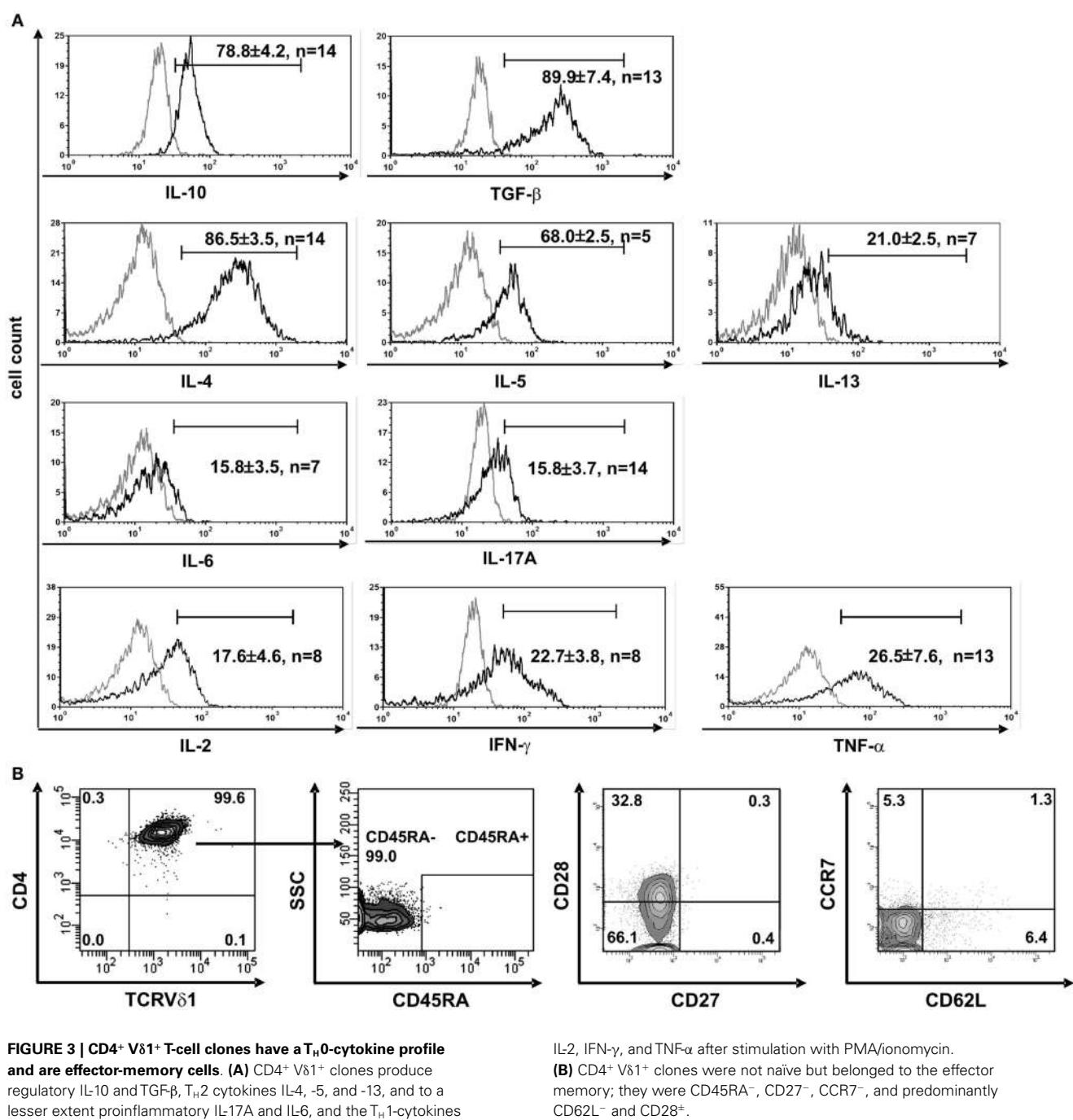
standard culture conditions, which mimic mild inflammation [blue: DAPI; Axiohot microscope (Zeiss)]. **(C)** Transcription factors expressed by CD4 $^+$ V δ 1 $^+$ T-cell clones, determined by real-time RT-PCR. GATA-3 (mean: 0.22 ± 0.03 SEM), T-bet (mean: 0.0009 ± 0.0002 SEM), Foxp3 (mean: 0.01 ± 0.006 SEM), and RORC (mean: 0.0001 ± 0.00008 SEM).

days or up to 5 months after the establishment of the cell culture [V δ 1 $^+$ clone K1: +3 months and +5 months (different culture plate), V δ 1 $^+$ clone P7: +3 months] (Figure 5A, top). The cells remained either SP CD4 $^+$ or changed their co-receptor from SP CD4 $^+$ to DP CD4 $^+$ /CD8 $^{\text{dim}}$. This led to pools of cells that were either SP CD4 $^+$ and/or SP CD8 $^+$ (Figure 5A, bottom). The CD4 $^+$ V δ 1 $^+$ T-cell clone-derived TCR $\alpha\beta$ $^+$ T-cell line K1.1 (K1.1- $\alpha\beta$ $^+$) almost exclusively expressed surface TRBV11-2 $^+$ and -4.1 $^+$, and T-cell line K1.2 (K1.2- $\alpha\beta$ $^+$) almost exclusively TRBV27 $^+$ and -19 $^+$ (Figures S2B,C in Supplementary Material). The clone P7-derived TCR $\alpha\beta$ $^+$ T-cell line did not express TCR V β families that are detectable with available TCR antibodies (data not shown). All CD4 $^+$ V δ 1 $^+$ T-cell clone-derived $\alpha\beta$ T cells were T10B9.1A-31 $^+$ (framework epitope of $\alpha\beta$ TCR heterodimer), WT31 $^+$ (CD3 ϵ), CD3 $^+$, V δ 1neg, γ TCRneg, CD25lo/neg, CD28neg, and were also terminally differentiated effector-memory RA-re-expressing T cells (TEMRA) (Figure 5B). The CD4 $^+$ V δ 1 $^+$ T-cell clone-derived $\alpha\beta$ T-cell lines lacked CD103, and therefore did not have a tissue-resident memory (TRM) phenotype (not shown). Furthermore, they did not transcribe TCR- δ segments, which is due to the deletion of the TCR- δ locus as a result of the recombination of the genes that encode the TCR- α chain. Nevertheless, the $\alpha\beta$ T-cell lines still transcribed rearranged γ chain segments (Figure 5C). Under standard culture conditions, the $\alpha\beta$ T cells derived from clone K1-V δ 1 $^+$ and P7-V δ 1 $^+$ produced IL-4 but not IFN- γ . In addition, they responded to (PMA)/ionomycin stimulation with a (subtle) increase in IL-4 release and with the substantial *de novo* production of IFN- γ , demonstrating their functionality (Figure 5D).

The $\alpha\beta$ T cells responded poorly to mitogenic stimuli (data not shown). The clone C3-23-derived $\alpha\beta$ T-cell line produced IFN- γ (41% of the cells) and IL-10 (55% of the cells) when stimulated with PMA/ionomycin. These are the same cytokines as those produced in lower quantities under standard culture conditions (not shown). $\alpha\beta$ T-cell lines derived from other clones produced mainly IFN- γ and IL-10.

DEVELOPMENTAL STEPS OF CD4 $^+$ V δ 1 $^+$ CLONE CELLS IN THE PROCESS OF TRANSDIFFERENTIATION

In order to study TCR re-organization during transdifferentiation, the expression of the constant region of the TCR chains, C δ , C γ , C β (BMA031) and C α (as a TCR- $\alpha\beta$ -associated framework epitope), in CD4 $^+$ V δ 1 $^+$ clones undergoing TCR change was monitored. CD4 $^+$ V δ 1 $^+$ T cells consistently downregulated the expression of the TCR- γ and TCR- δ chains, thus becoming V δ 1 $^{\text{dim}}$ /C γ $^{\text{dim}}$, and then V δ 1 $^{\text{lo/neg}}$ cells that lacked C γ (Figure 6A). V δ 1 $^{\text{lo/neg}}$ -C γ $^{\text{neg}}$ cells expressed C β on their cell surface. These V δ 1 $^{\text{lo/neg}}$ -C β -expressing cells were CD4 $^+$ CD8 $^+$ DP and eventually developed into V δ 1 $^{\text{neg}}$ -C β $^+$ SP CD4 $^+$ or SP CD8 $^+$ T cells (Figure 6A). In order to identify the number of $\alpha\beta$ T cells in the cultures, we stained for C β and TCR $\alpha\beta$ in V δ 1 $^+$ cell cultures and found that $84.6\% \pm 8.0$ SD of all V δ 1 $^{\text{lo/neg}}$ cells expressed C β , while $26.6\% \pm 6.64$ SD of all C β $^+$ V δ 1 $^{\text{lo/neg}}$ cells stained positive for TCR $\alpha\beta$ (Figure 6B). C β $^+$ cells were CD4 $^+$ CD8 $^+$ DP ($29.75\% \pm 15$ SD), or CD8 $^+$ ($8.37\% \pm 4.62$ SD), or CD4 $^+$ ($58.38\% \pm 11.21$ SD), and in rare cases DN ($3.5\% \pm 5.4$ SD) (an example of which is shown for one clone in Figure 6A). TCR $\alpha\beta$ $^+$



cells then upregulated $\alpha\beta$ TCR and could not be distinguished from peripheral $\alpha\beta$ T cells (not shown).

INFLAMMATION TRIGGERS DIFFERENTIATION OF V δ 1 $^+$ $\gamma\delta$ T CELLS INTO $\alpha\beta$ T CELLS

As TCR change occurred in only one out of 50 established CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones, we purified V δ 1 $^+$ $\gamma\delta$ T cells, including the DN, CD8 $^+$, and CD4 $^+$ subsets from peripheral blood mononuclear cells (PBMNCs) of healthy human donors. These cells (panV δ 1 $^+$) were subsequently used to study whether

inflammatory stimuli trigger transdifferentiation. For that two different inflammatory settings were compared: standard culture conditions were designated as mild inflammation, whereas stronger inflammatory stimuli were termed overt inflammation.

PanV δ 1 $^+$ T cells cultivated in the mild inflammatory environment (standard culture, see Materials and Methods) gave rise to a subset of T cells that transdifferentiated into TCR $\alpha\beta$ $^+$ T cells ($n = 12$) (Figure 7A) within 3–4 weeks. V δ 1 $^+$ cells sequentially changed their TCR, reorganized their V δ 1 $^+$ TCRint/lo phenotype to phenotype V δ 1int/lo/TCR- $\alpha\beta$ lo, and from the latter to

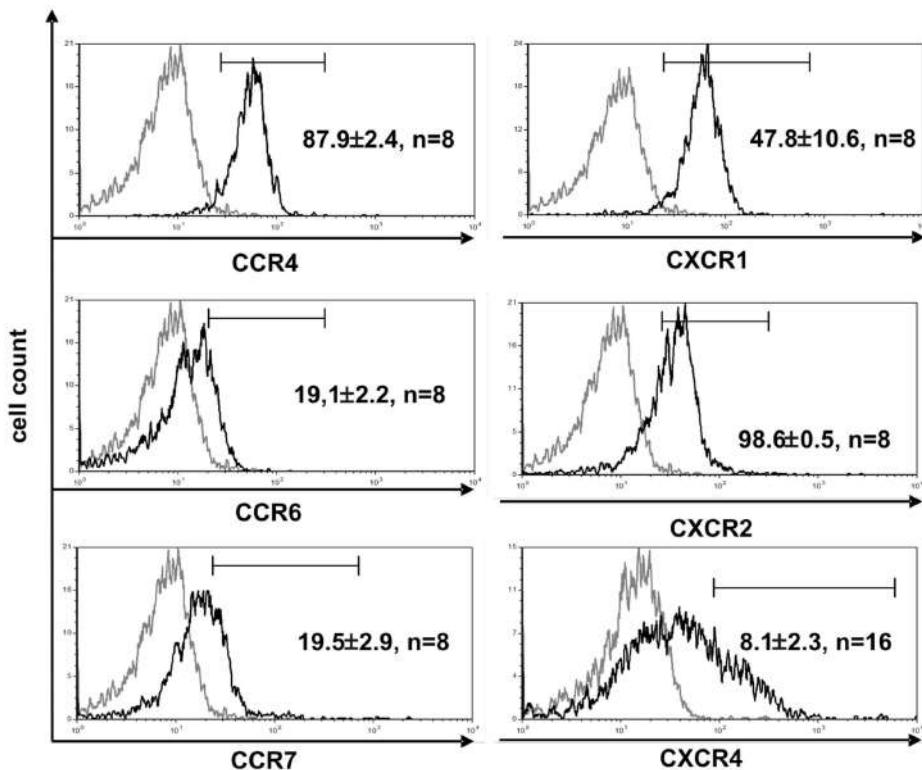


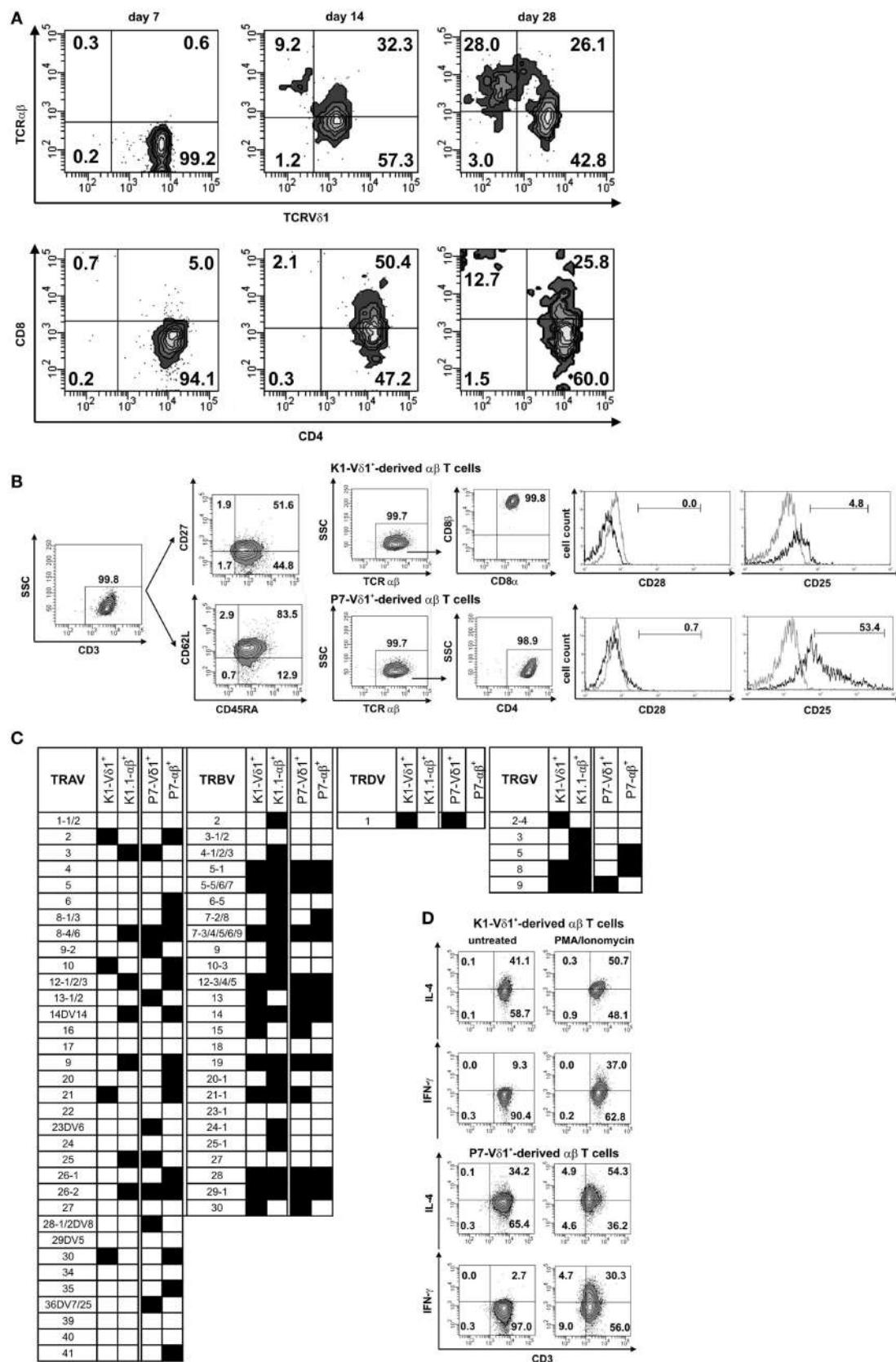
FIGURE 4 | CD4 $^+$ V δ 1 $^+$ T-cell clones express chemokine receptors associated with inflammation. V δ 1 $^+$ CD4 $^+$ clones show strong expression of CCR4, CXCR1, and -2, and express CCR6 $^{\text{lo}}$, CCR7 $^{\text{lo}}$, and CXCR4 $^{\text{lo}}$. Histogram marker shows cells that are positive for the antigen of interest.

Numbers indicate mean \pm SEM of CD4 $^+$ V δ 1 $^+$ T cells that stain positive for the respective marker (given in %). Each histogram shows one representative experiment of all clones tested. Numbers of clones tested are given in each histogram. Gray line: isotype control.

phenotype V δ 1 $^-$ γ δ $^-$ TCR- $\alpha\beta$ $^+$ (**Figure 7A**). The percentage of V δ 1int/lo/TCR- $\alpha\beta$ lo DP cells correlated exactly with the percentage of CD4 $^+$ cells in the initial panV δ 1 $^+$ T-cell pools. However, the number of V δ 1int/lo/TCR- $\alpha\beta$ lo DP cells did not correlate with the low number of $\alpha\beta$ T cell contaminants found in the initial culture pool (**Figure 7B**). The number of CD4 $^+$ V δ 1 $^+$ T cells within the panV δ 1 $^+$ T-cell pool varied greatly between individuals (mean: 0.926% of all V δ 1 $^+$ T cells; range: 0.1–3.0%), as did the number of $\alpha\beta$ T cells generated from panV δ 1 $^+$ cell pools (mean 1.82% of input V δ 1 $^+$ T cells; range 0.2–6.4%) (**Figure 7B**). Concomitantly with TCR change, RAG-1 and TdT mRNA was detected in panV δ 1 $^+$ T-cell pools. TdT is positively regulated by T β 4 of which large amounts are expressed by epithelial (V δ 1 $^+$) γ δ T cells (51) (**Figure 7C**). Additionally, mRNA isolated from the panV δ 1 $^+$ cultures showed that functionally rearranged TRBV and TRAV segments emerged at the same time as V δ 1int/lo/TCR- $\alpha\beta$ lo intermediates ($n = 5$) did (**Figures 8A,B**). The modulation of inflammatory culture conditions resulted in similar, overlapping TRAV expression patterns (**Figure 8C**). In contrast, TCR- α chain transcripts were not present in the aliquots of the initial panV δ 1 $^+$ T-cell pools. They were not present in panV δ 1 $^+$ and pan γ δ T-cell pools of peripheral blood of healthy donors either (not shown). The transcription rate of pre-T α was the same as in the controls with no template.

In the overt inflammatory approach panV δ 1 $^+$ T cells received a combination of cytokines that are pivotal in acute and chronic inflammation, and monocytes, which had been preactivated with the same cocktail for three days (see Materials and Methods: overt inflammation).

Fundamental phenotypic changes were observed at the protein and transcriptional level. A significant upregulation of CD4 (4.3-fold; $p = 0.0487$) created (more) V δ 1 $^+$ SP CD4 $^+$ cells and the novel phenotype of V δ 1 $^+$ CD4 $^+$ CD8 $^+$ DP T cells in five out of seven cultures (**Figures 9A,B**). Simultaneously, the CD8 co-receptor was downmodulated (**Figure 9B**) and the CD8 $\alpha\alpha$ homodimer was replaced by the CD8 $\alpha\beta$ heterodimer in a significant proportion of CD8 $^+$ V δ 1 $^+$ T cells (**Figure 9C**) ($p = 0.0452$, $n = 6$). Interestingly, panV δ 1 $^+$ T cells became weakly positive for V β chains on their surface (V β $^+$); most of them expressed TRBV30 ($n = 12$) as shown by FACS analysis (**Figure 9D**). In addition, other V δ 1 $^+$ T cells in the panV δ 1 T cell pool carried a varying number of individual TRBV chains on their surface (not shown). It is worth noting that strong inflammation (as indicated in the Section “Materials and Methods”) was not associated with the generation of DP V δ 1 $^+$ /TCR- $\alpha\beta$ $^+$ or $\alpha\beta$ T cells. Strong inflammation did not affect the transcription of RAG (**Figure 7C**), V β -segment usage (**Figure 8A**) and complexity score (native: 20.00 \pm 7.21 SEM, mild infl.: 7.67 \pm 2.96 SEM; overt infl.: 28.43 \pm 7.38 SEM). However,

FIGURE 5 | CD4 $^{+}$ V δ 1 $^{+}$ T-cell clones change their TCR into TCR- $\alpha\beta$ and can change their co-receptor.

(Continued)

FIGURE 5 | Continued

Phenotypic, molecular, and functional characterization of $\alpha\beta$ T cell lines exemplarily shown for lines derived from two different clones. (A) Process of transdifferentiation in a CD4 $^{+}$ V δ 1 $^{+}$ clone. Change of TCR expression (top), and change of co-receptor expression (bottom). (B) Resulting $\alpha\beta$ T-cell populations were heterogeneous. Exemplarily shown are two T-cell lines derived from two different clones. Their T-cell lines were terminally differentiated CD45RA-re-expressing T effector-memory cells (TEMRA). Clone

K1-V δ 1 $^{+}$ -derived $\alpha\beta$ T cells were CD8 $\alpha\beta$ $^{+}$ and did not express CD28 and CD25. Clone P7-V δ 1 $^{+}$ -derived $\alpha\beta$ T cells were CD4 $^{+}$, lacked CD28 but expressed CD25 $^{\text{int}}$. (C) Molecular analysis of TCR chain δ , γ , β , and α -transcripts in two CD4 $^{+}$ V δ 1 $^{+}$ clones (K1-V δ 1 $^{+}$ and P7-V δ 1 $^{+}$) and two of the derived $\alpha\beta$ T-cell lines (K1.1- $\alpha\beta$ $^{+}$ and P7- $\alpha\beta$ $^{+}$). The generated $\alpha\beta$ T-cell lines transcribe γ chain(s), but no δ chain. (D) Clone K1- and P7-V δ 1 $^{+}$ -derived $\alpha\beta$ T cells constitutively secrete IL-4, but not IFN- γ ; upon exposure to PMA/ionomycin they increase IL-4 secretion and secrete IFN- γ *de novo*. Gray line: isotype control.

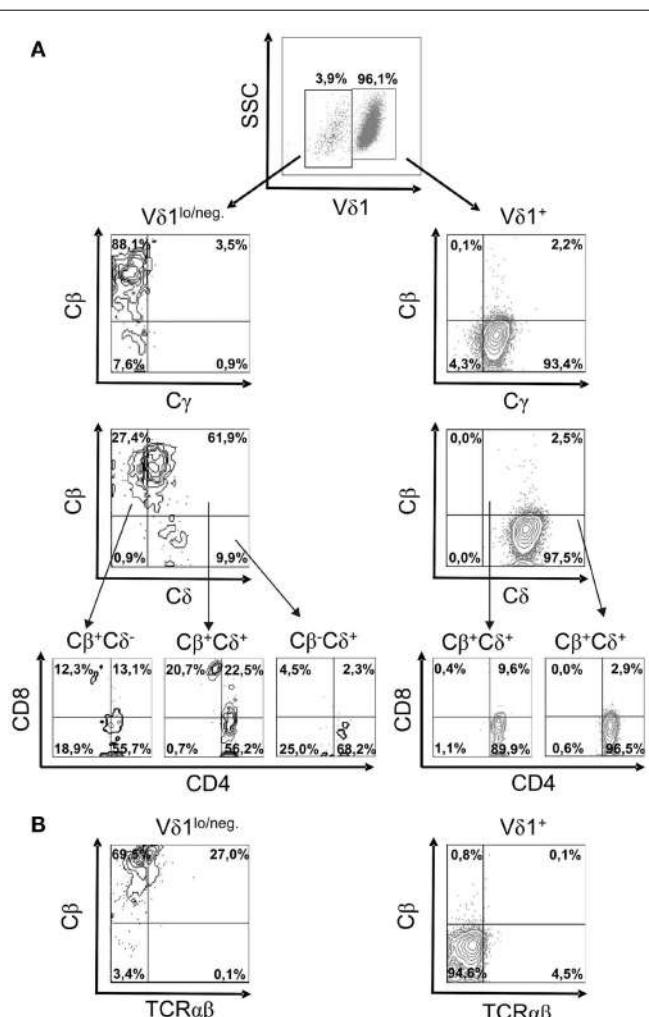


FIGURE 6 | Characterization of the subsets of a CD4 $^{+}$ V δ 1 $^{+}$ clone cells that changes TCR- $\gamma\delta$ into TCR- $\alpha\beta$. (A) V δ 1 $^{+}$ and V δ 1 $^{\text{lo/neg}}$ subpopulation of CD4 $^{+}$ V δ 1 $^{+}$ T-cell clones examined for the expression of C γ , C δ , C β , and C α (as a TCR- $\alpha\beta$ framework epitope) as well as co-receptors. A clone representative of seven analyzed clones is shown. (B) TCR- $\alpha\beta$ $^{+}$ cells in V δ 1 $^{+}$ and V δ 1 $^{\text{lo/neg}}$. C β^{+} -expressing V δ 1 $^{+}$ CD4 $^{+}$ T-cell clone fractions. A clone representative of seven analyzed clones is shown.

emergence of full-length transcripts of in-frame TRAV-26-2 and TRAV24 rearrangements in cultures of all donors demonstrated that the TCR- α locus had been accessible and was recombined ($n = 7$) (Figure 8B). TdT was also induced, evidenced through long stretches of N nucleotides in the TRAV24 sequences (Table 1).

V α rearrangements, both in panV δ 1 $^{+}$ and CD4 $^{+}$ V δ 1 $^{+}$ clone cultures, exactly followed the hierarchical order of the thymic rearrangement process that reconciles the sequential opening of the 3' end of the V region and the 5' end of the J region (52).

IDENTIFICATION OF THE V δ 1 $^{+}$ CD34 $^{\text{dim}}$ PRECURSOR *IN VIVO* AND ITS TRANSDIFFERENTIATION INTERMEDIATES IN PHYSIOLOGICAL AND INFLAMED TISSUE

In order to study the significance of the V δ 1 $^{+}$ CD34 $^{\text{dim}}$ precursor and its transdifferentiation *in vivo*, its frequency in the bone marrow was determined. Of all lymphocytes in the bone marrow, 0.039% were V δ 1 $^{+}$ CD34 $^{\text{dim}}$ precursors ($n = 8$, not shown), which correlated with the number of V δ 1 $^{+}$ CD34 $^{\text{dim}}$ T cells per V δ 1 $^{+}$ subset in the peripheral blood of diseased individuals, but was significantly different from their numbers in healthy PBMNCs (Figure 10A). However, the overall number of V δ 1 $^{+}$ T cells was significantly higher in the peripheral blood of diseased subjects [4.6-fold; range: healthy 0.3–2.0 ($n = 6$), diseased 0.6–8.6 ($n = 7$)] (Figure 10B), which influences the absolute number of CD34 $^{\text{dim}}$ cells in the periphery (Figure 10A). Our diseased cohort included individuals that suffered from viral infections ($n = 4$), lupus erythematosus ($n = 1$), vitiligo ($n = 1$), and a viral infection associated with chronic fatigue syndrome ($n = 1$). The V δ 1 $^{+}$ CD34 $^{\text{dim}}$ precursors in the peripheral blood were mostly DN (52.08%) and CD8 (42.66%) (not shown), which is consistent with the finding that CD4 $^{+}$ is upregulated by inflammatory stimuli (Figure 9A).

TCR- γ CHAIN EXPRESSION IN V δ 1 $^{+}$ T CELLS *IN VIVO*

As TCR change occurred in TCR- $\gamma^{\text{low/neg}}$ -expressing cells (C γ $^{\text{lo/neg}}$), we determined TCR- γ expression *in vivo* (freshly drawn peripheral blood) and in native healthy bone marrow. Unexpectedly, TCR- γ chain (C γ) expression was significantly higher in V δ 1 $^{+}$ than in V δ 2 $^{+}$ cells in the blood of healthy donors and in bone marrow. In contrast, there was a significantly higher expression of C γ in the V δ 2 $^{+}$ than in the V δ 1 $^{+}$ cells in the blood of diseased volunteers and in bone marrow. When comparing absolute C γ expression, the V δ 2 $^{+}$ subset showed a significant upregulation of TCR- γ chain expression during inflammation, whereas the V δ 1 $^{+}$ subset expressed the γ -chain constitutively in bone marrow and PBMNCs irrespective of inflammatory stimuli. Blood samples were taken from individuals suffering from uncharacterized viral infection ($n = 3$), pneumonia ($n = 1$), cystitis ($n = 2$), nephritis ($n = 1$); (C γ MFI mean: V δ 1 $^{\text{bone marrow}}$ 2360 ± 188 SEM versus V δ 2 $^{\text{bone marrow}}$ 1298 ± 99 SEM; V δ 1 $^{\text{healthy}}$ 2533 ± 129 SEM versus V δ 2 $^{\text{healthy}}$ 1711 ± 218 SEM; V δ 1 $^{\text{diseased}}$ 2606 ± 213 SEM versus V δ 2 $^{\text{diseased}}$ 3986 ± 256 SEM) (Figure 10C).

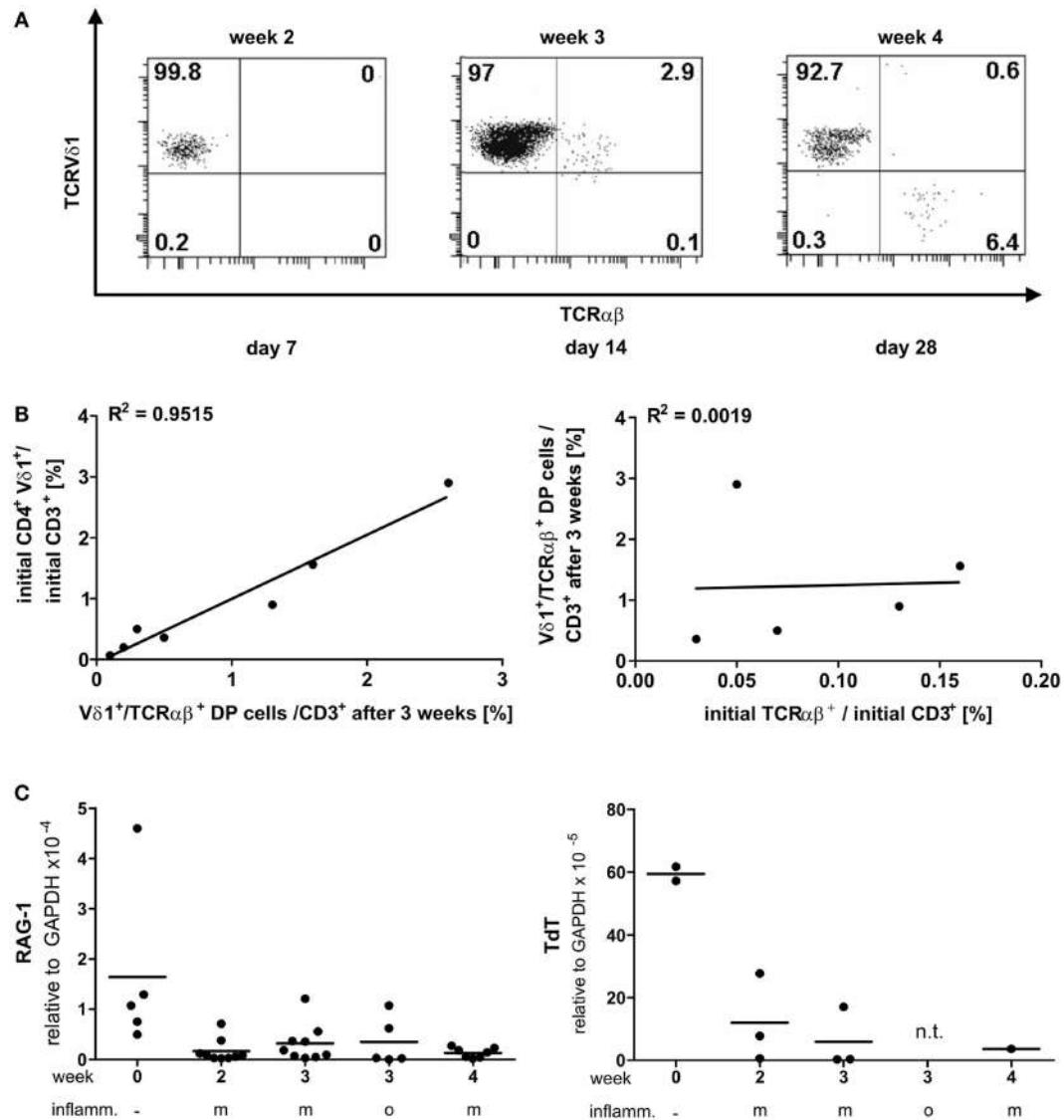


FIGURE 7 | V δ 1 $^{+}$ T cells differentiate into $\alpha\beta$ T cells. RAG and TdT expression in panV δ 1 $^{+}$ cultures, native and over time in different inflammatory culture conditions; correlation of TCR DP cells with CD4 $^{+}$ V δ 1 $^{+}$ and residual $\alpha\beta$ T-cell numbers in initial panV δ 1 $^{+}$ T-cell pools. **(A)** panV δ 1 $^{+}$ T cells cultivated in the presence of IL-7, PHA, IL-2, and irradiated allogeneic feeder cells gave rise to a cell fraction that co-expressed V δ 1 and TCR- $\alpha\beta$ after 3 weeks, before differentiating into TCR- $\alpha\beta$ T cells. Series of dot plots are representative of 12 independent experiments. **(B)** Number of V δ 1 $^{+}$ /TCR- $\alpha\beta$ $^{+}$ cells varied between individuals (mean: 0.926%/V δ 1 $^{+}$; range 0.1–3%) (right) as did the number of produced $\alpha\beta$ T cells (mean: 1.82%/V δ 1 $^{+}$; range 0.2–6.0%) (left); V δ 1 $^{+}$ /TCR- $\alpha\beta$ $^{+}$ double-positive cells in week 3 of culture corresponded with the number of

CD4 $^{+}$ V δ 1 $^{+}$ T cells in initial V δ 1 $^{+}$ T-cell pool (left). There was no correlation between residual TCR- $\alpha\beta$ $^{+}$ T cells after V δ 1 $^{+}$ cell separation and set-up of initial V δ 1 $^{+}$ T-cell culture and the number of V δ 1 $^{+}$ /TCR- $\alpha\beta$ $^{+}$ double-positive T cells after 3 weeks of culture (right). Each dot represents one independent experiment. **(C)** After initial depression, RAG-1 and TdT quantities subtly increased in V δ 1 $^{+}$ cell cultures and were detectable simultaneously with the appearance of V δ 1 $^{+}$ /TCR- $\alpha\beta$ $^{+}$ coexpressing cells. m, Mild inflammation (IL-7, PHA, IL-2, and irradiated allogeneic feeder cells); o, overt inflammation (exposure to strong inflammatory stimuli in week 3, conditions described in the Section “Materials and Methods”); week 0, freshly isolated, native V δ 1 $^{+}$ cells were analyzed.

TCR- β CHAIN EXPRESSION IN V δ 1 $^{+}$ T CELLS *IN VIVO*

TCR- β chain expression was determined for the V δ 1 $^{+}$ subset and the $\alpha\beta$ T-cell compartment in the peripheral blood of healthy and diseased individuals (Figure 11A). The percentage of V β $^{+}$ -expressing V δ 1 $^{+}$ cells differed highly significantly between healthy ($n=6$; $20.0\% \pm 5.98$ SEM) and diseased ($n=5$; $77.8\% \pm 13.34$ SEM) blood (Figure 11B).

V δ 1 $^{+}$ C β $^{+}$ TCR INTERMEDIATES IN PERIPHERAL BLOOD AND AT SITES OF INFLAMMATION

In order to assess whether the transdifferentiation of CD4 $^{+}$ V δ 1 $^{+}$ $\gamma\delta$ T cells is of physiological importance, we investigated body fluids of inflamed tissues for the presence of cellular intermediates of the transdifferentiation route. V δ 1 $^{+}$ C β $^{+}$ intermediates were present in significantly larger numbers in body fluids

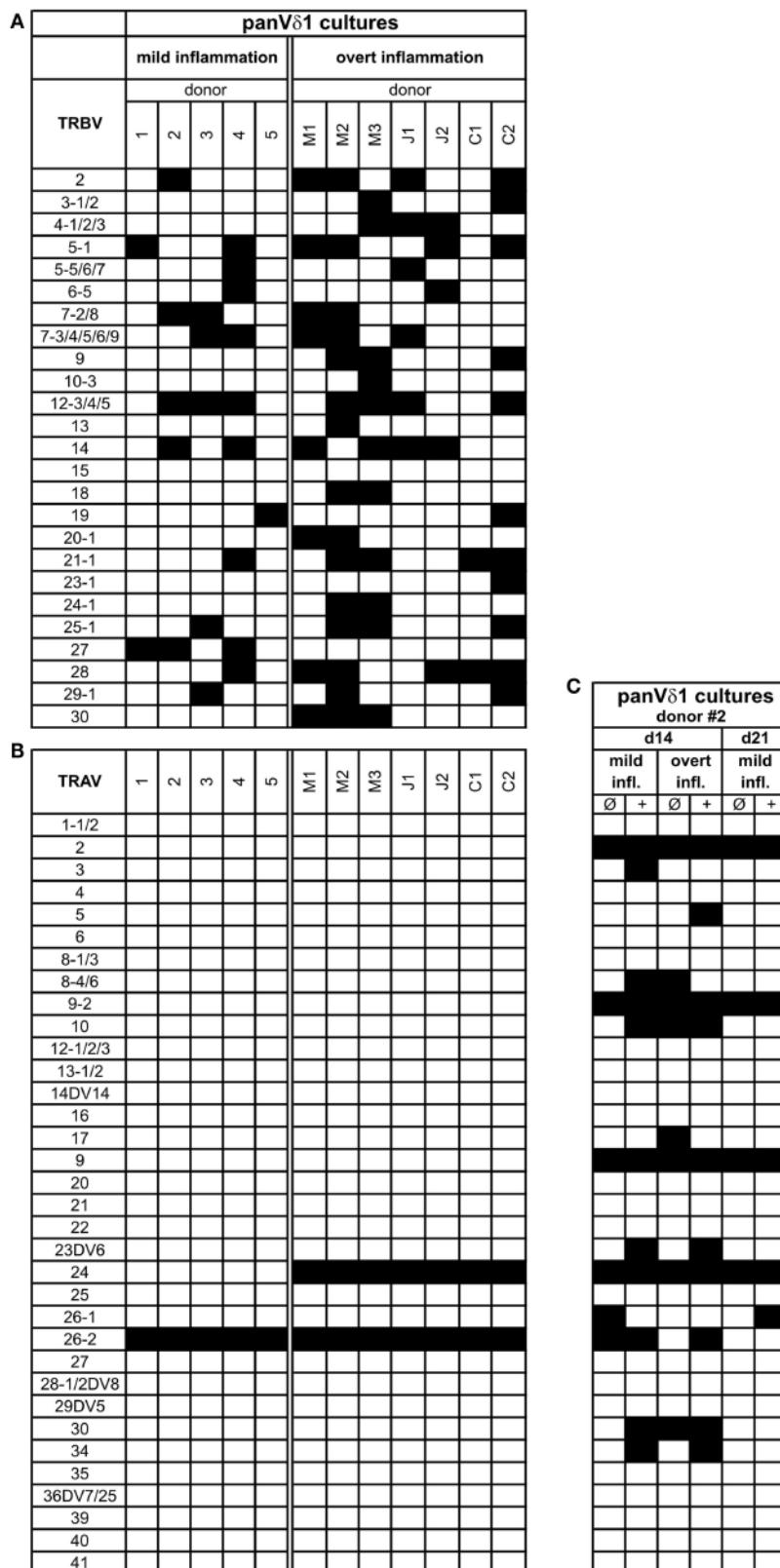
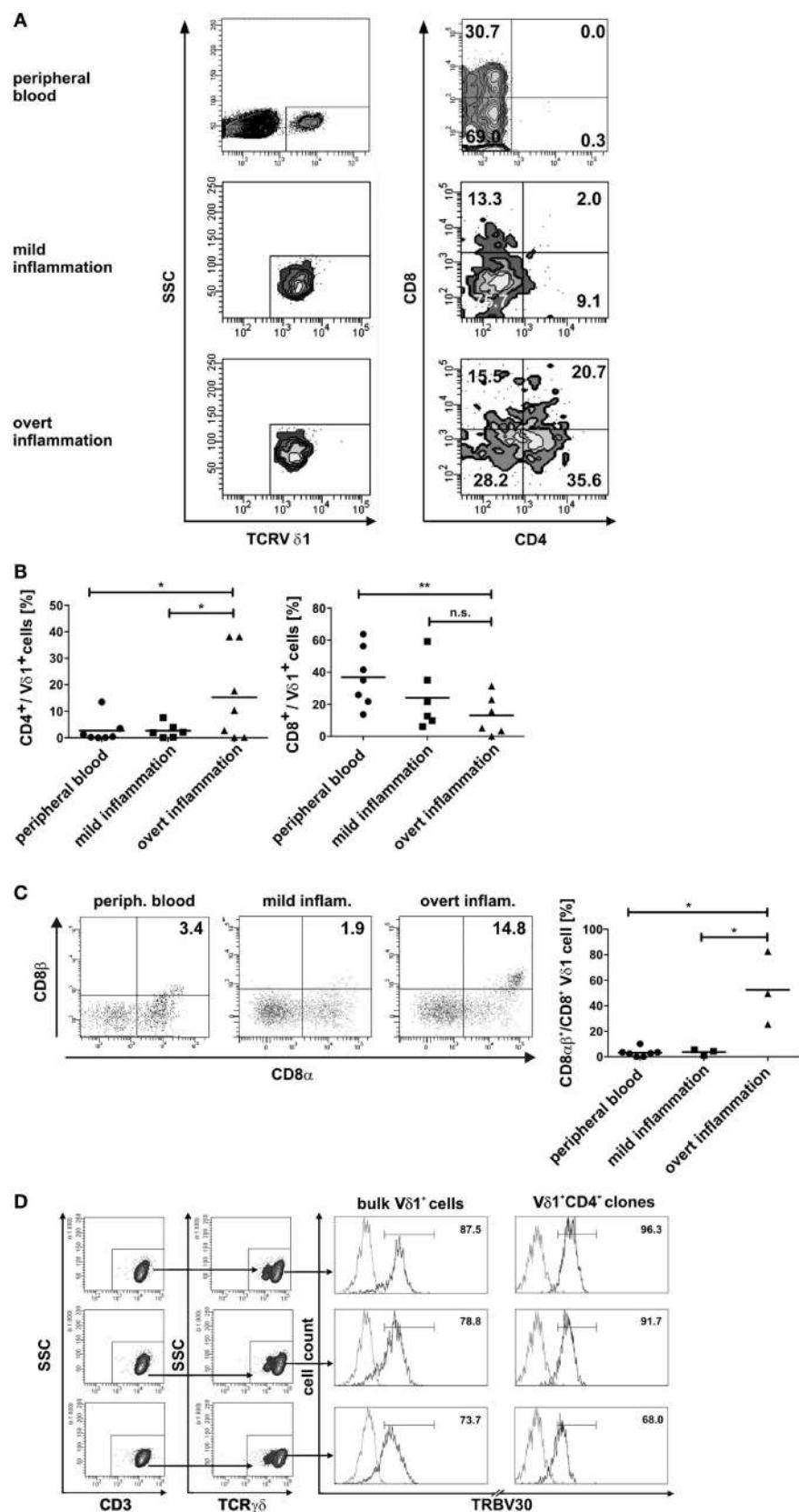


FIGURE 8 | Repertoire of fully rearranged and transcribed TRBV and TRAV segments in panV δ 1 $^{+}$ T cell pools that were cultivated in different inflammatory environments. **(A)** TRBV-segment usage and **(B)** TRAV-segment usage after 3 weeks of culture **(C)** Comparison of

V α -segment usage of panV δ 1 $^+$ T cells from one donor (#2) in different modified inflammatory environments. +, Treatment combined with anti-CD40; IL-15 (10 ng/mL) was added to all cultures. Black squares indicate expression of transcripts of rearranged TCR chains.

**FIGURE 9 |** Phenotypic changes of panV δ 1 $^{+}$ T cells in inflammatory conditions.

(Continued)

FIGURE 9 | Continued

(A) Inflammation-induced phenotype of DP CD4 $^+$ CD8 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells. **(B)** Ex vivo overt inflammatory conditions significantly increased the number of CD4 $^+$ T cells in panV δ 1 $^+$ cell cultures to an extent that was much higher than that seen in peripheral blood of healthy individuals ($p = 0.0493$) or in mild inflammation *in vitro* ($p = 0.0487$). In comparison to peripheral blood of healthy individuals, overt inflammation led to a significant reduction of the number of CD8 $^+$ V δ 1 $^+$ cells ($p = 0.0097$). **(C)** Overt inflammation induced the expression of the CD8 $\alpha\beta$ heterodimer in a significant proportion of CD8 $^+$ V δ 1 $^+$ cells ($p = 0.0452$) in 3/3 donors tested. **(D)** panV δ 1 $^+$ cells show TRBV30 chains

on the cell surface after 7 days of (both: mild and overt) inflammation (5/5 tested, shown are 3/5); TRBV30 was also observed on clones that were chronically exposed to mild inflammation (7/7 tested, 3/7 are shown). Gray line: isotype control. Histogram marker shows range of cells that stained positive for the antigen under investigation. Numbers indicate mean \pm SEM of V δ 1 $^+$ (CD4 $^+$) T cells that stained positive for the respective marker (given in %). The histograms show three representative experiments for panV δ 1 $^+$ T cells and CD4 $^+$ V δ 1 $^+$ clones stained with TRBV30. TRBV30 expression was determined in panV δ 1 $^+$ cultures with 4 different donors in 3 independent experiments each, and in 7 CD4 $^+$ V δ 1 $^+$ clones.

Table 1 | CDR3 region peptide composition of rearranged TRAV segments in panV δ 1 $^+$ T cells induced by mild and overt inflammation and CDR3 regions of rearranged TRBV and TRAV segments of $\alpha\beta$ T cells derived from CD4 $^+$ V δ 1 $^+$ clone K1-V δ 1 $^+$ (K1.1- $\alpha\beta$ $^+$) under mild inflammatory conditions.

| Donor | No. of exp. | TRAV | TRAJ | 3' of V-segment | CDR3 | J segment | Public motif type | Reference |
|---|-------------|------|------|-----------------|------------------|------------------|-------------------|-----------|
| MILD INFLAMMATION | | | | | | | | |
| 1 | 1 | 26-2 | 41 | Cl | LRGL | NSGYALNFKCKRT... | 2 | N/A |
| | 1 | 26-2 | 41 | Cl | LRGS | NSGYALNFKCKRT... | 2 | N/A |
| 2 | 1 | 26-2 | 41 | Cl | LRDW | NSGYALNFGKGT... | 3 | N/A |
| 3 | 1 | 26-2 | 41 | Cl | LRDW | NSGYALNFGKGT... | 3 | N/A |
| 4 | 1 | 26-2 | 41 | Cl | LRDL | NSGYALLFCKRT... | 3 | N/A |
| 5 | 1 | 26-2 | 41 | Cl | LRDL | NSGYALLFCKRT... | 3 | N/A |
| OVERT INFLAMMATION | | | | | | | | |
| 1 | 1 | 26-2 | 41 | Cl | LRGL | NSGYALNFGKGT... | 3 | N/A |
| 2 | 1 | 26-2 | 41 | Cl | LRDL | NSGYALNFGKGT... | 3 | N/A |
| 3 | 1 | 26-2 | 41 | Cl | LRDL | NSGYALNFGKGT... | 3 | N/A |
| 4 | 2 | 26-2 | 41 | Cl | LRDL | NSGYALNFGKGT... | 3 | N/A |
| 5 | 1 | 26-2 | 41 | Cl | LRDL | NSGYALNFGKGT... | 3 | N/A |
| 6 | 3 | 26-2 | 41 | Cl | LRGL | NSGYALNFGKGT... | 3 | N/A |
| 7 | 1 | 26-2 | 41 | Cl | LRDL | NSGYALNFGKGT... | 3 | N/A |
| 4 | 3 | 24 | 3 | LA | RRNDG | SASKIIFGSGT... | 3 | N/A |
| 5 | 2 | 24 | 3 | LA | RRNDG | SASKIIFGSGT... | 3 | N/A |
| 6 | 2 | 24 | 3 | LA | RRNDG | SASKIIFGSGT... | 3 | N/A |
| α CHAINS OF $\alpha\beta$T CELLS DERIVED FROM CLONE K1 UNDER MILD INFLAMMATORY CONDITIONS | | | | | | | | |
| K1.1- $\alpha\beta$ $^+$ | 3 | 5 | CA | VRD | DTGRRALTFGSGT | – | N/A | |
| | 14DV4 | 17 | CA | MREGRF | KAAGNKLTFGGGT... | 2 | (53) | |
| | 25 | 10 | CA | G | TGGGNKLTFGTG... | – | N/A | |
| | 26-2 | 52 | CQ | LSTARTPTP | SQDAVHSSISQGST | 1 | (54, 55) | |
| β CHAINS OF $\alpha\beta$T CELLS DERIVED FROM CLONE K1 UNDER MILD INFLAMMATORY CONDITIONS | | | | | | | | |
| K1.1- $\alpha\beta$ $^+$ | 11-2 | 1-1 | CAS | RRG | TEAFFGQGT... | 2 | (56), (57) | |
| | 19 | 2-7 | CASS | SNGQGV | YEQYFGPGT... | 1 | (58) | |
| | 27 | 2-3 | CASS | LGDRVA | DTQYFGPGT... | 2 | (59) | |

CDR3 regions of rearranged TRAV segments in panV δ 1 $^+$ cultures and of rearranged TRBV and TRAV segments in K1-derived $\alpha\beta$ T cells. (A) top: mild inflammation induced exclusively TRAV-26-2JC transcripts in panV δ 1 $^+$ T cells, transcripts of donors J, K, and P were sequenced; overt inflammation also induced TRAV24; TRAV-derived transcript sequence analysis is shown for all donors and all experiments. (B) CDR3 sequences of TRBV and TRAV segments, detected in spectratype analysis as single peaks, were determined from K1-derived $\alpha\beta$ T cells (K1-17): 3/18 single peaks of TRBV-segments, 4/4 single peaks of TRAV segments. Sequences were derived from cDNA transcripts after PCR-based spectratype analysis.

of inflamed tissue (synovia of arthritic joints, tumor-associated ascites, pleural effusion; mean expression $23\% \pm 5.38$ SEM; range 0.8–63.2%) than in the peripheral blood of healthy individuals (mean expression healthy 3.0%; ± 0.86 SEM; range 0.6–10.0%) (**Figure 11C**).

Given that the transdifferentiation intermediates express the δ 1- and a β -chain for a $\delta\beta^+$ heterodimer, which must occur

in the absence of the TCR- γ chain (60), and given that the α -chain is not yet rearranged and expressed, the expected resulting phenotype after FACS staining with the murine antibody that targets the $\gamma\delta$ TCR constant region (clone 11F2) and the $\alpha\beta$ TCR framework epitope (T10B9.1A-31) is CD3 $^+$, “TCRnegative,” CD4 $^+$ or CD8 $^+$. This phenotype was detected in all donors tested (**Figure 11D**).

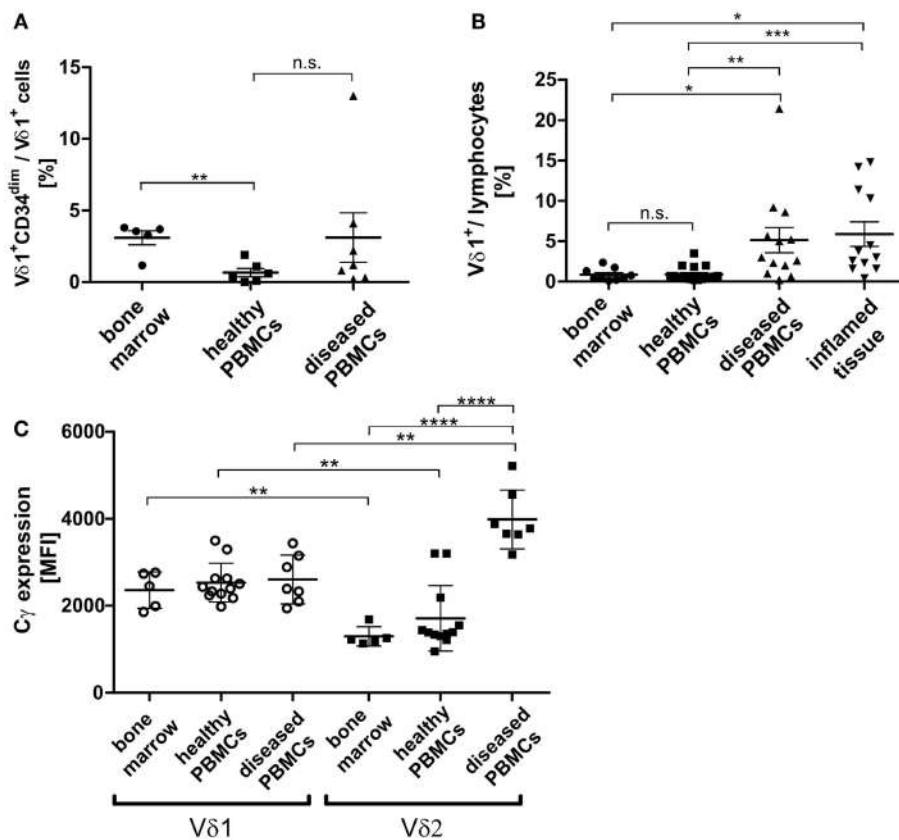


FIGURE 10 | V δ 1 $^+$ lymphocytes and V δ 1 $^+$ CD34 $^{\text{dim}}$ T cell population in blood and marrow. **(A)** Percentage of V δ 1 $^+$ CD34 $^{\text{dim}}$ T cells/V δ 1 $^+$ lymphocytes in peripheral blood of healthy ($n = 6$) and diseased donors ($n = 7$) and healthy bone marrow ($n = 5$). The percentage of V δ 1 $^+$ CD34 $^{\text{dim}}$ T cells/V δ 1 $^+$ lymphocytes was significantly higher in native bone marrow compared to peripheral blood from healthy donors ($p = 0.0017$). **(B)** The quantity of V δ 1 $^+$ lymphocytes was (highly) significantly increased in the peripheral blood of diseased ($n = 13$; mean: 5.13 ± 1.57 SEM; $p = 0.0096$) and inflamed tissue ($n = 12$; mean: 5.88 ± 1.52 SEM; $p = 0.0388$) compared to the quantity observed in the blood of healthy individuals ($n = 17$; mean:

0.89 ± 0.22 SEM). V δ 1 $^+$ lymphocytes were significantly more frequent in peripheral blood ($p = 0.0367$) and body fluids ($p = 0.0107$) of diseased individuals than in bone marrow ($n = 9$ mean: 0.85 ± 0.26 SEM).

(C) Absolute expression level of TCR- γ chain on V δ 1 $^+$ and V δ 2 $^+$ cells in healthy ($n = 12$) and diseased ($n = 7$) peripheral blood and bone marrow ($n = 5$). Blood samples included blood from patients with uncharacterized viral infection ($n = 3$), pneumonia ($n = 1$), cystitis ($n = 2$), nephritis ($n = 1$) (C γ MFI mean: V δ 1^{bone marrow} 2360 ± 188 SEM versus V δ 2^{bone marrow} 1298 ± 99 SEM; V δ 1^{healthy} 2533 ± 129 SEM versus V δ 2^{healthy} 1711 ± 218 SEM; V δ 1^{diseased} 2606 ± 213 SEM versus V δ 2^{diseased} 3986 ± 256 SEM).

Thus, our data provide evidence for the existence of a pathway for extrathymic $\alpha\beta$ T-cell development from the CD4 $^+$ V δ 1 $^+$ T-cell precursor as shown in Figure 12.

DISCUSSION

In this study, we identified the human V δ 1 $^+$ $\gamma\delta$ T-cell subset as a reservoir of a CD4 $^+$ cell entity that shows the cumulative expression of markers and molecules that are pivotal for T-cell progenitor phenotype and function namely CD34 $^{\text{dim}}$, FLT3 $^+$, c-kitlo, CD105 $^+$, and CXCR4 $^+$. Low level and simultaneous expression of cytokines characterizing multiple Th lineages conforms with the view that stem-cells possess a wide-open chromatin structure to maintain their multipotentiality, which is progressively lost during differentiation (61, 62). The combined expression of five different chemokine receptors that direct the movement of circulating T cells to sites of inflammation indicates a function of this progenitor in inflamed tissue. We provide phenotypic, transcriptional and functional evidence that initial CD34 $^{\text{dim}}$ CD4 $^+$

V δ 1 $^+$ $\gamma\delta$ T-cell clones can develop into functional $\alpha\beta$ T cells in *ex vivo* cultures. By monitoring surface expression of the constant region of the 4 different TCR chain loci γ , δ , β and α on CD34 $^{\text{dim}}$ CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cell clones, we identified the CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells' transdifferentiation into $\alpha\beta$ T cells as a sequential invariant process that is triggered by (mild) inflammatory stimuli (schematically shown in Figure 12). The re-organization of V δ 1 $^+$ TCR- $\gamma\delta$ into TCR- $\alpha\beta$ was associated with morphological and physiological changes of the CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells reproducing thymic T-cell development: cultured peripheral CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells increased in size, acquired an LGL-like morphology, downregulated TCR- γ and – to a lesser extent – TCR- δ ; they proliferated vigorously, expressed functionally rearranged TCR- β chains on their surface, thus forming the V δ 1 $^+$ C β $^+$ cellular intermediates that traversed into the CD4 $^+$ CD8 $^+$ DP stage. An increase in RAG and TdT activity preceded the induction of the rearrangement of the TCR α loci in exact thymic order before the V δ 1 $^+$ C β $^+$ intermediates shut down V δ 1 expression, expressed an

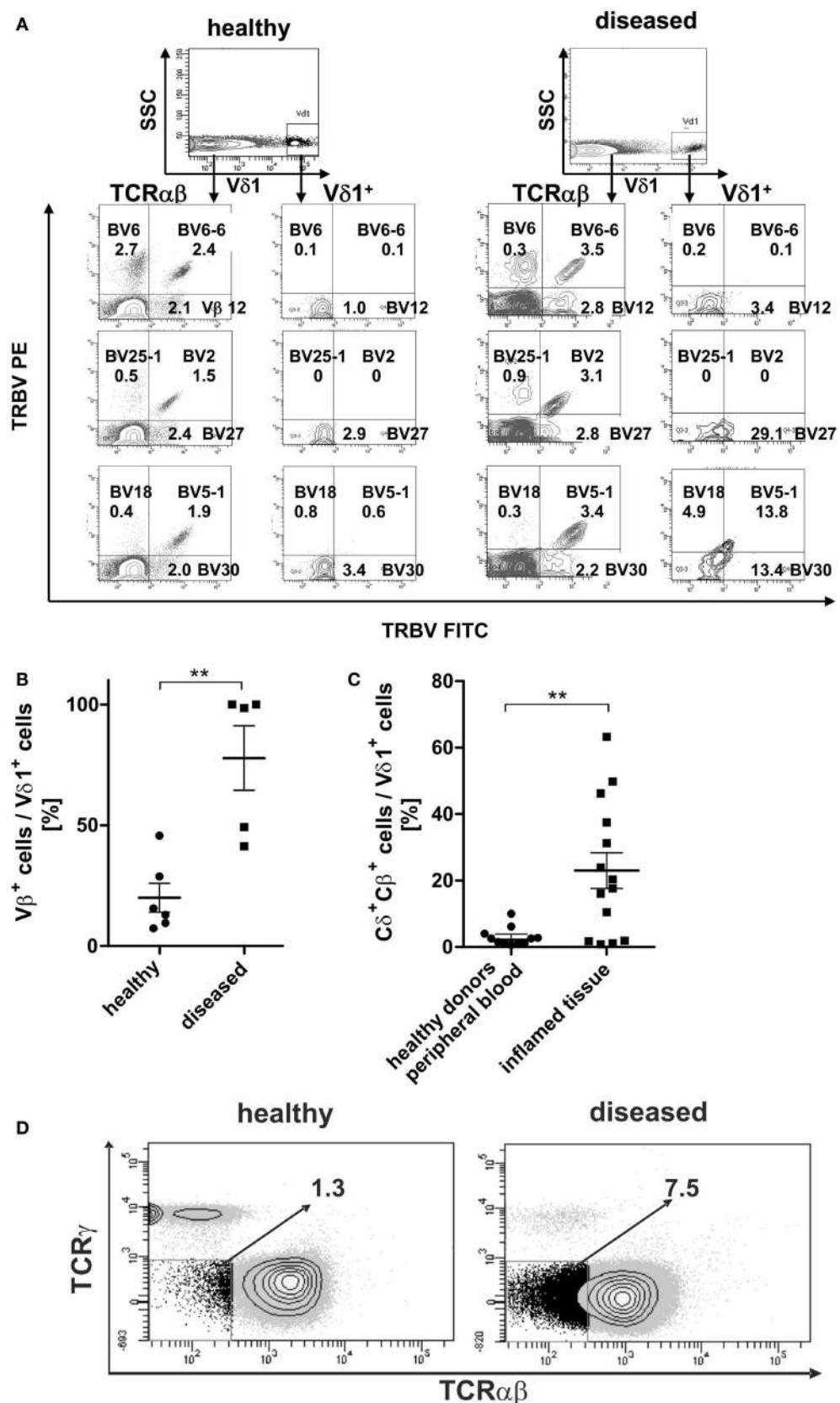


FIGURE 11 | Surface V β expression of $\alpha\beta$ T cells in comparison with peripheral V $\delta 1^+$ T cells in healthy and diseased individuals.

(Continued)

FIGURE 11 | Continued

(A) Representative V β family staining pattern (exemplarily shown are nine different V β families) on $\alpha\beta$ T cells and V δ 1 $^+$ T cells in peripheral blood MNCs shown for one healthy individual and for one individual suffering from viral infection. MNCs were stained with the TCR V β Repertoire Kit (Beckman Coulter); three different V β family expressions are assessed in the same tube by combining three monoclonal antibodies (mAb) with only two fluorophores

(see Materials and Methods for detailed information). **(B)** Percentage of V β $^+$ expressing cells in the V δ 1 subset in healthy ($n=6$) and diseased individuals ($n=5$). **(C)** Percentage of V δ 1 cells with a C α $^+$ C β $^+$ phenotype in healthy ($n=11$) tissue differs significantly from that of body fluids of inflamed tissue ($n=14$) ($p=0.0343$). **(D)** CD3 $^+$ cells that are C γ neg and TCR- $\alpha\beta$ framework-epitope neg (DN for TCR) in healthy ($n=11$) and diseased individuals ($n=4$). One representative blot is shown.

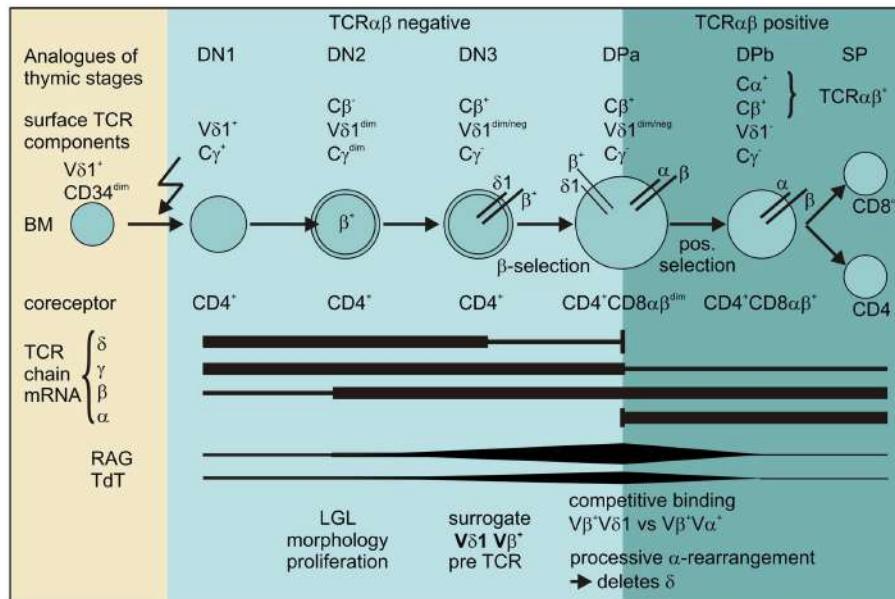


FIGURE 12 | Scheme for the transdifferentiation of V δ 1 $^+$ CD34 dim precursors into $\alpha\beta$ T cells inflammatory stimuli induce V δ 1 $^+$ CD34 dim precursors to move from the bone marrow into the periphery and express CD4 $^+$. Chemokine receptors guide V δ 1 $^+$ CD4 $^+$ precursors to the sites of inflammation where inflammation-associated stimuli induce their development into $\alpha\beta$ T cells: V δ 1 $^+$ CD4 $^+$ cells sequentially rearrange, transcribe and express V β chain intracellularly, while downmodulating TCR γ and to a lesser extent V δ 1 expression. The V β chains then appear

on the surface of C γ $^{lo/neg}$ cells and $\delta^+\beta^+$ precursors transit into the CD4 $^+$ CD8 $^+$ DP stage where processive rearrangement in the α locus – accompanied by the expression of RAG and TdT – deletes the δ -chain, leading to the successive loss of V δ 1 on V δ 1 $^{dim/neg}$ V β $^+$ cells. V β $^+$ chains preferentially pair with newly formed α chains in V δ 1 $^{dim/neg}$ V β $^+$ cells that become weakly positive for TCR- $\alpha\beta$ before they completely lose V δ 1 expression, upregulate TCR- $\alpha\beta$ and become mature and functional SP CD4 $^+$ or CD8 $^+$ $\alpha\beta$ T cells.

$\alpha\beta$ TCR and developed into functional SP CD4 $^+$ or CD8 $^+$ $\alpha\beta$ T cells.

In order to exclude these observations as artifacts of *ex vivo* culture systems, we compared our findings with established literature data, and in order to identify its physiological relevance, we aimed to show analogs of this new developmental pathway *in vivo*. Several groups of researchers have shown that around 10–20% of all peripheral $\gamma\delta$ T cells transcribe and express in-frame TCR- β rearrangements (63–65) that can guide $\alpha\beta$ T-cell development, resulting in fully functional, mature T cells (66). Consistent with these findings, CD4 $^+$ V δ 1 $^+$ clones transcribed in-frame TCR- β chain rearrangements, showed cytoplasmic protein expression of TCR- β chains, and expressed the TCR- β chain on their cell surface as they bound BMA031. This is in accordance with the findings of Miossec et al. (67) who showed that up to 45% of peripheral V δ 1 $^+$ cells bind C β region-specific monoclonal antibodies (mAb) BMA031. Using a set of mAbs that recognize the variable domain of 24 different human V β chains, we refined our analysis in order

to demonstrate that peripheral V δ 1 $^+$ T cells of healthy individuals expressed the full spectrum of V β chains. While Miossec and other researchers showed that the V δ 1 $^+$ variable region can substitute V α in functional T-cell receptor α -chains and thus serve as an agent for V β surface expression in the peripheral $\alpha\beta$ T cell subset (67–71), in the current study we identified a subset in every CD4 $^+$ V δ 1 $^+$ clone analyzed, which lacked surface C α – because the clone culture did not bind to T10B9.1A-31 that recognizes the TCR- $\alpha\beta$ framework epitope and did not transcribe a V δ 1C α rearrangement. Nevertheless, the subset still showed surface V β expression while at the same time being negative for TCR- γ . A T cell that lacks TCR- α , which is the preferred binding partner of TCR- β , can express TCR- β on its cell surface either in dimerized form with pre-T α (72–74) or as a homodimer, as had been suggested for thymic T-cell development before pre-TCR was identified (75). Given that V β $^+$ -expressing CD4 $^+$ V δ 1 $^+$ T cells are $\alpha\beta$ T-cell progenitors, they need to ensure a controlled developmental transition beyond the DN3 stage to the DP stage, which is limited to cells that have functionally rearranged

TCR- β chain genes that can pair with TCR- α . The complete lack of pre-T α mRNA in all clones and panV δ 1 $^+$ cultures investigated, along with the fact that TCR- δ has the same constant domain size and the same spacing of the basic residues in the transmembrane region as the TCR α chain (76), thus enabling TCR- δ to physically pair with TCR- β (60) in the absence of TCR- γ (60), suggests an intriguing scenario that does not involve a pre-TCR generation, which requires pre-T α , i.e., the pairing of TCR- β with TCR- δ for V δ 1 $^+$ V β $^+$ surrogate pre-TCR.

The idea of V δ 1 $^+$ V β $^+$ pairing conforms with the findings of Hochstenbach et al. who described V δ 1 $^+$ V β $^+$ heterodimers on the CD4 $^+$ T-cell fraction derived from a human V δ 1 $^+$ C γ neg Burkitt lymphoma (DND-41), established from pleural effusion (60). Thus, the formation of V δ 1 $^+$ V β $^+$ heterodimers on V δ 1 dim cells for surrogate pre-TCR seems completely feasible and would also make sense in terms of the quantity of pre-TCR surface expression, because the pre-TCR is expressed 50 to 100-fold lower than the TCR- $\alpha\beta$ on mature T cells, as is the pre-B cell receptor (BCR) on the surface of pre-B cells (77). Consistently, V δ 1 dim cells show low V δ 1 $^+$ and V β $^+$ expression. Accordingly, the V δ 1 $^+$ V β $^+$ heterodimer for pre-TCR would add to the list of other surrogate pre-TCRs that have been described to promote progression of DN thymocytes to the DP stage in various model systems, including $\gamma\delta$ (78–83), $\alpha\beta$ (84–87), $\alpha\gamma$ (88), and pT α/γ (89) heterodimers.

As V δ 1 $^+$ V β $^+$ pairing only occurs in the absence of the γ -chain, TCR change requires downregulation of γ -chain protein expression. Thymocytes progressively downregulate TCR- γ expression from the DN3 to the DP stage to the relatively low level found in mature peripheral $\alpha\beta$ T cells (4). CD4 $^+$ V δ 1 $^+$ clones, correspondingly to the markers that they expressed – which are consistent with DN3 thymocytes – showed a cell fraction that expressed V δ 1 $^+$ at low level, and were low/neg. for TCR- γ (C γ $^{lo/neg.}$). The significantly higher *in vivo* TCR- γ expression on V δ 1 $^+$ T cells compared to V δ 2 $^+$ T cells in bone marrow and peripheral blood was unexpected (**Figure 10C**). This observation was put into perspective during inflammation when TCR- γ expression was significantly increased in the V δ 2 $^+$ compared to the V δ 1 $^+$ T-cell subset in the peripheral blood of diseased individuals, while TCR- γ expression remained unchanged in V δ 1 $^+$ cells (**Figure 10C**).

When CD4 $^+$ V δ 1 $^+$ clone cultures were compared with $\gamma\delta$ $^+$ subsets *in vivo*, the TCR- γ expression levels of the clones (chronically exposed to inflammation) were identical to those observed in the V δ 2 $^+$ but not to those observed in the V δ 1 $^+$ subset in the blood of diseased individuals. In contrast, the CD4 $^+$ V δ 1 $^+$ clone fraction that underwent TCR change was identical to the peripheral V δ 1 $^+$ subset. This indicates that, in contrast to all other V δ 1 $^+$ cells, CD4 $^+$ V δ 1 $^+$ T cells have the capacity to modulate TCR expression, possibly due to the disulfide bond that links the constant region of C δ to the C γ 1 segment of their TCR, a similarity they share with the V δ 2 TCR. In addition, they can downregulate TCR- γ chain expression during the process of transdifferentiation (**Figure 6A**).

Presuming that the V δ 1 $^+$ V β $^+$ heterodimer is a surrogate pre-TCR in V δ 1 lo C γ $^{neg.}$ T cells, signals that trigger V β chain selection would require CXCR4 (90) and GATA-3 (91). Accordingly, CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T-cell clones express high amounts of GATA-3, which

allows them to control the translation of TCR- β mRNA into protein (92, 93), to increase in cell size, which is a feature that accompanies pre-TCR expression in DN3 (92, 93), and to traverse the conditional developmental arrest of the β -selection checkpoint into DN4 stage (92, 93). Moreover, GATA-3 positively regulates the transcription enhancer E α , which is crucial for the initiation of rearrangement and expression of TCR- α (94). In this context, it was not surprising that the regulatory regions of both the TRAV-26-2 segment and the TRBV30 segment share a GATA-3-binding cis element (95). Additionally, CD4 $^+$ V δ 1 $^+$ T-cell clones simultaneously express the hallmark molecules RAG and TdT that guide thymocytes from DN3 through to the DP stage.

These results substantiate the assumption that distinctive C β -expressing CD4 $^+$ V δ 1 $^{lo/neg.}$ C γ $^{neg.}$ T cells, CD4 $^+$ CD8 $^+$ DP V δ 1 $^{lo/neg.}$ C γ $^{neg.}$ T cells and TCR- $\alpha\beta$ $^+$ SP CD4 $^+$ or SP CD8 $^+$ V δ 1 $^{lo/neg.}$ C γ $^{neg.}$ T cells that are present in CD4 $^+$ V δ 1 $^+$ clone cultures are cellular intermediates resulting from the successful traversal of β -selection. Similar to the situation with immature CD4 $^+$ CD8 $^+$ -expressing “thymocytes” that express the antigen receptors and undergo positive and negative selection, which is the core process of $\alpha\beta$ T-cell development.

Our finding that newly generated CD4 $^+$ V δ 1 $^+$ clone-derived $\alpha\beta$ T cells underwent cell death when exposed to high-affinity ligands such as antibodies that specifically targeted surface V β chain, CD3 (soluble OKT-3), or CD3/CD28, supports the assumption that selection accompanies transdifferentiation (not shown).

Moreover – in analogy to the process of positive selection in the thymus – RAG expression was repressed in panV δ 1 $^+$ cell cultures after the emergence of $\alpha\beta$ T cells (**Figure 7C**). We then verified our findings with published molecular data. The finding that TRBV30 was expressed in clones and in panV δ 1 cultures as initial TCR- β chain, points to the outstanding role of TRBV30 segment regulation and function (59). The TRBV30 segment is unique as it is the only β segment located outside the main cluster, but downstream of the J and C segments and forming the 3' end of the locus. The TRBV30 segment has the opposite transcriptional orientation to the other segments, and must therefore be rearranged by inversion of the D β , J β , and C β gene segments. This is in contrast to the rearrangement of all other V β segments – and is not deletional (59). Thus, rearrangements involving TRBV30 open up the β locus, and enable the subsequent rearrangement of other segments.

Moreover, TRBV30 segment chromatin access is biallelic (previously shown for mV β 14, the ortholog of TRBV30 in mice), and recombinational accessibility is not downregulated by TCR β chains (96). TRBV30 transcripts were replaced by transcripts of other β rearrangements in clones and panV δ 1 cultures at later time points, corresponding to the occurrence of β chains on the cell surfaces in FACS analysis. This indicates subsequent secondary rearrangements that possibly involve mechanisms earlier described for allelic inclusion and TCR revision in CD4 and CD8 $\alpha\beta$ T cells (97–100). The peculiarities in the regulation of the TRBV30 segment – independence of the elements that control access and rearrangement in the main cluster (59), an increased accessibility in thymocytes that transit from DN to DP stage in contrast to all other V β segments (101), and the lack of allelic

exclusion and feedback inhibition (96) – has led to the assumption that the TRBV30/mV β 14 segment (59) has an entirely distinct function *in vivo*. Our data strongly support this argument.

Moreover, the *de novo* α rearrangement in V δ 1 $^+$ precursor cells followed the exact hierarchical order of thymocytes that begin α -locus recombination with the 3' end V α and 5' end J α segments, from where the process proceeds to the distal ends of the locus (52). PanV δ 1 $^+$ cultures rearranged the TRAV segments TRAV-26-2, 26-1, and -24, which corresponds exactly to encyclopedic knowledge about the initiation of TCR- α locus rearrangement (52). Moreover, as these segments are interspaced in the δ locus, and removed when a functional TCR- δ 1 chain is generated, *de novo* TCR- α rearrangements must occur on the chromosome where the α locus is still in germline configuration, rendering synchronous expression of TCR- α and TCR- δ possible. This does not exclude the possibility of TCR- δ genes replacing conventional TCR- α genes during the rearrangement of the α -locus, and forming hybrid V δ 1J α C α chains (67–71). V δ J α C α hybrid chain transcripts were detected in few panV δ 1 $^+$ T-cell and some CD4 $^+$ V δ 1 $^+$ clone cultures (not shown). Moreover, our data are also consistent with the view that the TCR- α locus underlies tight regulation and that the TCR repertoire is not a vast and chaotic morass, but rather a patterned and perhaps even predictable system (102). Coherently, we found the same α -chain segments rearranged in response to identical epigenetic stimuli in multiple donors, and overlapping sets of α -segments rearranged in response to slightly modulated triggers.

CD4 $^+$ V δ 1 $^+$ T cells also reveal an analogy with thymic TCR- α gene recombination, which lacks allelic exclusion but continues until a functional α chain can form an MHC-restricted TCR with the β chain (103) so that multiple V/J recombination events are able to occur on the same allele (11, 104). Likewise, the processivity that correlates with the lifespan of the precursor cell (105) was reproduced, CD4 $^+$ V δ 1 $^+$ T cells with superior viability (more than 1 year in uninterrupted culture) exhausted the α -locus. The clone-derived $\alpha\beta$ T-cell line K1.1 changed TCR months after the establishment and showed rearranged TRAV-26, -25, -14 and -3, $\alpha\beta$ T-cell line P7 rearranged TRAV-26-2, -26-1, -24, -19, -14, -10, and -2 segment.

CHARACTERIZATION OF THE $\alpha\beta$ T CELLS GENERATED

To test functionality, the CD4 $^+$ V δ 1 $^+$ -clone-derived TCR $\alpha\beta^+$ T-cell lines K1.1 and P7 that lacked CD28 were examined in greater detail. These T-cell lines revealed an impaired proliferative response to mitogenic stimuli and carried public TCRs, as reported previously for viral and autoimmune diseases (Table 1) (53–59). This is consistent with the CD28 $^-$ phenotypes' high frequencies of functional virus-specific memory CTL that mediate TCR-mediated lysis (55). Constitutive IL-4 secretion and significant production of IFN- γ upon TCR stimulation showed that K1.1 $\alpha\beta^+$ and P7 $\alpha\beta^+$ were functional (Figure 5D).

Other CD4 $^+$ V δ 1 $^+$ -clone-derived $\alpha\beta$ T cells had an effector-memory phenotype, some expressed CD28 while others did not, and a broad V β and V α repertoire (not shown). They had no TRM phenotype, were negative for CD103 and were also CCR7 lo or negative and expressed varying amounts of CD62L and CD28 (not shown). Thus, V δ 1 $^+$ -derived T cells had diverse but distinct

cell-surface phenotypes, a complex T-cell receptor repertoire, and produced diverse cytokines. They could thus be classified as “functional” Th type cells. The TCRs sequenced were identical to viral antigen-specific TCRs (Table 1). The findings suggest that $\alpha\beta$ T cells that arise extrathymically from V δ 1 $^+$ CD34 dim CD4 $^+$ progenitors have a memory phenotype that enables them to respond rapidly to environmental challenges.

PHYSIOLOGICAL RELEVANCE

To date the molecular determinants for thymic homing are missing, and it has been shown that neither the three-dimensional thymic microenvironment nor thymic epithelial cells are essential for T-cell development (106). Answers to the questions as to why the thymus provides a unique environment for T-cell differentiation and whether the differentiation of the broad range of TSP cells proceeds via a single canonical or via rather multiple pathways remain equally elusive (107, 108). Despite these uncertainties, the CD4 $^+$ V δ 1 $^+$ T-cell precursors show high consistency with the thymocytes' phenotypic and functional behavior and the transition through strictly defined stages on an invariable differentiation route. The underlying genetic and physiological processes are largely identical on the molecular level, and it is evident that transdifferentiation is an efficiently controlled and thus a significant developmental pathway. Moreover, CD4 $^+$ V δ 1 $^+$ T-cell precursors – which express markers of TSP, DN1-DN3, and ISP progenitors – were as effective in generating mature $\alpha\beta$ T cells as DP thymocytes are. DP cells, constituting more than 90% of thymocytes, are selected for an MHC-restricted receptor, which is thought to occur relatively infrequently (109, 110) and results in the differentiation of only 1–2% DP precursors into mature T cells. The number corresponds exactly with what we found, namely, which 1 in 50 CD4 $^+$ V δ 1 $^+$ clones changed the TCR, and panV δ 1 $^+$ cultures reproducibly generated 1.82% SP CD4 $^+$ and/or SP CD8 $^+$ $\alpha\beta$ T cells/panV δ 1 $^+$ cell pool. Importantly, while homeostatic expansion reduces the complexity of the $\alpha\beta$ TCR repertoire in relation to the total number of $\alpha\beta$ T cells, CD4 $^+$ V δ 1 $^+$ T-cell transdifferentiation creates greater complexity of the $\alpha\beta$ TCR repertoire as the progenitors *de novo* generate a broad spectrum of new $\alpha\beta$ TCRs in the process of transdifferentiation. This may help assure that even centenarians can acquire immunity to newly encountered antigens.

Moreover, elevated precursor numbers and V δ 1 $^+$ C β $^+$ transdifferentiation intermediates were found in the body fluids of inflamed tissue (peripheral blood, pleura, inflamed joints, and ascites), which is in line with the observation that the number of V δ 1 $^+$ $\gamma\delta$ T lymphocytes is expanded in human diseases, including infections (111–114), but also rheumatoid arthritis (115), multiple sclerosis and HIV (116). This indicates that transdifferentiation is a highly economical process that only takes place in inflamed tissues that require T cells with diverse and adaptive TCRs. The high consistency with thymocytes in terms of the developmental route and productive efficacy thus suggests that the replenishment of the peripheral $\alpha\beta$ T-cell pool through V δ 1 $^+$ -descendants is one strong principle in T-cell homeostasis.

In summary, this study describes the unique, previously unknown role of peripheral CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells as $\alpha\beta$ T cell

precursors that can respond to hematopoietic stressors such as inflammation by differentiating into functionally, mature $\alpha\beta$ T cells at the site required. We describe the expression of HSC and progenitor markers as this subset's peculiarity. We pinpoint the transdifferentiation of CD4 $^+$ V δ 1 $^+$ $\gamma\delta$ T cells as a process of TCR re-organization that is embedded in a developmental route similar to thymic $\alpha\beta$ T-cell development but distinguishable from the latter by V δ 1 $^+$ V β $^+$ C γ neg C α neg intermediates, which suggests the formation of V δ 1 $^+$ V β $^+$ heterodimers for surrogate pre-TCR. The conclusions drawn from the *in vitro* data are strongly supported by the results of *ex vivo* analyses of diverse body fluids, where the progenitor's C β -expressing V δ 1 dim C γ $^{lo/neg}$ transdifferentiation intermediates were detected in inflamed tissue.

Most importantly, the study provides a conceptual framework for a central goal of (developmental) immunology, namely, to understand how T-cell development is ultimately conducted in the absence of thymic function. The assignment of this fundamental role for $\gamma\delta$ T cells opens a new vista in immunology and requires reevaluation of adaptive immune responses in infection, autoimmunity and cancer.

MATERIALS AND METHODS

All procedures were carried out according to the Declaration of Helsinki and were approved by the Clinical Ethics Committee at the University of Tübingen (projects 38/2009B02 and 470/2013B02).

SEVEN-COLOR FLOW CYTOMETRY

Cells were pretreated with FcR Blocking Reagent (Miltenyi Biotec) according to the manufacturer's instructions, resuspended in flow cytometry buffer [PBS, 2% (vol/vol) FCS, 250 mM EDTA] and incubated for 15 min (8°C, in the dark) with one or more of the following monoclonal antibodies, all specific for human epitopes: FITC-anti-V δ 1, unconjugated anti-V δ 1 (TS8.2, Fisher Scientific), APC-anti-TCR $\gamma\delta$ (B1, BioLegend), PE-anti-TCR $\gamma\delta$ (recognizing C γ ; 11F2, BD Pharmingen), PE-anti-TCR $\gamma\delta$ (recognizing C δ ; SA6.E9, Life Technologies) PE-anti-TCR $\alpha\beta$ (T10B9.1A-31, BD Pharmingen), FITC-anti-TCR $\alpha\beta$ (WT31, BD Pharmingen), murine anti-human TCR- $\alpha\beta$ mAb (BMA 031, Behringwerke Marburg). PE-anti-V δ 2 (B6, BD Pharmingen), FITC/PE/PerCP-anti-CD4 (SK3, BD) or Vioblu-anti-CD4 (VIT4, Miltenyi Biotec), FITC/PE/PerCP/APC-anti-CD3 (SK7, BD) APC-anti-CD3 (MEM57, Exbio), APC-anti-CD8 β (2ST8.5H7, BD), and APC-H7-anti-CD8 α (SK1, BD). FITC/APC-anti-CD62L (LT-TD180, ImmunoTools), PE/APC-anti-CD25 (2A3, BD Pharmingen), PE-anti-CD135 (4G8, BD Pharmingen), PE-anti-CD127 (M21, BD Pharmingen), and APC-anti-CD132 (4G3, BD Pharmingen). All IgG isotypes and secondary antibody rat anti-mouse IgG 1-PE (X56) were purchased from BD Pharmingen. Cytokines were detected by intracellular staining using standard procedures, and with APC-conjugated anti-IL-2 (MQ-1-17H12), -IL-4 (MP4-25D2), -IL-5 (TRFK5), -IL-10(10JES3-19F1), -IL-13(JES10-5A2), -TNF- α (MAb11), -IFN- α (B27), all from BD Pharmingen, PE-anti-IL-17A from BioLegend, and biotinylated-anti-TGF- β (MAB240) from R&D Systems. Streptavidin conjugated with APC or PE (Invitrogen) was used

for the detection of biotinylated antibodies; the cells were transferred into new vials after each washing step in order to reduce background and exclude false positive results.

In order to exclude dead cells from being analyzed, all cells were subject to live/dead exclusion using the Alexa Fluor 350 NHS Ester kit. In order to exclude the possibility of contaminants from feeder cells, irradiated feeder cells were cultivated and analyzed as clone cultures and used as controls in all experiments performed. Cells were analyzed using LSR II or FACS Calibur systems, and the FACS Diva@software and CellQuest software programs were used for the acquisition and analysis of flow cytometric data.

TRBV repertoires were analyzed with the IOTest® Beta Mark Kit (Beckman Coulter), a multi-parametric analysis tool designed for the quantitative flow cytometric determination of the TCR V β repertoire of human T lymphocytes. Taking advantage of the fact that V β specificities may be grouped into mutually exclusive combinations, three V β expressions can be detected in the same tube using an innovative staining strategy that uses three mAb stained with two fluorophores only. One mAb is conjugated to a FITC molecule, the second to PE and the third one is a carefully balanced mixture of a PE- and a FITC-conjugated form.

IMMUNOFLUORESCENCE

V δ 1 $^+$ CD4 $^+$ T-cell clones were harvested from culture dishes and washed twice in sterile 1 × PBS. For TCR- β chain expression analysis, cells were applied to the slides by cytocentrifuge, fixed in -20°C acetone/methanol (50/50; 2 min), and stained with mouse-anti-human TCR Beta F1 (1:50) (8A3, Thermo Scientific). Cy™ 3-goat-anti-mouse IgG (1:400) (Jackson ImmunoResearch) was used to visualize TCR- β chain expression. Slides were analyzed with an Axiophot microscope (Zeiss) and the AnalySIS® 3.2 software (Soft Imaging Systems).

MOLECULAR METHODS

RNA isolation/cDNA synthesis

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized using the Superscript III First Strand Synthesis Super Mix formulation (Invitrogen). All cDNAs were tested for the expression of a 800 bp amplicon of β -actin.

Real-time PCR

cDNAs derived from CD4 $^+$ V δ 1 $^+$ clones and from panV δ 1 $^+$ cultures were analyzed with ABI TaqMan primer/probe sets for PTCRA (Hs00300125_m1), GAPDH (Hs02758991_g1), and RORC (Hs01076122_m1), all purchased from Life Technologies. All other primers are self-designed and are available upon request. Invitrogen's TaqMan assay reagent and BioRad's IQ Master Mix were used for qPCR. Gene expression was calculated using the change-in-threshold method [$\Delta C_{(T)}$] and GAPDH as reference.

TRAV chain analysis

Done in accordance with Han et al. (117).

TRBV spectratyping

Performed according to Gorski et al. (118) with minor modifications. 5'FAM-labeled BC primers were used, PCR amplicons were

detected using an ABI 3130xl Genetic Analyzer, the GeneScan 600 LIZ dye size standard, and the GeneMapper software (both Applied Biosystems).

$\gamma\delta$ immunoscope

Performed with discriminating primers obtained from Annik Lim and according to her protocol (personal communication).

Identification of TCR-CDR3 regions

The relevant PCR products required for spectratype analysis were reamplified with unlabeled C primer. Sequencing was performed with the 3130xl Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Sequencing Kit according to the manufacturer's protocol. Translation of the cDNA sequence into the protein sequence was conducted with the EMBOSS Transeq software, which is available free of charge (119, 120).

CELL CULTURE, SELECTION, AND CLONING EXPERIMENTS

Informed consent was obtained from all volunteers. Sterile heparinized peripheral blood was collected from normal, healthy donors ($n = 12$); healthy bone marrow were leftovers from HLA-typing of potential stem-cell donors ($n = 10$), aliquots of LPH were leftovers from quality control after graft generation ($n = 12$). All samples were obtained and handled according to the Declaration of Helsinki and the procedures were approved by the Clinical Ethics Committee at the University of Tübingen. PBMCs were isolated by density centrifugation (Ficoll-Hypaque). Up to 2×10^7 PBMCs were pretreated using FcR Blocking Reagent (Miltenyi Biotec) according to the manufacturer's protocol, stained with FITC-anti-V δ 1 (TS8.2, Fisher Scientific) using $10 \mu\text{l}$ antibody per 1×10^7 PBMCs (15 min, +8°C, in the dark). Subsequently, the Anti-FITC MultiSort Kit (Miltenyi Biotec) was used for the isolation of "dim" cells. The cells were separated with columns (Miltenyi Biotec). FACS analysis usually had a purity of >99% V δ 1 $^+$ T cells and less than 0.16% $\alpha\beta$ TCR $^+$ cells. Alternatively, V δ 1 $^+$ $\gamma\delta$ T cells were selected with the Anti-TCR γ/δ MicroBead Kit (Miltenyi Biotec).

For the establishment of V δ 1 $^+$ CD4 $^+$ T-cell clones, V δ 1 $^+$ T cells were enriched for CD4 using the Dynal® CD4 Positive Isolation Kit (Invitrogen). All procedures were carried out according to the manufacturer's instructions. V δ 1 $^+$ CD4-enriched T cells were cloned using the limiting dilution procedure (0.1 cells/well) in round bottom 96-well plates in the presence of 200 IU/mL IL-2 (ImmunoTools), 1.0 ng/mL IL-7 (ImmunoTools), 0.25 $\mu\text{g}/\text{mL}$ phytohemagglutinin (Oxoid), and 1×10^5 allogeneic, 80 Gy-irradiated feeder cells. After 21 days, V δ 1 $^+$ CD4 $^+$ T-cell clones were identified by FACS analysis and kept under standard culture conditions – which represents mild inflammation – until further manipulation and analysis. Cloning strategy routinely yielded 5–10% of initially seeded V δ 1 $^+$ CD4 $^+$ T cells (0.1 cells/well). For panV δ 1 culture assays, 1,000 V δ 1 $^+$ cells were seeded per well in round bottom 96-well plates and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, L-glutamine (4 mM), and penicillin (50 U/mL)/streptomycin (50 $\mu\text{g}/\text{mL}$), all purchased from Biochrom GmbH.

Overt inflammation was mimicked in panV δ 1 cultures by adding the following cytokines to the standard culture medium in week 3: IL-1 β (10 ng/mL; ImmunoTools), IL-18 (10 ng/mL; R&D Systems), IL-6 (50 ng/mL; ImmunoTools), sIL-6R (100 ng/mL; ImmunoTools), and IL-12 (10 ng/mL; ImmunoTools). Monocytes that had been preactivated for 3 days in the presence of these inflammatory cytokines were also added to the culture. Monocytes were generated from PBMCs by plastic adherence in cell-culture flasks for 2 h (PBMC density $1.5 \times 10^6/\text{mL}$ in RPMI 1640 standard medium; 5% CO₂, water-saturated atmosphere, 37°C). Non-adherent cells were subsequently removed along with the supernatant; adherent cells were washed twice with warm PBS, and new culture medium containing inflammatory cytokines was added. After 3 days, the monocytes were removed from the bottom of the culture flask with a cell scraper, counted, centrifuged, resuspended in conditioned medium, irradiated (80 Gy), and 1×10^5 monocytes/well added to panV δ 1 $^+$ cell cultures.

FUNCTIONAL ANALYSIS

$\alpha\beta$ T cells derived from transdifferentiated V δ 1 $^+$ CD4 $^+$ $\gamma\delta$ T-cell clones were stimulated for 5 h with PMA (50 ng/mL) and ionomycin (750 ng/mL, Sigma). Brefeldin A (10 $\mu\text{g}/\text{mL}$) was added for the last 60 min of incubation. Cytokine production was measured by intracellular staining and FACS analysis as described above.

STATISTICAL ANALYSES

Statistical analyses were performed with the GraphPad Prism software V5.0 (GraphPad Software). Statistical differences were analyzed using the parametric student *t*-test, error bars in the graphs depict the SEM. A *p* < 0.05 was considered as statistically significant.

AUTHOR CONTRIBUTIONS

Hendrik Ziegler, Christian Welker, and Marco Sterk contributed equally to the work, and performed most of the experiments, contributed to and established methodology; Jan Haarer contributed and established methodology, and performed experimental work; Hans-Georg Rammensee and Rupert Handgretinger contributed to experimental design and Rupert Handgretinger provided essential material. Christian Welker prepared all figures and helped editing the manuscript; Karin Schilbach initiated all work on V δ 1 $^+$ CD4 $^+$ cells, proposed and identified V δ 1 $^+$ CD4 $^+$ cells as a CD34 $^+$ T cell precursor, planned, designed, supervised all experiments *in vitro* and *ex vivo*, interpreted data, identified transdifferentiation as the re-organization of the TCR- $\gamma\delta$ into TCR- $\alpha\beta$ via a V δ +V β +V γ ^{neg}:V α ^{neg} intermediate, established the developmental scheme, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00645/abstract>

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Phosphoantigen presentation to TCR $\gamma\delta$ cells, a conundrum getting less gray zones

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The mechanistic requirements of antigen recognition by T cells expressing a $\gamma\delta$ TCR has revealed important differences with those of $\alpha\beta$ TCR cells and, despite impressive new data generated in the very recent years, they remain poorly understood. Based on the structure of the TCR chains and the tissue distribution, $\gamma\delta$ cells are represented in a variety of populations. The major subset of human peripheral blood $\gamma\delta$ cells express V γ 9V δ 2 TCR heterodimers and are all stimulated by phosphorylated metabolites (commonly called phosphoantigens). Phosphoantigens are molecules with a very small mass and only stimulate V γ 9V δ 2 cells in the presence of antigen-presenting cells, suggesting a strict requirement for dedicated antigen-presenting molecules. Recent studies have identified butyrophilin (BTN) 3A1 as the molecule necessary to stimulate V γ 9V δ 2 cells. BTN3A1 extracellular, transmembrane, and cytoplasmic domains have different functions, including cognate interaction with the V γ 9V δ 2TCR, binding of the phosphoantigens, and interaction with cytoplasmic proteins. This review mainly discusses the known molecular mechanisms of BTN3A1-mediated antigen presentation to $\gamma\delta$ cells and proposes a model of phosphoantigen presentation, which integrates past and recent studies.

Keywords: antigen presentation, $\gamma\delta$ TCR, butyrophilin 3A1, infection control, tumor surveillance

INTRODUCTION

Two types of TCR can be expressed in a mutually exclusive manner by T cells made up of either $\alpha\beta$ or $\gamma\delta$ chains. Both receptors are heterodimers, and while the TCR γ and β genes are encoded in different loci, the TCR δ genes, being nested within the TCR α locus, are subject to deletion when the TCR α genes are rearranged. T cells expressing TCR homologous to the human TCR γ and TCR δ genes have been described in many species, indicating an important function of $\gamma\delta$ TCR cells. Despite the enormous number of studies on $\gamma\delta$ TCR cells, a series of issues remain poorly solved. They include the evolutionary necessity for two separate populations of T lymphocytes (1), the nature of the antigens that stimulate $\gamma\delta$ TCR cells, and the mechanisms of antigen presentation and the role of these cells in immune response. This paper will only touch upon some of these points to review in greater detail the published studies on the most abundant population of circulating human $\gamma\delta$ cells, their antigen specificities, and the modes of presentation of these antigens.

THE STRUCTURE OF THE $\gamma\delta$ TCR

Both $\alpha\beta$ and $\gamma\delta$ TCRs are heterodimers linked by disulfide bonds. Some rare $\gamma\delta$ TCR, namely those using the constant $\gamma 2$ chain, lack a critical cysteine and thus form non-covalently linked heterodimers. Whether this structural difference has important functional effects remains poorly investigated.

The structures of human $\gamma\delta$ TCR have been solved and showed pairing of the γ and δ chains, resembling that of $\alpha\beta$ TCR heterodimers (2). The complementarity-determining region 3 (CDR3) regions of both γ and δ genes form quite large bulges on

the top of the receptor, suggesting a direct involvement in antigen recognition (3). A second main aspect is that the human TCR composed of the V γ 9 and V δ 2 chains is characterized by an elbow angle at the C–V junction, which is different from that of the $\alpha\beta$ TCR of immunoglobulins (2). It was speculated that other $\gamma\delta$ TCR composed of non-V γ 9 and non-V δ 2 chains also show similar angles, as the residues found in the V–C interface of the solved V γ 9V δ 2 TCR are conserved in most human and mouse γ and δ genes (4). Although this unique structure might have important functional implications, no study has directly addressed this aspect.

The CDR loops of the TCR γ and TCR δ chains closely resemble those of other TCR genes, though important differences are present. The CDR1 and CDR2 loops of V γ and the CDR1 loop of V δ are positioned in a manner similar to those of V β and V α , respectively, and they are two residues longer than their $\alpha\beta$ TCR counterparts. In addition, in the CDR2 loop of V δ , the C'' strand pairs with the C' strand of the inner β -sheet of the domain, whereas in the V α chain CDR2 loop, the C'' strand pairs with the D strand of the outer β -sheet. These structural characteristics contribute to a jagged surface of the $\gamma\delta$ TCR, which is very different from that of a $\alpha\beta$ TCR that binds MHC-peptide complexes, implying that the surface of the antigen-presenting molecule interacting with the V γ 9V δ 2 TCR is very different from that of an MHC molecule.

Other studies have investigated the structure of different types of $\gamma\delta$ TCR and their mode of interaction with respective ligands. The V δ 1 chain of an MICRA-reactive T cell showed a surprisingly flat surface, which is not found in other TCR structures (3), implicating an interaction very different from that of $\alpha\beta$ TCR with MHC molecules.

A TCR recognizing the MHC class I-like molecule T22 showed dominance in this interaction of the germline-encoded residues of the junctionally recombined CDR3 δ , which bound to the α helices of T22 (5), a mode of antigen recognition different from that of antibodies and MHC-restricted TCR.

THE ANTIGENS STIMULATING $\gamma\delta$ CELLS

The conservation of the γ and δ genes throughout the primate lineage suggests that these distinctions probably have important functional consequences.

Since the initial discovery of the T cell population expressing the $\gamma\delta$ TCR, it was clear that the identification of the nature of stimulatory antigens was an important step to understanding the function of $\gamma\delta$ TCR during immune responses. A variety of antigens have been identified and their nature has been recently reviewed in Ref. (6). Here, we briefly describe the published studies reporting antigens, which stimulate TCR $\gamma\delta$ cells and discuss them according to their nature and expression on target cells.

SURFACE MOLECULES STIMULATING $\gamma\delta$ TCR CELLS

The list of cell surface molecules that may establish cognate interactions with the $\gamma\delta$ TCR is large and is continuously increasing (Table 1). All of the identified surface molecules stimulate a small

percentage of $\gamma\delta$ cells and raise the important question of whether these specificities are occasional or instead should be considered as an important part of the antigenic repertoire of $\gamma\delta$ cells. MHC molecules were found to stimulate alloreactive responses of $\gamma\delta$ cells (7–10), and mutagenesis studies on the MHC molecule indicated that the topology of $\gamma\delta$ TCR interaction with the MHC was distinct from that of $\alpha\beta$ T cells (11).

CD1 molecules have been found to interact with the human $\gamma\delta$ TCR. The first isolated human T cell clone showed CD1c autoreactivity (12) and other CD1c-autoreactive $\gamma\delta$ TCR cells were found later (13, 14). Rare CD1d-restricted $\gamma\delta$ TCR cells were identified by staining with CD1d tetramers loaded with α -Galactosylceramide (15) or with sulfatide (16, 17). V δ 1 was the V δ chain used in both types of $\gamma\delta$ cells and it was the predominant chain interacting with CD1d. The V γ chain was found contacting α -Galactosylceramide (15), whereas in the case of sulfatide recognition, the V γ chain interacted neither with CD1d nor with sulfatide (17).

Another surface molecule with a CD1d-resembling structure, which stimulates a rare population of $\gamma\delta$ cells is the endothelial protein C receptor (EPCR) (18), a lipid-binding protein expressed by endothelial cells. One T cell clone expressing a V δ 5–V γ 4 TCR interacted with low affinity with EPCR, and this interaction was facilitated by CMV infection. The enhancing effect of CMV was not related to changes in EPCR lipid binding and remains poorly characterized.

Other MHC-like molecules interacting with mouse $\gamma\delta$ cells are TL 27b (19), TL 10b (11, 20), and T22 (21). The structure of the specific TCR bound to the T22 molecule showed that germline-encoded residues of the CDR3 δ loop were responsible for binding T22 in an orientation different from that seen in $\alpha\beta$ TCR binding to MHC-peptide complexes (5). Human V δ 1-expressing cells were also found to interact with the MICA molecule (22). The V δ 1 TCR of these cells and the NKG2D, another MICA receptor, bound MICA in a mutually exclusive manner. The analysis of the binding kinetics suggested a model in which the initial contact between the $\gamma\delta$ cells and the target cell is established by fast binding of MICA to NKG2D, followed by a much-prolonged interaction with the TCR.

Two other proteins were reported to stimulate human V γ 9V δ 2 cells, namely “an entity related to the mitochondrial F1 ATPase” expressed by some tumor cells and a delipidated form of apolipoprotein A-I (Apo A-I) (23). A soluble V γ 9V δ 2 TCR was found to interact with Apo A-I and bovine F1 ATPase in the low micromolar range by surface resonance (SPR) studies. When different V γ 9V δ 2 clones were compared, only some were positively influenced by the presence of Apo A-I, indicating a redundant role of this molecule.

A last set of proteins that were reported to stimulate human $\gamma\delta$ cells are heat-shock proteins (24–26). Surface expression of these molecules was associated with targeting of $\gamma\delta$ cells on different tumor cells (27).

All these findings suggest that antigen recognition by $\gamma\delta$ T cells is different from that of $\alpha\beta$ T cells. The different length of both V γ and V δ CDR3 loops also supports this conclusion. These loops are often critical for antigen binding in Ig and significantly contribute to peptide binding in $\alpha\beta$ TCRs. When the CDR3 regions of Ig H and L chains are compared with those of TCR α , β , γ , and δ chains, the one from Ig H and TCR δ are the most variable in size and

Table 1 | Antigens stimulating $\gamma\delta$ cells.

| $\gamma\delta$ TCR/cell type | Antigens/restriction molecules | Reference |
|--|---|--------------|
| HUMAN | | |
| V γ (several) V δ 1 | MICA, MICB | (3, 22) |
| V γ (several) V δ 1 | CD1c, CD1d | (12–17) |
| V γ 4V δ 5 | EPCR | (18) |
| V γ 9V δ 2 | F1 ATPase, Apo A-I | (23) |
| V γ 9V δ 2 | GroEL homolog on Daudi Burkitt's lymphoma cells | (25) |
| V γ 9V δ 2 | Hsp60, Hsp65 | (24, 26, 27) |
| V γ 9V δ 2 | IPP, HMBPP | (43–45) |
| | Tetanus toxoid | (30, 31) |
| | DXS2 or Rv2272 peptides | (40) |
| V δ 1 clones | HLA-A24, HLA-A2 | (9, 10) |
| V γ 4V δ 1 | HLA-B27 | (32) |
| V γ 3V δ 2 | Histidyl-tRNA synthetase | (39) |
| MOUSE | | |
| Hybridoma G8 | T10, T22, T27 | (5, 11, 21) |
| Hybridoma KN6 | T27 | (11, 19) |
| Hybridoma LBK5 | IE κ | (11) |
| V γ 1V δ 8 (NX6) | Cy3 | (41) |
| V γ 4V δ 4 (1G9) | NP | (41) |
| V γ 1–J γ 4/V δ 5 (MA2) | PE | (42) |

Apo A-I, apolipoprotein A-I; CD1c or CD1d, cluster of differentiation 1 isoforms; Cy3, cyanine 3; DXS2, mycobacterium 1-deoxy-D-xylulose-5-phosphate-synthase 2; EPCR, endothelial protein C receptor; HLA, human leukocyte antigen; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; Hsp, heat-shock protein; IPP, isopentenyl pyrophosphate; MICA or MICB, MHC class I chain-related protein A or B; NP, 4-hydroxy-3-nitrophenylacetyl; Rv2272, mycobacterium transmembrane protein; T10, T22, or T27, MHC class Ib proteins; PE, algae protein phycoerythrin.

are significantly longer than Ig L and TCR γ chains, respectively (28). In contrast, TCR α and β pairing occurs with chains of nearly identical average CDR3 lengths. These important TCR structural differences have been related to a type of antigen recognition by $\gamma\delta$ TCR similar to that of Ig (29).

SMALL MOLECULES STIMULATING $\gamma\delta$ TCR CELLS

Several studies have identified both human and mouse $\gamma\delta$ T cells that were activated by small peptides, carbohydrates, and haptens. Initial studies showed the existence of human and mouse $\gamma\delta$ cells recognizing MHC-peptide complexes expressed on the surface of antigen-presenting cells (APC) (10, 30–32). In contrast with human alloreactive $\alpha\beta$ TCR cells, the number of alloreactive $\gamma\delta$ cells remains quite rare. H2-peptide-specific $\gamma\delta$ TCR cells were found in mice, after inhalation of ovalbumin (33). Whether these $\gamma\delta$ cells recognize intact ovalbumin or its peptides was not investigated.

Mouse $\gamma\delta$ TCR hybridomas recognizing peptides from heat-shock proteins were also described. Interestingly, these peptides were presented by non-MHC molecules and their recognition was sensitive to amino-acid changes in the peptide sequences and to the type of $V\gamma$ chain expressed by specific hybridomas (34). Another study described mouse $\gamma\delta$ cells recognizing glycosylated peptides in which MHC class I-bound peptides with two or three sugars were stimulatory (35). The central position of the sugar in the peptide sequence was critical for stimulation (36), probably because of direct interaction with the $\gamma\delta$ TCR.

Several studies have shown that in some patients with myopathies, $\gamma\delta$ cells infiltrate the affected area and are associated with acute pathology (37). In one study, the infiltrating T cells were cloned and found to be oligoclonal (38). The TCR of these cells was composed of a $V\gamma 1.3-J\gamma 1-C\gamma 1/V\delta 2-J\delta 3$ heterodimer, which is not frequent in normal donors. This TCR recognized histidyl-tRNA synthetase, an antigen also recognized by anti-Jo-1 autoantibodies (39). The $\gamma\delta$ TCR target epitope was strictly conformational, independent of post-translational modification, exposed on the surface of the intact protein, and mutagenesis studies showed that a short alpha-helical loop constituted part of the $\gamma\delta$ stimulating epitope.

Recent studies reported the identification of two mycobacterial proteins, namely 1-deoxy-D-xylulose 5-phosphate synthase 2 and Rv2272 protein, which activated $\gamma\delta$ T cells isolated from patients with pulmonary tuberculosis (40). Two peptides, 12 amino-acid long from each of these proteins, retained the capacity

of stimulating a major population of $\gamma\delta$ cells expressing a unique CDR3 δ segment. Whether this recognition occurred by cognate interaction of the TCR with the peptides or via a dedicated presenting molecule was not investigated.

More recently, mouse $\gamma\delta$ cells specific for small haptens have been identified (41). These T cells recognized cyanine 3, a synthetic fluorescent molecule, and 4-hydroxy-3-nitrophenylacetyl, a classical hapten. Another small molecule stimulating both murine and human $\gamma\delta$ TCR cells is the alga protein phycoerythrin (PE) (42). The TCR of isolated cells directly interacted with the whole PE, thus indicating a B cell-like antigen recognition capability.

Another type of small antigens stimulating $\gamma\delta$ cells is represented by phosphorylated metabolites generated in the mevalonate pathway in eukaryotic cells or in the methyl erythritol pathway in bacteria and in some eukaryotes. The most representative stimulatory molecules are isopentenyl pyrophosphate (IPP) (43, 44) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (45) (Figure 1). Both are very small molecules with a molecular weight of 245 and 262, respectively, and are composed of one isoprene unit to which a diphosphate is attached. Small changes in the structure of these ligands, either on the phosphate or the isoprene moieties, profoundly affect the $\gamma\delta$ cell stimulatory capacity. Several analogs of these compounds have been synthesized with an intermediate stimulatory capacity between that of IPP and of HMBPP [reviewed in Ref. (46)]. The same population of $\gamma\delta$ cells also recognizes some tumor cells. This recognition is ascribed to the abnormally elevated production of IPP by tumor cells, as result of changes in the regulation of their mevalonate metabolic pathway (47). All of these findings show that $\gamma\delta$ cells may cross-react to phosphorylated metabolites accumulating inside tumor cells and to metabolites released by bacterial cells in the microenvironment. Importantly, when bacteria infect target cells, they induce alteration of the host mevalonate pathway by subverting several regulatory mechanisms (48). These alterations lead to a transient and acute accumulation of IPP, which is then responsible for the activation of $\gamma\delta$ cells. In conclusion, $\gamma\delta$ cells may recognize (i) tumor cells that accumulate IPP; (ii) bacterial metabolites such as HMBPP, and (iii) cells accumulating IPP following infection with bacteria not producing HMBPP.

An important finding is that these metabolites stimulate the human cells expressing the $V\gamma 9V\delta 2$ heterodimer and also other primate $\gamma\delta$ cells, which express TCR heterodimers closely resembling the $V\gamma 9V\delta 2$ TCR (49). The role of the CDR3 sequences of

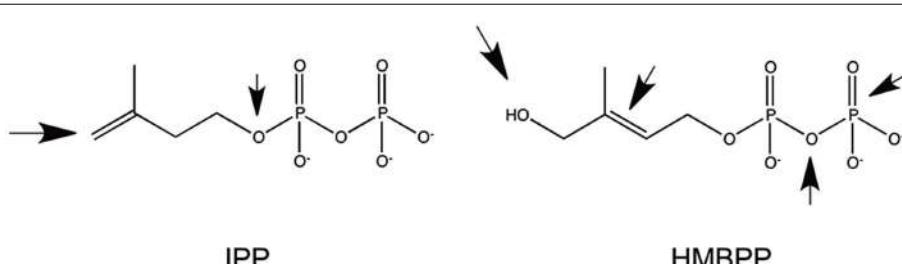


FIGURE 1 | Structure of the two most common phosphorylated metabolites stimulating $V\gamma 9V\delta 2$ cells. The arrows indicate the parts of the molecules whose modifications significantly reduced immunogenicity.

$V\gamma 9V\delta 2$ TCR in antigen recognition remains debated. Sequence analysis of T cell clones reacting to IPP and HMBPP (50–54) showed CDR3 regions of different length and sequences, thus indicating that they are unlikely to be involved in direct contact with such small antigens. In addition, most peripheral, but not thymic $V\gamma 9V\delta 2$, clones contained Val or Leu or Ile amino acids at position 97 of the CDR3 (55), raising the possibility of a selection for hydrophobic residues during expansion in peripheral blood. The conclusions of these investigations were in line with more recent data in which the mutation of the TCR $V\gamma 9$ and $V\delta 2$ genes suggested that a large TCR footprint is involved in antigen stimulation (56), probably because the TCR interacts with molecules much larger than the small stimulatory metabolites.

Although the stimulation of $\gamma\delta$ cells by phosphoantigens has been described for many years, important cell biology aspects of how the $V\gamma 9V\delta 2$ TCR engages these molecules remained poorly defined. Classical studies of antigen presentation were performed in different laboratories and provided useful information to the understanding of how this interaction may occur. APC were necessary for this activation and only human APC could activate $V\gamma 9V\delta 2$ cells, suggesting that antigen-presenting molecules or surface-expressed co-stimulatory molecules were necessary (57). These observations were confirmed in other studies (58, 59). Cells from many different human tissues (47), from different donors, and APC neither expressing MHC class II molecules nor $\beta 2$ -microglobulin (57) were also stimulatory indicating that the required presenting/accessory molecule is ubiquitous, non-polymorphic, and species-specific.

Fixed APC maintained the capacity to stimulate $V\gamma 9V\delta 2$ cells as shown by lymphoma Daudi cells (25), which accumulate endogenous IPP (47). Fixed APC remained stimulatory also when exogenous IPP was added after fixation (60), indicating that metabolically inactive APC remain stimulatory. This information also suggested that the exogenous antigen does not require specific internalization into APC and that the presenting molecule is already expressed in a stimulatory form on Daudi cells before fixation and so it remains after fixation. In one study, the responsiveness of $V\gamma 9V\delta 2$ cells to a crude mycobacterial lysate was investigated (61). When monocytes were first pulsed and then fixed, they retained the stimulatory capacity, whereas they were not stimulatory if pulsed after fixation. This study also showed that chloroquine increased the antigen-presenting capacity of APC and this effect was not associated with inhibition of lysosome acidification. It was suggested that chloroquine facilitates antigen presentation by decreasing the degradation or turnover of surface presenting molecules. These studies underlined the importance of surface molecules that can be fixed in a stimulatory state upon antigen binding.

Several laboratories also reported that phosphoantigens cannot be pulsed on APC, i.e., when APC are incubated with phosphoantigens, they immediately lose their stimulatory capacity upon washing (58, 60), probably caused by a weak binding to, and a fast dissociation from, the presenting molecule. This behavior is incompatible with a cytoplasmic stimulatory activity of phosphoantigens, and is instead in agreement with an extracellular role of a presenting molecule on APC. Consistent with a weak extracellular antigen binding, association of prenyl pyrophosphate antigens

with the surface of APCs was reported only in the presence of 50- to 1000-fold higher concentrations of antigen than those required to stimulate $\gamma\delta$ cells (62).

Another important finding was that the addition of the antigen to APC is immediately followed by T cell response. This was observed in several kinetic studies (58, 63, 64). Using cytosensor microphysiometry, $\gamma\delta$ T cell activation was detected in <9 s after antigen addition (63). As charged compounds passively pass the plasma membrane with great difficulty (65) and require an active endocytic process, it is unlikely that phosphoantigens added to the extracellular milieu may accumulate in the cytoplasm of APC in <9 s. Such a fast T cell response is in agreement with the possibility that the antigen binds to surface molecules almost immediately and that internalization into the APC is not necessary.

Other clues to the nature of antigen binding comes from studies of stimulatory and inhibitory capacity of IPP (66) and HMBPP (63) analogs. Antagonistic activity was found with some ligands, and in one case, $\gamma\delta$ cells became unresponsive after a very brief interaction (<5 min) with the antagonist compounds (66). This state of unresponsiveness was fully reversible but lasted at least 3 days even after removal of the antagonist, thus indicating that the interaction with the antagonist induced a significant change in $\gamma\delta$ cells. This observation is in agreement with a model of antigen recognition in which antigenic molecules exposed on the cell surface bind phosphorylated metabolites and, according to their structure, an agonist or antagonist signal is induced in the interacting $\gamma\delta$ cells. It remains intriguing that when competition studies were performed using methylene diphosphonates, higher doses of competitors were required to inhibit stimulation with IPP than with BrHPP (63) despite the fact that IPP is a weaker agonist than BRHPP (67). Further investigations are required to explain this unexpected result.

A significant finding was that the $V\gamma 9V\delta 2$ TCR enters the immune synapse in the absence of antigen (68), thus suggesting that this TCR may interact with molecules already exposed on the APC surface in the absence of phosphoantigens. This interaction is enough to form an APC-T cell synapse, although it is not capable of inducing a full $\gamma\delta$ cell response, which instead requires the presence of the antigen.

THE ROLE OF BUTYROPHILINS IN STIMULATING $\gamma\delta$ CELLS

An important advancement was made by the identification of Butyrophilin (BTN) 3A1 (BTN3A1) as the molecule required to stimulate $V\gamma 9V\delta 2$ cells by phosphoantigens (69, 70). This molecule belongs to the family of BTN (Figure 2), which have been attributed a series of functions including immunomodulation (71, 72) and induction of maturation of mouse thymic $V\gamma 5V\delta 1$ cells (73). BTN proteins can be involved in milk fat globule formation in the lactating mammary tissue of cows (74), or in dampening and inhibiting immune reactivity (71, 72).

The BTN3 family is conserved together with the TCR $V\gamma 9$ and $V\delta 2$ genes in higher primates and in some species of placental mammals, but not in rodents (75). The common occurrence or loss of these three genes suggested their co-evolution based on a functional relationship.

Butyrophilins are structurally very similar to molecules of the B7 family. A major characteristic shared among the B7,

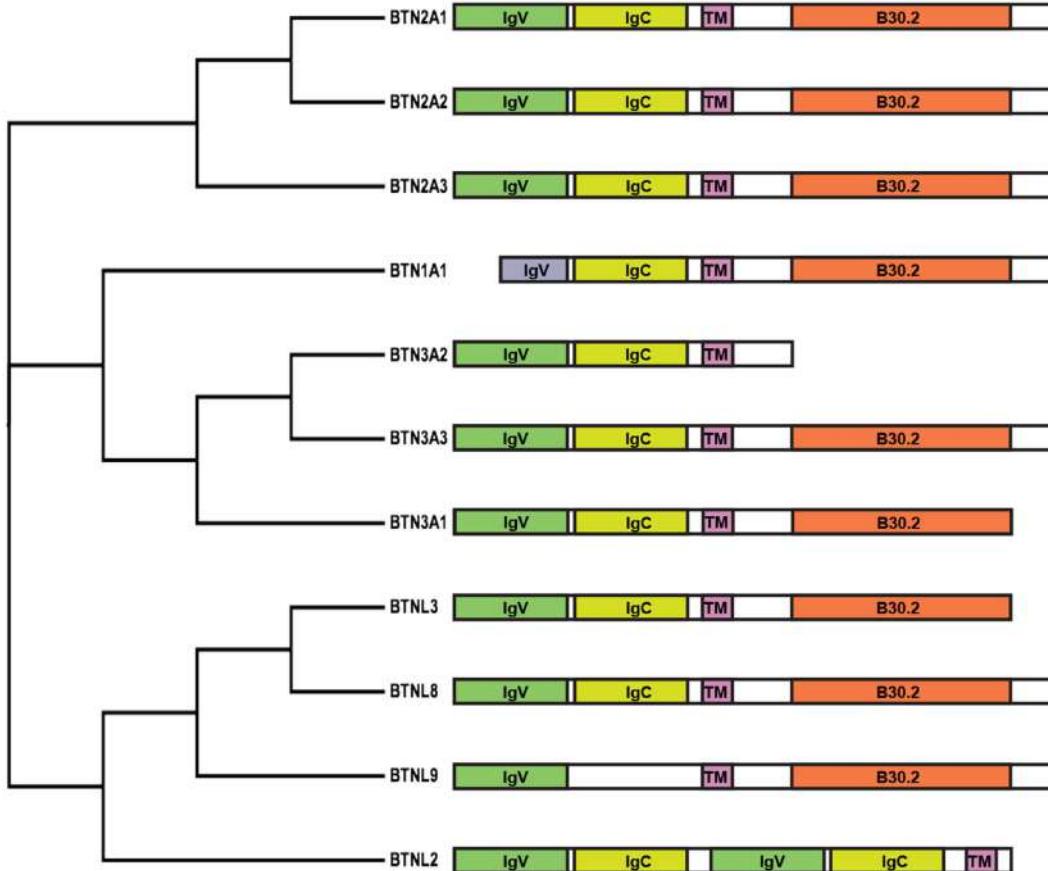


FIGURE 2 | Cladogram and schematic diagram of human BTN and BTNL family members. Protein sequences were aligned with ClustalV2 and the tree rendered using FigTree software (tree.bio.ed.ac.uk/software/figtree). Diagram shows the domain organization of members belonging to the BTN family consisting of two extracellular Ig-like domains, IgV and IgC, a

single-pass transmembrane domain (TM) and an intracellular B30.2 domain. Exceptions include BTN1A1, consisting of a short IgV-like domain; BTN3A2, lacking a B30.2 domain; BTNL9, containing a single Ig-like domain; and BTNL2, consisting of four extracellular Ig-like domains and lacking a B30.2 domain.

BTN, and butyrophilin-like (BTNL) families is the two extracellular immunoglobulin-like domains – one IgV-like and one IgC-like domain. Some members, like BTNL2 and B7 homolog 3 (B7H3), instead comprise four extracellular immunoglobulin-like domains. Like several members of the B7 family (76), BTN proteins have also been found to form dimers (77), although their occurrence on the surface of APC remains to be confirmed. Structural studies with soluble BTN3 molecules have shown the formation in solution of two types of BTN3 dimers (77). One type is formed by juxtaposition of the C-like domains (head-to-head dimer), resembling the dimers formed by B7 family members. In contrast, the second type of dimer was characterized by an asymmetric head-to-tail binding. Fluorescence resonance energy transfer experiments suggested that the head-to-head dimers are more frequently formed, at least in solution.

Importantly, when reconstitution experiments were performed by transfecting the BTN3A1 gene in mouse cells, it was found that this protein alone is not sufficient to restore the stimulation of V γ 9V δ 2 cells (69, 70, 78), suggesting that additional molecules are necessary.

A common feature of the BTN and BTNL families (with the exception of BTN3A2 and BTNL2) is the presence of intracellular domains with a structure resembling the B30.2 domain found in more than 150 other human proteins (79). This domain forms protein–protein interactions with various cytoplasmic molecules with different activities (80).

One study describing the stimulatory role of an anti-BTN3A1 monoclonal antibody (mAb) (69) provided early insights into the mechanism of BTN3A1–phosphoantigen presentation. When this antibody was added to target cells expressing BTN3A1, it induced the activation of V γ 9V δ 2 cells independently of the presence of phosphoantigens. A second anti-BTN3A1 mAb was instead inhibitory. These findings raised a series of new questions associated with the occurrence of both stimulatory and inhibitory anti-BTN mAbs. This issue was further studied by resolving the structure of BTN3A in association with the two mAbs (77). Indeed, while the inhibitory antibody bound to the distal part of the V-like domain, the stimulatory antibody bound to a more membrane proximal region of the V-like domain. The activatory antibody was also found to be compatible with *in vitro* formation of BTN3

homodimers in which the C-like domains of two BTN3 molecules interact with each other, as reported for other B7-like molecules. The authors speculated that the capacity of this antibody to facilitate this type of dimers was associated with the stimulatory capacity of this mAb, whereas the inhibitory mAb prevented BTN3 homodimerization.

A second study used a genetic approach to identify the chromosomal loci encoding the gene required for stimulation of V γ 9V δ 2 cells (70). By using a panel of mouse–human somatic cell hybrids, the telomeric region of human chromosome 6 was identified as important. By using a second series of somatic hybrids with truncations in this region, a closer genetic mapping identified 14 candidate genes, and among those BTN3A1 was found necessary for stimulating $\gamma\delta$ cells. Transfection and knock out studies confirmed that while BTN3A1 was important, BTN3A2 and BTN3A3 had no apparent role in stimulating V γ 9V δ 2 cells. Additional experiments investigated the mechanism of BTN3A1 stimulation. A recombinant BTN3A1 protein containing only the V-like domain showed binding to IPP and HMBPP. This was investigated using three different approaches, namely SPR, mass spectrometry of intact BTN3A1–antigen complex, and structural analysis of BTN3A1–IPP and HMBPP complexes. These studies showed a weak interaction of the two phosphoantigens with BTN3A1 and indicated their mode of binding. Additional studies addressed the important issue of whether the V γ 9V δ 2 TCR makes cognate interaction with the BTN3A1–phosphoantigen complexes. This aspect was initially investigated by SPR and then by surface-enhanced Raman scattering (SERS), a technique capable of detecting very weak protein–protein interactions. These studies revealed that only a soluble V γ 9V δ 2 TCR interacted with the complex, and neither soluble V γ 9V δ 1 TCR nor $\alpha\beta$ TCR used as controls. The V γ 9V δ 2 TCR weakly interacted with the recombinant BTN3A1 in the absence of phosphoantigens and this interaction was enhanced by addition of IPP (70).

Another important finding was that when the cytoplasmic B30.2 domain of BTN3A1 was grafted on the non-stimulatory BTN3A3 molecule, stimulation of V γ 9V δ 2 was restored (69). Thus, both the extracellular and the cytoplasmic domains of BTN3A1 were required (Figure 3). The importance of intracellular domains has been already reported in the field of antigen presentation. Indeed, the cytoplasmic domains of other antigen-presenting molecules, for example, CD1 molecules, are involved in proper internalization, endosomal recycling, and in the physiological presentation of lipid antigens (81). The cytoplasmic domains of several presenting molecules associate with different protein partners and each of these interactions contribute to antigen presentation and productive T cell activation.

In more recent studies, binding of IPP and HMBPP to the B30.2 domain and not to the V-like domain of BTN3A1 was reported (82, 83), and mutagenesis studies of the B30.2 domain of the non-stimulatory BTN3A3 where an amino-acid change in the putative antigen binding pocket to that of BTN3A1 conferred binding of HMBPP and $\gamma\delta$ cell stimulatory capacity (82). In this latter study, no binding of the TCR to the V-like domain of BTN3A1 was detected and it was proposed that the B30.2 domain is important because it binds phosphoantigens and with unknown mechanisms it induces the activation of $\gamma\delta$ cells. Although interesting,

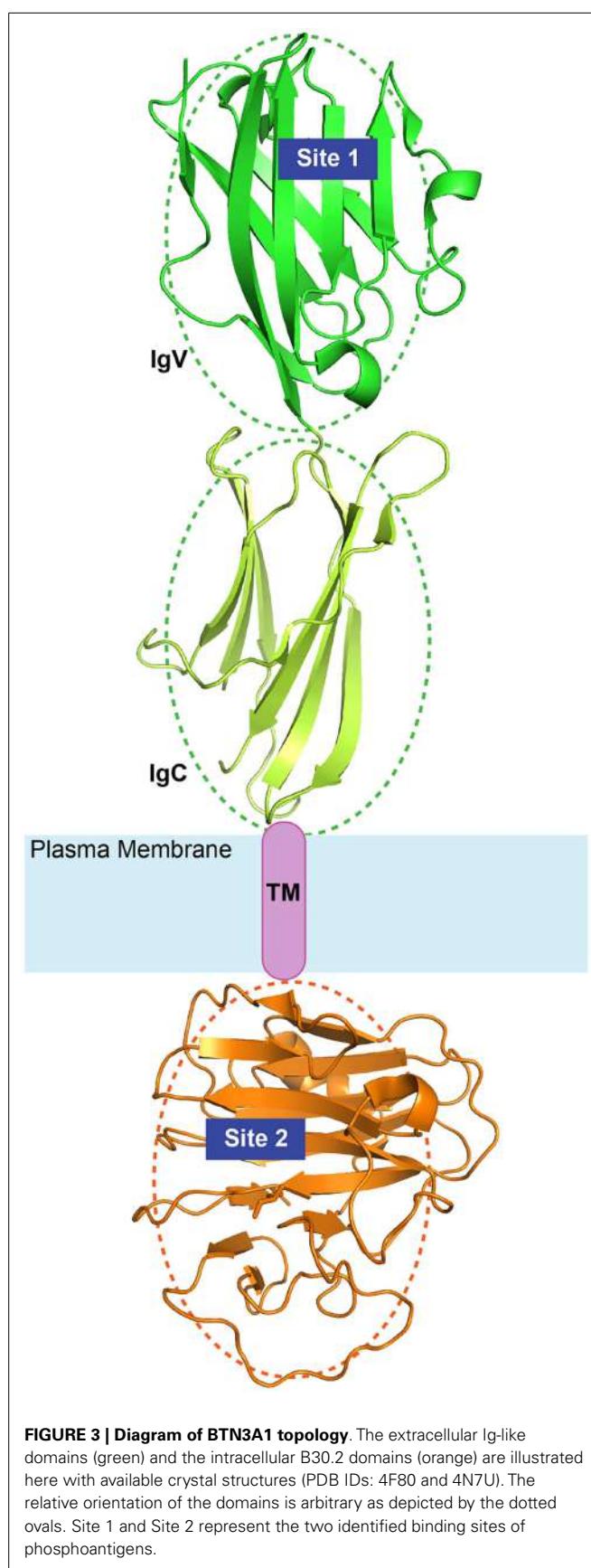


FIGURE 3 | Diagram of BTN3A1 topology. The extracellular Ig-like domains (green) and the intracellular B30.2 domains (orange) are illustrated here with available crystal structures (PDB IDs: 4F80 and 4N7U). The relative orientation of the domains is arbitrary as depicted by the dotted ovals. Site 1 and Site 2 represent the two identified binding sites of phosphoantigens.

this hypothesis is inconsistent with the published literature discussed above. The incapacity of detecting phosphoantigen and TCR binding to the V-like domain of BTN3A1 might be ascribed to technical reasons, for example, utilization of techniques not capable of detecting weak protein–protein interactions and lack of adequate control of the proper conformation of recombinant molecules studied.

As described above, a large number of data obtained in several laboratories indicated that phosphoantigens must be present outside the APC to stimulate $\gamma\delta$ cells and these data are not compatible with a model in which exogenous phosphoantigens must first be internalized into the cytoplasm to become active. The fact that the B30.2 domain of BTN3A1 binds the phosphoantigens *in vitro* is not proof that similar binding occurs *in vivo*. This is a main issue that has not been experimentally tested, but is fundamental to proposing the hypothesis of the intracellular mode of phosphoantigen activity. As there is common agreement that the B30.2 domain has a major role in activation of V γ 9V δ 2 cells, other possibilities should be considered to explain its mode of function. One hypothesis is that the B30.2 domain binds to one or several cytoplasmic proteins instrumental for the correct display of BTN3A1. A second possibility is that the B30.2 domain is necessary for the correct recycling of BTN3A1 to endosomal compartments. According to this latter possibility, the B30.2 domain would resemble the cytoplasmic tails of other antigen-presenting molecules that bind to cytoplasmic signaling partners and direct BTN3A1 trafficking to compartments where the antigen is loaded and unloaded. These alternative mechanisms of action require the presence of unique motifs in the B30.2 domain. It is possible that some of these important motifs could have been lost in the mutagenesis study (82).

On the basis of all these data, we propose a model of activation of V γ 9V δ 2 cells by phosphoantigens whereby the V γ 9V δ 2 TCR makes cognate interaction with the BTN3A1 molecule (Figure 4). The interaction of the TCR with the V-like domain of BTN3A1 is positively influenced by the presence of phosphoantigens through two possible mechanisms. The first one takes into account that the phosphoantigens interact with the TCR, thus increasing the overall affinity of interaction as suggested by SERS experiments (70). Alternatively, phosphoantigen binding may induce a conformation change in BTN3A1, which in turn interacts with the TCR, leading to a full activation of $\gamma\delta$ cells. Both these possibilities are in line with the inhibitory function of antagonist analogs (63, 66). The presence of an extracellular V-like domain is not sufficient to activate V γ 9V δ 2 cells, as shown by the relevance of the B30.2 cytoplasmic domain (69). This domain could also bind phosphoantigens (82), and it remains difficult to envisage how $\gamma\delta$ cell activation is induced upon this interaction. One hypothesis is that upon phosphoantigen binding to the B30.2 domain, the extracellular domains of BTN3A1 assume a new conformation, which promotes stable contact with the $\gamma\delta$ TCR. Alternatively, phosphoantigen binding to the B30.2 domain could facilitate proper trafficking and membrane localization of BTN3A1. It is important to underline that binding to the V-like and B30.2 domains may not be necessarily mutually exclusive. Phosphoantigen binding to both domains may be needed for appropriate $\gamma\delta$ cell stimulation. A new series of mutagenesis and reconstitution

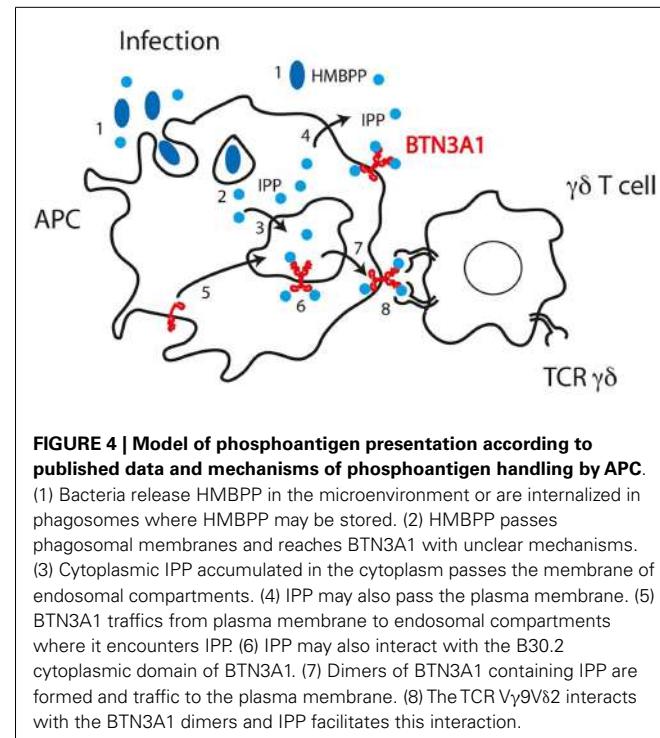


FIGURE 4 | Model of phosphoantigen presentation according to published data and mechanisms of phosphoantigen handling by APC.

(1) Bacteria release HMBPP in the microenvironment or are internalized in phagosomes where HMBPP may be stored. (2) HMBPP passes phagosomal membranes and reaches BTN3A1 with unclear mechanisms. (3) Cytoplasmic IPP accumulated in the cytoplasm passes the membrane of endosomal compartments. (4) IPP may also pass the plasma membrane. (5) BTN3A1 traffics from plasma membrane to endosomal compartments where it encounters IPP. (6) IPP may also interact with the B30.2 cytoplasmic domain of BTN3A1. (7) Dimers of BTN3A1 containing IPP are formed and traffic to the plasma membrane. (8) The TCR V γ 9V δ 2 interacts with the BTN3A1 dimers and IPP facilitates this interaction.

experiments in BTN3-deficient APC are required to properly address this issue.

An important aspect is how endogenous ligands, such as IPP that is synthesized in the cytosol, are loaded onto the extracellular V-like domain of BTN3A1. Such an event implies that IPP crosses an endosomal membrane or the plasma membrane. Data in our laboratory indicate that a dedicated transporter is required for presentation of endogenous IPP. Remarkably, the transporter is required only when IPP accumulates within the APC and not when it is provided exogenously.

Although great advancement has been achieved in understanding the mechanisms of V γ 9V δ 2 cell activation and phosphoantigen presentation, many questions remain. For example, how is the surface expression of BTN molecules regulated, in which cellular compartment do they traffic, do they associate with other molecules intracellularly and/or on the plasma membrane, and how do phosphoantigen antagonists inhibit BTN3A1? As members of the BTN3 family have been found to interact with other surface proteins, which are not $\gamma\delta$ TCR and are not broadly expressed (84), it will be also important to identify these other partners and investigate their role in T cell responses.

The light shed by BTN3A1 on the conundrum of antigen presentation to V γ 9V δ 2 cells has exposed only the tip of an iceberg. It will be very interesting to follow the evolution of this field and the implications of BTN in stimulating other types of $\gamma\delta$ cells.

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Sensing of pyrophosphate metabolites by V γ 9V δ 2 T cells

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The predominant population of $\gamma\delta$ T cells in human blood express a T cell receptor (TCR) composed of a V γ 9 (V γ 2 in an alternate nomenclature) and V δ 2 domains. These cells came into the limelight when it was discovered they can respond to certain microbial infections and tumorigenic cells through the detection of small, pyrophosphate containing organic molecules collectively called “phosphoantigens” or “pAgS.” These molecules are intermediates in both eukaryotic and prokaryotic metabolic pathways. Chemical variants of these intermediates have been used in the clinic to treat a range of different cancers, however, directed optimization of these molecules requires a full understanding of their mechanism of action on target cells. We and others have identified a subclass of butyrophilin-related molecules (BTN3A1-3) that are directly involved in pAg sensing in the target cell, leading to engagement and activation of the T cell through the TCR. Our data and that of others support the pAg binding site to be the intracellular B30.2 domain of BTN3A1, which is the only isoform capable of mediating pAg-dependent stimulation of V γ 9V δ 2 T cells. Here, we review the data demonstrating pAg binding to the B30.2 domain and our studies of the structural conformations of the BTN3A extracellular domains. Finally, we synthesize a model linking binding of pAg to the intracellular domain with T cell detection via the extracellular domains in an “inside-out” signaling mechanism of the type characterized first for integrin molecule signaling. We also explore the role of V γ 9V δ 2 TCR variability in the CDR3 γ and δ loops and how this may modulate V γ 9V δ 2 cells as a population in surveillance of human health and disease.

Keywords: V γ 9V δ 2, phosphoantigens, T cells, T cell receptor, butyrophilins, B30.2

Gamma delta T cells represent a conundrum when trying to understand the mechanisms of T cell ligand recognition that results in T cell activation. T cells expressing $\alpha\beta$ T cell receptors (TCRs) that develop normally in the thymus recognize all antigens with the requirement of an antigen-presenting molecule belonging to the MHC superfamily. This MHC requirement encompasses conventional $\alpha\beta$ T cell recognition of classical class I and class II MHC molecules as well as innate-like or semi-invariant T cell recognition of non-classical or MHC-like molecules. Examples include Type I invariant Natural Killer T (iNKT) and Type 2 NKT cell recognition of CD1d, Mucosal Associated Invariant T (MAIT) cell recognition of MR1, and non-conventional $\alpha\beta$ T cell recognition of the human Group 1 CD1s (1). While these different T cell types recognize their various MHC ligands with diverse footprints, the fact remains that they are all “restricted” to recognizing antigens in the context of their respective MHCs.

This same MHC requirement does not appear to hold true for $\gamma\delta$ T cells. While defining ubiquitous antigens for this lineage of T cells have been challenging, a clearer perspective has started to emerge with recent breakthroughs in antigen definition for these cells. While a comprehensive survey of these results is not the focus of this review and has been discussed elsewhere (2, 3), what has emerged from these studies is that $\gamma\delta$ T cell are specific for both MHC and non-MHC proteins. To first understand

this conundrum it is important to emphasize that $\gamma\delta$ T cells cannot be grouped together as a whole. Instead, $\gamma\delta$ T cells are divided into many different populations with different antigen reactivities, effector functions, and tissue residence (4, 5). Another important point is that there is little, if any, homology between $\gamma\delta$ T cell populations in mice with those in humans, suggesting that these cells have rapidly adapted to different antigenic stimuli or immunological environments in the two different hosts. This is supported by the observed rapid evolution of many of the V γ genes within the primate lineage (6, 7). Recent work from our laboratory has focused on two very different $\gamma\delta$ T cell populations in humans: first, those that do recognize antigens in the context of CD1 molecules, which has been reviewed elsewhere (8); and secondly a population that appears to be MHC-independent and instead responds to small pyrophosphate antigens called “phosphoantigens (pAgS).” This second population, called “V γ 9V δ 2”, “V γ 2V δ 2”, or “ γ 2 δ 2” by different groups, called V γ 9V δ 2 here, is the topic of this review. Recent breakthroughs by several groups have started to reveal the complex mechanism behind pAg regulation of this cell population. These findings have led to a shift in the paradigm of what specificities regulate T cell activity and a better understanding of the molecular mechanisms behind regulation of this important T cell population in humans.

V γ 9V δ 2 T cells are the major subset of $\gamma\delta$ T cells found in human blood, comprising up to 5% of the T cells in healthy individuals and expanding to 20–50% during infection or disease (9). These cells play important roles in mediating immunity against microbial pathogens, including *Mycobacterium tuberculosis* and *Mycobacterium leprae* [the causative agents of tuberculosis and leprosy, respectively, reviewed in Ref. (10)], and can respond potently against certain types of tumor cells (11, 12). No homologous pAg-reactive V γ 9V δ 2 T cell population has been identified in rodents or lagomorphs, however, genes homologous to both V δ 2 and V γ 9 have been identified in other placental mammalian species including sloth, armadillo, lemur, aye aye, bottlenose dolphin, killer whales, and horse (13). Furthermore, expression of V γ 9V δ 2 TCRs was demonstrated in alpacas. This suggests that V γ 9V δ 2 T cells are present in species outside the primate lineage and likely predate the split of the placental mammals. The lack of V γ 9V δ 2 T cells in rodents and lagomorphs demonstrate that this lineage has been lost in some species, perhaps compensated by selection for alternative T cell subtypes.

As mentioned above, V γ 9V δ 2 T cells represent an important departure from the classical T cell recognition paradigm, in that no MHC or MHC-like molecules have been implicated in their activation (14). Instead, the aforementioned pAgs (Figure 1), which are pyrophosphate containing metabolites, are the key trigger (15–18). Amongst these, isopentenyl pyrophosphate (IPP) (16, 19) is generated from the endogenous mevalonate (MVA) pathway (HMG-CoA, cholesterol biosynthesis) and accumulates intracellularly during dysregulated metabolism in many types of tumor cells. Addition of aminobisphosphonates like zoledronate (NBP) or alkylamines also causes intracellular IPP accumulation through inhibition of farnesyl pyrophosphate synthase (12, 20, 21); this strategy is used frequently in studies of V γ 9V δ 2 T cell stimulation. A much more potent set of pAgs (i.e., HDMAPP/HMBPP; hydroxy-methyl-butyl-pyrophosphate) are microbial metabolites from the isoprenoid pathway (17) and represents “non-self” pathogen signals. A synthetic pAg, bromohydrin pyrophosphate (BrHPP) also strongly activates V γ 9V δ 2 T cells and is often used in *in vitro* functional experiments (22). The pAg-induced recognition of target cells is TCR dependent, as V γ 9V δ 2 TCR transfected Jurkat cells become activated by pAgs (23). While no direct interaction has been detected between pAgs and the $\gamma\delta$ TCR, cell-to-cell contact is necessary in pAg-induced $\gamma\delta$ T cell activation (14, 24), indicating that molecules expressed on the cell-surface of target cells or $\gamma\delta$ T cells are required for activation.

FOCUS ON BUTYROPHILINS AS KEY PLAYERS IN THE V γ 9V δ 2 T CELL RESPONSE TO pAgs

A major breakthrough in our understanding of V γ 9V δ 2 T cell activation came with the identification of the butyrophilin-3 (BTN3) protein family as a key mediator in this process (25). BTN3 proteins, also known as CD277, are type I membrane proteins with two immunoglobulin (Ig)-like extracellular domains (IgV and IgC) (26, 27) (Figure 2A) with close structural homology to the B7-superfamily of proteins. BTN3A molecules are members of a much larger butyrophilin superfamily with diverse roles in host homeostasis (28, 29). A key factor in the initial discovery of the role of BTN3A in V γ 9V δ 2 activation was the serendipitous discovery that

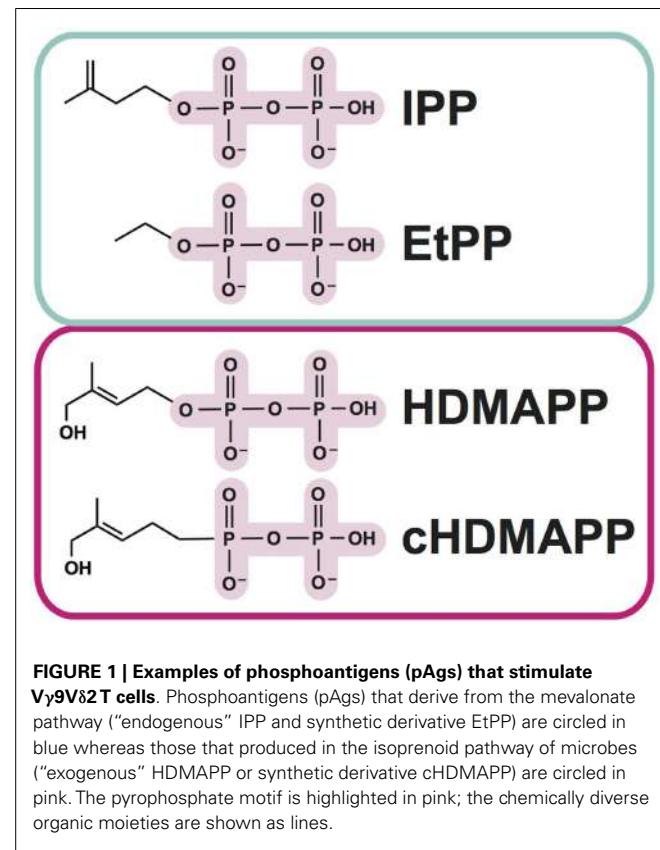
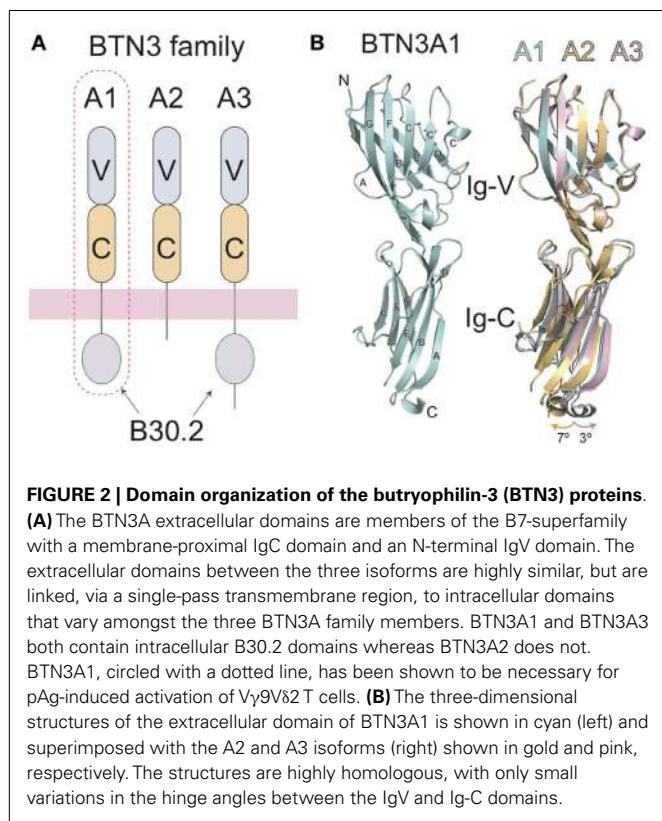


FIGURE 1 | Examples of phosphoantigens (pAgs) that stimulate V γ 9V δ 2 T cells. Phosphoantigens (pAgs) that derive from the mevalonate pathway (“endogenous” IPP and synthetic derivative EtPP) are circled in blue whereas those that produced in the isoprenoid pathway of microbes (“exogenous” HDMAPP or synthetic derivative cHDMAPP) are circled in pink. The pyrophosphate motif is highlighted in pink; the chemically diverse organic moieties are shown as lines.

a mouse antibody (clone 20.1), raised against human BTN3A molecules, caused a surprising proliferation and expansion of $\gamma\delta$ T cells in IL-2 supplemented peripheral blood mononuclear cell (PBMC) cultures (25). The presence of this antibody elicited production of IFN- γ , TNF- α , and upregulation of the activation marker CD69 and has recently been shown to elicit very similar intracellular signaling in V γ 9V δ 2 T cells as pAgs (30). This phenomenon was restricted to the V γ 9V δ 2 population in PBMCs, with no effect on $\alpha\beta$ T cells or those $\gamma\delta$ s not expressing a V γ 9V δ 2 TCR. Consistent with the lack of MHC requirement for stimulation of this $\gamma\delta$ T cell population, addition of the 20.1 antibody to a panel of human tumor/transformed cell lines, some of which lack MHC surface expression, induced potent activation of responding V γ 9V δ 2 T cells (25). To rule out an effect of BTN3A expressed on V γ 9V δ 2 T cells, murine V γ 9V δ 2 TCR transductants, which do not express BTN3A molecules, were used as effector cells and shown to also respond to these 20.1 treated target cells. This experiment also confirms the requirement for the V γ 9V δ 2 TCR, supporting previous studies in V γ 9V δ 2 Jurkat transfectants (23). Similar results with the 20.1 Ab were found by another group (31). Other approaches also confirmed the role of BTN3A molecules in V γ 9V δ 2 T cell activation; taking a genetic approach, Vavassori et al. mapped genetic elements required for V γ 9V δ 2 T cell activation to the 3- to 27.4-Mb interval of human chromosome 6 and further refined their candidates by screening only those coding regions that had a predicted transmembrane element (32). Included within these candidates were the BTN3A molecules.



Three isoforms of BTN3A are present in humans, BTN3A1, BTN3A2, and BTN3A3, each encoded by a separate gene (26). The extracellular domains of the BTN3A molecules are highly sequence and structurally homologous, with only minor variations observed in the hinge angle between the IgV and IgC domains of their crystal structures when the three extracellular domain structures are superimposed (27) (Figure 2B). All three BTN3A isoforms are recognized by the 20.1 antibody and can mediate 20.1 mAb-induced activation of V γ 9V δ 2 T cells (25, 27), suggesting that a shared epitope on BTN3A molecules is involved in the process of V γ 9V δ 2 stimulation. Curiously, a different BTN3A specific antibody, 103.2, had an antagonistic effect on pAg-mediated V γ 9V δ 2 stimulation after addition to target cells, suggesting that it either blocks an epitope on the BTN3A extracellular domain or induces or stabilizes a non-stimulatory conformation of BTN3A on the cell-surface (25).

In the crystal structures of the BTN3A extracellular domains, two dimeric interfaces were observed (27), one that would generate a symmetric V-shaped homodimer positioning the C-terminal transmembrane domains close together (Dimer 1, Figure 3) and the other a head-to-tail homodimer with an asymmetric dimer interface, requiring the BTN3A molecules to lay flat, parallel to the cell-surface (Dimer 2, Figure 3). Both dimer interfaces were of appreciable size, Dimer 1 buried \sim 1520 Å 2 whereas Dimer 2 buried \sim 1080 Å 2 . Both dimer interfaces were also highly conserved between the three BTN3A isoforms; only 2 out of the 18 interface residues in Dimer 2 differed between the BTN3A isoforms. However, the Dimer 2 interface was observed in the crystal structures of all three BTN3A isoforms indicating these

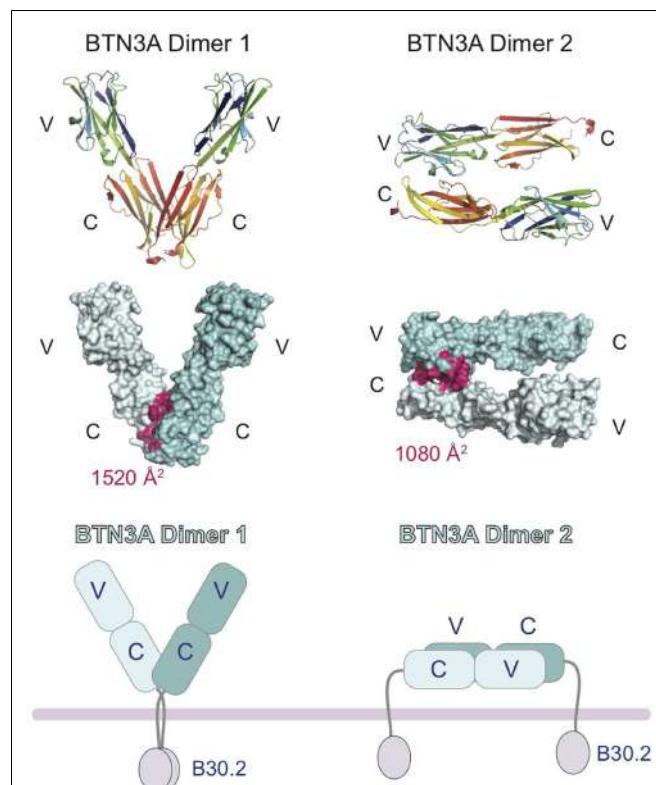


FIGURE 3 | Cartoon representation of the domain organization of the butyrophilin-3 (BTN3) proteins. Structures of the extracellular domains of the BTN3A1 proteins shown in the two dimeric states present in the crystal lattice. Dimer 1 (left) associates via the IgC domains and forms a V-shaped dimer, placing the intracellular B30.2 domains in close proximity to each other. Dimer 2 (right) associates in an head-to-tail fashion with the IgV domain of one BTN3A monomer interacting with the IgC domain of another. This would result in the dimer laying parallel to the cell-surface, with the intracellular B30.2 domains separated. The interface contact residues are colored pink and shown on the surface representation of the two dimeric forms (middle panel). The buried surface area (BSA) is shown for both dimers.

differences were tolerated. Residues involved in the Dimer 1 interface differed at three positions across the three BTN3A isoforms although examination of the contacts in this interface revealed that these interactions involved only main chain atoms, thus tolerating variation in the composition of the side chain residues. This suggests that these extracellular domains can form heterodimers adopting both dimeric conformations when co-expressed on the cell-surface. Using soluble extracellular domains, we were able to establish that BTN3A molecules exist as stable homodimers in solution and, using a FRET approach, that the dimer conformation in solution was Dimer 1 (27). This does not, however, rule out the possibility that both dimers can exist on the cell-surface, perhaps stabilized through the transmembrane or intracellular domains not present in the soluble molecules.

Insight into the binding sites and mode of action of the 20.1 and 103.2 antibodies was revealed with the complex crystal structures of single-chain versions of these antibodies (containing just the antigen-binding V domains) in complex with BTN3A1 (27).

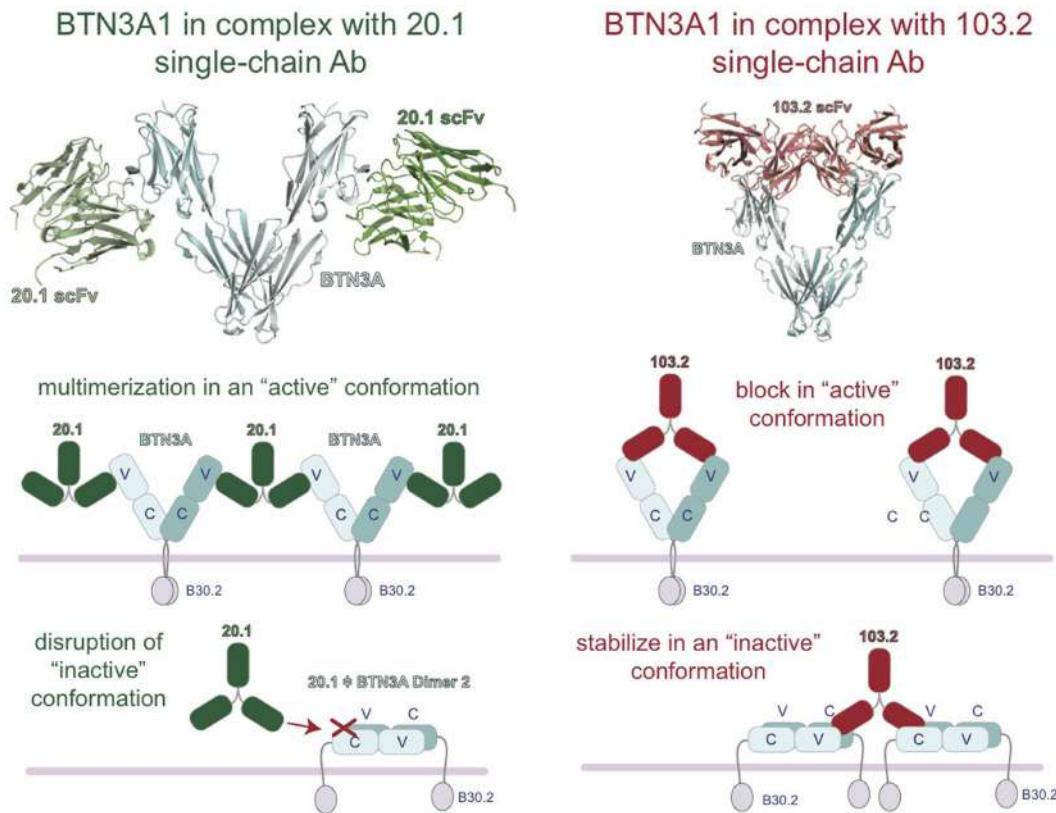


FIGURE 4 | Model of the regulation of BTN3A architecture by the agonist 20.1 and antagonist 103.2 antibodies. Structures of the extracellular domains of the BTN3A proteins (cyan) in complex with agonist (20.1, green) and antagonist (103.2, red) antibody single chains (scFv). The 20.1 antibody cannot "reach" across a BTN3A dimer 1 to occupy both binding sites and therefore is likely to multimerize BTN3A

molecules on the cell-surface (left). The 20.1 antibody binds to the Dimer 2 interface of the IgV domain and therefore would disrupt the Dimer 2 conformation on the cell-surface. The 103.2 antibody can bind both Dimer 1 and Dimer 2 conformations, either potentially blocking the activating Dimer 1 form or stabilizing the "inactive" Dimer 2 form on the cell-surface.

These complex structures demonstrated that these two antibodies bind to separate epitopes on the BTN3A surface (Figure 4), a result confirmed by competition-binding assays performed by Surface Plasmon Resonance (SPR). Curiously, the 20.1 antibody binding site positions the antibody such that it cannot bind bivalently to one BTN3A dimer as the two binding sites are too distant. For both 20.1 antibody binding sites to be occupied in the Dimer 1 conformation would require engagement of two separate BTN3A homodimers. Thus, binding of the 20.1 antibody could effectively cross-link these molecules on the cell-surface. Also interesting was the finding that the 20.1 binding site overlaps with that of the Dimer 2 interface, suggesting that binding of the 20.1 antibody would compete with the Dimer 2 conformation (Figure 4) and instead select for, and stabilize, the Dimer 1 conformation. The 103.2 epitope is accessible in both Dimer 1 and Dimer 2 conformations; in contrast to the 20.1 antibody, 103.2 would likely bind with both binding sites to one BTN3A Dimer 1, but would have to cross-link BTN3A molecules in the Dimer 2 conformation.

These results lead us to propose a model whereby these two dimeric states are related to the stimulatory potential of the cell upon which they are expressed. In normal, non-stimulatory

conditions, BTN3A molecules would exist in the Dimer 2 state (head-to-tail) and thus not be in a state to provide a stimulatory signal to surveying V γ 9V δ 2 T cells. Upon addition of the 20.1 antibody, BTN3A molecules in the Dimer 2 conformation would be converted to Dimer 1; these would be cross-linked on the cell-surface via binding of one 20.1 antibody to two BTN3A dimers, and thus be converted into a "stimulatory" conformation permissible to stimulate V γ 9V δ 2 cells (Figure 4). The potential ability of the 20.1 antibody to cross-link BTN3A molecules in this model is consistent with the observed immobilization of BTN3A molecules via Fluorescence Recovery after Photobleaching (FRAP) that occurs during conversion of a cell from a non-stimulatory to stimulatory state (25). This model also proposes that addition of 103.2 antibody could either block a site on BTN3A required for V γ 9V δ 2 cells activation or stabilize the Dimer 2 conformation on the cell-surface (Figure 4), thus leading to the inhibitory activity observed when this antibody is added in conjunction with pAg.

But what is the role of pAg in this process? Failed efforts to show a direct interaction between the V γ 9V δ 2 TCR and pAg early on suggested additional players were involved in this process; the requirement of cell-cell contact for V γ 9V δ 2 T cell stimulation

also supported this hypothesis (14). Based on recent published results, two general models have been proposed to explain how pAg functions to stimulate V γ 9V δ 2 T cells. The first model is tantalizingly simple; it describes the extracellular domain of BTN3A molecules as “antigen-presenting” whereby BTN3A molecules associate with pAg and “present” it directly to the V γ 9V δ 2 TCR (32). While this model would fit well with the requirement of an antigen-presenting molecule for $\alpha\beta$ T cell recognition of antigen, this model has met with controversy and is not supported by data generated from several groups and discussed further below. Model 2 is based on the finding that only one of the three BTN3A isoforms (BTN3A1) can support pAg-mediated V γ 9V δ 2 activation. This was demonstrated through siRNA knock-down experiments and reintroduction of individual BTN3A1, BTN3A2, or BTN3A3 isoforms; BTN3A1 alone was found to be pAg-reactive (25). This suggests that there is a unique element to this isoform that alone can initiate stimulation in a pAg specific way. Domain deletion and swapping experiments gave the first indication of the identity of this unique element: BTN3A1 lacking its intracellular domain failed to mediate pAg-mediated V γ 9V δ 2 stimulation but was highly stimulatory upon addition of the 20.1 antibody. BTN3A3, which cannot support pAg-mediated stimulation of V γ 9V δ 2 T cells, was made pAg stimulatory by swapping of its intracellular domain with that of A1 (31, 33). These data strongly support a pivotal role of the intracellular domain of the BTN3A1 isoform in pAg-mediated V γ 9V δ 2 stimulation. Model 2 is based on these findings and focuses on the intracellular domain of BTN3A1 as the pAg sensor.

The three BTN3A molecules differ substantially in their intracellular domains; A1 and A3 each contain a B30.2 domain

(also known as PRY/SPRY domains) whereas A2 lacks this domain (**Figure 2**). The B30.2 domains found in A1 and A3 are highly homologous, with 87% amino acid identity between the two (**Figure 5**). The intracellular region of A3, however, has a unique 70 amino acid extension C-terminal to its B30.2 domain (**Figures 2 and 5**). B30.2 domains are classified as protein–protein interaction domains and are found in other butyrophilin family members as well as non-related proteins (over 50 genes in the human genome have predicted B30.2 domains). Many B30.2 domain-containing proteins have been reported to be important in immune function, including the TRIM and pyrin families (34), although in most cases the binding partners have not been characterized. The importance of the B30.2 domain in pAg sensing was first demonstrated through swapping of just this domain between the A1 (capable of pAg activation) and A3 (incapable of activation) isoforms (33). Introduction of the A1 B30.2 domain into the A3 isoform converted this isoform to stimulatory for V γ 9V δ 2 T cell in the presence of pAg, whereas, the reverse swap (A3-B30.2 into A1 isoform) abrogated its ability to stimulate V γ 9V δ 2 T cells in a pAg-dependent fashion.

INTRACELLULAR B30.2 DOMAIN OF BTN3A1 AS THE pAg SENSOR

Direct interactions between both endogenous and exogenous pAgS with the B30.2 domain of BTN3A1 were measured with a highly sensitive technique called Isothermal Titration Calorimetry (ITC), which measures the heat absorbed or lost during binding events (33, 35). The affinities calculated from these techniques ($KD = \sim 1 \mu M$ for exogenous pAg, $\sim 1 mM$ for endogenous pAg) also reflected the functional potency of these compounds.

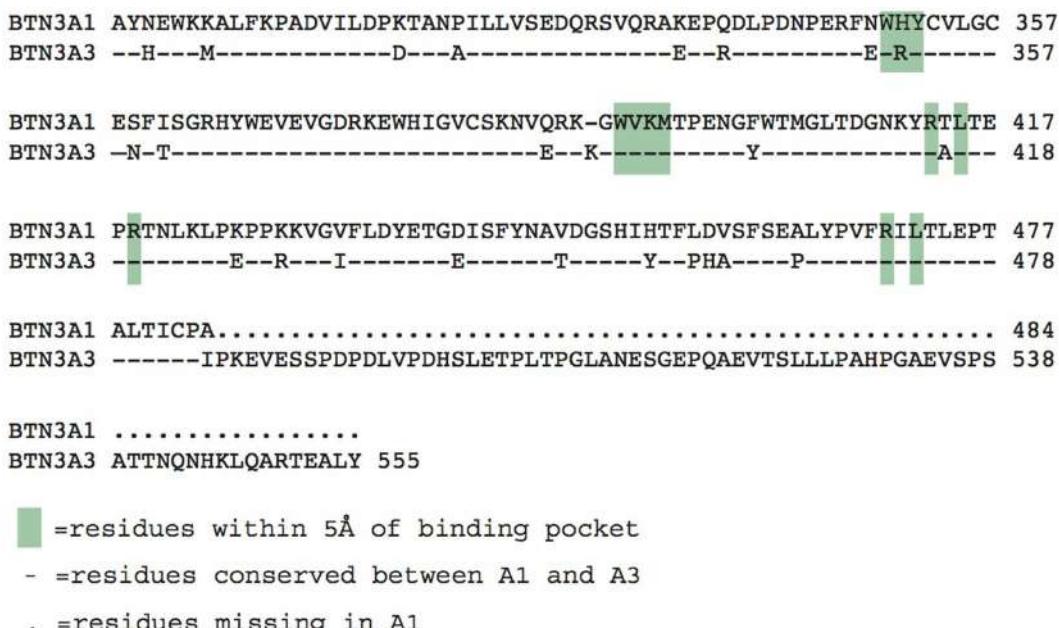
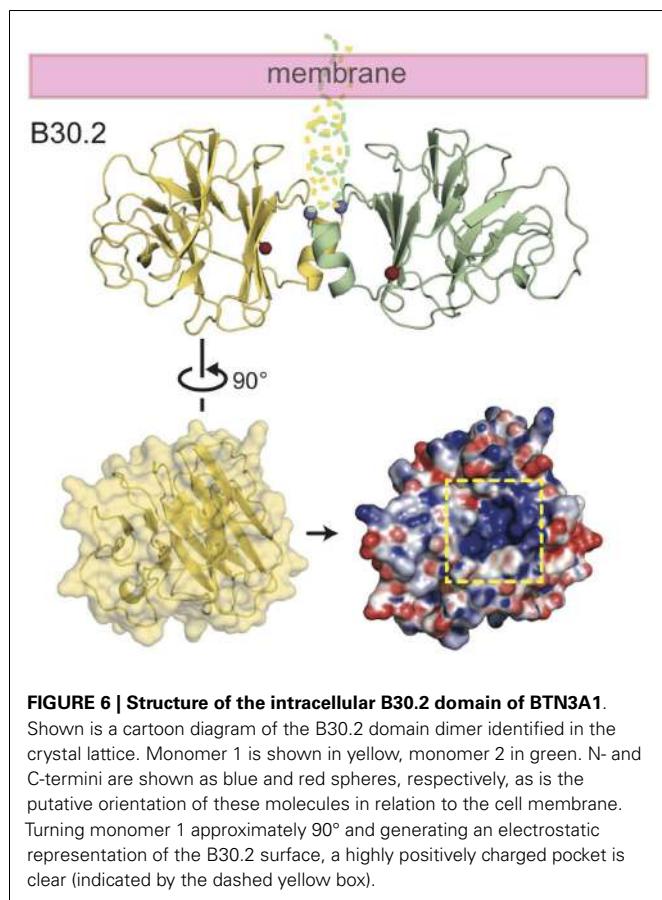


FIGURE 5 | Alignment of the intracellular B30.2 domains from BTN3A1 and BTN3A3. Amino acids are shown in the single letter designations, BTN3A1 is the consensus. “-” indicates positions of identity with BTN3A1.

differences are shown in their single letter abbreviations. Green boxes indicate residues within 5 Å of the phosphoantigen binding pocket. BTN3A3 has an additional polypeptide extension.



in mediating stimulation of V γ 9V δ 2 T cells. The endogenous IPP pAg is typically 1,000-fold weaker potency than that of the exogenous HMBPP (36). Association studies with the HMBPP pAg were also shown via chemical shift perturbations (CSP) via Nuclear Magnetic Resonance (NMR), an equally sensitive technique (35). Of note, no association of pAg could be measured with the BTN3A3-B30.2 domain, or to the extracellular domains of BTN3A1, A2 or A3, with either of these techniques (33, 35).

The crystal structure of the B30.2 domain of BTN3A1 (Figure 6) was highly informative in deciphering the pAg binding site (33). The structure of BTN3A1 B30.2 domain was highly homologous to previously reported B30.2 domains, in particular the B30.2 domain of Trim21, an intracellular Fc receptor (37). Importantly, specific to the BTN3A1 B30.2 domain was a highly positively-charged (basic) pocket nestled in Sheet A of the structure (Figure 6). This pocket was lined with basic residues including arginines (R412, R418, and R469), histidines (H351 and H378) and a lysine (K393) (Figure 7). The charge complementarity between the B30.2 positively charged pocket and the negative charge of pAg made this an excellent candidate for pAg binding.

Charge swapping mutagenesis studies, where the basic residues were mutated to acidic (negatively charged), completely abrogated pAg binding and reactivity in cell stimulation assays, providing compelling evidence that this indeed was the pAg binding pocket (33). However, these results did not explain entirely the differences of pAg binding to the A1 versus A3-B30.2 domains. Close

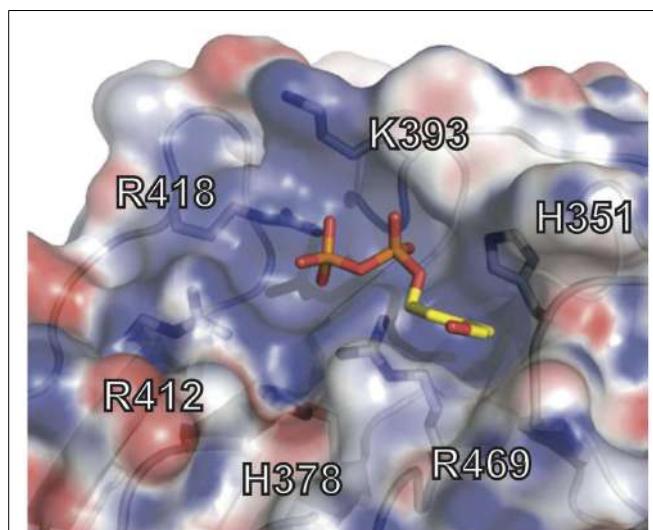


FIGURE 7 | Phosphoantigen binding pocket of B30.2 domain. Close-up view of the B30.2 pAg binding pocket with the side chains lining the pocket shown under the semi-transparent surface. The positions are labeled with the numbering relative to the full-length BTN3A1 molecule. The pAg is shown as sticks, modeled into the binding pocket; phosphates are colored orange and red (oxygen) and the organic moiety is shown in yellow.

examination of the amino acid differences between these isoforms revealed a single amino acid difference that lay within the binding pocket: position 351 was a histidine in A1 and an arginine in A3 (Figure 5). Swapping of this single amino acid difference between the domains (i.e., mutating the H to R in A1 and R to H in A3) transferred both pAg binding ability and functional ability to stimulate V γ 9V δ 2 T cells. Position 351 is quite buried within the pAg binding pocket; it is likely that the size and shape of the side chain difference from an H to an R changes the architecture of the binding pocket such the pAg thus characterized do not bind to A3. This raises the possibility that there are other pyrophosphate compounds yet to be described that may preferentially bind to A3 over A1. It is unclear as to the role, if any, of the A2 and A3 isoforms in V γ 9V δ 2 T cell activation; their potential ability to form heterodimers with A1, assemble with alternative antigens via their B30.2 domain (in the case of A3) and modulate cell-surface assembly with other protein binding partners is an area of active investigation.

Additional insight into the mechanics of pAg binding to the B30.2 domain, pursued through crystallization and NMR experiments, have revealed evidence for a conformational change induced in the B30.2 domain upon pAg binding. The first insight into this was during our pursuit of a complex structure between the B30.2 domain and pAg where we attempted to “soak” pAg into already existing crystals (33). (This is a common approach for studying small molecule binding sites in proteins.) While protein crystals appear solid, they contain a substantial amount of liquid that forms solvent channels between the protein molecules. Thus, small molecules (such as pAg) can move freely in the crystal lattice and bind to their appropriate binding site in the protein as it is locked in the crystal lattice. This methodology assumes

that binding of the small molecule does not induce changes in the conformation of the protein as this can disrupt the packing of the protein in the crystal lattice and cause the crystals to dissolve. Soaking of B30.2 domain crystals with pAgS did just this, causing the crystals to dissolve immediately upon addition. Locking of the protein–protein contacts within the crystal lattice via covalent crosslinking via glutaraldehyde preserved the crystal structure and allowed a complex structure of pAg and B30.2 to be resolved. Within this structure there is clear tetrahedral electron density for the beta- and alpha- phosphates of the pAg within the binding pocket, however, the organic portion of the pAg could not be resolved (33). More direct evidence for a conformational change is seen in CSP observed in the Heteronuclear single quantum coherence spectroscopy (HSQC) of apo (empty) B30.2 versus that with added HMBPP (35). Thus, it is clear that binding of pAg to the B30.2 domain induces structural rearrangements/conformational changes that we hypothesize is the first in a cascade of intracellular and extracellular events leading to target cell transmission of a stimulatory signal to the V γ 9V δ 2 TCR.

This model of intracellular sensing of pAgS is consistent with the fact that many of the physiologically relevant pAgS are first generated and accumulate inside target cells. Endogenous pAgS, such as IPP or DMAPP, are intermediates of the MVA pathway, which is conserved in eukaryotes and archaea for isoprenoid biosynthesis (38). It has been reported that these pAgS accumulate intracellularly during dysregulated metabolism in many types of human tumor cells. For example, overexpression of HMG-CoA reductase, the rate limiting enzyme of the MVA pathway, in the non-Hodgkin B cell lymphoma cell-line Daudi and mammary cancer cells such as breast adenocarcinoma cells, can cause an increased level of IPP that is then recognized by V γ 9V δ 2 T cells (12, 39). Manipulation of the MVA pathway by various synthetic drugs (such as statins and aminobisphosphates) or short hairpin RNAs targeted to enzymes either upstream or downstream of IPP production can trigger or suppress pAg-induced T cell activation, supporting the idea that V γ 9V δ 2 T cells can sense intracellular IPP levels (12, 20, 21, 40). Another set of pAgS derive from exogenous microbial sources and are also much more potent. These metabolites, such as HMBPP from the microbial methylerythritol phosphate (MEP) pathway, exist in bacteria and several photosynthetic eukaryotes (38). Some microbes producing these pAgS are intracellular pathogens such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, which can enter and survive within the host cells (41, 42). Immune cells such as monocytes, macrophages and dendritic cells can also engulf these pathogens and elicit pAg-dependent T cell responses (43–46). Extracellular pathogens like *Escherichia coli* can also be phagocytosed by immune cells like neutrophils (47). The subsequent V γ 9V δ 2 T cell response is strictly dependent on the ability of the phagocytosed pathogens to produce HMBPP. A more recent study also demonstrated that after T cell priming, these pathogen-harboring neutrophils develop an antigen-presenting phenotype (48). These studies suggest that the presence of exogenous pAgS, *intracellularly*, is critical for V γ 9V δ 2 T cell activation and function. Admittedly, exogenous pAgS can also be secreted by some extracellular pathogens or immune cells like neutrophils, and administration of soluble pAgS in the presence

of BTN3A-expressing antigen-presenting cells trigger V γ 9V δ 2 T cell activation, as was demonstrated in early studies of these T cells (24). It is unknown how these extracellular pAgS get internalized, giving that their negatively charged features render direct membrane permeability unlikely. Possible mechanisms for passing through the plasma and endocytic membranes may include specific membrane transporters or charge neutralization by ester formation.

PHOSPHOANTIGEN STRUCTURE AND BIOACTIVITY

The variable chemical structures of pAgS relate directly to their bioactivity. The pyrophosphate moiety is central since its strong negative charge can make electrostatic contacts with the positively charged binding pocket of the B30.2 domain. Indeed, monophosphate substituents have significantly reduced specific activity compared with their pyrophosphate counterparts (49). On the other hand, large chemical groups like AMP can be added without affecting the bioactivity of pAgS (49) and, in fact, some natural pAgS are nucleotidic conjugates (50, 51) that may be processed by specific antigen-presenting cells. More intriguingly, it has been found that hydrolysis of the pyrophosphate moiety is associated with pAg bioactivity and non-hydrolyzable analogs of pAgS can even inhibit the T cell activation (52). These features suggest that either pre- or post-processing of pAgS can occur before or after association with the B30.2 domain and may have important implications for T cell activation.

Since the pyrophosphate moiety is essentially the same for every pAg, the potency of pAgS is largely dependent on their organic moieties. Changes in the length of the alkyl chain and positions of the double bond, even though very subtle, can lead to dramatic change in potency (49). Notably, the structural difference between endogenous ligand IPP and exogenous HMBPP only lies in the additional hydroxyl group on HMBPP, yet the potency and binding affinity of this strong ligand increases by about 1000-fold. One possible explanation that was explored early on was the chemical reactivity of pAgS. The polarizability of the C3 substituent correlates with bioactivity: an increased specific activity for T cells was observed upon addition of a halohydrin to the C3 position (52). This leads to an interesting speculation that a covalent reaction may occur between the pAg and its binding partner. More recently, some studies have pointed out that the chirality of the pAgS may also play a role in their bioactivity. It has been found that E-stereoisomers are much more potent antigens for T cell activation (53). Potential intramolecular hydrogen-bonding states of these isomers affect the overall shape of the ligand and may be important for binding to the B30.2 domain. Most of the studies regarding the structure of pAgS were conducted before the identification of the B30.2 domain of BTN3A1 as the binding partner; optimization of binding to this domain has already begun with exciting implications for pAg-based therapeutics (35).

PHOSPHOANTIGENS SIGNAL THROUGH AN INSIDE-OUT MECHANISM VIA THE B30.2 DOMAIN

Of course, the detection of pAgS intracellularly needs to translate to a signal that can be detected by surveying V γ 9V δ 2 T cells. We and others have proposed an inside-out signaling model

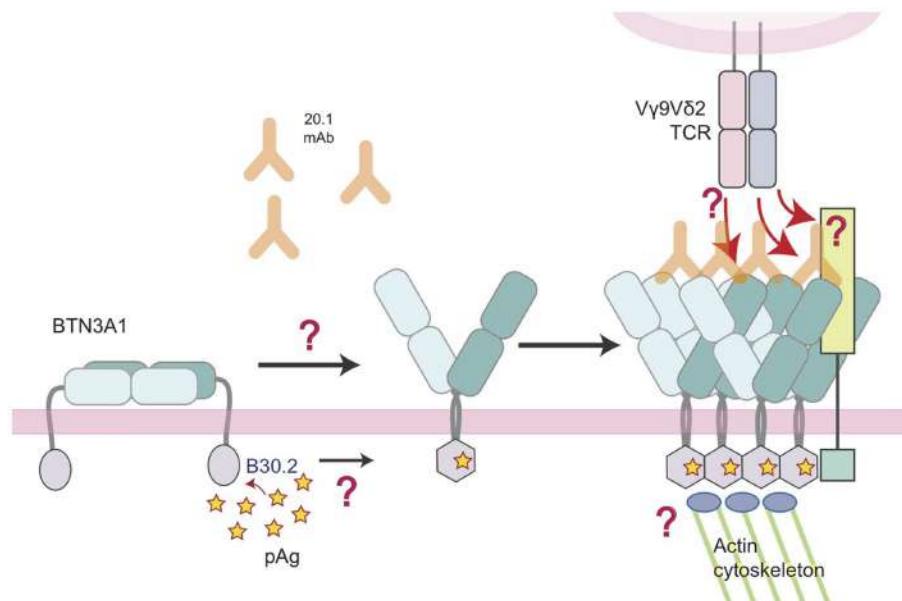


FIGURE 8 | Model of the molecular changes that occur in BTN3A molecules upon addition of 20.1 antibody or detection of accumulating intracellular pAg. In this model, we propose that BTN3A molecules exist on the surface of a healthy, unaffected cell in an inactive state, perhaps in a conformation similar to the dimer 2 visualized in the crystal lattice (left). Upon addition of the 20.1 antibody, dimer 2 is destabilized and BTN3A molecules are converted/stabilized in the dimer 1 conformation. An increase in the intracellular pAg concentration has a similar effect except the dimer conversion is mediated by changes in the intracellular B30.2 domain, which

undergoes a conformational change upon pAg binding (hexagonal shape). This conformational change induces structural reorganization of BTN3A molecules, either via immobilization through B30.2 association with the actin cytoskeleton or B30.2 multimerization of BTN3A molecules, which is then signaled by an inside-out mechanism to change the architecture of the BTN3A extracellular domains. This architectural change could alone be the signal that V γ 9V δ 2 TCRs recognize, or recruitment of an additional, human-specific accessory molecule (lime-green square) occurs, which directly engages the V γ 9V δ 2 TCR.

(31, 33) whereby the binding of pAg intracellularly is translated extracellularly for detection by the V γ 9V δ 2 TCR. This could be through several means that work individually or in concert to initiate TCR recognition: (1) immobilization/clustering of BTN3A that increases the avidity for the TCR, (2) a conformational change of the BTN3A extracellular domains from non-stimulatory (Dimer 2) to stimulatory (Dimer 1), or (3) the two previous situations resulting in the recruitment of an additional factor that directly engages the V γ 9V δ 2 TCR. Number 1 and 2 invoke a direct interaction between V γ 9V δ 2 TCRs and the extracellular domains of BTN3A whereas version 3 involves an unknown accessory protein that is the true V γ 9V δ 2 TCR ligand (Figure 8). Whether BTN3A is directly recognized by the V γ 9V δ 2 TCR is controversial; Vavassori and colleagues were able to measure an interaction between the IgV domain of BTN3A1 and a V γ 9V δ 2 TCR used in their studies (32) while we have not been able to do the same with the G115 V γ 9V δ 2 TCR and the full-length extracellular domain of BTN3A1 (33). In addition, we have not been able to stimulate V γ 9V δ 2 T cells with murine transfected BTN3A1 either through 20.1 antibody addition or through pAg treatment (33) whereas others, using a different V γ 9V δ 2 TCR have observed stimulation via 20.1 Ab treatment of BTN3A1 transfected Chinese-hamster ovary (CHO) cells (54). pAg-mediated stimulation was only observed in CHO cells containing human Chromosome 6. These conflicting results represent probably the most important conundrum in the V γ 9V δ 2 T cell field at present. BTN3A

molecules are necessary but are they sufficient for V γ 9V δ 2 recognition? Evidence for other molecules playing a role in V γ 9V δ 2 recognition and activation such as HSP-60 (55) and F1-ATPase with or without ApoA1 (56) suggest this system could be a complicated coordination of many molecular players. Further coordination through adhesion molecules, activating and inhibitory Natural Killer receptors, Toll-like receptors (TLRs), and Fc Receptors [reviewed in Ref. (57)] also fine-tune V γ 9V δ 2 activation thresholds and functional responses.

A feature of V γ 9V δ 2 TCRs that may be important in answering the question regarding a direct interaction between the TCR and BTN3A is that despite the fact that this population uniformly use a V γ and a V δ 2 chains in their TCR, there exists considerable diversity within the V γ -J γ and V δ -D δ -J δ rearrangement, which translates into significant amino acid diversity within the CDR3 γ and CDR3 δ loops of these TCRs (58–60). These loops have been shown via mutagenesis to be important for V γ 9V δ 2 T cell activation (60) and sequence variation of these loops between T cell clones (controlled for expression of Natural Killer receptors) translates into a range of different, graded, reactivity (61). It is thus possible that use of V γ 9V δ 2 TCRs with different sequences, and thus different affinities for BTN3A, may be playing a role in the differing abilities to measure interactions between the V γ 9V δ 2 TCR and BTN3A. Alternatively there may be a requirement for additional co-factors to be expressed on target cells in conjunction with BTN3A to fully engage the majority of the V γ 9V δ 2 T cell repertoire.

Clearly, we have just begun to pull the curtain back on the inner-workings of V γ 9V δ 2 T cell ligand recognition and activation; identification of the B30.2 domain as the pAg sensor has already resulted in compounds developed with higher potency *in vitro* (35). Identification of additional players in this process will lead to important therapeutic targets with implications both for the treatment of tumors and microbial infections, but also for minimizing immune-related side effects during the use of bisphosphonates for the treatment for osteoporosis and cancer-related bone fractures.

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Molecules and mechanisms implicated in the peculiar antigenic activation process of human V γ 9V δ 2 T cells

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In human beings, as well as in most non-human primates, the major peripheral $\gamma\delta$ T cell subset, which accounts several percent of the whole lymphoid cells pool in adults, carries an heterodimeric TCR composed of V γ 9 and V δ 2 chains. V γ 9V δ 2 T cells are specifically and strongly activated by small organic pyrophosphate molecules termed phosphoantigens (phosphoAg). These low molecular weight compounds are metabolites that are produced by either microbes or endogenously, as intermediates of the mammalian mevalonate pathway, and can accumulate intracellularly during cell stress like transformation or infection. Despite the characterization of numerous natural and synthetic phosphoAg, the mechanism(s) underlying the unique and specific antigenic activation process induced by these compounds remains poorly understood. Activation is both TCR- and cell-to-cell contact-dependent, and results of previous studies have also strongly suggested a key contribution of membrane-associated molecules of primate origin expressed on target cells. The recent identification of B7-related butyrophilin (BTN) molecules CD277/ BTN3A, and more precisely their BTN3A1 isoforms, as mandatory molecules in the phosphoAg-induced recognition of target cells by V γ 9V δ 2 T cells opens important opportunities for research and applications in this field. Here, we review the unusual and complex antigenic reactivity of human V γ 9V δ 2 T cells. We highlight the recent advances in our understanding of this process, and propose a model that integrates the type I glycoprotein BTN3A1 and its intracellular B30.2 domain as a physical intermediate implicated in the detection of dysregulated intracellular levels of phosphoAg and the sensing of cell stress by V γ 9V δ 2T cells. A better understanding of this mechanism will help optimize novel immunotherapeutic approaches that utilize the unique functional potential of this major $\gamma\delta$ T cell subset.

Keywords: human $\gamma\delta$ T lymphocytes, functions, antigenic activation, phosphoantigens, butyrophilin

$\gamma\delta$ T CELLS AS MANDATORY T CELL SUBSETS OR EVOLUTIONARY RELICS?

T cells are critical component of the adaptive immune system and are essential for defense against foreign organisms and self-dysregulation. T cells can be divided into two lineages, according to the composition of their T cell receptor (TCR). While most $\alpha\beta$ T cells recognize peptidic antigens (Ag) bound to highly polymorphic major histocompatibility complex (MHC) molecules and are qualified as conventional T cells, several T cell subsets react against conserved non-peptidic Ag. Like other adaptive immune effectors, these latter non-conventional or innate-like T cells express clonally distributed Ag recognition receptors, made of either $\alpha\beta$ or $\gamma\delta$ TCR subunits, which are associated to subunits of the CD3 signal transduction complex at the cell membrane. The ontogenetic and developmental features of $\gamma\delta$ T cells, which have been extensively reviewed elsewhere (1–4), will be summarized here. During thymic ontogeny, $\gamma\delta$ T cells emerge before $\alpha\beta$ T cells and are predominant at early stages of fetal development (embryonic days from 14 to 18 in mice). One of the striking hallmarks

that distinguish $\gamma\delta$ from $\alpha\beta$ T cell subsets is their potential for TCR structural diversity, which is considerable despite a highly restricted V gene repertoire. However, this very limited combinatorial diversity of $\gamma\delta$ TCR is efficiently counterbalanced by extensive junctional diversity, due to removal or addition of non-germline-encoded nucleotides at V-(D)-J junctions and alternate D segments reading frames, which allows the generation of a $\gamma\delta$ TCR diversity greater than that of conventional $\alpha\beta$ T cells (theoretically $\sim 10^{16}$ in rodents and human). The extent of TCR junctional diversity can greatly vary from one $\gamma\delta$ T cell subset to another, with several populations expressing highly conserved so-called “invariant” TCRs in some tissue locations, as it is the case for murine V γ 5V δ 1 or V γ 6V δ 1 T cell subsets. An important feature of $\gamma\delta$ T cells is the preferential expression of TCR V regions in distinct tissue locations. In human beings, V γ 9V δ 2 T cells are preferentially found in the peripheral blood where they represent more than 80% of $\gamma\delta$ T cell pool, and about 5% of the CD3 $^+$ cells in healthy adults. By contrast, V δ 2 $^-$ subsets, such as V δ 1 $^+$ and V δ 3 $^+$ subsets, are mainly detected in epithelial tissues (e.g., epidermis and

mucosa), spleen, thymus, and liver. The preferential localization of $\gamma\delta$ T cell subsets might be explained by their capacity to be activated and to expand upon recognition of specific ligands whose expression would be restricted to particular tissues, or to specific homing capacities acquired during intrathymic development (5). In agreement with a peripheral tissue expansion process, absolute numbers of human V γ 9V δ 2 T cells steadily increase in blood during the first years of life as the diversity of their TCR repertoire decreases.

Strikingly and in contrast to mice and humans, $\gamma\delta$ T cells often represent the major circulating lymphocytes in cattle, sheep, pigs, and birds (6), which strengthen the idea that $\gamma\delta$ T cells play a critical role in immune responses to cell stress and infections. However, since the initial characterization of $\gamma\delta$ T cells in the late 1980s, the *raison d'être* of this mysteriously distinct subset of CD3 $^{+}$ T cells within evolved vertebrate species (such as primates and rodents), which already carry innate and adaptive immunity cell subsets, remains unclear. A recent study has revealed that the genetic programs for two primordial T cell-like lineages, oddly similar to $\alpha\beta$ and $\gamma\delta$ T cells, and one B cell-like lineage are found in several species of jawless vertebrates devoid of RAG recombinase and MHC molecules (7). It is not known whether a tripartite adaptive immune system was already present in a common vertebrate ancestor 500 millions years ago, and diverged since then along two distinct phylogenetic lineages, or it appeared two times independently by convergent evolution. Yet this finding strongly argues for a unique role of $\gamma\delta$ T cells, as effectors of the transitional immunity endowed of unique functional properties and/or antigenic specificities.

FUNCTIONAL FEATURES AND ANTIGENIC SPECIFICITIES OF $\gamma\delta$ T CELLS

$\gamma\delta$ T cells have been characterized for their ability to deliver a broad array of effector functions upon activation *in vitro* and *in vivo*. Numerous studies mostly performed in mice, human beings, and non-human primates, indicate that $\gamma\delta$ T cells are implicated in the control of infectious (e.g., bacteria, virus, parasite) diseases, tumor development, homeostasis, wound healing, and tissue repair. The functional features of $\gamma\delta$ T cells have been recently and extensively reviewed elsewhere (3, 4, 8) and will not be detailed in this review.

$\gamma\delta$ T cells, including human V γ 9V δ 2 T cells, can directly kill and eliminate infected, activated, or transformed cells and contribute to pathogen clearance. In these physiopathological contexts, activated $\gamma\delta$ T cells engage pathways associated to the release of cytotoxic and bacteriostatic molecules such as perforin, granzymes, granulysin, and defensins, death-inducing receptors and TNF-related apoptosis-inducing ligand receptors. $\gamma\delta$ T cells are also able to produce modulatory cytokines that play a key role against intracellular pathogens and the promotion of inflammation (e.g., TNF- α , IFN- γ), extracellular bacteria, fungi (e.g., IL-17), and parasites (e.g., IL-4, IL-5, IL-13). $\gamma\delta$ T cell-released cytokines and factors might have beneficial or deleterious effects according to the physiopathological context. As an example, activated $\gamma\delta$ T cells are able to downmodulate immune responses (e.g., TGF- β , IL-10), to promote tissue healing, epithelia repair, and cell survival. Interestingly, studies have shown that activated $\gamma\delta$ T cells can promote dendritic cells (DC) maturation through the release

of type I and type II IFNs, which underlies their adjuvant role played for the control of infections (9–12). Unexpectedly, some $\gamma\delta$ T cell subsets, like human V γ 9V δ 2 T cells, could also constitute a novel type of professional APC that would elicit CD8 $^{+}$ /CD4 $^{+}$ $\alpha\beta$ T cell responses through the acquisition of a DC-like phenotype upon antigenic activation (13). Whether or not such function is found in other human and murine $\gamma\delta$ T cell subsets remains to be assessed. To date, none of the broad functional features described for $\gamma\delta$ T cells is specific to this T cell subset. Conjugated attempts of many laboratories failed to clearly establish and define common functional features of $\gamma\delta$ T cells that would basically distinguish them from conventional and innate-like $\alpha\beta$ T cells. Taken together, these observations suggest that most of the key contribution of the functional responses displayed by activated $\gamma\delta$ T cells might rather rely on the tight regulation of their kinetics of activation as well as the ability of these innate-like T cell subsets to be present “at the right time, in the right place.” The unique Ag specificities of $\gamma\delta$ T cells could also significantly account for their “programed” distribution within organs and tissues and their striking evolutionary conservation aside from T and B cell subsets which also assemble their Ag-receptor genes through recombinatorial rearrangement.

One particularly attractive hypothesis to account for the remarkable species and inter-individual conservation of $\gamma\delta$ T cells, as well as the lack of functional redundancy with $\alpha\beta$ T and B cells, is that this former subset, like an intermediate “T–B hybrid” cell type, might be rather designed for an efficient and unique mode of recognition of a broad set of conserved native Ag (e.g., proteins, lipids, carbohydrates) or complexes. In such contexts, this set of Ag either directly interact with $\gamma\delta$ TCR or are presented by non-polymorphic MHC-related or yet unknown presenting molecules. In line with this hypothesis, the structure of $\gamma\delta$ TCR heterodimers suggests that these molecules display immunoglobulin (Ig)-like recognition features, which strengthen the idea for alternative modes of Ag recognition by $\gamma\delta$ TCRs (14). This view is supported by both the diversity and the nature of $\gamma\delta$ TCR agonist molecules already identified, as well as by the direct reactivity of $\gamma\delta$ T cells and B cells against similar native molecules (e.g., F₀-F₁ ATP synthase, phycoerythrin) (15, 16).

$\gamma\delta$ T cells are key players in the immune surveillance of cellular distress, owing to their general ability to recognize Self determinants that are frequently upregulated in contexts of inflammation, infection, or cancer. While $\gamma\delta$ TCR contribute to detection of danger-associated molecular patterns, cognate interactions between $\gamma\delta$ TCR and defined Ag have been reported in a few cases only. As for some other innate-like cell subsets, $\gamma\delta$ T cells can be activated, in a cell-to-cell contact but classical MHC-independent manner, by small molecules or intact proteins without requirement for a processing similar to the conventional $\alpha\beta$ T cells Ag. Strikingly, while $\gamma\delta$ T cells can respond to different stimuli in various physiopathological contexts, a substantial fraction of the physiological murine and human $\gamma\delta$ TCR ligands characterized so far fall into the large MHC class I-related molecules family (e.g., T10-T22, MICA/B, CD1c, CD1d, EPCR) (17–21). Moreover the activation of $\gamma\delta$ T cells subsets by some of these molecules might be dependent on their ability to present specific ligands such as lipids (e.g., EPCR, CD1d).

THE HISTORY OF PHOSPHOANTIGENS AND THE FIRST IDENTIFICATION OF NATURAL HUMAN V γ 9V δ 2 T CELL AGONIST MOLECULES

In humans, as well as in most non-human primates, the major peripheral $\gamma\delta$ T cell subset in adults carries a TCR composed of a V82 chain systematically paired to a V γ 9 chain (using the nomenclature of Lefranc and Rabbitts (22), also referred to as V γ 2 by using the Seidman and colleagues nomenclature). This $\gamma\delta$ T cell subset represents about 5% of CD3 $^{+}$ cells within human peripheral blood and more than 80% of the peripheral $\gamma\delta$ T cell population in healthy adults. After the fortuitous discovery of $\gamma\delta$ T cells and the subsequent generation of monoclonal antibodies (mAbs) specific for human γ or δ TCR chains, high frequencies of V γ 9V δ 2 T cells were reported within peripheral blood or lesions from patients infected by a variety of bacteria (e.g., *Mycobacterium leprae*, *Mycobacterium tuberculosis*) and protozoans. Accordingly, V γ 9V δ 2 T cells efficiently responded to mycobacterial extracts *in vitro*, which contained small non-peptidic compounds (protease-resistant) that also bind to lectins. The isolation and characterization of the stimulatory fractions within these extracts led to the identification of various agonist low molecular weight carbohydrate compounds that were structurally related to phosphoesters and depend on the presence of phosphate moieties for their bioactivity (23–26). IPP (isopentenyl pyrophosphate) from *M. smegmatis* was identified as the first natural agonist for V γ 9V δ 2 T cells. This compound, and its isomer DMAPP (dimethylallyl pyrophosphate), were called phosphoAg, a family of compounds including the *E. coli* and *mycobacteria*-derived (2E)-1-hydroxy-2-methyl-but-2-enyl-4-diphosphate (HDMAPP), also known as (E)-4-hydroxy-3-methyl-2-but enyl pyrophosphate (HMBPP), and additional natural molecules produced by many microorganisms and plants (27–30) (Figure 1). Reports also indicated that V γ 9V δ 2 T cells are efficiently activated by a broad array of human tumor cells, such as lymphoma and carcinoma. The V γ 9V δ 2 T cells antigenic molecules from tumor cells were identified as IPP and DMAPP phosphorylated metabolites of the isoprenoid mevalonate (MVA) biosynthesis pathway, which is implicated in cholesterol synthesis (31). Accordingly, pharmacological MVA pathway inhibitors acting upstream (e.g., statins) or downstream (e.g., alkylamines; aminobisphosphonates, NBP) of phosphoAg biosynthesis, respectively, decrease or increase V γ 9V δ 2 T cell activation (32). Whereas most eukaryotic cells, fungi and archeabacteria produce isoprenoids through the MVA pathway, most of eubacteria, cyanobacteria, and algae protozoa synthesize isoprenoids through a related biosynthesis pathway referred to as the 1-deoxy-D-xylulose-5-phosphate (DOXP) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (33). HDMAPP produced through the DOXP pathway remains the most potent natural V γ 9V δ 2 activator so far identified (bioactivity of 0.1 nM, which is 30,000 times more potent than IPP). This suggests that this highly potent microbial phosphoAg, rather than endogenous phosphoAg such as IPP, would account for the highly sensitive sensing by V γ 9V δ 2 T cells of infected cells or pathogens using the DOXP/MEP pathway. The efficiency of the microorganisms recognition process by $\gamma\delta$ T cells was linked to their DOXP/MEP isoprenoid synthesis pathway (34). Accordingly, the genetic manipulation of the DOXP/MEP pathway in bacteria, similarly to the pharmacologic

| Phosphoantigens | EC50 | Molecule | Type |
|--|----------------|----------------|--|
| IPP Isopentenyl pyrophosphate | 50-500 μ M | | Natural (Vertebrate and microorganism) |
| DMAPP Dimethylallyl pyrophosphate | 0,23 μ M | | Natural (Vertebrate and microorganism) |
| HDMAPP / HMBPP (2E)-1-hydroxy-2-methyl-but-2-enyl-4-diphosphate / (E)-4-hydroxy-3-methyl-2-but enyl pyrophosphate | 39-70pM | HDMAPP | Natural (Microorganism) |
| C-HDMAPP (2E)-1-hydroxy-2-methylpent-2-enyl-pyrophosphate <i>Picostim</i> | 91pM | | Synthetic |
| BrHPP Bromohydrin of isopentenyl pyrophosphate <i>Phosphostim</i> | 20-50nM | | Synthetic |

FIGURE 1 | Examples of characterized phosphoantigens that induce V γ 9V δ 2 T cell activation. These listed phosphoAg are either from natural or synthetic origin. They induce different range of activation with EC₅₀ value for V γ 9V δ 2 T cell activation that can vary between different human donors. From Ref. (36, 39, 64, 97).

manipulation of the MVA pathway in human cell lines, regulates the production of these microbial phosphoAg which resulted in the modulation of the antigenic activation of V γ 9V δ 2 T cells (34, 35).

Research groups have next attempted to define the chemical basis and structure-to-functions relationships for the antigenicity of phosphoAg. These studies first reported that the substitution of phosphate for the pyrophosphate moiety or the elimination of the double bond reduced antigenic bioactivity of these compounds (24). As synthetic phosphoAg have been further produced and tested (e.g., BrHPP for bromohydrin pyrophosphate), these various molecules were compared to the natural ones (Figure 1) and next classified from an antagonist or weak, medium to a strong agonist bioactivity on V γ 9V δ 2 T cell activation (27, 36). These studies indicate that agonist or antagonist bioactivity of these compounds correlates with the extent and kinetics of pyrophosphate dephosphorylation (also referred to as β -phosphate cleavage) and alteration of their organic segment. Interestingly, a fast extracellular acidification measured after cell exposure to soluble agonist phosphoAg was reported (37, 38). As the phosphonate moieties are less susceptible to chemical or enzymatic hydrolysis than their corresponding phosphate counterparts, some groups have also synthesized phosphonate and pyrophosphonate analogs that could have improved pharmacological properties linked to an increased stability in biological media (39).

The specific and efficient activation of primate V γ 9V δ 2 T cells induced by isoprenoid phosphorylated compounds provides an attractive unified explanation for the unique and broad reactivity of this T cell subset in infectious and tumor contexts. However, the mechanism(s) of this highly conserved and uncommon specific antigenic recognition process by V γ 9V δ 2 T cells has remained puzzling and ill defined.

A PECULIAR MODE OF V γ 9V δ 2 T CELL ACTIVATION INDUCED BY PHOSPHOANTIGENS?

The unique ability of primate V γ 9V δ 2 T cells to specifically sense very subtle variations of phosphoAg levels in various physiopathological contexts such as infections and cancer highlighted the importance of the role played by the V γ 9V δ 2 TCR molecules in this process. Indeed, correlative studies have initially reported the specific activation of human T lymphocytes expressing a V γ 9V δ 2 TCR by Daudi Burkitt's lymphoma cells and mycobacteria (40, 41) and a direct contribution of germline residues specific to V γ 9, V δ 2, and J γ P elements to this recognition process (42). Altogether, these studies suggested that the antigenic activation of V γ 9V δ 2 T cells induced by phosphoAg is mediated by germline-encoded segments of the $\gamma\delta$ TCR. The $\gamma\delta$ TCR dependence of phosphoAg-induced T cell reactivity, also supported by the effects of $\gamma\delta$ TCR blocking mAbs (23, 24, 26), has been finally demonstrated by the results of V γ 9V δ 2 TCR transfer experiments (43). Indeed, the transfection of a V γ 9V δ 2 TCR in human Jurkat T cells enabled these cells to produce IL-2 in response to Daudi human tumor cells, mycobacterial extract, and soluble phosphoAg. Taken together with additional observations (44–46), this indicated that expression of V γ 9V δ 2 TCR is necessary and sufficient for the efficient sensing of identical, or structurally related, Ag from mycobacterial extracts, tumor cells, NBP-sensitized cells and synthetic soluble phosphoAg. Accordingly, events and signaling pathways classically triggered in T lymphocytes upon TCR-dependent activation (e.g., Erk and p38 MAPK pathways) were also reported for phosphoAg-induced activation of human V γ 9V δ 2 T cells (47–50).

Various mechanisms have been proposed to account for the V γ 9V δ 2 TCR-dependent sensing of phosphoAg that triggers strong activation of $\gamma\delta$ T lymphocytes. In a first hypothesis, soluble phosphoAg, as fully conserved native molecules, which are released by microbes or mammalian target cells (e.g., from tumor or NBP-treated cells) could directly, and specifically, interact with V γ 9V δ 2 TCR heterodimers to trigger $\gamma\delta$ T lymphocyte activation. This process would rely on both the bioactivity and the concentration of the released compounds. This direct interaction model is supported by: (i) the 3D structural features of a V γ 9V δ 2 TCR together with the results of $\gamma\delta$ TCR transfer/mutagenesis experiments that revealed the existence of a putative “binding” groove that could accommodate small (1–3 kDa) negatively charged phosphoAg (14) and, (ii) a mandatory role of the junctional region of the TCR γ chain (51) and the contribution of key conserved residues such as: lysine residue in the γ chain CDR3 loop, an arginine residue in CDR2 δ , and an aliphatic amino acid residue in CDR3 δ (52, 53). However, all attempts to biophysically or biochemically demonstrate any direct phosphoAg/V γ 9V δ 2 TCR interactions have failed so far (14, 54) and additional key observations, such as the cell-to-cell contact requirement for the activation of V γ 9V δ 2 T cells by soluble phosphoAg (55, 56) do not favor a direct recognition process.

THE HUNT FOR UNKNOWN PHOSPHOANTIGEN-PRESENTING MOLECULE(S)

On the basis of these last observations, non-exclusive molecular events were proposed to account for the necessity of cell-to-cell contacts for phosphoAg-induced V γ 9V δ 2 T cell activation, such

as: (i) a “stabilization” of native or modified membrane-bound phosphoAg on target cells surface that would be required for protection against degradation by extracellular phosphatases, (ii) a topological clustering/aggregation of phosphoAg and/or their co-localization with key membrane-associated molecules (e.g., adhesion, NKR) for an efficient cross-link of the $\gamma\delta$ TCR.

The critical contribution of species-specific Ag-presenting cells has been evidenced, meaning that only cell-to-cell contact with human, and some non-human primate cells, are able to trigger a phosphoAg reactivity of human V γ 9V δ 2 T cells (57, 58). This restriction indirectly suggested the requirement for species-specific determinants and, as primate phosphoAg-sensitized cell lines lose their ability to efficiently activate V γ 9V δ 2 T cells upon protease treatments, these key surface determinants were characterized as membrane-associated proteins (59). Several adhesion and costimulation molecules (e.g., ICAM-1, CD166) have been proposed to account, at least partially, for these requirements (57, 58). However, human V γ 9V δ 2 TCR transfer into murine T cell lines was shown to be sufficient to trigger phosphoAg reactivity against human, but not rodent target cells (60, 61) (C. Harly & E. Scotet, *unpublished observations*). Additionally, macaque V γ 9V δ 2 TCR tetramers were shown to bind to human, but not mouse cell surface upon phosphoAg treatment. In agreement with the contribution and the protein nature of key cell surface determinants, this binding was abrogated by protease pre-treatments of the target cells (59). Altogether, these results underlined the critical requirements of primate cell membrane-expressed determinants of protein nature, specifically recognized by V γ 9V δ 2 T cells. The contribution of these unknown molecule(s) was further supported by a study uncovering the role of all the CDRs of V γ 9V δ 2 TCR in the recognition of phosphoAg-sensitized cells, thus suggesting a large contact surface with a putative antigenic complexes/molecules, and not only small phosphorylated compounds (62). Additionally, the existence of “phosphoAg-presenting molecules” was supported by the characterization of photoaffinity prenyl pyrophosphate Ag (analogs of HMBPP), designed to form covalent bounds with close proximity molecules after UV-treatment. This study suggests that such molecules stably associate to broadly distributed, functionally non-polymorphic, and not known Ag-presenting molecule(s) on human target cells and activate V γ 9V δ 2 T cells (63). At last, the generation and the functional characterization of various synthetic compounds analogous to phosphoAg has revealed that some of them were not only unable to efficiently activate V γ 9V δ 2 T cells, but could specifically interfere with the stimulating activity of phosphoAg (37, 63, 64). Similarly, IPP could efficiently inhibit the stable association of photoaffinity prenyl pyrophosphate Ag to primate target cells (63). These observations could reflect the existence of competition mechanisms established between phosphoAg (e.g., IPP) and analogs for a limited number of binding sites on the same unidentified molecule(s) expressed by target cells.

PhosphoAg recognition has been described to be an extremely rapid process (~10 s) and is not abrogated by glutaraldehyde fixation of the target cells (36, 55). In contrast, the antigenic activation of V γ 9V δ 2 T cells by NBPs or alkylamines is indirect and mediated by the intracellular accumulation of IPP (31, 65, 66). It remains unclear how intracellular IPP is detected at the cell surface.

Whether phosphoAg are transported, exported (e.g., intracellular IPP) outside or internalized inside (e.g., extracellular HMBPP) the target cells, specifically or not, anchored or adsorbed on target cell surface, whether they interact with yet unknown ubiquitous surface molecule or whether they rapidly regulate the expression/conformational changes of determinants that are detected by V γ 9V δ 2 TCR remains open.

Taken together, the previous findings would tend to rule out a “simple” direct recognition process of phosphoAg by V γ 9V δ 2 T cells, and lead to hypothesize the existence of a “phosphoAg-presenting molecule(s)” of protein nature, ubiquitously expressed on the surface of primate cells. However, such molecule(s) was never identified, and, as we will see in the second part of this review, more recent works strongly suggest an alternative model for phosphoAg sensing by human V γ 9V δ 2 T cells.

BTN3A1, AN UBIQUITOUS CELL SURFACE-EXPRESSED HUMAN BUTYROPHILIN MOLECULE, PLAYS A MANDATORY ROLE FOR THE ACTIVATION OF HUMAN V γ 9V δ 2 T CELLS INDUCED BY PHOSPHOANTIGENS

Recently, our group has clearly demonstrated for the first time the specific and mandatory role played by BTN3A1, a type I glycoprotein expressed on target cells, in the phosphoAg-induced reactivity of human V γ 9V δ 2 T cells (60). BTN3A1, -A2, and -A3 constitute the BTN3A (also known as CD277) subfamily of butyrophilins (BTN) molecules. These three isoforms are encoded by three distinct genes, found in human and some non-human primates. *Btn* genes constitute a subgroup of at least 10 genes in most species. Eleven of them have been identified in mouse, and 13 in human, where they are located in the MHC class I region of chromosome 6p. Notably, BTN family is member of the Ig superfamily and shares structural homology with B7 family members at extracellular domain level (mostly composed of Ig-like domains referred to as IgV and IgC domains) (67). Phylogenetically, BTN molecules share a common ancestor with the B7 family, initially suggesting that they could have immunological functions (68).

The functions of BTN molecules as well as their molecular partners remain ill defined. The eponymous BTN gene, *BTN1A1*, is highly expressed in the lactating mammary glands (secretory epithelium) and represents the major protein associated with fat droplets in milk. *BTN1A1* has been reported to mainly play a role in the regulation of the amount of lipids and size of droplets expressed in milk of mammals (69). This function is linked to its intracellular B30.2 domain, which has been shown to bind xanthine oxydoreductase and stabilize its association with the milk fat globule membrane (70). A few studies have reported immunological functions for some human BTN members such as *BTN1A1*, *BTN2A2* and *BTN3A* molecules that can regulate cellular immunity and T/NK cells activation (67, 71–75). As compared to *BTN1A1*, human *BTN2A* and *BTN3A* are widely expressed in many tissues (76). The ectodomains of the three isoforms of *BTN3A* consist of two domains, so-called IgV and IgC that have a very high homology (>95%). On the other hand, the intracellular domain B30.2 (PRY/SPRY) is only present in *BTN3A1* and *BTN3A3* and poorly conserved. *BTN3A1* is ubiquitously expressed in human beings (75, 77) and homologs are found in all primates carrying V γ 9V δ 2 T cells (78). Strikingly, *BTN3A* orthologs are

not found in the rodent lineage, which also lacks V γ 9V δ 2 T cell counterparts specific for phosphoAg. The emergence of TCR V γ 9, V δ 2, and *BTN3* genes with eutherian placental mammals has been recently reported (79). This recent study suggests a strong evolutionary functional link between the expression of V γ 9V δ 2 TCR and *BTN3* proteins.

Our study shows a mandatory role for the *BTN3A1* isoform in the specific detection of human distressed cells (e.g., tumor and mycobacteria infected cells) by human V γ 9V δ 2 T cells, which strongly suggested that *BTN3A1* molecule represent a major species-specific determinant regulating the antigenic reactivity of human V γ 9V δ 2 T cells (60). This work demonstrates that *BTN3A1* does not act as a key costimulatory or adhesion molecule but as a mandatory protein for the V γ 9V δ 2 TCR-dependent phosphoAg-mediated recognition of target cells. Furthermore, our data indicate that *BTN3A1* expression is necessary for the recognition of human target cells by baboon V γ 9V δ 2 T cells, and that antigenic activation of baboon V γ 9V δ 2 T cells, induced by either human or baboon cells is abrogated by a blocking anti-BTN3 mAb (C. Harly & E. Scotet, *unpublished observations*), which is fully in line with the evolutionary functional link between the expression of V γ 9V δ 2 TCR and *BTN3* proteins. It will be interesting to extend these observations to other V γ 9V δ 2 expressing species, including the non-primate ones, such as alpacan (79) to either strengthen or challenge this functional link. Importantly, several groups (61) (C. Harly & E. Scotet, *unpublished observations*) have reported that the expression of *BTN3A1* is probably not sufficient to induce the recognition of rodent target cells by human V γ 9V δ 2 T cells, even when co-expressed with key human adhesion molecules (e.g., ICAM-1). In contrast, transferring a 27.4 megabases region of the human chromosome 6p including *BTN3A1* could confer to a mouse cell line the ability to activate V γ 9V δ 2 T cells in the presence of phosphoAg (61, 80). This suggests that additional species-specific partner molecules encoded on the human chromosome 6p, together with *BTN3A1*, are required for ensuring its functional activity.

THE INTRACELLULAR B30.2 DOMAIN OF BTN3A1 BINDS PHOSPHOANTIGENS AND MEDIATES THEIR SENSING BY V γ 9V δ 2 T CELLS

While other molecules involved in the phosphoAg-mediated recognition of target cells by V γ 9V δ 2 T lymphocytes remain to be identified, the physical and chemical features of *BTN3A1* already shed light on possible mechanism(s) taking part to this process. BTN molecules comprise two extracellular Ig-like domains, a single pass transmembrane domain, and, for some members including *BTN3A1* and *BTN3A3*, a B30.2 (SPRY/PRY)-related intracellular domain (67). The mandatory role played by *BTN3A1* isoform during antigenic activation of V γ 9V δ 2 T cells, but not *BTN3A2* or *BTN3A3*, first suggested that the highly conserved extracellular region (>95%) of *BTN3A1* unlikely accounts for its functional specificity in phosphoAg-mediated activation. In line with this assumption, we next showed, by swapping the domains between *BTN3A* isoforms, that the intracellular portion of *BTN3A1* is necessary for *BTN3A1* to mediate phosphoAg stimulation of V γ 9V δ 2 T cells, and is sufficient to confer this properties to the inactive isoform *BTN3A3* (60). Therefore this finding highlights a crucial

and specific contribution for the intracellular B30.2 domain of BTN3A1.

These results have been very recently confirmed and extended by a joined study from the group of E. J. Adams and ours. In this work, a set of structural, molecular and cellular approaches has been performed to unambiguously demonstrate the direct interaction between phosphoAg and the N-terminal part of the intracellular domain B30.2 of BTN3A1, through a positively charged pocket (81). Each of the pyrophosphate compound that activate V γ 9V δ 2 T cells bind the B30.2 domain of BTN3A1, but not the B30.2 domain of BTN3A3, and their agonist potency is directly correlated with their binding intensity. Strikingly, comparative sequence analysis between the B30.2 domains of BTN3A1 and BTN3A3 isoforms identified a putative Histidine residue specific to BTN3A1 (replaced by an Arginine at this position in BTN3A3). Structural, biochemical, and functional assays show that this residue is required for the binding of phosphoAg to BTN3A1 B30.2, and their V γ 9V δ 2-stimulating activity. These observations, which have been confirmed by other groups (64, 82), indicate that internal sensing of changes in phosphoAg metabolite concentrations by BTN3A1 molecules represent a critical step in V γ 9V δ 2 T cell detection of infection and tumorigenesis.

PHOSHOANTIGENS ACT INTRACELLULARLY AND THEREFORE ARE NOT *BONA FIDE* V γ 9V δ 2 T CELL ANTIGENS

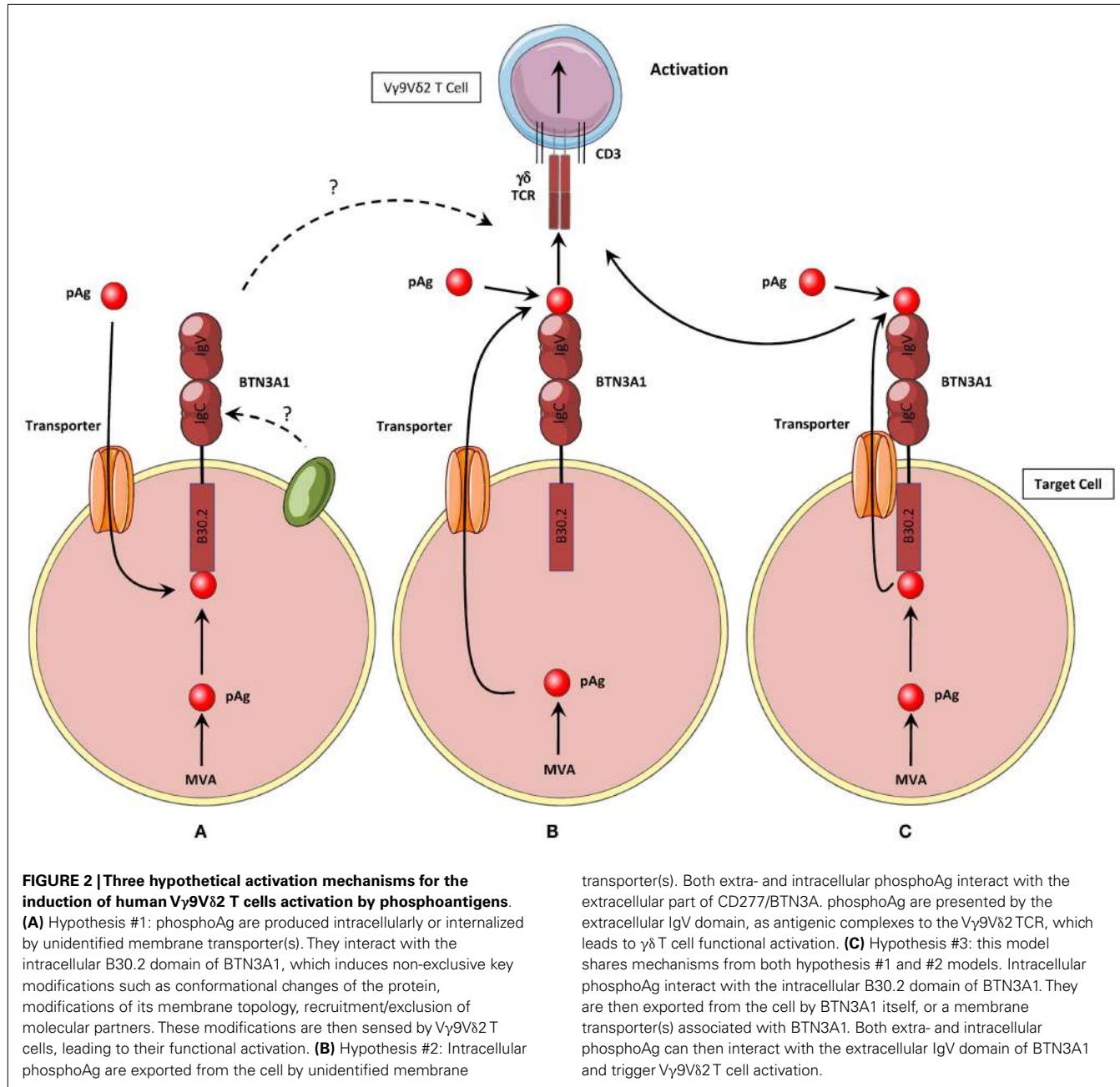
The direct physical and functional link established between the intracellular phosphoAg and the transmembrane protein BTN3A1 represents a tremendous advance in deciphering how primate V γ 9V δ 2 T cells can exquisitely sense the dysregulation of intracellular phosphoAg levels by scanning the surface of distressed target cells. However, many key issues remain to be solved to fully understand the fine modalities of this process, summarized in **Figure 2A**. How is the intracellular interaction between pyrophosphate compounds and intracellular B30.2 domain translated to the cell surface? Which cell surface determinants are finally detected by V γ 9V δ 2 T cells? How exogenous phosphoAg efficiently “sensitize” target cells through BTN3A1, while the negative charge of their phosphorylated moieties unlikely allows them to passively cross the plasma membrane, and their mode of action was proposed to be independent of any active processing machinery (55, 63)?

A recent study from the group of G. De Libero has proposed an attractive model for BTN3A1 function, which would resolve some of these issues. Endogenous and exogenous phosphoAg would be directly first loaded on the extracellular portion of BTN3A1 and this complex would next directly interact with the V γ 9V δ 2 TCR (80) (**Figure 2B**). This study provide molecular and cellular evidences for a direct interaction between phosphoAg, the extracellular IgV domain of BTN3A1, and the V γ 9V δ 2 TCR therefore supporting the appealing assignment of BTN3A1 as a classic Ag-presenting molecule for pyrophosphate compounds. However, this model appears to be insufficient to explain several key experimental observations, such as the specific requirement for BTN3A1 isoform, but not for the BTN3A2 and BTN3A3, whereas the extracellular domain of the three proteins is highly homologous, especially within the area composed of the candidate binding residues for phosphoAg, which are shared between BTN3A1 and BTN3A2. More importantly, this model also does not take in

account the mandatory role of the intracellular B30.2 domain of BTN3A1 in this process. A hypothesis that could reconcile some of the features seemingly contradictory between these two models would be that BTN3A1 itself is a transporter molecule, or closely associated with one. In this model, BTN3A1 would interact with both intracellular and extracellular phosphoAg through, respectively, its B30.2 domain and its extracellular IgV domain. This latter one would next serve for the presentation of both type of phosphoAg to V γ 9V δ 2 T cells (**Figure 2C**). However, the ability of the extracellular part of BTN3A1 to bind phosphoAg and interact with the V γ 9V δ 2 TCR has not been confirmed by either biochemical or functional analysis (61, 64, 81, 82) (C. Harly & E. Scotet, *unpublished observations*). Taken together, the studies performed so far indicate that phosphoAg-induced sensing of target cells is mediated by the BTN3A1 intracellular B30.2 domain for both endogenous and exogenous phosphoAg (**Figure 2A**). As these results failed to demonstrate any direct contribution (e.g., interactions) of the extracellular region of BTN3A1 in this process, they would rather support a model in which phosphoAgs are not directly recognized by V γ 9V δ 2 T cells, and therefore are not *bona fide* V γ 9V δ 2 Ag.

An important issue raised from this assumption concerns the requirements for the internalization of exogenous phosphoAg within the cells, through the plasma membrane. Early evidence did not support the existence of such a process: (i) the negative charge of phosphoAg does not allow them to passively cross the plasma membrane, (ii) phosphoAg do not seem to require any active internalization or processing for triggering V γ 9V δ 2 T cell activation (55, 63), (iii) the very fast kinetics of V γ 9V δ 2 T cell activation induced by phosphoAg suggest they are readily active at the extracellular level (37, 38, 47, 49). The mode of intracellular action of negatively charged exogenous phosphoAg has been recently addressed in a cellular approach (64). In this elegant study, synthetic pro-phosphoAg was designed to allow their passive diffusion through the plasma membrane, in an inactive protected state. After cleavage of the protective groups by intracellular esterases, active phosphoAg were released and were unable to leave the cytoplasm. The results clearly show that these highly bioactive compounds bind the intracellular domain of BTN3A1. Additionally, the intracellular uptake of pro-phosphoAg was confirmed by a long-term effect of the pro-drug on target cell sensitization after washes. Therefore, these results strongly support an unified mode of action of phosphoAg to trigger V γ 9V δ 2 T cell activation, regardless of their endogenous, exogenous, intracellular, and extracellular origin.

Thus, phosphoAg would act intracellularly in target cells and their direct interaction with the B30.2 domain of BTN3A1 would be next sensed by V γ 9V δ 2 T cells. This model, as well as the alternative ones, relies on the existence and the contribution of additional molecular player(s), involved in either the uptake of extracellular phosphoAg, or the release of intracellular phosphoAg (**Figure 2**). Whether this process is active or not, phosphoAg-specific or not, and can impact the quality and the magnitude of the functional activation of V γ 9V δ 2 T cells remains to be determined. The identification of yet unknown membrane-associated transporter molecule(s) should greatly help understanding of the mode of phosphoAg action.



RE-ORGANIZATION OF CELL SURFACE MOLECULES INDUCED BY INTERACTIONS BETWEEN PHOSPHOANTIGENS AND THE BNT3A1 INTRACELLULAR B30.2 DOMAIN?

This model of intracellular detection of phosphoAg raises the key question of the nature of molecular events implicated in the sensing of this process by V γ 9V δ 2 T cells. This binding of phosphoAg to the B30.2 domain of BTN3A1 should be somehow “translated” to the cell surface of target cells, in order to be detected by V γ 9V δ 2 T cells. An interesting clue for deciphering this mechanism comes from the observation that some mAbs specific of the extracellular region of BTN3A can precisely mimic the phosphoAg-induced recognition of target cells by V γ 9V δ 2 T cells (60, 83). Additionally,

transporter(s). Both extra- and intracellular phosphoAg interact with the extracellular part of CD277/BTN3A. phosphoAg are presented by the extracellular IgV domain, as antigenic complexes to the V γ 9V δ 2 TCR, which leads to V γ δ T cell functional activation. **(C)** Hypothesis #3: this model shares mechanisms from both hypothesis #1 and #2 models. Intracellular phosphoAg interact with the intracellular B30.2 domain of BTN3A1. They are then exported from the cell by BTN3A1 itself, or a membrane transporter(s) associated with BTN3A1. Both extra- and intracellular phosphoAg can then interact with the extracellular IgV domain of BTN3A1 and trigger V γ 9V δ 2 T cell activation.

some anti-BTN3 mAbs abrogate both phosphoAg- and agonist anti-BTN3 mAbs-induced recognition of target cells by V γ 9V δ 2 T cells. This suggests similar mechanisms of sensitization of target cells induced by both phosphoAg and agonist anti-BTN3 mAbs. Interestingly, the sensitization of target cells induced by agonist anti-BTN3 mAbs requires neither the presence of phosphoAg nor the expression of intracellular B30.2 domain. Accordingly, each BTN3 isoform, comprising or not a B30.2 domain, was equally able to trigger the functional activation of V γ 9V δ 2 T cells (60, 82). The structural analysis of extracellular region of BTN3A1 with agonist ScFv mAb revealed a ~20 Å displacement as measured between V domains, induced upon binding of the agonist mAb

(78). Altogether these observations strongly suggest that agonist anti-BTN3 mAbs can probably induce some key BTN3A structural modifications, similar to the effects triggered by phosphoAg. However, agonist anti-BTN3 mAbs in complex with the extracellular part of BTN3, are neither sufficient to activate V γ 9V δ 2 T cells when plastic-coated or expressed on the cell surface of rodent cells, nor able to interact with V γ 9V δ 2 TCR in solution (60, 78, 81). Though still conflicting (61, 80), these results suggest that the direct recognition of either anti-BTN3 mAbs in complex with extracellular BTN3 or conformational changes/crosslinking of the extracellular domain of BTN3 induced after binding of these agonist anti-BTN3 mAbs might not be sufficient to fully account for the antigenic activation of V γ 9V δ 2 T cells.

Thus, the topological remodeling of some cell surface key determinants, involving the aggregation or the exclusion of specific membrane proteins likely represents an important step for this stress-sensing process by V γ 9V δ 2 T cells. This is supported by recent FRAP (*Fluorescence Recovery After Photobleaching*) experiments showing that anti-BTN3 mAbs significantly decreases the mobility of BTN3A isoforms on the cell surface, regardless of the composition of their intracellular domain (60, 81). Importantly, similar effects were observed on human target cells with NBP-induced intracellular accumulations of phosphoAg, in correlation with the expression of a functional B30.2 domain. Hence, these results provide a strong link between the reduced surface mobility of BTN3A on human target cells and their detection by V γ 9V δ 2 T cells. The mechanism(s) implicated in such membrane diffusion alterations and the physiological relevance of these events still need to be experimentally further addressed. It would be interesting to determine whether human BTN3A molecules, when expressed on the cells surface of rodent cells, are also immobilized after phosphoAg or anti-BTN3 mAbs treatments, despite the inability of such cells to trigger functional activation of human V γ 9V δ 2 T cells. Such experiments should help further understand the link between BTN3A1 reduced mobility induced by phosphoAg and V γ 9V δ 2 T cell activation, as well as determine whether some key primate-specific molecules are required for: (i) translating the phosphoAg/B30.2 interactions into this reduced BTN3A mobility process, (ii) BTN3A immobilization itself, or, (iii) V γ 9V δ 2 T cell activation induced following sensing of this alteration of BTN3A mobility.

These FRAP experiments present essential evidence that the simple interaction(s) of small intracellular metabolites, like phosphoAg, with the ubiquitously expressed intracellular BTN3A1 B30.2 domain can substantially affect the global topological organization of this cell surface-expressed type I glycoproteins. The observation that BTN3A1 B30.2 crystals dissolve upon phosphoAg soaking (81) raises the possibility that the interaction(s) between phosphoAg and this B30.2 domain could result in conformational rearrangements leading to this membrane mobility alterations. This hypothesis is supported by nuclear magnetic resonance spectroscopy experiments revealing some major chemical shift perturbations upon phosphoAg binding in the B30.2 domain, not only within the B30.2 domain, but also in the upstream membrane proximal region (64). Therefore, these recent results open the possibility that such induced conformational changes could be translated from the intracellular domain of BTN3A1

to its extracellular domain, similarly to the “inside-out” signaling process already observed with integrins (84), and result in a global cell surface remodeling (Figure 3).

WHICH ARE “REAL” TCR LIGANDS FOR HUMAN V γ 9V δ 2 T CELLS ?

While a lot of attention has recently been brought onto the mechanism(s) linking phosphoAg and BTN3A1, the molecule(s) that could finally interact with the V γ 9V δ 2 TCR and trigger T cell activation remain(s) unidentified. It is particularly unclear whether BTN3A1 is directly recognized, alone or in molecular complexes, or if this molecule indirectly plays a key role for the recognition of V γ 9V δ 2 TCR ligand(s) at the target cell surface. Several candidate molecules have been previously proposed as V γ 9V δ 2 T cells Ag accounting for the specific recognition of tumor or infected cells, in various pathological contexts. These candidate molecules were from bacterial [e.g., GroEL HSPs (85)], SEA (86), viral [e.g., HSV-1 glycoprotein I (87), or Self origin [e.g., F₀–F₁ ATP synthase (15)], ULBP4 (88), MSH2 (89), HSP60 (90)]. Mitochondrial F₀–F₁ ATP synthase was a particularly interesting candidate as the ectopic cell surface expression of this mitochondrial complex was reported on tumor cells. F₀–F₁ ATP synthase also binds a delipidated form of apolipoprotein A-I, which is required for optimal V γ 9V δ 2 T cell activation. Further studies showed the direct recognition of these molecules by the V γ 9V δ 2 TCR (15), a process that can be modulated by MHC class I molecules (91). Because F₀–F₁ ATP synthase was shown to interact with some phosphoAg, this molecule was proposed as a presenting molecule for phosphoAg (92) but this property has not been yet confirmed. Interestingly, the recent characterization of V γ 9V δ 2 T cell subsets amplified *in vitro* following contact with *M. BCG*-infected DCs has shown that only a subset of phosphoAg-responsive V γ 9V δ 2 T cells were actually responsive to *M. BCG* (93). TCR repertoire analysis of this T cell subset revealed a restricted diversity in V δ 2 CDR3 sequences, as compared to IPP-reactive V γ 9V δ 2 T cells. This study suggested that the reactivity of these V γ 9V δ 2 T cell subsets toward *M. BCG* could be mediated by non-phosphoAg molecules. Similar observations were made in pulmonary tuberculosis patients (94). The specificity of these subsets was next characterized which led to the identification of new protein/peptides of mycobacterial origin (e.g., OXYS, DXS2, Rv2272) (94, 95) that can interact with V γ 9V δ 2 TCRs and activate a large fraction of V γ 9V δ 2 T cells isolated from peripheral blood of tuberculosis infected patients but not $\gamma\delta$ T cells of healthy donors.

Therefore, these recent studies provide attractive evidences that some V γ 9V δ 2 TCRs might not be designed for the unique detection of phosphoAg/BTN3A1-linked stress signals. However, antagonist anti-BTN3 mAbs have been shown to abrogate the recognition of *M. BCG*-infected cells (60). Even if this result does not directly address the essential requirement for BTN3A1 expression, and its intracellular B30.2 domain, in the reactivity of V γ 9V δ 2 T cells against *M. BCG*, it strongly underlines an important function for this BTN in this process. As the “polyreactivity” of various V γ 9V δ 2 T cell clones toward various antigenic molecules has not been determined so far, it is therefore difficult to exclude or include them in a general model of V γ 9V δ 2 T cell activation by phosphoAg. An interesting hypothesis is that each of these V γ 9V δ 2

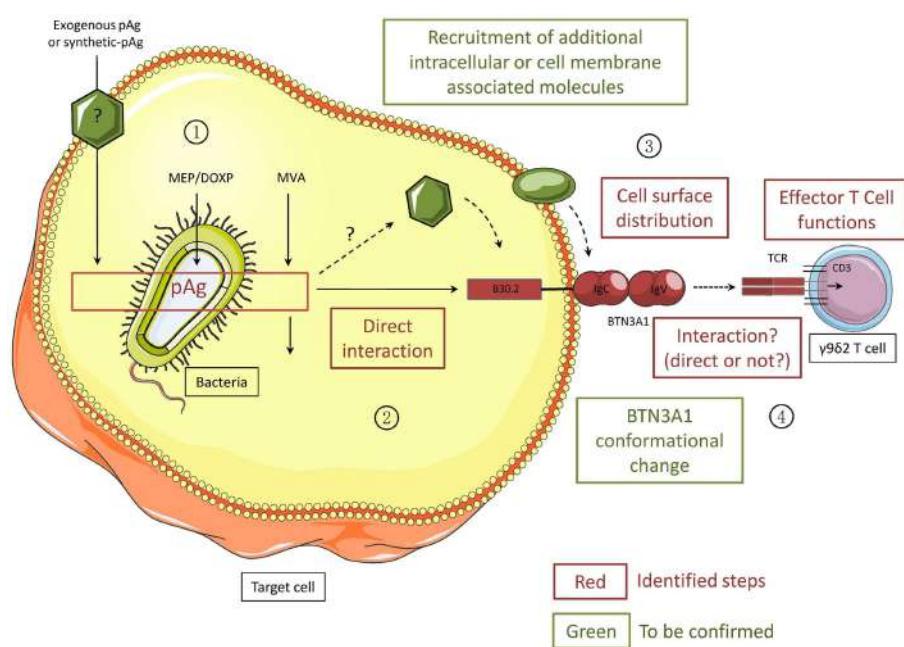


FIGURE 3 | Different steps of the activation process of human V γ 9V δ 2 T cells induced by phosphoantigens. All the steps/molecules colored in red have been identified while the green ones will need to be confirmed. PhosphoAg (pAg)s accumulate intracellularly as metabolites of the endogenous mevalonate pathway (MVA) or the microorganism MEP/DOXP pathway. Exogenous pAg can also be internalized through yet undefined membrane transporter(s). PhosphoAg directly interact with the N-terminal portion of the

intracellular B30.2 domain of CD277/BTN3A1. Intracellular partner molecules could be recruited either by BTN3A1 or by phosphoAg. Following PhosphoAg/B30.2 interaction, the conformation of BTN3A1 is altered and the cell surface distribution of BTN3A1 together with yet unknown other molecular partners is modified. These exquisite signals are sensed by V γ 9V δ 2 T cells, in a TCR-dependent manner, leading to the functional activation of V γ 9V δ 2 T cells. Whether the V γ 9V δ 2 TCR directly interacts with BTN3A1 remains unclear.

TCR ligands expressed on the surface of stressed target cells is not individually detected by V γ 9V δ 2 T cells, but become activator as part of a complex involving several V γ 9V δ 2 TCR ligands, as suggested by the results from the study reporting the recognition of the F₀-F₁ ATP synthase/ApoA1 complex (15). In agreement with this idea, BTN3A1 could regulate the formation or the topology of V γ 9V δ 2 antigenic complexes on the cell surface.

PHYSIOLOGICAL IMPLICATIONS FOR THE DETECTION OF BOTH MODIFIED-SELF AND NON-SELF BY V γ 9V δ 2 T CELLS

V γ 9V δ 2 T cells have the ability to discriminate between self and modified-self, by detecting subtle changes of phosphoAg levels, due to increased MVA pathway activity and/or uptake of exogenous phosphoAg, within stressed, transformed, or infected target cells. How V γ 9V δ 2 T cells can make this subtle distinction is not well understood, but a fine control of such capacities is essential to avoid any deleterious effect linked to the strong effector functions of V γ 9V δ 2 T cells (e.g., cytotoxicity) in case of an inappropriate activation. Importantly, as also suggested for other T cell subsets, the recognition of altered determinants, from either self or non-self origin, by the TCR complex does not fully reflect the “true” reactivity of these T lymphocytes and the ability of these ligands/TCR interactions to trigger functional responses. Indeed, the TCR-mediated reactivity of V γ 9V δ 2 T cells against target cells not solely depends on the expression levels of Ag but also relies on the fine tuning of their activation threshold by

costimulatory/adhesion molecules and activating/inhibitory NK receptors (47, 57, 58, 96).

The interaction of phosphoAg with the intracellular B30.2 domain of the ubiquitous BTN3A1 molecule, at a 1:1 molar ratio (64, 81), which would be subsequently translated to the cell surface, offers a simple option for the quantitative sensing of endogenous phosphoAg within target cells by V γ 9V δ 2 T cells. However, the MVA pathway, which produces the endogenous phosphoAg metabolites (e.g., IPP), is constitutively engaged at different levels of activity in healthy cells, according to their physiological functions. For example, highly proliferating cells such as activated proliferating T cells display some metabolic characteristics that are very similar to those of stress-altered cells, including an upregulated MVA pathway. As several additional molecular players remain to be identified to fully decipher the mechanisms of phosphoAg sensing by V γ 9V δ 2 T cells (Figure 3), the complexity of the currently proposed model already offers many levels of regulation, such as the expression levels of molecules regulating phosphoAg/B30.2 interactions and/or BTN3A1 conformational/topological changes, as well as the availability of BTN3A1 or V γ 9V δ 2 TCR ligands themselves. Therefore, following their identification, it will be critical to study the expression of each of these molecules in both physiological and pathological contexts. As an hypothesis, BTN3A isoforms that are devoid of any phosphoAg-binding B30.2 domain could exert inhibitory functions by competing with BTN3A1 for the interaction with

molecular partners (e.g., ectopic F_0 - F_1 ATP synthase, ApoA1 or other TCR ligands). To first test this, the expression levels of the different isoforms of BTN3A will need to be further measured and compared, not only in different healthy cell subsets (75) but also in various altered contexts.

Besides their ability to quantitatively sense variations of endogenous phosphoAg levels in target cells, V γ 9V82 T cells also efficiently discriminate between weak agonist phosphoAg that are produced by the endogenous MVA pathway (e.g., IPP) and strong agonist ones which are produced by the microorganism DOXP/MEP pathway (e.g., HMBPP). Isothermal titration calorimetry has shown that the potency of phosphoAg correlates with their binding affinity for the intracellular B30.2 domain of BTN3A1 (64, 81). According to biochemical and biophysical studies, the binding of phosphoAg on the B30.2 domain would rely on their mandatory phosphate moiety, which interacts with a unique binding site in the B30.2 domain, and could be modulated by the nature of their organic solvent (64, 81). It remains unclear how such binding affinities are in turn quantitatively translated to the target cell surface and next detected by V γ 9V82 T cells. One possibility is that the stability of these interactions could directly impact on the stability of the induced BTN3A1 conformational/topological changes. Another hypothesis is that phosphoAg directly or indirectly recruit partner molecules, according to their biochemical properties. As the chemical reactivity of phosphoAg has been reported as a key requirement for V γ 9V82 T cells activation and the kinetics of the dephosphorylation step correlates with their bioactivity, the importance of the contribution of this β -phosphate cleavage step will also need to be further analyzed. Interestingly, some phosphoAg analogs that are resistant to β -phosphate cleavage are not only unable to activate V γ 9V82 T cells but also abrogate their activation induced by phosphoAg (37). These observations suggest that phosphoAg and their analogs compete for binding/interacting sites (e.g., B30.2 domain) and that β -phosphate cleavage is required for an additional step still to be identified.

CONCLUDING REMARKS

Primate V γ 9V82 T cells are endowed with unique reactivity patterns against a broad range of stressed cell targets, including infected and tumor cells. This property has been mainly attributed to a specific recognition of dysregulated phosphoAg in various pathological contexts, as well as an apparently diversified set of proteins and peptides of Self and non-Self origins that could directly interact with the V γ 9V82 TCR. Despite the lack of obvious common feature between all these antigenic determinants, several V γ 9V82 TCR ligands of Self origin have been linked to the cell metabolism, and their expression or localization could be regulated by transformation or infection. Because metabolic dysregulation represents a general feature of various cell distress events, including transformation, infection and injury, the activation of V γ 9V82 T cells induced by this type of antigenic determinants would represent a unique and efficient strategy allowing the early detection of many pathological contexts. It will be crucial in future studies to understand by which mechanisms such molecules of diverse nature and origin can trigger and regulate V γ 9V82 T cell antigenic activation, and whether all these molecules are

recognized individually, or as part of a general mechanism of detection of distressed-self and non-self, involving phosphoAg, BTN3A1, and various other molecules yet to be identified. Our attempts to integrate the results of previous experimental data on the modalities of V γ 9V82 T cell activation induced by phosphoAg, highlighted by the recent identification of BTN3A1 as mandatory player in this process, led to an incomplete, yet already complex model of phosphoAg sensing by V γ 9V82 T cells. Against all expectations, phosphoAg sensing by V γ 9V82 T cells is an indirect process (Figure 3), involving several intermediate players that remain to be identified.

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The V γ 9V δ 2 T cell antigen receptor and butyrophilin-3 A1: models of interaction, the possibility of co-evolution, and the case of dendritic epidermal T cells

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Most circulating human gamma delta T cells are V γ 9V δ 2 T cells. Their hallmark is the expression of T cell antigen receptors (TCR) whose γ -chains show a V γ 9-JP (V γ 2-J γ 1.2) rearrangement and are paired with V δ 2-containing δ -chains, a dominant TCR configuration, which until recently seemed to occur in primates only. V γ 9V δ 2 T cells respond to phosphoantigens (PAg) such as (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which is produced by many pathogens and isopentenyl pyrophosphate (IPP), which accumulates in certain tumors or cells treated with aminobisphosphonates such as zoledronate. A prerequisite for PAg-induced activation is the contact of V γ 9V δ 2 T cells with cells expressing butyrophilin-3 A1 (BTN3A1). We will first critically review models of how BTN3 might act in PAg-mediated V γ 9V δ 2 T cell activation and then address putative co-evolution of V γ 9, V δ 2, and BTN3 genes. In those rodent and lagomorphs used as animal models, all three genes are lost but a data-base analysis showed that they emerged together with placental mammals. A strong concomitant conservation of functional V γ 9, V δ 2, and BTN3 genes in other species suggests co-evolution of these three genes. A detailed analysis was performed for the new world camelid alpaca (*Vicugna pacos*). It provides an excellent candidate for a non-primate species with presumably functional V γ 9V δ 2 T cells since TCR rearrangements share features characteristic for PAg-reactive primate V γ 9V δ 2 TCR and proposed PAg-binding sites of BTN3A1 have been conserved. Finally, we analyze the possible functional relationship between the butyrophilin-family member Skint1 and the γ 8 TCR-V genes used by murine dendritic epithelial T cells (DETC). Among placental mammals, we identify five rodents, the cow, a bat, and the cape golden mole as the only species concomitantly possessing potentially functional homologs of murine V γ 3, V δ 4 genes, and Skint1 gene and suggest to search for DETC like cells in these species.

Keywords: γ 8 T cell, V γ 9V δ 2 T cell, phosphoantigen, BTN3, alpaca, co-evolution, DETC and Skint1

$\alpha\beta$ T CELLS AND $\gamma\delta$ T CELLS

Jawed vertebrates (Gnathostomata) possess lymphocytes expressing Ig-domain containing antigen-receptors, whose highly diverse antigen-binding sites are generated by RAG-dependent somatic recombination of genes encoding for an antigen-binding variable-domain. Such receptors are broadly classified into three types. $\alpha\beta$ and $\gamma\delta$ T cell antigen receptors (TCR), which are encoded by $\alpha\beta$ and $\gamma\delta$ genes, respectively, and the B cell antigen receptors, which are encoded by immunoglobulin heavy and light chain genes (1–3). The exact sequence of emergence of the antigen-receptor genes is controversial. One view is that the $\gamma\delta$ TCR encoding genes originally constituted a receptor for soluble antigens, which is

primordial to the MHC-restricted $\alpha\beta$ TCR, BCR, and antibodies (2, 4). Alternatively, $\alpha\beta$ and $\gamma\delta$ TCR-genes may have emerged from a common ancestor (5). An interesting case of convergent evolution has occurred in jawless vertebrates (Agnatha). Their lymphocytes express antigen-receptors completely distinct from those of Gnathostomata both in terms of molecular composition (leucine rich repeats instead of Ig domains) and genetic basis of diversity generation [Cytidine deaminase (AID) dependent gene conversion]. Three distinct lineages of lymphocytes expressing distinct antigen receptors variable leukocyte receptors (VLR) (6, 7) have been identified: T-like lymphocytes maturing in the thymus express VLRA and VLRC while B-like lymphocytes produce VLRB as soluble antigen-receptors (8).

Among T cells, those which confer adaptive immunity are MHC-restricted T cells. They express $\alpha\beta$ TCR, which bind to complexes of polymorphic MHC molecules and peptide antigens. Their diversity is generated by RAG-mediated recombination of V(D)J genes of both chains. The diversity of their third complementarity-determining regions (CDR3) which are encoded by the V α J α and V β D β J β transition is further increased by joining

Abbreviations: aa, amino acids; BTN, butyrophilin; BTN3A1-ED, butyrophilin-3 A1 extracellular domain; BTN3A1-ID, butyrophilin-3 A1 intracellular domain; Chr. 6, chromosome 6; DETC, dendritic epidermal T cells; DOXP, 1-deoxy-D-xylulose 5-phosphate pathway; FPPS, farnesyl diphosphate synthase; HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP, isopentenyl pyrophosphate; mAb, monoclonal antibody; MEP, 2-C-methyl-D-erythritol 4-phosphate pathway; PAg, phosphoantigen; VLR, variable leukocyte receptors.

flexibility and insertion of P and N-nucleotides. The final composition of TCR specificities (T cell repertoire) is shaped by intrathymic positive and negative selection guided by anatomically controlled presentation of peptide-MHC complexes and avidity of binding of the emerging TCR to those complexes (9). A highly conserved (2, 3), although not absolute feature in all vertebrate species (10), is the division of mature T cells as in MHC-class I restricted CD8 T cells, which exert killer function and MHC-class II restricted CD4 T cells, which regulate immune functions (2, 3). In spite of a likely co-evolution of the peptide presenting MHC molecules with $V\alpha$ and $V\beta$ TCR genes, it is not possible to predict MHC-class restriction or antigen-specificity of a given T cell from usage of certain TCR genes (11–13).

Alongside “conventional” MHC-restricted $\alpha\beta$ T cells, several T cell populations exist with specificities for ligands other than MHC-peptide complexes. These “non-conventional” T cells can express $\alpha\beta$ or $\gamma\delta$ TCR. The generation of their TCR diversity follows the same genetic mechanisms as for MHC-restricted $\alpha\beta$ TCR but in contrast to MHC-restricted T cells TCR-gene usage can be predictive for ontogeny, homing, and effector functions and is used to define distinct T cell subpopulations (12, 14–17). The best understood population of non-conventional T cells are the CD1d-restricted invariant NKT cells (17, 18). Their TCR α chains are invariant with a characteristic $V\alpha J\alpha$ rearrangement and pair with β -chains of restricted $V\beta$ gene usage. The iNKT TCRs bind in a highly conserved manner to complexes of the non-polymorphic MHC-class I like CD1d molecule and microbial or host cell glycolipids. Thus, functionally they resemble pattern recognition receptors of innate immune cells, which do not discriminate between highly variable antigens but recognize molecular patterns (17, 18). In contrast to MHC-restricted $\alpha\beta$ T cells, which at least in mammals differentiate into CD8 killer T cells and CD4 helper and regulatory T cells, subpopulations of non-conventional T cells vary strongly between phylogenetic groups, e.g., many mammals lack iNKT cells as well as the restricting CD1d molecule (19, 20) but even closely related species such as mouse and rat differ dramatically in terms of iNKT-cell frequency although the respective genes for iNKT TCR and CD1d are highly conserved (21).

$\gamma\delta$ T cells differ strongly between taxa. This difference can be rather global as in case of $\gamma\delta$ T cells whose frequencies among blood T cells vary between 1 and 5% in man and mouse to 50% in ruminants (22). The presence or absence of entire populations defined by their TCR gene usage is even more striking (14, 23–25). Two such cases are addressed in this review: at first the human V γ 9V δ 2 T cells, which have so far only been found in higher primates (26). For their function, associated with various microbial and host metabolites, the molecule butyrophilin-3A1 is mandatory and this article will address the putative function of BTN3A1 and current evidence for a co-evolution of V γ 9V δ 2 TCR and BTN3A1. Secondly, we provide a first analysis of phylogeny of V γ and V δ genes constituting the TCR of murine dendritic epidermal T cell receptor (DETC) (27–30) and *Skint1*, a member of the butyrophilin family, which is critical during the ontogeny and function of these cells (31).

V γ 9V δ 2 T CELLS: TCR AND PHOSPHOANTIGEN REACTIVITY

The vast majority of human blood $\gamma\delta$ T cells respond to so called “Phosphoantigens” (PAg) (32–34). Their TCR share a characteristic V γ 9JP (alternatively designated as V γ 2J γ 1.2) rearrangement (35–38) and V δ 2-containing chains (35) bearing one of the hydrophobic amino acids (38, 39): Leucine (L), Isoleucine (I), Valine (V), or Glycine (G) at position 97. Unless explicitly mentioned PAg-reactive T cells and V γ 9V δ 2 T cells will be used as synonyms in this article.

Freshly isolated V γ 9V δ 2 T cells share functional features with TH1 cells, CD8 T cells, and NK cells (40) but upon culture they can differentiate to TH17 like (41), and to professional antigen-presenting cells (42). Apart from killing or cytokine release, they promote and regulate immune responses by crosstalk with dendritic cells (43), NK cells (44), and monocytes (45). Numerous preclinical and clinical studies demonstrated their therapeutic potential for treatment of tumors (46, 47) and infection (48, 49). The antigen-dependent activation of V γ 9V δ 2 T cells is strongly modulated by additional receptors (50) including inhibitory and activating NK-cell receptors (51, 52). In case of NKG2D even a direct triggering of some effector functions is possible (53).

PAg are products of isoprenoid synthesis, which specifically activate V γ 9V δ 2 T cells. The building blocks of isoprenoid synthesis are isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate. Both are weak PAg (54, 55) and 1000- to 10000-fold less potent than the strongest naturally occurring PAg (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (26, 55, 56). HMBPP is the immediate precursor in the synthesis of IPP by the non-mevalonate pathway also known as DOXP, MEP, or Rohmer pathway. The non-mevalonate-pathway is restricted to eubacteria, cyanobacteria, plants, and apicomplexan protozoa (26, 56), which may have adopted this pathway by endosymbiosis of precursors of chloroplasts and apicoplast, respectively (57, 58). HMBPP is the driving force of a massive V γ 9V δ 2 T cell expansion in infections with HMBPP producing parasites or bacteria, which can lead to an increase of V γ 9V δ 2 T cells from 1 to 5% of blood T cells to more than 50% (26, 49). In mammals and most other animals, IPP is synthesized via the mevalonate pathway whose manipulation can render human cells into V γ 9V δ 2 T cell activators. Cells pulsed with aminobisphosphonates (e.g., zoledronate or pamidronate) become potent activators of primary V γ 9V δ 2 T cells (59, 60) and of V γ 9V δ 2 TCR transductants (61) most likely in consequence of IPP accumulation after inhibition of the IPP metabolizing farnesyl diphosphate synthase (FPPS) (59, 62). The same effects are seen by inhibiting expression of FPPS (63, 64) and of isopentenyl diphosphate isomerase (64). Other modes of activation by IPP accumulation are activation (65) or over-expression of HMG-CoA reductase (66), which is the rate-controlling enzyme upstream of IPP synthesis, and finally inhibition of FPPS by alkylamines (67, 68).

Altogether the V γ 9V δ 2 TCR acts as a kind of pattern recognition receptor, which senses microbial infections as well as metabolic changes found in transformed, infected, or drug treated host cells (69). This reactivity can be harnessed clinically since remission of certain tumor entities after V γ 9V δ 2 T cell activation was observed in clinical trials (46, 47, 59, 60) and it may even contribute

to the beneficial effects of zoledronate seen upon treatment of premenopausal women with early-stage breast cancer (70).

Some tumors such as the human B cell lymphoma Daudi (71, 72) spontaneously activate V γ 9V δ 2 T cells. This activation depends on intracellular accumulation of IPP and can be abolished by statins, which inhibit the HMG-CoA reductase and consequently also IPP synthesis (66). In contrast, still unclear despite intensive investigation is the contribution and the mechanistic basis of V γ 9V δ 2 T cell activation by the pro-apoptotic IPP metabolite ApppI (triphosphoric acid 1-adenosin-50-yl ester 3-(3-methylbut-3-enyl) ester), which is synthesized from IPP and AMP by aminoacyl-tRNA-synthetases (73, 74). Also not clear is how PAg-action is associated with the reported binding of the V γ 9V δ 2 TCR G115 to ectopically expressed F1-ATPase (74, 75).

BTN3 IS MANDATORY FOR PAg-MEDIATED ACTIVATION OF V γ 9V δ 2 T CELLS

PAg act not as soluble molecules but need to be “presented” by cells of human or primate origin (76). This can be seen as evidence for species-specific molecules, which could be PAg-presenting molecules and/or for molecules with special co-stimulatory or cell–cell interaction mediating properties (77, 78). Attempts to identify PAg-binding cell surface molecules by biochemical means, e.g., with the help of photo-activated PAg analogs have failed so far (79, 80) although direct binding of tetramers of rhesus monkey V γ 9V δ 2 TCR to HMBPP pulsed primate dendritic cells was reported (81).

The major breakthrough in defining species-specific molecular requirements for V γ 9V δ 2 T cell activation by PAg was the identification of the pivotal role of Butyrophilin 3 (BTN3) in modulating PAg induced responses (82). BTN3 – also named CD277 – is a membrane protein, which belongs to the butyrophilin family and shares similarity with the B7 family (83). In human beings, three isoforms of BTN3 named BTN3A1, BTN3A2, and BTN3A3 exist whose genes localize to the telomeric end of the MHC complex on the short arm of human chromosome 6 (84). The extracellular domain of BTN3 (BTN3-ED) consists of an N-terminal IgV-like domain (V-domain) and a membrane proximal IgC-like domain (C-domain). The BTN3-ED of the three isoforms is extremely similar and cannot be discriminated by the available antibodies (82, 83). The intracellular domain (ID) of BTN3A1 and BTN3A3 belongs to the B30.2 family (85, 86) and is often found in proteins involved in innate immunity. This includes even molecules encoded by genes found in the hypothetical “proto MHC” of placozoa, which form the most basal branch of Metazoa (87). The BTN3A2 molecule has a truncated intracellular B30.2-negative domain. So far the binding of BTN3 to V γ 9V δ 2 TCR is controversial and a natural ligand or counter-receptor of the BTN3-extracellular domain has not been found yet. Moreover, CD277 antibodies modulate not only responses of V γ 9V δ 2 T cells but also those of $\alpha\beta$ T cells and NK cells (88, 89).

The first evidence for a critical role of BTN3 in V γ 9V δ 2 T cell activation came from the agonistic action of the CD277-specific monoclonal antibody (mAb) 20.1 on V γ 9V δ 2 T cells and other CD277-specific antibodies, such as mAb 103.2, being antagonists for PAg-mediated activation (82). Co-cultures of V γ 9V δ 2 T cells or human V γ 9V δ 2 TCR transductants with mAb-pulsed BTN3

positive cells suggested that mAbs work by binding to stimulatory or target cells and not to the human $\gamma\delta$ T cells. This was formally proven by demonstrating that mAb 20.1 activates BTN3-negative murine V γ 9V δ 2 TCR transductants in co-cultures with human Raji cells (82). These reporter cells were either mouse hybridoma BW58 (or C58 $\alpha\beta$ -) or the rat/mouse T cell hybridoma 53/4-transduced with a V γ 9V δ 2 TCR (TCR-MOP) (82, 90, 91). Interestingly, the PAg-induced IL-2 production by both cell types depends strictly on provision of strong co-stimulatory signal. This can be achieved via ligation of a chimeric rat/mouse CD28 overexpressed on the surface of the reporter cell by CD80 or CD86 on the stimulatory human cell, which can be of natural origin or introduced by gene-transduction (91).

Activation by mAb 20.1 of TCR-MOP transductants but also of primary responder cells is usually less efficient than PAg-induced activation. Nevertheless, it has been shown for primary $\gamma\delta$ T cells that PAg- and mAb 20.1-induced activation are quite similar with respect to TCR-mediated signals and phenotypic changes of the cells (92). Furthermore, activation by mAb 20.1 is resistant to statins and not accompanied by accumulation of IPP (80, 82).

MODELS OF PAg-ACTION: ALLOSTERIC CHANGE VS. ANTIGEN PRESENTATION

At present, two major hypotheses to explain PAg-mediated activation of V γ 9V δ 2 T cells compete with each other. One suggests that PAg-binding to the ID of BTN3A1 provokes changes in the BTN3A1-ED either directly or with help of molecules recruited by the ID. These events are accompanied by a reduced mobility of cell surface BTN3A1 and are mandatory for binding of the V γ 9V δ 2 TCR to BTN3A1 or associated molecule(s) alone or in complex with BTN3. We will refer to this concept as the “allosteric model” (82, 93, 94). The other concept suggests a direct binding of BTN3A1-PAg complexes to the V γ 9V δ 2 TCR and describes BTN3A1 as an antigen-presenting molecule. This will be referred to as the “antigen-presentation model” (95).

THE ALLOSTERIC MODEL

The work of Harly et al. (82) describes not only the general importance of BTN3 for the PAg response but also that BTN3 isoforms differently support PAg-dependent V γ 9V δ 2 T cell activation while no such differences are found for mAb 20.1-induced activation. Evidence was obtained by comparing BTN3 isoform specific knockdown cells and transductants for their capacity to induce PAg-dependent stimulation of primary V γ 9V δ 2 T cells.

The same pattern was found for the reduction in BTN3-cell surface motility. In photobleaching experiments cells expressing BTN3A1 or BTN3A2 constructs were compared. Aminobisphophonate (pamidronate) pulsed BTN3A1 but not BTN3A2 transductants showed a clear mevastatin-sensitive decrease in cell surface motility suggesting a PAg-induced BTN3 isoform related effect. In contrast, for mAb 20.1 no differences between the isoforms were revealed although it decreased motility even stronger than PAg. Further important insights came from structural and functional characterization of the BTN3-ED (93). In this study, soluble and crystallized BTN3-ED revealed no major difference between the three BTN3 isoforms and could consequently not help to explain their functional difference. More interestingly,

these structural analyses revealed two types of BTN3-homodimers. One had a symmetric paralleled structure where both chains were fixed by interaction of their C-domains. The other one had an asymmetric head to tail conformation with contacts between V- and C-domain, respectively. Importantly, the agonistic but not the antagonistic antibody favors formation of the symmetric dimer. In addition, co-crystals with single chain (sc)20.1 revealed a conformational shift of the BTN3 dimer while sc103.2 has no such effect. These results together with other data lead to model where BTN3 and mAb 20.1 binds with a 2:1 stoichiometry and formed a BTN3 lattice at the cells surface. In contrast, intact mAb 103.2 binds with 1:1 stoichiometry and is expected to inhibit formation of such lattices while no inhibitory effects were seen with sc103.2.

Important clues how IDs might control PAg-dependent stimulation comes from recent crystallographic and functional studies (94). This study first identified by deletion and amino acid exchange mutants a region in the B30.2 domain as critical for

PAg-action. Crystallographic analysis identified then a positively charged pocket in this region (the presumed contacts with the PAg are marked in **Figure 1B**), which could accommodate a PAg. Isothermal calorimetry demonstrated PAg-binding to recombinant BTN3A1-ID, which was considerably stronger for HMBPP than for IPP and was extinguished by the same mutants, which abolished $V\gamma 9V\delta 2$ TCR activation by respective BTN3 transducants. The importance of the pocket is further corroborated by a single aa mutant in BTN3A1 (H351R), which destroyed the PAg-binding to BTN3A1-ID, the PAg-dependent activation of $V\gamma 9V\delta 2$ T cells and the zoledronate-induced reduction of cell surface motility. Altogether, evidence is provided that changes induced by PAg-binding to BTN3A1-ID correlate with changes seen in BTN3A1 cell surface motility and PAg-induced $V\gamma 9V\delta 2$ T cell activation (82, 93, 94). For illustrating the key points of this hypothesis a simplified version of the “allosteric model” is depicted in **Figure 2A**.

| A | | IgV | | | | | | | | | | | | | |
|----------|--|-----|---|---|----|---|-------|---|-----|---|---|---|---|---|---|
| Hum | AQFSVLGPSPGILAMVGEDADLPCHLFPTMSAETMP <u>LK</u> WVSSSLRQVVNVYADGKEVEDRQSAPYRGRTSILRDGITAGKAALRIHNVTASDSGKYLC <u>X</u> FQDGDF <u>E</u> KAL | . | . | . | TG | . | K.TPE | . | D.A | . | R | . | N | . | I |
| Aye | -----S.....R.....R.....TG.....K.TPE.....D.A.....R.....N.....N.....I | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| GML | ...A.H..P.....S.....R.....A.....G.....K.TPE.....V..D.....R.....N.....N.....I | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| PTa | ...A.H..P.....S.S.N.....R.....M.E.....M.KL.TE.....L.....V.....Y.R.....N.....N | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Alp | ...A.I..P..V.VI....E.....S.K.....Q.....FM.G.....I.E.....E.....D.....V.....R.....N.....N | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Kwh | ...A.I..P.....E.....K.....N.....T.R.....FM.Q.....T.E.....E.....Q.S.R.....N.....V.....N | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hor | ...I..P.....T.....SLK.....M.R.....E.YE.....NE.....Q.M.E.....E.T.....YG.RV.....N.....EN.....M | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Arm | ...A.IR.PEA.V.....E.....S.K.....LDS.D.W.K.R.....DL.PG.R.A.AK.ADE.....V.....SVAER.V.....S.R.....H.....DN.....M | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Slo | -----A.SKITTSEP---LTVEETTYVIY.MTTSMKSTRGAEG | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| B | | IgC | | | | | | | | | | | | | |
| Hum | VELKVAALGSDLHV DVKG YKDGG IHL ECRST GWP PQP QI QWSNNKGENI PTVEAPVVADGV GLYAVA ASVIM RGS SGE GV SCTIR SLL GLEKT ASIS IADP FFRSA | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Aye |M.AE..ME.....H..RDA..I..A.A.....V.N..QQ..R..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| GML |A..LEM..E.....HV..RDA..V..A.A.....E..M.....N..QQ..R..V..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| PTa |E.RS.E.....KDA..T.ATA.....R.....L..I..N..Q..R..TE..MNS | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Alp |IEM..H.....G.T.S.....RDV..Q..M..A..A..LA..A..T..TS..L..VKD..A..E..IVKNP..NQ..R..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Kwh |IEM..HE.....G.T.....RDA..Q..M..A..A..LPAT..A..TS..LTVKS.....I..NP..NQ..Q..IR.. | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hor |C..F.IE..S.EA.E.....MY.....IDA..D..A..A..L..ERA.....VVK.....E..I..NP..SK..R..V..WR.. | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Arm | | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Slo |C.....IEM..E.....K..V.N.....KDA..K..ALAS.L..A.....TA.V..G.A.....IV.N..Q..KV..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| B | | IgC | | | | | | | | | | | | | |
| Hum | ADVI LPKTAN PILLVSE DQR SVQRAKE PQL PDN PER FNW H YCVLG CES FIS GRH WEVE VGDR KEW HIGVCS KNV QRKG W K MTP ENGF WT MGL TDGN KY R TL TE PR | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| GML |DS.....L..L..L..E.K..L.I.....H..K..T.....Q..RR..E.....I..Y..QS GE.N..A..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Aye |L..L..L..E.E.....L..S.R.....R..T.....Q..RR..E.....I.Q..QS GE.N..A..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| PTa |L..E.....I.....E..N..I..D.R.....N.T.....WR..E.....Y..S..Y..N..A..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Alp |N.....N..L..DKR.N.....D.....K..T.....R..QE..E..C.....V..S..A..SD.. | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| BND |N.....D..L..DKR.N.....K..D.....K..T.....V..RE..E..R..I.....S..D..A..D.. | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hor |N.....N.....L.WV DRR..N..RK..DS..S.....T..F.....N..V..ME..E..YSICRA..K.....E..SN..ED..QA..AHF.. | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Arm | -TWSWT..PRRT..SWWPPRTGGACGGQRGGVCATT..SDSPGM TARWAARASRR..ETFLGGGG.....CTS..QRG..REE..VLGQDD..RERM LDH..AERRER LPRPH PAAD | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hum | NLKLPKPKK VGVFLDYETGDISFYNAVDGSHIHTFLDV SFSE ALYPVF <u>R</u> ILTL EPTALTICPA | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| GML | K.T.AG..R.....EV..M.....Y..PHT..GP.C..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Aye | K.T.TQ..G.....EV..M.....Y..PHT..CGP.C..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| PTa | K.T.D.A.TR.....EV..M.....Y..HT..P.C..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Alp | K.TVAN..QR.....EV..M.....Y..PHTF..GP.W..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| BND | K.TIAN..Q..I.....EV..A..VY..PHT..GP.W..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hor | K.TIAN..QR.....N.EV.....Y..PQT..GP.C.I.G.S..D..... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Arm | QAARGRA.QTG..RVPGPRD..RGVLLRRCRRLPPLHLP AHL LGP..WT.....V..V... | . | . | . | . | . | . | . | . | . | . | . | . | . | . |

FIGURE 1 | Alignment of human BTN3A1 (V-C) and B30.2 domain.

(A) ClustalW2 amino acid alignment of V-C domains of human BTN3A1 extracellular domain with respective homologous sequence identified from WGS database at NCBI. Underlined bold amino acids of human BTN3A1-ID were predicted to interact with PAg (95). **(B)** ClustalW2 amino acid alignment

of intracellular B30.2 domains of human BTN3A1 with respective homologous sequence. Underlined bold amino acids were of human BTN3A1-ID were predicted to interact with PAg (94). Species were abbreviated as Hum, Human; Aye, Aye-aye; GML, Gray mouse lemur; PTa, Philippine tarsier; Alp, Alpaca; Kwh, Killer whales; Hor, Horse; Arm, Armadillo; Slo, Sloth.

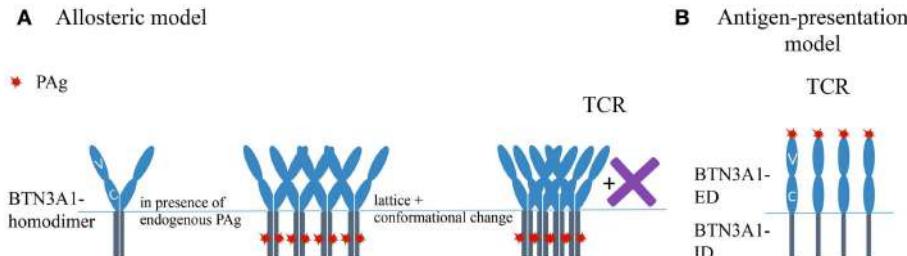


FIGURE 2 | Simplified sketches depicting the “allosteric model” and the “antigen-presenting model.” Note that both models can be partially combined. For reasons of simplicity involvement of hypothetical additional molecule(s) (X) is only depicted where absolutely necessary. **(A)** Sketch

representing the allosteric model describing possible events involved in PAg mediated activation of $V\gamma 9V\delta 2$ TCR. X – represents unidentified molecules, which might involve in mechanism. **(B)** Schematic representation of antigen-presentation model. TCR, T cell receptor; PAg, phosphoantigen.

THE ANTIGEN-PRESENTING MODEL

Vavassori et al. used a different approach to identify the species-specific factor mandatory for PAg-mediated activation and describe *BTN3A1* as an *antigen-presenting molecule* (95). Their approach takes advantage of the fact that mouse lacks a *BTN3* ortholog. At first, murine reporter cells expressing a $V\gamma 9V\delta 2$ TCR were used to screen mouse-human hybrid cell lines for their capacity to mediate PAg-dependent stimulation with the aim to map this trait to a part of the human genome. By analysis of several of such mouse-hybrid cell lines the telomeric 3–27 Mb region of the human chromosome 6p was found to be mandatory for PAg-presentation. This region comprises the entire MHC as well as the *BTN3A1* and *BTN3A2* but not the *BTN3A3* gene. Thus, genomic localization of the mandatory gene(s) is fully consistent with previously published data that *BTN3A1* is mandatory for PAg-mediated activation. The genetic evidence for *BTN3A1* as candidate for the molecule involved was further confirmed by knock down and over-expression experiments.

Interestingly, the reporter cells used in this study were not $V\gamma 9V\delta 2$ TCR-transduced murine hybridoma cells as described above but $V\gamma 9V\delta 2$ T lymphocytes generated from RAG knock-out mice transgenic for the $V\gamma 9V\delta 2$ TCR B2G9, which were matured *in vivo* by administration of anti-CD3 mAb (95, 96). An important difference between data obtained with primary murine reporter cells expressing the $V\gamma 9V\delta 2$ TCR B2G9 and $V\gamma 9V\delta 2$ TCR-MOP transduced reporter cells is that the agonistic mAb 20.1 was not stimulatory but inhibitory for the transgenic mouse cells. First results of our group obtained with TCR transductants suggest that this difference reflects variation of the TCR clonotypes, which stands against the idea of mAb 20.1 being a general activator of $V\gamma 9V\delta 2$ T cells. Nevertheless, to our knowledge, there is no published data on determination of frequencies of mAb 20.1 vs. PAg-reactive cells or direct comparison of sensitivity of different TCR clonotypes for either stimulus supporting this notion. If TCR clonotypes do indeed differ in their sensitivity to both types of stimuli, it would affect models on PAg or mAb 20.1 action. Our interpretation of the presumed clonal differences would rely on substrate competition and inherent qualities of different TCR clonotypes. In the former case, we hypothesize that upon treatment of cells with PAg or mAb 20.1 *BTN3A1* adopts a new conformation, which somehow allows binding of $V\gamma 9V\delta 2$ TCR

to *BTN3-ED-PAg* or mAb complex or to *BTN3-ED-associated* cell surface molecules(s). This conformation could differ to some extent after exposure of the cell to PAg or mAb 20.1 whereby mAb 20.1 might inhibit conversion into the PAg induced conformation. As a result, some TCR clonotypes cannot bind to the mAb 20.1-induced conformation. Indeed, one could imagine that mAb 20.1-binding “freezes” *BTN3-ED* in a conformation (93), which is distinct from the PAg-induced one (93, 95). Considering inherent qualities of TCR clonotypes as the basis for their differential capacity in recognizing *BTN3A1-ED-PAg* complex or *BTN3-mAb* complex, we propose or speculate that some $V\gamma 9V\delta 2$ TCR, e.g., TCR B2G9 preferentially bind to a complex of PAg bound to the *BTN3A1-ED*, whereas others would preferentially bind to the conformationally changed *BTN3A1* whose ED does not need to be in complex with the PAg. Consistent with this model would be that the area covered by the mAb 20.1 is rather near to the hypothetical PAg-binding site discussed in the next paragraph. Consequently for some TCR mAb 20.1 would compete with binding of the $V\gamma 9V\delta 2$ TCR to a *BTN3A1-PAg* complex while for others mAb 20.1 would still be stimulatory.

De Libero and coworkers (95) provide also a wealth of data in favor of a direct binding of PAg to *BTN3A1-ED* and of binding of *BTN3A1-PAg* complexes to the $V\gamma 9V\delta 2$ TCR: (i) IPP and HMBPP induce a substantial IFN γ secretion by the murine reporter cells cultured in *BTN3A1-V* domain coated culture plates. (ii) Mass spectrometry data of *BTN3A1-V* incubated with IPP is consistent with a *BTN3A1-IPP* complex of 1:1 stoichiometry. (iii) Plasmon resonance analysis of PAg binding to *BTN3A1-V* domain allowed calculation of K_d values. These are considerably lower than that of MHC-peptide complexes: 66.9×10^{-6} M for binding of *BTN3A1-V* to IPP and 3.06×10^{-6} M for binding of *BTN3A1-V* to HMBPP. (iv) Crystal structures of complexes generated from *BTN3A1-V* and IPP or HMBPP, respectively, identify a shallow Ag-binding groove. The amino acids proposed to interact with HMBPP or IPP respectively are marked in Figure 1A. (v) Plasmon resonance analysis revealed a low affinity binding of recombinant $V\gamma 9V\delta 2$ TCR multimers (dextramers) (TCR G2B9) to immobilized eukaryotic recombinant *BTN3A1-ED* (vi) Surface enhanced Raman scattering demonstrates binding of monomeric TCR to *BTN3A1* with a K_d of 34×10^{-5} M in the presence and 93×10^{-5} M in the absence of IPP. All these data can be interpreted

as evidence that BTN3A1 serves an antigen-presenting molecule (95). A simplified version of this hypothesis is depicted in **Figure 2B**.

The results and conclusions (95) on BTN3-binding to PAg and V γ 9V82 TCR summarized in the previous paragraph are heavily disputed by proponents of the “allosteric model” (94). In their recent publication, the groups of Adams and Scotet/Bonneville, respectively, give detailed account on failed attempts to detect PAg-binding to the BTN3-ED and of V γ 9V82 TCR (TCR-G115) dextramers to immobilized BTN3-ED. Furthermore, their paper opposes the interpretation of the BTN3-V domain crystallized in presence of PAg as BTN3-PAG complex (94). Based on their analyses of the B30.2 domain and their own negative data on PAg- and TCR-binding, BTN3A1 is refuted as an “antigen-presenting molecule.” Instead, they propose in the discussion section: “a model where PAg binding to the BTN3A1 ID results in recruitment of additional primate-specific factors and/or rearrangement of the BTN3A1 extracellular domain that generates a stimulatory signal directly detected by the V γ 9V82 TCR. This is similar to “Model 2” proposed by Morita and colleagues” (94). The paper of the Morita group (80) mentioned here describes their failed attempts to show binding to BTN3-ED by the use of photo-activatable HMBPP and develops models to explain lacking PAg-binding to BTN3-ED despite its crucial role for the activation of V γ 9V82 T cells.

Some of the data and interpretations reported by the proponents of either model can be reconciled and others not. For example, both agree on the importance of the ID for BTN3A1 mediated PAg activation, and in principle the data on PAg-binding to the BTN3-ID could be adapted in a model with BTN3A1 as an antigen-presenting molecule in following ways: (1) PAg-binding to the BTN3A1-ID may result in intracellular trafficking, e.g., recruitment of a molecule which enables loading of PAg to the BTN3A1-ED and/or involved in proper cell surface distribution of BTN3A1 with the PAg loaded BTN3A1-ED. (2) Another possibility could be that PAg-binding to the BTN3A1-ID induces a conformational change facilitating formation of the BTN-ED-PAG complex or stabilization of such a complex. Concerning the dissent on crystallographic and binding data it would be most desirable – and important for the research community – if controversies on the validity of experimental procedures and their interpretation were solved, e.g., by collaborative attempts of both parties to reproduce data with exchanged reagents, e.g., the different BTN3 preparations and the different TCR clonotypes (TCR-G115 vs. TCR-G2B9). In addition, completely different experimental approaches such as the identification of cofactors controlling PAg-mediated activation by genetic methods might lead to new insights and perspectives and help to solve this controversy.

EVIDENCE FOR CHR 6 ENCODED GENE(S), WHICH IN ADDITION TO BTN3A1 ARE MANDATORY FOR PAg-MEDIATED ACTIVATION

A first hint that BTN3A1 alone is not the only molecule expressed in primates required for PAg mediated activation comes from experiments where BTN3A1-transduced murine cells pulsed with zoledronate failed to activate human V γ 9V82 T cells (94). Nevertheless, these negative results need to be met with caution since in this xenogeneic setting co-stimulatory receptors and adhesion

molecules required for V γ 9V82 TCR-mediated activation might miss their partners on the murine cell and activation may be incomplete.

To solve this problem, murine reporter cells provide a valuable tool. We tested mouse-human hybridomas generated in our laboratory for their capacity to activate reporter cells (TCR-MOP transductants) (97) similarly, as it was done in (95). Consistent with the localization of *BTN3A1* on chromosome 6 (Chr. 6) only hybridoma carrying the human Chr. 6 were able to induce PAg-dependent V γ 9V82 TCR responses. To test whether *BTN3A1* alone or *BTN3A1* and other genes on Chr. 6 allow the PAg-mediated stimulation and whether the same accounts for mAb 20.1-induced activation, different types of Chinese hamster ovary (CHO) cells were tested. We compared CHO cells and CHO cells containing human Chr. 6 and the *BTN3A1* transductants of either cell type. In a nutshell, Chr. 6 was found to be sufficient and mandatory to induce activation in the presence of HMBPP and zoledronate while *BTN3A1* alone allowed mAb 20.1-induced activation even in the absence of Chr. 6 (97).

These data as well inhibition studies with BTN3-specific antibodies are in full agreement with the pivotal role of *BTN3A1* in PAg-mediated activation but imply also the existence of one or more human gene(s) controlling PAg-action, which are missing in rodent cells. Such gene(s) could be involved in PAg-loading onto *BTN3A1* analogous to the TAP being mandatory for MHC-class I mediated peptide-presentation. Other possibilities would be, e.g., control of *BTN3A1* related signaling pathways steering cellular distribution of *BTN3A1* or a factor X directly associated with *BTN3A1*. In any case identification of these molecules in primates will be essential for the design of rodent models with functional V γ 9V82 T cells, which can be expected to provide fresh insights of V γ 9V82 T cell physiology and facilitate preclinical research on V γ 9V82 T cells and V γ 9V82 T cell-activating drugs (97). The search for such molecule(s) could be facilitated by the murine reporter cells (95, 97), which allow functional screens for the missing genes, e.g., of rodent-human radiation hybrids containing fragments of human Chr. 6 or human expression libraries.

An important implication of the finding that mAb 20.1 permits V γ 9V82 TCR-mediated activation by *BTN3A1* expressing rodent cells is that species (human) specific gene(s) can be dispensable for PAg-independent V γ 9V82 TCR-mediated activation. We suggest that mAb 20.1 induces a conformational change of *BTN3A1*, which allows the V γ 9V82 TCR to directly interact with *BTN3A1* or a highly conserved molecule, which upon mAb 20.1 incubation interacts with *BTN3A1* and is then recognized by the TCR.

We tested also CD69 up-regulation of human V γ 9V82 T cells with PBMC co-cultured with different CHO cell variants pulsed with zoledronate. Only co-cultures with Chr. 6 consomic CHO cells induced a V γ 9V82 T cell specific activation. Interestingly, we failed to detect CD107a induction suggesting that in this setting V γ 9V82 TCR-mediated activation is incomplete (97). This is of interest since Sandstrom et al. (94) who tested another xenogeneic setting namely co-culture of primary V γ 9V82 T cells with zoledronate or mAb 20.1 pulsed *BTN3A1*-transduced murine cells used CD107a expression as read out and might have missed a partial activation. Not excluded can be that *BTN3A1*-transduced CHO cells, which are of hamster origin might express species- or

cells-specific factors that are missing in murine BTN3A1 transductants (94) and allowed activation of our reporter cells (TCR-MOP transductant).

V γ 9V δ 2 TCR: THE NEGLECTED INTERACTION WITH PROTEIN ANTIGENS

The identification of PAg as activator for the vast majority of V γ 9V δ 2 T cells does not exclude other physiological modes of TCR-triggered V γ 9V δ 2 T cell activation. In a series of studies He and colleagues investigated tumor infiltrating $\gamma\delta$ T cells, i.e., especially malignancies of ovarian origin, and identified specific ligands whose recognition depended largely on the CDR3 δ and CDR3 δ flanking regions of the TCR (98–103). This was demonstrated with help of TCR transductants but also with Ig constructs with implanted CDR3 δ , which bind to tumors *in vitro* and trigger tumor elimination in xenografted mice (103). Among the proposed antigens were MutS homolog 2, hsp 60 (99), and the NKG2D ligand ULBP-4 (100). In one case tumor-specific and PAg-mediated activation were compared and mutagenesis of amino acid 97 in the CDR3 δ abolished PAg but not an anti-tumor response (102). This CDR3 δ -controlled recognition is reminiscent of that of T22 molecules by murine $\gamma\delta$ TCR (12, 104) and could also be important for other human $\gamma\delta$ T cells. In any case, apart from its therapeutic potential the possibility of PAg-independent tumor recognition by V γ 9V δ 2 T cells demonstrates a function of V γ 9V δ 2 T cells beyond that of a sensor of metabolic aberrations or of microbial metabolites (69). Indeed, it is rather likely that such (oligo)clonal responses might have been missed in the analysis of PAg-responding cells given the presumed low frequency of such protein antigen-specific V γ 9V δ 2 TCR in comparison to PAg-reactive cells. Furthermore, these oligoclonal PAg-independent responses might contribute to the changes in the V γ 9V δ 2 T cell repertoire seen during infections with HIV or with *Mycobacterium tuberculosis* (49, 105).

V γ 9V δ 2 TCR AND BTN3 IN PRIMATES

Monkeys including hominids possess V γ 9V δ 2 TCR and functional BTN3A1 genes (80, 93, 106, 107). PAg reactivity of V γ 9V δ 2 T cells has been directly demonstrated for simian species (simiiformes), which include the new world monkey Nancy Ma's night monkey (*Aotus nancymaae*) (108) and the common marmoset (*Callithrix jacchus*) (109) and the old world monkeys rhesus macaque (*Macaca mulatta*) (110) and cynomolgous macaque (*Macaca fascicularis*) (111), which bear a hydrophobic amino acid at position 97 of delta chain and V γ 9JP rearrangements. Human and most primate species have a K(Lysine)KIK motif in JP, which is sometimes changed to R(Arginine)KIK without apparent consequences for PAg reactivity (107, 110).

An update in September 2014 of our previous searches (107) identified translatable V γ 9, V δ 2, and BTN3A1 sequences in lower primates for the representative of non-simiiforme haplorrhini, the philippine tarsier (*Carlito syrichta*). The same was found for aye aye (*Daubentonia madagascariensis*) and the gray mouse lemur (*Microcebus murinus*) representing the two lemur clades of the strepsirrhini suborder. The BTN3A1 sequences were largely conserved in the proposed PAg-binding motif of the BTN3-ED and in the BTN3-ID. The BTN3-ID of aye aye and tarsier showed the

H351R substitution, which abrogates PAg-binding of BTN3A3 (Figure 1). Therefore, it would be interesting to learn whether this also leads to loss of PAg-binding or is compensated by other sequence changes. If PAg-binding of the BTN3-ID was indeed lost and if PAg involvement is needed to maintain the typical V γ 9V δ 2 TCR repertoire then one would expect consequential effects on this repertoire, e.g., less or no V γ 9JP rearrangements.

V γ 9, V δ 2, AND BTN3 CO-EMERGED AND MAY HAVE CO-EVOLVED IN PLACENTAL MAMMALS

Studies on rodent genomes and on TCR expressed in farm animal species (cow, pig, horse) provided no evidence for V γ 9 or V δ 2 homologs, and it was generally assumed that V γ 9V δ 2 T cells might be restricted to (higher) primates. Taking advantage of increasing number of public data genome data bases, especially the 29 mammals project, which covered all mammalian orders (112), we blasted against genomes of Eutherian mammalian species in search for genes homologous to human V γ 9, V δ 2, and BTN3-ED. We asked for at least 80% sequence coverage and 70% nucleotide identity to human genes in order to identify new species as potential V γ 9V δ 2 T cell carriers. Homology was confirmed by reverse blasting and different types of phylogenetic trees were generated. Most of these results have been published recently (107) but we take the opportunity of this paper to present changes resulting from recent updates in the database and from inclusion of the BTN3-ID in our analysis (Figure 1).

Truly surprising results were obtained from the analysis of non-primate species. The timing of the origin of placental mammals is subject of a protracted debate and has been estimated to occur between 165 and 65 million years ago (113–115). Nevertheless, there is consensus that Xenathra – represented in the databases by the nine-banded armadillo (*Dasypus novemcinctus*) and the two fingered sloth (*Choloepus hoffmanni*) belong to a clade distinct from Boreoeutheria, which represents the other placental mammals. Thus, detection of a gene in a species of Xenathra and Boreoeutheria proves its presence in a common placental predecessor. Exactly, this is seen for V γ 9, V δ 2, and BTN3-ED since they are found in sloth and armadillo. While TCR-V genes were rather conserved (107), Figure 1 shows that BTN3-ED of sloth lacks a major part of the V domain while that of armadillo lacks parts of the C domain. A BTN3-ID like domain could not be identified for sloth and the B30.2 domain of armadillo was identified as a homolog of BTN2-ID. With the current knowledge on BTN3-structure-function relationship and PAg-mediated V γ 9V δ 2 T cell activation, it seems unlikely that these molecules could function as proposed for human BTN3. Consequently, we would not expect maintenance of typical V γ 9V δ 2 T cells (e.g., dominance of the characteristic V γ 9JP rearrangements) but if such cells were found, then they should be tested for PAg-reactivity or -binding to other ligands. Testing is especially relevant for armadillo, since it is a natural host *Mycobacterium leprae*, an important human pathogen and potential target for V γ 9V δ 2 T cells (116).

Many of the tested genomes had lost all three genes. This was true for lagomorphs and most rodents and explains why PAg-reactive cells have never been observed in the classical small animal models (rat, mouse, guinea pig, and rabbit). A notable exception could be the 13-lined squirrel (*Spermophilus tridecemlineatus*),

which conserved a translatable V γ 9 and a presumably functional BTN3 (**Figure 1**) but lacks a V δ 2. Therefore, it will be of special interest to learn whether this V δ 2 is truly missing or whether the searched database is incomplete. Species of other orders such as those representing Bovidae (cow, sheep, goat, tibetan antelope) kept all three genes but at least one of them was either not translatable (e.g., *BTN3-ID* of tibetan antelope) or otherwise non-functional as in the case of the horse (Perissodactyla) whose V γ 9 and V δ 2 genes lacked one of the cysteine required for Ig domain disulfide bond (107).

It is very striking that all species (11 species representing 9 families representing 3 of the 4 mammalian superorders) with translatable V γ 9 and V δ 2 TCR gene possess translatable *BTN3-ED*. The inverse correlation is not as strict, since *BTN3A1* of horse (Equidae), white rhinoceros (Rhinocerotidae), several bats (Vespertilionidae), and the thirteen-lined squirrel (Sciuridae) are translatable, although the species lack either functional V γ 9 and/or V δ 2 genes. However, inspection of *BTN3-ID* (**Figure 1**) of horse, rhinoceros and bats shows considerable differences in the proposed PAg-binding site suggesting loss of PAg-related function. These species show non-conservative substitutions in the proposed extracellular and intracellular PAg-binding sites while the other species with a complete and translatable *BTN3-ED* showed no or only conservative changes (K to R and vice versa). Altogether, the data suggest an interdependence in conservation of function between V γ 9, V δ 2, and *BTN3* genes and indicate molecular co-evolution of V γ 9V δ 2 TCR and *BTN3* (117).

ALPACA (*VICUGNA PACOS*) AS MODEL TO STUDY V γ 9V δ 2 T CELLS IN A NON-PRIMATE SPECIES

Of the nine species originally found to possess translatable V γ 9, V δ 2, and *BTN3-ED* genes (107), alpaca was the only one accessible for further analysis. V γ 9-C γ PCR products from PBMC cDNA were cloned and although different J γ segments were identified, 90% of the clones showed a JP rearrangement. The primate KKIK or RKIK motif is largely conserved in the three alpaca JP, which have a KTIK or RTIK motif. In contrast to human V γ 9V δ 2 T cells, the V δ 2 gene rearranged (25 out of 25 clones) always with a single J δ gene, which is highly homologous to human J δ 4 (TRD)*04. The amino acids L, I, V, and G at position 97, which are typical for PAg reactive human V γ 9V δ 2 TCR were found in 8 out of 17 clones bearing alpaca V δ 2 chains. Similar to human, alpaca V δ 2 chains also show high diversity in CDR3 lengths ranging from 11 to 18 amino acids (38, 107), while CDR3 γ length is rather restricted (107). Transduction of 58 α - β -cells (BW58) with full length alpaca V γ 9 and V δ 2 TCR chain genes led to their cell surface expression as assessed by surface staining for murine CD3 and detection of IL-2 upon culture with immobilized anti-CD3 antibodies. Due to lack of appropriate reagents, the formal proof of cells concomitantly expressing V γ 9 and V δ 2 TCR chains is still missing.

Nevertheless, at least with respect to *BTN3A1* and the TCR genes, there is currently no obvious reason to assume that PAg could not activate alpaca V γ 9V δ 2 T cells. Indeed, the *BTN3A1* has been cloned and expressed and showed complete identity with human *BTN3* in the published intracellular- and extracellular PAg-binding sites (118). The functionality with respect

to PAg-responses will be tested with $\gamma\delta$ TCR transductants and camelid stimulator cells but also with alpaca PBMC.

If PAg-reactivity can be confirmed, alpaca will provide an outgroup allowing identification of common denominators of PAg-reactivity of V γ 9V δ 2 T cells, and it will allow analysis of conservation of the molecular mechanisms of PAg-dependent stimulation or presentation, respectively. If PAg-reactivity is missing, then interesting questions arise. Are there alternative modes of V γ 9V δ 2 T cell activation and are they also *BTN3A1*-dependent? If there is a *BTN3*-dependent V γ 9V δ 2 T cell activation, new questions arise such as, did other molecules take over the role of PAg and can introduction of human genes (favorably encoded on chromosome 6) generate a *BTN3A1*-dependent PAg-response similar to that seen in humans?

IDENTIFYING CANDIDATE SPECIES FOR DENDRITIC EPIDERMAL T CELLS BY INVESTIGATING $\gamma\delta$ TCR GENES AND *Skint1* HOMOLOG IN MAMMALS

At the end of this review, we want to discuss a possible co-evolution of $\gamma\delta$ TCR genes and their relation to another member of the butyrophilin family. Dendritic epidermal T cells (DETC) present an extreme case of a highly specialized $\gamma\delta$ T cell population, which so far has only been found in mice and rats (28, 119, 120). As the name indicates these T cells have dendritic shape and reside in the epidermis. They appear as the earliest T cells during development in the fetal thymus and subsequently migrate to skin. In the skin, they fulfill TCR-dependent and TCR-independent functions in body barrier surveillance including control of tumor development, skin repair, and allergy control. A hallmark of these cells is expression of a single TCR, which with unique rearrangements containing V γ 3 and V δ 4 (IMGT-nomenclature; other common nomenclatures are V γ 3V δ 1 and V γ 5V δ 1). Canonical DETC can be replaced by cells with a polyclonal TCR repertoire but these do not fulfill all of their functions (15, 25, 30). Mandatory for the development of canonical DETC is the molecule Skint1, which is a member of the butyrophilin family. It consists of a V-C domains containing extracellular domain and a three times transmembrane-spanning domain (31, 121, 122). Its role in DETC development was discovered by analysis of FVB/N mice from Taconics laboratories. These mice showed changes in the DETC TCR repertoire, which were correlated with a skin phenotype (spontaneous ear inflammation and exaggerated irritant contact dermatitis response to tetradecanoylphorbol acetate). The genetic basis of this phenotype is a termination mutation in codon 324 of Skint1 immediately upstream of the third transmembrane domain of the molecule (31, 121). In a recent study on structure-function relationship of Skint1, it was found that a tightly regulated cell surface expression on medullary thymic epithelial cells was mandatory for efficient DETC development. Furthermore, mutagenesis and domain exchange proved that each Skint1 domain is non-redundant, including a unique decamer specifying V-domain processing (122) (marked in **Figure 3**).

Prompted by the striking concomitant conservation of TCR-V genes and *BTN3A1* and the success with identification of alpaca as a candidate for a V γ 9V δ 2 T cell positive species, we decided to search for homologs of DETC TCR-V genes V γ 3 and V δ 4 and of *Skint1* as a gene controlling DETC development using the same methods as for *BTN3* and V γ 9V γ 2 TCR genes (107). Homologs

A

| | |
|-----|---|
| Mou | EVKITA INLOVOT HVPPNTKG VIVECHSGG FWPRPLM QWRD RGRGE VIPAA SKHS QGRD KLNF NMKIS LLI SEFF QKV ICCL QNPL TGQEE |
| Rat | ...V.....R.....E....S.....G.R.....D.S..IT.....G |
| CGM | .L.V..T..DI..V....I..L..N.E..Q..S..NN..IM.SVFT.Y.MDSA..S..MT..LKDKSYRN.T..... |
| PVo | ...V..VS.....L...N....Q..E..S.....DEG..I.MI..R..SS..... |
| GHa | ...V..VS.....L...N....Q..E..S.....DEG..I.MI..R..SS..... |
| CHA | ...V..VG.....N....K..E..S.....T.....DEG..I.MI..RD.SSP..T.Y.....Q |
| Cow | ...V..TS..ET..L.....LL..N....Q.Q.E..S.E.I..PS.....DT.....MT..QS-THGN.T.Y.R..V..... |
| GHB | ...V..TS..EI..L.N.....LL..S.E....Q.Q.E..S..I..YT.L.....DT.....H..MT..LRHH..HRN.T.....SV..... |

B

| | |
|-----|---|
| Sk1 | SEPFIVNGLEGPVPLASLGGNNLELSQLSQQPQQAQHMEIRWFERNLYTEPVHLYRDKGKDFGEIISKYVERTELLKDGGIGEGKVTLRIFNVTVDGGDSYHCVFKDGDGFYEEH |
| Sk2 | ..K.T.T..QR...P...V.....R.R..Y..N..LH..T.....D..K.....KL.A.....V.E..... |
| Sk3 | ..Q.TIT..R...P..I.....N.Q.....R..Y..N..LH..T.....HD..K.....V.K.....I..... |
| | |
| Sk1 | ITEVKITAINLOVOIHVHPNPKGVIVECHSGGWFPRLMQWRDRRGEVIPAASKSHSQGRDKLFNMKISLLISESFFQKVICCLQNPLTGQE |
| Sk2 | ..I.V...TSSVMY.LMQ..I..ML.....Q.H.E..N.K.NI..T..A..DEN.....TMT..EA.SHRSIT.Y..L..H.. |
| Sk3 |V..TSSDIK.IM..I..ML..R..Q.H.E..SN.Q..T..Q..DEN.....TMN.FADVGLH.I.T.YI..L..H.. |

FIGURE 3 | Alignment of human BTN3A1 (V-C) and B30.2 domain.

(A) ClustalW2 amino acid alignment of V-C domains of mouse Skint1 extracellular domain with respective homologous sequence identified from WGS database at NCBI. Underlined bold 10 amino acids stretch is predicted to confer functionality for mouse Skint1 (122). **(B)** ClustalW2 amino acid

alignment of V-C domains of mouse Skint1 extracellular domain with its paralogs. Underlined bold 10 amino acids stretch is predicted to confer functionality for mouse Skint1 (122). Species were abbreviated as Mou, Mouse; CGM, Cape golden mole; PVo, Prairie vole; GHa, Golden hamster; CHa, Chinese hamster; GHB, Greater horseshoe bat.

for at least one of the three genes were identified in 69 species, all of them were Eutheria. No hits were found for Xenathra but the cape golden mole was identified as an afrotherian species being “triple positive.” In case of the TCR-V genes, the assignment was always clear. In case of *Skint1*, forward blasting of the V-C domains let to identification of genes as *Skint1* homolog, which in reverse blast turned out to be *Skint2* or *Skint3* homologs. We marked this in our table (Table S1 in Supplementary Material) but the limited knowledge about Skint structure and mode of action does not allow to make any predictions to which extent the Skint homologs in these species are redundant – which at least in mouse they are not (122). Also statements on translatability have to be taken with some caution since transmembrane encoding exons were not included in the search.

Nevertheless, the retrieved data (Table S1 in Supplementary Material and **Figure 3**) could help to identify non-murine species as candidates for a search for rearranged DETC TCR genes and functional Skint: prime candidates are rodent species, which do not belong to the family of muridae but are still phylogenetically not too distant. These would be the two hamster species. Also interesting but unfortunately not yet sequenced at genomic level is the cotton rat (*Sigmodon hispidus*) and therefore not to be found in the Table S1 in Supplementary Material and **Figure 3**. This “new world mouse” species is a well-established animal model for a number of infectious diseases (123). Triple positive members were also found in other mammalian superorders than the rodent-containing Euarchontoglires. These are Afrotheria with the golden

cape mole (*Chrysocloris asiatica*) and Laurasiatheria species with the greater horseshoe bat (*Rhinolopus ferrumequinum*) and the cow (*Bos tauris*). Nearly, all bats (Chiroptera) carry translatable V γ 3, V δ 4, and either Skint1 or Skint2. Finally, the cow was also triple positive and was the only Artiodactyla species with a translatable Skint. Given the limits of our data-base search, however, at first it needs to be tested whether the latter is still functional. If expression of a Skint1 can be confirmed, it will be of interest to test whether a cow DETC population might be hidden among other populations such as the circulating polyclonal dermal γ 8 T cells or the small epidermal γ 8 T cell population (124).

Nevertheless, despite all possible pitfalls in database analysis, we are confident that comparative analysis of TCR genes and genes of putative TCR ligands or of molecules controlling development and function of non-conventional T cells will allow to identify genetic or functional homologs to human non-conventional T cells. The identification of such homologous populations in phylogenetic distant species or species with different life style could help to identify common themes on preservation and flexibility of genes and of functions of such cells. Going back to human beings (or mice) and seeing old acquaintances in a new light may help for a better understanding of the human system and identification of targets of genetic and immune-intervention.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00648/abstract>

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$\gamma\delta$ T lymphocytes as a first line of immune defense: old and new ways of antigen recognition and implications for cancer immunotherapy

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Among $\gamma\delta$ T cells, the V δ 1 subset, resident in epithelial tissues, is implied in the defense against viruses, fungi, and certain hematological malignancies, while the circulating V δ 2 subpopulation mainly respond to mycobacteria and solid tumors. Both subsets can be activated by stress-induced molecules (MIC-A, MIC-B, ULBPs) to produce pro-inflammatory cytokines and lytic enzymes and destroy bacteria or damaged cells. $\gamma\delta$ T lymphocytes can also recognize lipids, as those associated to *M. tuberculosis*, presented by the CD1 molecule, or phosphoantigens (P-Ag), either autologous, which accumulates in virus-infected cells, or microbial produced by prokaryotes and parasites. In cancer cells, P-Ag accumulate due to alterations in the mevalonate pathway; recently, butyrophilin 3A1 has been shown to be the presenting molecule for P-Ag. Of interest, aminobisphosphonates indirectly activate V δ 2T cells inducing the accumulation of P-Ag. Based on these data, $\gamma\delta$ T lymphocytes are attractive effectors for cancer immunotherapy. However, the results obtained in clinical trials so far have been disappointing: this review will focus on the possible reasons of this failure as well as on suggestions for implementation of the therapeutic strategies.

Keywords: $\gamma\delta$ T cells, aminobisphosphonate, BTN3A1, NKG2D ligands, ADAM proteins

$\gamma\delta$ T CELLS AND ANTIGEN RECOGNITION

Human $\gamma\delta$ T lymphocytes comprise different subsets defined by their T-cell receptor (TCR), the most prominent of which is present in circulating blood, representing 3–5% of T lymphocytes, and is composed of cells expressing the V γ 9V δ 2 TCR (V δ 2T cells). The subset bearing the V δ 1 chain of the TCR is <1–2% of circulating T cells and is mostly represented in the mucosal-associated lymphoid tissue, known to play an important role in the first-line defense against viral, bacterial, and fungal pathogens (1–5). $\gamma\delta$ T cells recognize a wide variety of antigens, such as lipids, proteins, and phosphoantigens (P-Ag), without the need of HLA-restricted antigen presentation (6–9): circulating V δ 2 T lymphocytes are involved in the response to mycobacteria, EBV, and some solid tumors, while resident V δ 1 T cells contribute to the immunity against *Listeria monocytogenes*, CMV, and certain hematological malignancies (2–4, 10). Both $\gamma\delta$ T-cell subsets can interact with stress-induced MIC-A, MIC-B, and ULBPs; the recognition is mediated through the NKG2D receptor, also expressed by $\alpha\beta$ T lymphocytes (3, 11–13). In $\gamma\delta$ T cells, NKG2D seems to work in association with the TCR that also binds to these stress molecules: upon its engagement, an activating signal is delivered in $\gamma\delta$ T lymphocytes that promptly exert their effector function, by proliferating, producing pro-inflammatory and antimicrobial cytokines, such as interferon-gamma (IFN)- γ or tumor necrosis factor (TNF)- α , or releasing lytic enzymes to destroy bacteria or infected cells, as a response to damage signals (10–13). A similar mechanism can be exploited by $\gamma\delta$ T lymphocytes to face transformed cells that also overexpress NKG2D ligands (−L) due to the stress-inducing

transformation, like in solid tumors or in hematological malignancies (14–19). Of note, these ligands can also be upregulated at the cell surface by drugs, including all-trans-retinoic acid or sodium valproate, commonly used in anti-leukemic therapeutic schemes, thus improving $\gamma\delta$ T cell-mediated anti-cancer capacity (20–23). Another potent stimulus for $\gamma\delta$ T cells of the V δ 2 subset, acting through the TCR, is represented by low molecular weight P-Ag (4–8). Consistent with the stress-surveillance model, P-Ag may be autologous, such as isopentenylpyrophosphate (IPP), which accumulates in many virus-infected or transformed cells, or microbial, such as hydroxymethyl but-2-enyl pyrophosphate (HMBPP), a metabolic intermediate specific to many prokaryotes and parasites (4–8). Of clinical interest, aminobisphosphonates (N-BPs), which are widely prescribed for osteoporosis and malignancy, indirectly activate V γ 9V δ 2 cells by inhibiting farnesyl-pyrophosphate synthase, which provokes IPP accumulation (24–28).

POSSIBLE ANTIGEN-PRESENTING MOLECULES FOR $\gamma\delta$ T CELLS

Thus, the types of Ag recognized by $\gamma\delta$ T lymphocytes may vary in size, composition, and molecular structure, much more than those recognized by $\alpha\beta$ T cells, and include soluble or cell surface proteins, small peptides, phospholipids, prenyl-pyrophosphates, and sulfatides. The mode of antigen recognition by $\gamma\delta$ T cells has been a controversial issue for several years, as they apparently do not need Ag presentation by specialized cells. The TCR that these lymphocytes are equipped with, display some peculiar features such as a limited diversity compared to the $\alpha\beta$ TCR, and a type of interaction

with the Ag that rather resembles that of the B-cell receptor. This hypothesis is based on structural and functional findings: indeed, CDR3 regions of the $\gamma\delta$ TCR resemble immunoglobulin (Ig) CDRs in terms of length and variability, as the TCR δ and γ chain have long or short CDR3, respectively, as is the case of Ig heavy and light chains (29, 30). In contrast, length and conformation of TCR α and β CDR3s are similar to each other, which may be a requirement for the docking on the surface of MHC molecules and the recognition of MHC-bound peptides. In some cases, however, small Ag may be presented to $\gamma\delta$ T cells as well, in general in the case of soluble small molecules unable to induce a TCR cross-linking (31). A still unsolved question seems to be the Ag-presenting molecule recognized by $\gamma\delta$ T cells. In mice, the non-classical or truncated MHC molecules T10/T22, not constitutively expressed at the cell surface but induced by stress signals, have been shown to bind to $\gamma\delta$ TCR, that makes an angle using CDR3 δ amino-acid side chains for the interaction (32, 33).

Other structures described to be potentially responsible for Ag presentation to $\gamma\delta$ T cells are the group1 CD1 molecules. CD1 comprises a family of non-polymorphic genes located outside the MHC complex and encodes proteins structurally related to MHC class-I molecules (34, 35). In humans, products of four of the five CD1 genes, designated CD1a, CD1b, CD1c, and CD1d, have been identified as type 1 integral membrane proteins associated with β 2-microglobulin and are expressed on antigen-presenting cells. A direct evidence for CD1 proteins as antigen-presenting molecules was provided by isolation of a human CD4 $^-$ CD8 $^-$ T-cell line that proliferated in response to *M. tuberculosis*-derived antigens: the purification of the CD1b-restricted antigens revealed a subset of mycolic acids, a family of free fatty acids present in the outer cell wall of mycobacteria and several other bacteria. Soon after, some glycolipids, such as phosphatidylinositol-containing lipoglycans and glycosylated mycolates, that are also associated with the mycobacteria cell wall, were identified as CD1b-presented antigen (36, 37). The CD1-restricted presentation of lipid and glycolipid antigens to T cells was strengthened by the three-dimensional structure of the mouse CD1d protein determined by X-ray crystallography (35, 38), showing a putative antigen-binding groove, which is remarkably different from that found in MHC molecules. Subsequent characterization of mycobacteria-derived antigens revealed a remarkable ability of human group 1 CD1 (CD1a, CD1b, CD1c) to mediate presentation of lipid and glycolipid antigens to T cells, including $\gamma\delta$ T cells.

It has been unknown for many years whether and how prenylpyrophosphates are presented to $\gamma\delta$ T cells. In the last two years, a number of papers have been published identifying butyrophilin (BTN)3A1 as the molecule that can directly bind P-Ag for presentation. BTNs are type 1 trans-membrane molecules containing two Ig-like domains in their extracellular portion (39). Some BTNs carry a B30.2 domain. In humans, the BTNs genes are clustered on chromosome 6 in the MHC class-I region containing three related genes: BTN3A1, BTN3A2, and BTN3A3 (40, 41). The former molecule seems the only one containing a B30.2 domain, forming a basic pocket, which is essential for N-BPs-mediated activation of $\gamma\delta$ T cells, although the authors did not show evidence for direct binding of P-Ag to BTN3A1 (40). More recently, such direct binding has been demonstrated to occur to the V-like domain of

BTN3A1 and the complex has been crystallized (42). It is still not clear how intracellularly generated P-Ag (e.g., those derived upon N-BPs treatment) can be associated to BTN3A1: one possibility is that P-Ag are secreted and then bind to the basic groove of BT3A1 or, alternatively, the B30.1 basic domain binds to P-Ag with low affinity and induces a conformational change in the external portion of the molecule that, in turn, is recognized by $\gamma\delta$ T cells (39, 42, 43).

$\gamma\delta$ T CELLS AND ANTI-CANCER SURVEILLANCE

Since their discovery in the late 1980s, $\gamma\delta$ T cells have been extensively studied and different characteristics, including MHC-unrestricted cytotoxic activity against malignant cells, have made these cells a promising potential therapeutic tool (3, 4, 10, 15, 44–46). It is now clear that $\gamma\delta$ T lymphocytes are good mediators of a stress-related response: for example, they can recognize directly stress-induced ligands, such as MIC-A, MIC-B, or ULBPs, through the NKG2D receptor or be activated by P-Ag derived by the isoprenoid pathway used by several microorganisms or by the mevalonate pathway in infected or transformed cells (1–4). However, NKG2D-L can be released, due to the action of the disintegrin-and-metalloproteinases ADAM10/17 or the disulfide-isomerase ERp5, overexpressed in solid and hematologic tumors (47–52). In their soluble form (sNKG2D-L), these ligands hinder the recognition of membrane-bound MIC-A/B or ULBPs by NKG2D receptor; in turn, sNKG2D-L are not able to trigger an activating signal in effector lymphocytes that cannot exert their anti-tumor activity (46–51). Moreover, serum levels of sNKG2D-L have been related to the outcome and progression of several neoplastic diseases (18, 23, 52–54).

$\gamma\delta$ T cells can also be indirectly activated by pro-inflammatory cytokines or by toll-like receptors (TLR) that bind to viral or bacterial products (1–4). Another activation signal can be delivered via CD16 through the interaction with the Fc of IgG: this binding initiate the antibody-dependent cell cytotoxicity (ADCC) exerted to destroy opsonized cells or microorganisms (2). Upon one of the mentioned stimuli, $\gamma\delta$ T lymphocytes expand, acquire cytotoxic function, and secrete an array of Th1 pro-inflammatory cytokines, such as IFN- γ or TNF- α . Another important feature of T lymphocytes expected to interact with cancer cells is their capacity to infiltrate tumors. Accordingly, tumor-infiltrating gamma delta T lymphocytes were detected in a broad spectrum of malignancies (2–4, 10).

For all these aspects of their function, $\gamma\delta$ T cells have been considered attractive for anti-cancer therapies: of note, ADCC can be exploited by the use of therapeutic monoclonal antibodies (mAbs) (44, 45, 55). In addition, various selective agonists, including P-Ag, for human $\gamma\delta$ T lymphocytes have been synthesized, allowing the launch of several clinical trials for patients with follicular lymphoma, multiple myeloma (MM), and acute myeloid leukemia, as well as non-hematological malignancies, such as renal cell (RCC), breast, and prostate carcinomas.

EVALUATION OF $\gamma\delta$ T CELL-BASED CLINICAL TRIALS

Given the demonstrated *in vitro* anti-cancer activity of $\gamma\delta$ T cells and their *in vivo* potential as anti-tumor effectors, numerous clinical trials have been performed in the last years to exploit the

properties of these cells for cancer immunotherapy (44, 56–64). Two methods have been applied so far: adoptive transfer of autologous $\gamma\delta$ T lymphocytes expanded *in vitro* and then reinfused to patients and direct administration of drugs or substances able to stimulate $\gamma\delta$ T cells *in vivo* (44, 56–58). The *in vitro* stimulation and expansion of this cell population is achievable using P-Ag, N-BPs, or immobilized anti- $\gamma\delta$ TCR antibodies, and allows the optimization and control of the effector cells obtained (7, 8, 24, 56). However, this method requires specialized laboratories and expertise and is rather expensive. In turn, the administration of N-BPs or synthetic P-Ag in combination with cytokines has been used as a cheaper and straight-forward therapeutic alternative. The third generation of N-BPs as zoledronate is the most commonly used for both *in vitro* activation and *in vivo* administration; the EC₅₀ for $\gamma\delta$ T cells is favorable (0.003 μ M) and a single dose of 4 mg leads to plasma levels (1–5 μ M) shown to be effective in activating $\gamma\delta$ T cells *in vitro* (56, 60). As an alternative, the synthetic phosphate-containing molecule bromohydrin pyrophosphate (BrHPP) is used for either *in vitro* expansion or *in vivo* stimulation of $\gamma\delta$ T lymphocytes and also upregulates their ability to mediate rituximab-induced ADCC (56, 61). Together with zoledronate or BrHPP, interleukin-2 is used for *in vitro* expansion of this T-cell population, and also added to the therapeutic schemes in different cancers; however, IL-2 is toxic at high doses (those that are commonly effective), leading to vascular leakage, hyperpyrexia, severe hypotension whereas low, and well-tolerated doses are much less effective *in vivo* (28, 56).

A preliminary pilot study by Wilhelm's team examined toxicity, *in vivo* activation of $\gamma\delta$ T cells, and anti-lymphoma efficacy of pamidronate/IL-2 in 19 patients with relapsed/refractory low-grade non-Hodgkin lymphomas (NHL) or MM (44). The authors demonstrated that pamidronate administered with low-dose IL-2 is well tolerated and induces a specific $\gamma\delta$ T-cell expansion; furthermore, the clinical response observed in the patients, i.e., stabilization or partial response, is linked to $\gamma\delta$ T-cell proliferation *in vivo*. A second study was reported by Dieli's group, showing that zoledronate induced the *in vivo* development of V γ 9V δ 2 cells producing IFN- γ and exerting strong anti-tumor responses (62). Therefore, a pilot study on the effects of zoledronate and IL-2 was conducted in the United States by Malkovsky's group in 12 patients with metastatic RCC (63). Adverse events typical of IL-2 monotherapy were observed in all patients, without partial or complete responses. In the following years, phase-I clinical trials were performed in metastatic hormone-refractory prostate cancer and in several patients with solid tumors using BrHPP (56, 64). Given BrHPP's safety profile, a multicentric phase-II study using the drug was launched in relapsed follicular lymphoma patients who had previously received previous lines of therapy, using rituximab at least once (56, 61). The treatment induced strong and specific amplification of TCRV γ 9V δ 2 T lymphocytes showing a Th1 and cytotoxic effector-memory cell profile (IFN- γ and TNF- α production), expressing Fc γ RIIIa (CD16) and displaying rituximab-mediated ADCC (56, 61). The combination of BrHPP and rituximab in immunotargeted therapy produced very encouraging results, particularly for follicular lymphoma patients with unfavorable Fc γ RIIIa gene polymorphisms (F/F or V/F, 95% of the patients). Thus, the initial evaluation of clinical trials leads

to the conclusion that $\gamma\delta$ T cell-based immunotherapy is more effective in hematological rather than in solid tumors.

POSSIBLE IMPROVEMENT OF $\gamma\delta$ T CELL-BASED IMMUNOTHERAPY

In the above cited review by Fisher and coworkers (56), 12 clinical trials involving 157 patients have been analyzed for the evaluation of the efficacy and/or failure of $\gamma\delta$ T cell-based immunotherapy, and some conclusions can be drawn. First, patients with solid tumors have been treated mostly with adoptive $\gamma\delta$ T-cell transfer, while patients with hematological cancers were mainly treated with $\gamma\delta$ T cell-expanding drugs. Second, as the trials reviewed were either phase-I, phase-II, or feasibility studies, all patients had already received previous treatments, as chemotherapy or other types of immunotherapy (IL-2 alone). Moreover, in some trials testing $\gamma\delta$ T cell-stimulating drugs, the combination with IL-2 led to high toxicity with low therapeutic effects. In adoptive transfer studies, different culture conditions and times as well as distinct cell sources (leukapheresis vs. peripheral blood), represent additional variables that render difficult the overall evaluation of the efficacy of these treatments. As the *in vitro* expansion of $\gamma\delta$ T lymphocytes is feasible and efficient, an accepted conclusion is that leukapheresis in general is not needed to obtain a sufficient amount of activated effectors to reinfuse. Some evidences emerge from the comparison of clinical responses to $\gamma\delta$ T cell-immunotherapy with standard-of-care second-line therapies in three selected cancer types, RCC, non-small cell lung carcinoma (NSCLC), and prostate cancer. The proportion of objective responses among patients treated with $\gamma\delta$ T cell-based immunotherapy is higher than that achieved with recommended second-line therapy in advanced prostate cancer (33.3% with $\gamma\delta$ T cells vs. 25.2% with prednisolone + docetaxel) and advanced RCC (4.8% with $\gamma\delta$ T cells vs. 1.8 with everolimus), but not in advanced NSCLC (7.6% with erlotinib, 12.2% with docetaxel, 0% with $\gamma\delta$ T cells) (56, 65–67). In general, the clinical response to $\gamma\delta$ T-cell immunotherapy in solid tumors is disappointing. There are several possible explanations for this and we will try to consider some of them. First, there might be a considerable difference in $\gamma\delta$ T-cell expansion capacity among patients, patients with hematologic malignancies being more responsive than those with solid tumors (44, 56–64). A considerable inter-individual variation in expansion capacity has been observed among patients with MM, NHL, or chronic lymphocytic leukemia (CLL) with an inverse correlation between the frequency of circulating regulatory T cells and the ability of $\gamma\delta$ T cells from cancer patients to proliferate in response to P-Ag (44, 56–64). Another possible inhibiting factor is represented by transforming growth factor (TGF) β that is known to decrease the NKG2D expression on lymphocytes reducing their activation (52, 68, 69). Moreover, sNKG2D-L released by cancer and accessory cells in the tumor microenvironment can impede the interaction of effector lymphocytes with tumor target cells. (48–52) In addition, other inhibitory signals, such as that delivered by PD-1 or via CTLA-4, can lead to a general inhibition of $\gamma\delta$ T-cell function at the tumor site (70, 71). Thus, a possible strategy to overcome inhibitory signals would be the use of mAbs blocking either CTLA-4, such as ipilimumab, or PD-1 or neutralizing TGF β (56, 69–71). In addition, inhibiting the enzymes responsible for sNKG2D

ligands, including ADAM10 and ADAM17 (71–76), with specific compounds, would push the balance toward $\gamma\delta$ T-cell activation; along this line, the combination of stimulating molecules, such as bisphosphonates, and therapeutic tumor-targeting antibodies, as the anti-CD20 rituximab or the anti-ERBB2 trastuzumab, should improve the efficacy of $\gamma\delta$ T-cell anti-tumor effect (56, 58). A different immuno-evasion mechanism exerted by tumor microenvironment may be represented by mesenchymal stromal cells (MSC) that are known to down-regulate T-cell effector functions (77, 78). We recently reported that LN-MSC derived from NHL patients impair the anti-tumor activity of V δ 2T lymphocytes, selectively inhibiting NKG2D-mediated lymphoma cell killing (79). Of note, N-BPs can prevent this effect by reducing TGF β and increasing IL-15 production by LN-MSC, and drive the differentiation of V δ 2 T lymphocytes into effector-memory cells producing Th1-type cytokines (79). Moreover, N-BPs do not alter the efficiency of V δ 2 T cells to exert rituximab-mediated ADCC. To be successful, $\gamma\delta$ T cell-based cancer immunotherapy will require protocols updated to limit most of the different immuno-escape mechanisms occurring at the tumor site.

PERSPECTIVES

Response rates to $\gamma\delta$ T cell-based immunotherapy, either as adoptive transfer or as stimulating drugs, are not satisfactory (10% of objective responses); however, about 39% of patients achieved disease stabilization, indicating a clinical benefit and suggesting the possibility of improving the efficiency of such therapeutic tool (56, 58, 80). Advantages of this type of anti-cancer therapy would be the safety of drugs and substances known to stimulate $\gamma\delta$ T cells, beside their efficiency in $\gamma\delta$ T-cell stimulation.

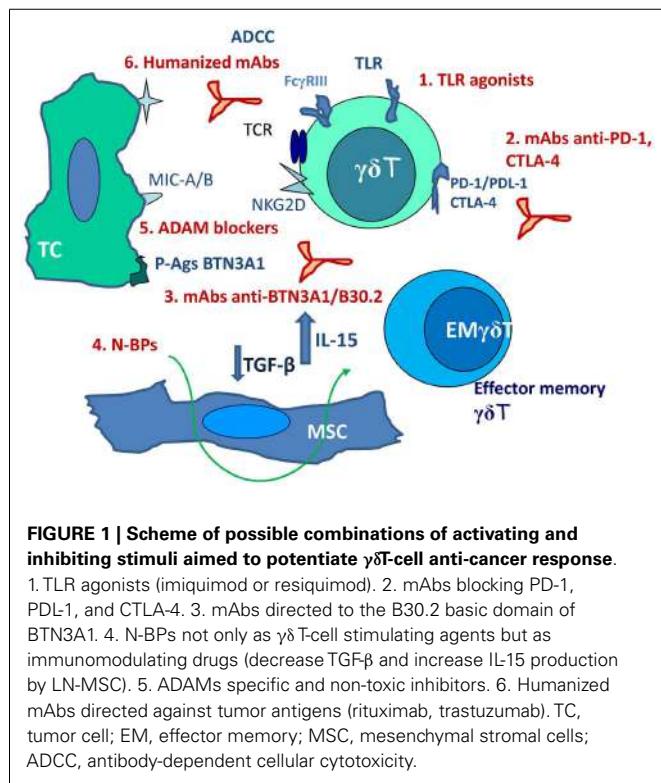


FIGURE 1 | Scheme of possible combinations of activating and inhibiting stimuli aimed to potentiate $\gamma\delta$ T-cell anti-cancer response.

1. TLR agonists (imiquimod or resiquimod). 2. mAbs blocking PD-1, PDL-1, and CTLA-4. 3. mAbs directed to the B30.2 basic domain of BTN3A1. 4. N-BPs not only as $\gamma\delta$ T-cell stimulating agents but as immunomodulating drugs (decrease TGF- β and increase IL-15 production by LN-MSC). 5. ADAMs specific and non-toxic inhibitors. 6. Humanized mAbs directed against tumor antigens (rituximab, trastuzumab). TC, tumor cell; EM, effector memory; MSC, mesenchymal stromal cells; ADCC, antibody-dependent cellular cytotoxicity.

Drawbacks are mainly represented by immuno-evasion. This can be counteracted (Figure 1) by including in the therapeutic protocols non-specific stimulators as TLR agonists (imiquimod or resiquimod) or the BCG vaccine (81). Recently approved clinical trials include mAbs blocking PD-1, PDL-1, and CTLA-4 (58, 70, 71) aimed to inhibit negative signals. Cancer-specific TCR gene transfer has been proposed in the last years to gain efficiency and specificity in the anti-cancer response; $\alpha\beta$ TCR engineered $\gamma\delta$ T cells have been shown to exert anti-tumor activity *in vitro* and may be considered as an alternative strategy for adoptive T-cell transfer (82, 83).

The recent identification of BTN3A1 as an essential molecule in P-Ag presentation to $\gamma\delta$ T cells opens new possible ways of interventions: both stimulating and inhibiting mAbs directed to the B30.2 basic domain of the molecule have been described (39–41, 84). These antibodies might be used differently to induce or regulate $\gamma\delta$ T-cell response to P-Ag, representing an additional tool in the design of immunotherapeutic protocols.

In addition, we propose the use of N-BPs not only as $\gamma\delta$ T-cell stimulating agents but as immunomodulating drugs (79). Finally, the development of ADAMs specific and non-toxic inhibitors would contribute to the improvement of NKG2D-mediated recognition of stress-induced molecules at the surface of tumor cells. Thus, such combined therapeutic protocols, including stimulating molecules, mAbs, and inhibitory substances acting on enzymes, which favor tumor immuno-evasion, may represent the new frontier of anti-cancer immunotherapy.

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Cancer immunotherapy using $\gamma\delta$ T cells: dealing with diversity

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The broad and potent tumor-reactivity of innate-like $\gamma\delta$ T cells makes them valuable additions to current cancer immunotherapeutic concepts based on adaptive immunity, such as monoclonal antibodies and $\alpha\beta$ T cells. However, clinical success using $\gamma\delta$ T cells to treat cancer has so far fallen short. Efforts of recent years have revealed a striking diversity in $\gamma\delta$ T cell functions and immunobiology, putting these cells forward as true “swiss army knives” of immunity. At the same time, however, this heterogeneity poses new challenges to the design of $\gamma\delta$ T cell-based therapeutic concepts and could explain their rather limited clinical efficacy in cancer patients. This review outlines the recent new insights into the different levels of $\gamma\delta$ T cell diversity, including the myriad of $\gamma\delta$ T cell-mediated immune functions, the diversity of specificities and affinities within the $\gamma\delta$ T cell repertoire, and the multitude of complex molecular requirements for $\gamma\delta$ T cell activation. A careful consideration of the diversity of antibodies and $\alpha\beta$ T cells has delivered great progress to their clinical success; addressing also the extraordinary diversity in $\gamma\delta$ T cells will therefore hold the key to more effective immunotherapeutic strategies with $\gamma\delta$ T cells as additional and valuable tools to battle cancer.

Keywords: $\gamma\delta$ T cells, cancer immunotherapy, $\gamma\delta$ T cell diversity, innate-like lymphocytes, $\gamma\delta$ TCR

IMMUNOTHERAPY TO TREAT CANCER: THE ERA IS NOW

Current treatment options to fight cancer heavily rely on pharmaceutical and radiological interventions that are accompanied by substantial off-tumor toxicity and lack of clinical efficacy. Cancer immunotherapy aims to capture the specificity and memory of the immune system and holds the promise of truly targeted treatment with durable clinical responses. Recent advances in clinical trials and the approval of more and more immunotherapeutic agents by international regulatory agencies have given the field considerable momentum, a fact that is mirrored by the announcement of cancer immunotherapy as the breakthrough of the year 2013 by *Science* (1).

So far, the vast majority of efforts aimed at utilizing the immune system to reject cancer have focused on components of adaptive immunity, including monoclonal antibodies and $\alpha\beta$ T cells. The human immune system can theoretically generate up to 10^{11} unique antibodies and some 10^{15} unique $\alpha\beta$ T cell receptors ($\alpha\beta$ TCRs) (2), and controlling this vast diversity in antigen specificity for targeted immune interventions has been a major challenge for clinical implementation. Although immunoglobulins are still used in clinical practice for untargeted protection against viral infections, such as in patients with general B-cell deficiencies, the real breakthrough in clinical immunotherapy came with mastering the genetic profile of defined monoclonal antibodies. Among the first therapeutic antibodies to directly target cancer were anti-CD20 (Rituxan or Rituximab) and anti-Her2 (Herceptin or Trastuzumab) antibodies to treat B cell leukemias and breast cancer, respectively. Treatment with these antibodies, recognizing one particular antigen with a defined affinity, has underscored the therapeutic potential of truly antigen-targeted immunotherapy, as

impressive clinical benefit has been reported across studies covering the last decade (3, 4). The clinical success of these pioneering agents has in recent years led to the development and regulatory approval of additional antibodies to target various cancers (5), propelling antigen-specific antibody-based immunotherapy into mainstream cancer treatment. Similar to the evolution of clinical antibody treatment, first evidence for the anti-tumor potential of adoptively transferred $\alpha\beta$ T cells originated from the transfer of a very diverse immune population, the so called donor lymphocyte infusions, in the early 1990s, when allogeneic donor $\alpha\beta$ T cells that were infused in patients after allogeneic stem cell transplantation demonstrated potent anti-leukemia responses (6). By now, these data have been complemented by remarkable clinical results obtained with strategies that aim to mobilize the tumor-reactivity of autologous T cells in cancer patients, either by the adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) (7, 8) or the infusion of monoclonal antibodies that stimulate T cell activity, such as the recently approved anti-CTLA4 antibody Ipilimumab (9, 10). Additionally, the genetic engineering of T cells with tumor-reactive $\alpha\beta$ TCRs (11, 12) or antibody-based chimeric antigen receptors (CARs) (13) has gained increasing interest in recent years, and the first clinical trials using adoptive transfer of such gene-modified T cells have demonstrated potent and lasting anti-tumor responses in selected patients (14–18).

Importantly, understanding the diversity of adaptive immune repertoires and utilizing very defined specificities for therapeutic interventions has so far been not only the success but also the downside of such therapies, resulting in highly personalized cancer care that depends on antibody-based strategies (including CAR-engineered T cells) with limited numbers of targetable tumor

antigens and $\alpha\beta$ T cell products that are only clinically applicable to HLA-matched patient populations. Moreover, clinical anti-tumor efficacy of $\alpha\beta$ T cell-based approaches is so far mainly restricted to particularly immunogenic tumor types, such as melanoma. Thus, there is a compelling need to call to arms alternative immune components for novel cancer immunotherapeutic concepts.

$\gamma\delta$ T CELLS: THE PROMISING OUTSIDERS

Unconventional $\gamma\delta$ T cells, a second lineage of T cells that express a unique somatically recombined $\gamma\delta$ TCR, possess unique features to confront the limitations of adaptive-based immunotherapeutic strategies. $\gamma\delta$ T cells are rapidly activated upon encounter of pathogen-derived antigens or self molecules that are upregulated on infected or stressed cells, resembling the activation of innate immune cells that sense molecular stress signatures (19, 20). Importantly, $\gamma\delta$ T cells are set apart from conventional $\alpha\beta$ T cells by the fact that activation of $\gamma\delta$ T cells does not depend on antigen presentation in the context of classical MHC molecules. A preferential usage of distinct TCR γ and δ chains, which together have the potential to form a tremendous repertoire of $\sim 10^{20}$ uniquely recombined $\gamma\delta$ TCRs (2), has formed the basis for the identification of two major $\gamma\delta$ T cell subsets. $\gamma\delta$ T cells that carry $V\gamma 9V\delta 2^+$ TCRs are primarily found in peripheral blood, where they constitute a minor fraction of total T cells and respond to non-peptidic intermediates of the mevalonate pathway called phosphoantigens. Other $\gamma\delta$ T cells express mainly $V\delta 1^+$ or $V\delta 3^+$ chains paired with diverse γ chains (also called $V\delta 2^{\text{neg}}$ $\gamma\delta$ T cells) and are highly enriched at mucosal sites and epithelial tissues. The effector mechanisms of $\gamma\delta$ T cells are highly similar to those of $\alpha\beta$ T cells and involve the secretion of high levels of cytokines and lysis of target cells by the release of granzymes and perforin and the engagement of FAS and TRAIL death receptors. Thus, by combining the potent effector functions of adaptive $\alpha\beta$ T cells with recognition modes that target unique classes of antigens in an innate-like manner, $\gamma\delta$ T cells are regarded as valuable sentinels that bridge innate and adaptive immunity.

Underlying the interest in $\gamma\delta$ T cells for use in cancer immunotherapy is a long-standing body of evidence indicating that $\gamma\delta$ T cells play important roles in tumor immunosurveillance. Human $\gamma\delta$ T cells display potent *in vitro* cytotoxicity toward a surprisingly large array of tumors, including cells derived from both solid and hematological origin (20–22). Importantly, $\gamma\delta$ T cells are also capable of targeting chemotherapy-resistant leukemic cells (23) and to kill leukemic and colon cancer stem cells (24) and Sebestyen and Kuball, unpublished observation). *In vivo* evidence for the non-redundant relevance of $\gamma\delta$ T cells in tumor immune surveillance stems from studies showing that $\gamma\delta$ T cell-deficient mice are more susceptible for developing cancer (25–27). Moreover, tumor-infiltrating $\gamma\delta$ T cells ($\gamma\delta$ TIL) have been observed in cancer patients with various cancers, and isolated $\gamma\delta$ TILs were shown to efficiently kill autologous tumors *ex vivo*, while leaving healthy cells unharmed (28–32). Important roles for $\gamma\delta$ T cells in tumor host defense are furthermore suggested by clinical data showing that high numbers of $\gamma\delta$ TILs in tumors of melanoma patients and elevated levels of circulating $\gamma\delta$ T cells in leukemia patients correlate with increased cancer-free survival (33, 34). Taken together, these studies have established a wealth

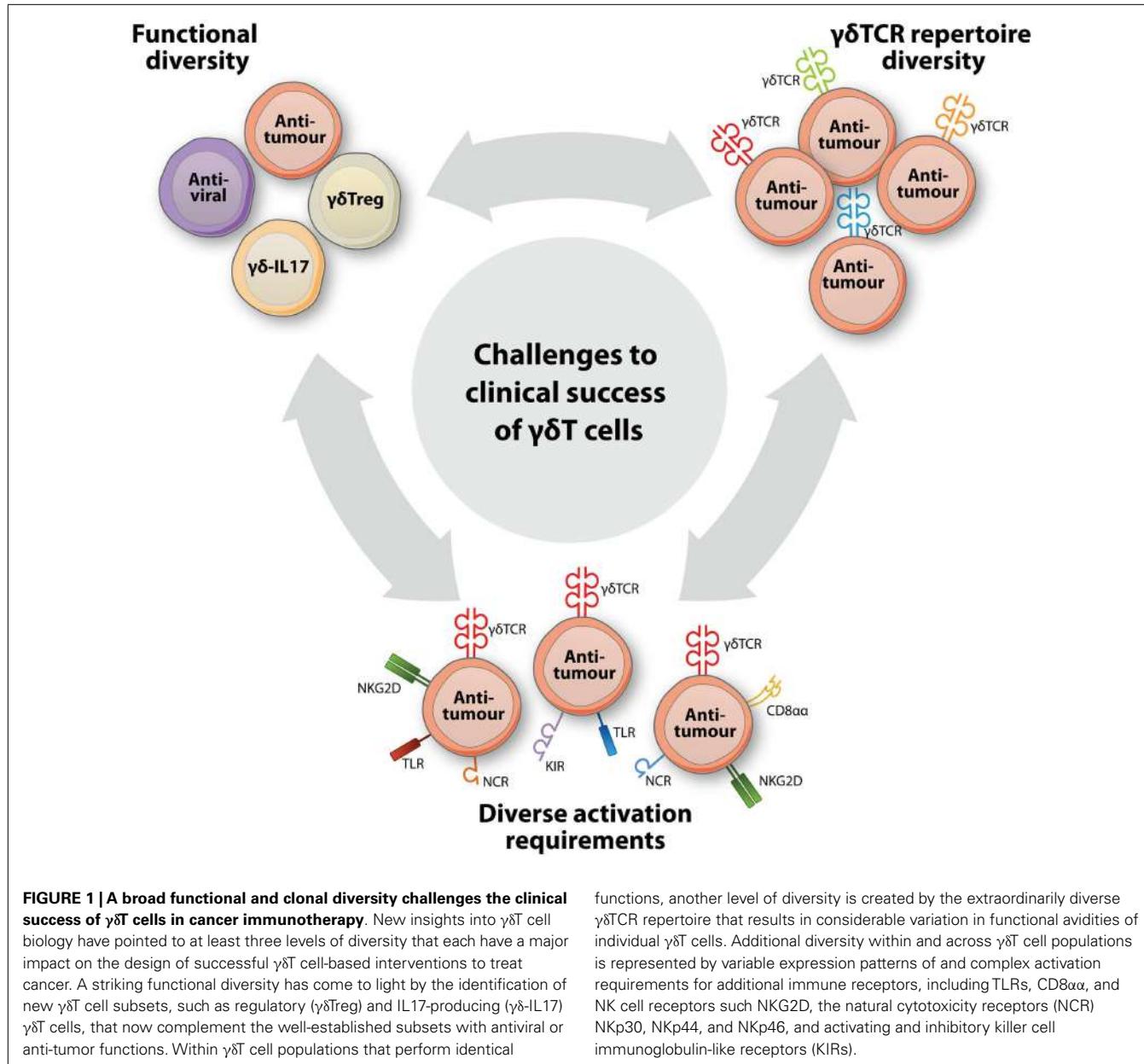
of evidence for the broad tumor-targeting capabilities of $\gamma\delta$ T cells and have sparked great interest in their application in cancer immunotherapy.

CLINICAL SUCCESS OF $\gamma\delta$ T CELLS: STUCK IN DIVERSITY?

Given the broad recognition of unique classes of tumor antigens by $\gamma\delta$ T cells combined with their potent killing capacity, it is no surprise that $\gamma\delta$ T cells have been the focus of attempts to design novel cancer immunotherapeutic strategies. Of the two major $\gamma\delta$ T cell subsets, clinical trials conducted so far have exclusively focused on the stimulation of autologous $V\gamma 9V\delta 2^+$ $\gamma\delta$ T cells that were either activated *in vivo* using so-called aminobisphosphonate compounds that specifically activate $V\gamma 9V\delta 2^+$ $\gamma\delta$ T cells, or expanded *ex vivo* and reinfused into patients. Protocols for the *in vivo* mobilization of $V\gamma 9V\delta 2^+$ T cells generally involved repeated cycles of intravenous injection of synthetic phosphoantigen (35) or aminobisphosphonates such as pamidronate (36) or zoledronate (37–40), in combination with multiple IL2 injections per cycle. In trials that explored the adoptive transfer autologous $V\gamma 9V\delta 2^+$ T cells, patient PBMCs were cultured *ex vivo* for 2 weeks in the presence of aminobisphosphonates (41–43) or synthetic phosphoantigen (44, 45) in combination with IL2. Even though these conditions promoted the expansion of $V\gamma 9V\delta 2^+$ T cells, *ex vivo* expanded cell products contained rather low (on average 50–60%) and highly variable percentages of $V\gamma 9V\delta 2^+$ T cells, and no additional purification of $V\gamma 9V\delta 2^+$ T cells was performed prior to reinfusion into patients. Patients received repeated infusions of expanded cells, in some trials in combination with IL2. Treatment using $\gamma\delta$ T cells was generally found to be safe using both *in vivo* and *ex vivo* stimulation protocols, but clinical responses varied widely across trials and were generally limited, even in patients with cancers generally sensitive to immune responses such as renal cell carcinoma [reviewed in Ref. (46–48)]. Important limitations included (a) the need for a preselection of patients due to a wide variability in *in vitro* cytotoxicity of patient $\gamma\delta$ T cells against autologous tumor tissue (36, 41, 44), and (b) limited *in vivo* or *ex vivo* expansion potential of patient $\gamma\delta$ T cells (40, 41, 44, 45, 49). Moreover, anti-tumor efficacy of $\gamma\delta$ T cells showed only marginal improvement over standard treatment options (46). Thus, despite the fact that these trials have established the anti-tumor potential of $\gamma\delta$ T cells in cancer immunotherapy, current therapeutic strategies using these cells clearly suffer from major shortcomings that have so far prevented $\gamma\delta$ T cells to live up to their clinical promise.

A REMARKABLE DIVERSITY HAMPERS APPLICATION OF $\gamma\delta$ T CELLS IN CANCER IMMUNOTHERAPY

Recent years have seen important progress in the understanding of $\gamma\delta$ T cell immunobiology and have uncovered a striking diversity in $\gamma\delta$ T cell functions and subsets. These new insights have important implications for the use of $\gamma\delta$ T cells in the treatment of cancer. To date, however, a profound appreciation of this $\gamma\delta$ T cell diversity has lacked from $\gamma\delta$ T cell-based clinical concepts and this is likely to contribute to the limited clinical results observed so far. At least three levels of $\gamma\delta$ T cell heterogeneity can be distinguished (Figure 1), including (a) a multitude of immune functions mediated by $\gamma\delta$ T cells, (b) a diverse $\gamma\delta$ TCR repertoire that, also for similar antigen-specificities, mediates different affinities, and (c)



the complex and diverse molecular needs for target recognition within the same and across different $\gamma\delta$ T cell populations. A thorough consideration of these features will be of central importance to improving the clinical efficacy of $\gamma\delta$ T cells in treating cancer.

$\gamma\delta$ T CELL FUNCTIONS: THE MORE THE BETTER?

$\gamma\delta$ T cells have, as discussed above, been attributed important and valuable functions in tumor immunosurveillance, but reactivity toward tumors is far from the only part that $\gamma\delta$ T cells play in immunity. By now, it is evident that $\gamma\delta$ T cells perform a plethora of functions that underline their involvement in diverse pathophysiological conditions other than cancer, including host defense against infectious pathogens such as bacteria, viruses, and parasites, the modulation of the activity of other immune cells, and promoting tissue regeneration after injury (20, 50).

Rapid expansions of $\gamma\delta$ T cells are observed in human beings infected with a variety of viruses or bacteria and $\gamma\delta$ T cells possess a potent capacity to directly kill infected cells (51). Moreover, a proportion of $\gamma\delta$ T cells contribute to pathogen clearance by the secretion of anti-microbial peptides such as granulysin and cathelicidin (52–54). Intriguingly, the recognition of pathogens may have important implications for $\gamma\delta$ T cell-mediated cytotoxicity against cancers, as subsets of $\gamma\delta$ T cells that respond to cytomegalovirus (CMV) infection have been reported to cross-recognize solid (55) as well as hematological (56) tumor cells *in vitro*. A role for virus-induced $\gamma\delta$ T cells in the protection from cancer *in vivo* is supported by observations that CMV infection in kidney transplant recipients was observed to associate with increased levels of $\gamma\delta$ T cells and concomitantly a reduced risk of developing cancer (57). Also in leukemia patients treated with

hematopoietic stem cell transplantation, CMV infection associates with lower incidence of leukemic relapse after transplantation (58, 59) and work from our laboratory has demonstrated that tumor surveillance by CMV-induced $\gamma\delta$ T cells is likely to play a major role in this (56), emphasizing the clinical value of such dual-reactive $\gamma\delta$ T cells in immunotherapy.

In addition to their strong reactivity to a wide variety of tumors and pathogens, a valuable feature of $\gamma\delta$ T cells is their capability to broaden immune responses by recruiting and activating additional immune cell populations. For example, activated $\gamma\delta$ T cells have the potential to orchestrate adaptive $\alpha\beta$ T cell responses, both directly by functioning as antigen-presenting cells (60–62) as well as indirectly via the interaction with dendritic cells (56, 63, 64). In addition, $\gamma\delta$ T cells have been reported to secrete cytokines to provide B cell help in the production of antibodies (65, 66), to prime NK cells to kill tumor cells (67), to rapidly recruit neutrophils via the secretion of IL-17 (68, 69), and to synergize with monocytes to mount anti-microbial $\alpha\beta$ T cell responses (70). However, in addition to the immunostimulatory roles of $\gamma\delta$ T cells, their modulatory function may be of regulatory nature as well, suggesting complex implications of $\gamma\delta$ T cells in mediating broader immune responses. For example, depending on antigenic exposure, $\gamma\delta$ T cells may suppress rather than promote antibody production by B cells (71, 72). Similarly, $\gamma\delta$ T cells can strongly inhibit the proliferation of activated $\alpha\beta$ T cells (73, 74), and a suboptimal maturation of DCs by $\gamma\delta$ T cells (56) may induce tolerogenic rather than cytotoxic $\alpha\beta$ T cell responses. Importantly, human and mouse IL17-producing $\gamma\delta$ T cells have recently been demonstrated to facilitate tumor growth by recruiting myeloid-derived suppressor cells to tumor sites (75, 76). With the recent identification of bona fide Foxp3-expressing regulatory $\gamma\delta$ T cell subsets (77), it is thus becoming clear that, depending on their local or temporal cytokine milieu, activated $\gamma\delta$ T cells may suppress instead of activate local immune responses (78). Indeed, even though the presence of $\gamma\delta$ T cells may correlate with increased survival of cancer patients in some studies (see above), their infiltration into tumor sites may also associate with worse clinical outcome of patients due to a immunosuppressive phenotype of local $\gamma\delta$ T cells (79–81).

A VERY DIVERSE $\gamma\delta$ TCR REPERTOIRE PRODUCES RECEPTEORS WITH VARIABLE ANTI-TUMOR AFFINITIES

Like $\alpha\beta$ TCRs and B cell receptors, $\gamma\delta$ TCRs are generated during T cell maturation through the somatic recombination of germline-encoded variable (V), diversity (D), and joining (J) gene segments. Despite the fact that the number of germline $V\gamma$ and $V\delta$ genes is far more limited than the repertoire of $V\alpha$ and $V\beta$ genes, more extensive junctional diversification processes during TCR γ and δ chain rearrangement leads to a potential $\gamma\delta$ TCR repertoire that is roughly 10⁵-fold larger than that of $\alpha\beta$ TCRs (2). Despite this extensive $\gamma\delta$ TCR repertoire, the diversity of antigens that are recognized by $\gamma\delta$ TCRs appears to be surprisingly limited. The vast majority of $V\gamma9V\delta2^+$ TCRs on circulating $\gamma\delta$ T cells are restricted to sensing elevated levels of phosphoantigens (22, 82), a process that has recently been demonstrated to involve the butyrophilin family member BTN3A1 (83, 84). Similarly, all antigens of $V\delta2^{\text{neg}}$ $\gamma\delta$ TCRs identified so far, including MICA/B (85), CD1 (86, 87), and EPCR (88), belong to the family of non-classical

MHC homologs, although additional antigens are likely to still be identified and may include MHC-unrelated molecules.

An important question is why this rather narrow antigen restriction of $\gamma\delta$ T cells is confronted with such a broad $\gamma\delta$ TCR diversity, instead of a rather oligoclonal or invariant repertoire as expressed by for example NKT cells (89). One possible explanation may be that the extensive $\gamma\delta$ TCR repertoire of $\gamma\delta$ T cells allows an important fine-tuning of $\gamma\delta$ TCR-mediated target cell recognition. Indeed, we have shown recently that phosphoantigen-responsive $V\gamma9V\delta2^+$ $\gamma\delta$ T cell clones differed widely in their functional avidity toward tumor cells (90). $\gamma\delta$ TCR transfer and mutation experiments showed that this variability in the ability to respond to tumor cells was mediated primarily through diverse sequence compositions that dictate the affinities of individual clone-derived $V\gamma9V\delta2^+$ TCRs. A similar $\gamma\delta$ TCR-mediated heterogeneity in anti-tumor specificity can be observed in the $V\delta2^{\text{neg}}$ subset of $\gamma\delta$ T cells, as we recently demonstrated that individual $V\delta1^+$ $\gamma\delta$ T cell clones display $\gamma\delta$ TCR-mediated reactivity against diverse arrays of tumor cells (56). Moreover, $\gamma\delta$ TCRs of other $V\delta1^+$ clones were not involved in tumor recognition but mediated interactions with dendritic cells, demonstrating that a diverse $\gamma\delta$ TCR repertoire can mediate not only a fine-tuning of anti-tumor avidity but also different functions. Accordingly, diverse $\gamma\delta$ T cell functions that segregate with $\gamma\delta$ TCR composition have been observed for the human $V\gamma9V\delta2^+$ and $V\delta2^{\text{neg}}$ subsets, as $V\gamma9V\delta2$ $\gamma\delta$ T cells have been generally ascribed potent cytotoxic effector functions, while $V\delta2^{\text{neg}}$ $\gamma\delta$ T cells rather have immunomodulatory roles (91, 92). However, these observations are contrasted by reports showing a superior tumor-homing and -killing capacity of $V\delta2^{\text{neg}}$ $\gamma\delta$ TILs over $V\gamma9V\delta2$ $\gamma\delta$ TILs in some cancers (30, 93), further underlining the heterogeneous and context-dependent nature of both $\gamma\delta$ T cell subsets.

$\gamma\delta$ T CELL ACTIVATION: A COMPLEX INTERPLAY BETWEEN RECEPTORS

Alongside the $\gamma\delta$ TCR, $\gamma\delta$ T cells can be activated through a variety of activating and inhibitory NK receptors (48, 94) and toll-like receptors (TLR) (95), emphasizing the innate-like nature of these unconventional T cells. Depending on the pathophysiological context, these receptors can provide costimulation to $\gamma\delta$ TCR-mediated activation signals or can activate $\gamma\delta$ T cells independent of $\gamma\delta$ TCR triggering, adding yet another level of heterogeneity and complexity to $\gamma\delta$ T cell biology. The best-studied receptor with dualistic roles in $\gamma\delta$ T cell activation is NKG2D, a natural cytotoxicity receptor (NCR) that is expressed on NK cells, most $\gamma\delta$ T cells and $CD8^+$ $\alpha\beta$ T cells. NKG2D recognizes the non-classical MHC homologs MICA/B and ULBPs, the expression of which is upregulated on many different tumors (96, 97). On $V\gamma9V\delta2^+$ $\gamma\delta$ T cells, NKG2D can amplify $\gamma\delta$ TCR-mediated effector functions in response to MICA/B-positive target cells (98, 99). In other cases, however, sole signaling through NKG2D has been proposed to be sufficient for activating $\gamma\delta$ T cells, without requiring $\gamma\delta$ TCR engagement (100, 101). However, as most of these studies have used TCR blocking antibodies and not receptor gene-transfer experiments, the impact of TCR affinity and signaling in NKG2D-triggered $\gamma\delta$ T cell activation might have been underestimated (Gründer and Kuball, unpublished observation). Factors that determine the directly stimulatory versus costimulatory

function of NKG2D are not known, but may involve signaling by polymorphic receptors such as inhibitory NK receptors (100). Apart from serving as ligand for NKG2D, MICA/B is also recognized by selected V δ 1 $^{+}$ $\gamma\delta$ TCRs (85). In fact, overlapping binding epitopes for NKG2D and $\gamma\delta$ TCRs on MICA/B result in competitive binding of both receptors for MIC ligands, suggestive of complex, temporally regulated interactions of both receptors for MIC ligands (102). Similarly, engagement of the NCRs NKp30, NKp44, and NKp46 on $\gamma\delta$ T cells can be sufficient for eliciting anti-tumor cytotoxicity, but interestingly only after expression of these receptors on $\gamma\delta$ T cells has been induced via triggering of the $\gamma\delta$ TCR (103). Differential involvement of the $\gamma\delta$ TCR and additional receptors has also been reported in pathophysiological processes other than cancer, as work by us and others has demonstrated that reactivity of $\gamma\delta$ T cells against CMV-infected cells may involve $\gamma\delta$ TCR-dependent (55, 104) and -independent (56) pathways, suggesting multimodal pathogen-sensing mechanisms that may involve NK receptors (48).

Recently, we have found additional evidence for a complex interplay between receptors in the response of $\gamma\delta$ T cells against tumor cells by demonstrating that CD8 $\alpha\alpha$, that serves as coreceptor for selected $\gamma\delta$ TCRs as reported by us recently (56), mediates $\gamma\delta$ TCR costimulation in a manner that depends on the particular tumor cell target (Scheper and Kuball, unpublished observation). Expression of CD8 $\alpha\alpha$ on T cells engineered to express a tumor-reactive $\gamma\delta$ TCR was a prerequisite for recognition of all tested tumor cell lines, but coexpression of signaling-deficient CD8 α variants or mutants with single residue substitutions in the extracellular domain of CD8 α alongside the $\gamma\delta$ TCR differentially impacted T cell reactivity toward the different tumor targets. Even though CD8 $\alpha\alpha$ $^{+}$ $\gamma\delta$ T cells were first identified over 20 years ago, when CD8 $\alpha\alpha$ was found to be commonly expressed on V δ 1 $^{+}$ $\gamma\delta$ T cells in the intestine but not circulating V γ 9V δ 2 $^{+}$ T cells (105, 106), the functional implications of CD8 $\alpha\alpha$ expression on $\gamma\delta$ T cells have remained rather controversial. A number of studies have reported regulatory functions for CD8 $\alpha\alpha$ $^{+}$ $\gamma\delta$ T cells, being capable of for example inhibiting inflammatory responses in celiac disease (107) but also to suppress $\alpha\beta$ T cell-mediated responses against tumor cells (80). On the other hand, and in line with our data (56), stimulated CD8 $\alpha\alpha$ $^{+}$ $\gamma\delta$ T cells have been reported to be as capable as CD8 $\alpha\alpha$ $^{-}$ $\gamma\delta$ T cells of secreting high levels of Th1 cytokines such as IFN γ (108). Moreover, cytokines produced by CD8 $\alpha\alpha$ $^{+}$ but not CD8 $\alpha\alpha$ $^{-}$ $\gamma\delta$ T cells have been implicated in the controlling of R5-tropic HIV replication and persistence (109). Thus, CD8 $\alpha\alpha$ $^{+}$ $\gamma\delta$ T cells appear to perform diverse functions depending on the context in which they are activated.

Taken together, the emerging insights into the molecular requirements for $\gamma\delta$ T cell activation and the interplay between different receptors in this process have substantially furthered our understanding of the response of $\gamma\delta$ T cells against cancer cells, but also unveil substantial challenges to the design of uniform $\gamma\delta$ T cell-based strategies for cancer immunotherapy.

SUCCESSFUL TRANSLATION USING $\gamma\delta$ T CELLS: PICKING THE RIGHT ONES

Beyond doubt, the implications of the functional and clonal heterogeneity of $\gamma\delta$ T cells for their application in the treatment of

cancer are substantial, and a failure to fully recognize this diversity in clinical concepts and trial designs is likely the most important contributing factor in the limited clinical results observed with $\gamma\delta$ T cells to date. Current clinical protocols based on the broad activation of unselected $\gamma\delta$ T cells are likely to induce $\gamma\delta$ T cell populations with diverse specificities, avidities, and functions, including regulatory. Consequently, high-avidity $\gamma\delta$ T cells with strong tumor-reactivity and a desired functional profile may represent only a relatively minor population of such cell products. In addition, stimulation of $\gamma\delta$ T cells using agents that primarily depend on strong $\gamma\delta$ TCR-mediated activation, such as the use of aminobisphosphonate and phosphoantigen compounds to expand V γ 9V δ 2 $^{+}$ $\gamma\delta$ T cells in trials pursued to date, most likely selects for $\gamma\delta$ T cells with low affinity V γ 9V δ 2 $^{+}$ $\gamma\delta$ TCRs and thus, low activity on primary tumor cells. Moreover, $\gamma\delta$ TCR-based activation strategies do not necessarily mobilize $\gamma\delta$ T cells that express a repertoire of NK receptors and TLRs required to potently respond to the multimolecular stress signature of tumor cells. Thus, the selection of optimally tumor-reactive $\gamma\delta$ T cell populations will likely be a critical parameter in the design of improved cancer immunotherapeutic concepts. In principal, this would favor strategies aimed at *ex vivo* rather than *in vivo* expansion of $\gamma\delta$ T cells, since the first allows a careful monitoring and culture-dependent skewing of $\gamma\delta$ T cell phenotype and functionality that is far more challenging to accomplish using *in vivo* stimulation protocols. With the clinical data available so far, it is difficult to corroborate this by comparing clinical responses observed in both types of trials, as studies using adoptive transfer of *ex vivo* generated $\gamma\delta$ T cells have so far relied on similar stimulation protocols (aminobisphosphonate or phosphoantigen in combination with IL-2) and the potential for extended *in vitro* manipulation for enhanced anti-tumor efficacy has not yet been investigated (41–45, 49, 110). Importantly, *ex vivo* manipulation of patient $\gamma\delta$ T cells could also include a valuable enrichment of tumor-specific $\gamma\delta$ T cells with high functional avidity, for instance using selection techniques based on the upregulation of activation markers or the production of cytokines such as IFN γ by $\gamma\delta$ T cells after *in vitro* coculture with autologous tumor cells. Nevertheless, $\gamma\delta$ TCR repertoires vary widely among individuals (111, 112), and generating sufficient numbers of $\gamma\delta$ T cells that recognize tumors with high avidity may therefore be challenging in certain patients. Similarly, NK receptor and TLR repertoires as well as CD8 α expression levels differ considerably between $\gamma\delta$ T cell subsets (56, 103, 105, 113) and between individuals (95, 114, 115), putting additional constraints on the generation of $\gamma\delta$ T cell products potently capable of rejecting cancer.

To overcome the limitations of patient $\gamma\delta$ T cell repertoires, $\gamma\delta$ TCRs with broad tumor-specificity could be identified *in vitro* and genetically introduced into patient-derived immune cells. Recent work by our group has demonstrated that gene-transfer of tumor-specific V γ 9V δ 2 $^{+}$ and V δ 1 $^{+}$ $\gamma\delta$ TCRs can be used to efficiently reprogram conventional $\alpha\beta$ T cells to recognize a wide variety of tumor cells (56, 90, 97). By exploiting the abundance and superior proliferation potential of $\alpha\beta$ T cells, large numbers of autologous $\gamma\delta$ TCR-engineered T cells with defined tumor-specificity can be generated *ex vivo* and subsequently reinfused into cancer patients. In contrast to $\alpha\beta$ TCR gene-transfer strategies,

introduced TCR γ and δ chains do not dimerize with endogenous $\alpha\beta$ TCR chains (97) and therefore do not lead to the formation of unwanted TCRs with unpredictable, and potentially dangerous, specificities. Moreover, since antigen recognition by $\gamma\delta$ TCRs does not depend on classical MHC molecules, well-characterized $\gamma\delta$ TCRs that mediate superior anti-tumor functional avidities can be applied to a broad patient population without the requirement for HLA matching. Additionally, transgenic expression of $\gamma\delta$ TCRs downregulates surface expression of endogenous $\alpha\beta$ TCR chains (56, 90, 97), enabling the use of engineered cell product even in an allogeneic “off-the-shelf” fashion. The *ex vivo* generation of $\gamma\delta$ TCR-engineered T cells furthermore allows additional manipulation of cell products, such as the selection of T cells with highest $\gamma\delta$ TCR expression levels or T cells which express beneficial TLRs or NK receptors. Importantly, such strategies can take advantage of the valuable lessons that have been learned from efforts to apply conventional $\alpha\beta$ T cells and their receptors in cancer immunotherapy, such as evidence for the effect of the differentiation status on *in vivo* persistence and function of clinical T cells (116). Our group has initiated the first clinical trial using $\gamma\delta$ TCR-gene-modified T cells to treat cancer patients (scheduled to start in 2015). Donor T cells engineered with a well-characterized tumor-reactive V γ 9V δ 2 $^{+}$ $\gamma\delta$ TCR (90) will be administered to leukemia patients after allogeneic stem cell transplantation as part of an engineered donor lymphocyte infusion. *Ex vivo* manipulations of gene-modified T cell products will include the depletion of cells that express only low levels of the clinical $\gamma\delta$ TCR and adapted culturing conditions to prevent terminal differentiation of engineered T cells before infusion into patients.

CLOSING REMARKS

Even though $\gamma\delta$ T cells have traditionally been regarded as a homogeneous immune population, important advances in the understanding of $\gamma\delta$ T cell immunobiology have revealed a striking diversity in functionality and molecular activation modes. These new insights are generally met with great enthusiasm as they give acclaim to $\gamma\delta$ T cells for their non-redundant involvement in so many pathophysiological and homeostatic processes. However, this pleiotropy of $\gamma\delta$ T cells is likely an important factor that stifles the clinical success of their application to treat cancer. As for adaptive immune interventions, it may be absolutely mandatory to carefully consider the plethora of $\gamma\delta$ T cell functions, the diversity in $\gamma\delta$ TCR specificities and affinities as well as the complex requirements for proper $\gamma\delta$ T cell activation. At the end, such broadly tumor-reactive $\gamma\delta$ T cells might be highly effective only under very defined molecular and pathophysiological conditions and therefore less broadly applicable as initially thought, though a valuable addition to current therapeutic options. This new concept represents a major challenge in the design of next generation $\gamma\delta$ T cell-based immunotherapies, and clinical trials that incorporate these exciting insights will need to be pursued to confirm the clinical potential of $\gamma\delta$ T cells in the treatment of cancer.

AUTHOR CONTRIBUTIONS

Wouter Scheper, Zsolt Sebestyen, and Jürgen Kuball wrote the manuscript; all authors agreed on the final manuscript.

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Histological analysis of $\gamma\delta$ T lymphocytes infiltrating human triple-negative breast carcinomas

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Breast cancer is the leading cause of cancer death in women and the second most common cancer worldwide after lung cancer. The remarkable heterogeneity of breast cancers influences numerous diagnostic, therapeutic, and prognostic factors. Triple-negative breast carcinomas (TNBCs) lack expression of HER2 and the estrogen and progesterone receptors and often contain lymphocytic infiltrates. Most of TNBCs are invasive ductal carcinomas (IDCs) with poor prognosis, whereas prognostically more favorable subtypes such as medullary breast carcinomas (MBCs) are somewhat less frequent. Infiltrating T-cells have been associated with an improved clinical outcome in TNBCs. The prognostic role of $\gamma\delta$ T-cells within CD3⁺ tumor-infiltrating T lymphocytes remains unclear. We analyzed 26 TNBCs, 14 IDCs, and 12 MBCs, using immunohistochemistry for the quantity and patterns of $\gamma\delta$ T-cell infiltrates within the tumor microenvironment. In both types of TNBCs, we found higher numbers of $\gamma\delta$ T-cells in comparison with normal breast tissues and fibroadenomas. The numbers of infiltrating $\gamma\delta$ T-cells were higher in MBCs than in IDCs. $\gamma\delta$ T-cells in MBCs were frequently located in direct contact with tumor cells, within the tumor and at its invasive border. In contrast, most $\gamma\delta$ T-cells in IDCs were found in clusters within the tumor stroma. These findings could be associated with the fact that the patient's prognosis in MBCs is better than that in IDCs. Further studies to characterize these $\gamma\delta$ T-cells at the molecular and functional level are in progress.

Keywords: $\gamma\delta$ T-cells, breast cancer, triple-negative breast cancer, histology, paraffin material

INTRODUCTION

Worldwide, breast cancer is the principal cause of cancer related deaths in women in developed and in developing countries (1). Breast cancer is a heterogeneous disease of numerous tumor subtypes with different biological characteristics and clinical prognosis (2). One subgroup with a particularly poor prognosis are triple-negative breast carcinomas (TNBCs) characterized by lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. TNBCs account for 10–17% of all breast carcinomas, depending on the sensitivity of tests used to define the ER, PR, and HER2 status (3) and frequently, these tumors contain marked lymphocytic infiltrates (4). TNBCs are generally high-grade tumors and mostly invasive ductal carcinomas (IDCs), although other types of breast cancers can also be triple negative such as the medullary breast carcinoma (MBC) (5, 6). MBCs represent only 3–5% of all breast cancers and are characterized by a well-circumscribed margin, a poorly differentiated nuclear grade, a high-mitotic rate, prominent syncytial growth in more than the 75% of the tumor area and a diffuse lymphoid infiltrate without intraductal components or micro-glandular features (7). And although the MBC's aggressive histological characteristics are very similar to those of high-grade

triple-negative IDCs, MBCs have generally a remarkably better prognosis than IDCs (7–9).

The association between tumor-infiltrating lymphocytes (TILs) and the clinical outcome has been well established in many different cancers and these findings initiated an increasing interest in valid markers of tumor behavior and treatment response (10–13). However, the numbers and composition of TILs may vary depending on the types of immune responses and antigens (14). Prominent infiltration by CD8⁺ T-cells has been generally associated with a better prognosis and response to therapies (15–18). In contrast, a predominance of some CD4⁺ T-cell subsets within TILs has been linked to a poorer outcome while the prognostic significance of increased numbers of regulatory T-cells (Tregs) remain controversial and may depend on the type of tumor (19, 20).

In this context, $\gamma\delta$ T-cells have been studied in distinct cancers as an interesting and intriguing part of the tumor microenvironment with demonstration of cytotoxicity *in vitro* against both solid and hematological malignancies (21–25). However, the identification and relevance of the different $\gamma\delta$ T-cell subsets within the tumor microenvironment remain poorly characterized. V γ 9V δ 2 T lymphocytes are the main subset in the human adult peripheral blood, where $\gamma\delta$ T-cells typically constitute about 5% of CD3⁺

lymphocytes. Besides V γ 9V δ 2 T-cells, lymphocytes expressing V δ 1 are typically found in human tissues (26, 27) such as intestine, mucosa, and skin. Even though they constitute only a small population of lymphocytes, $\gamma\delta$ T-cells may play a non-overlapping role in some human infections, autoimmunity (28), and tumor microenvironment (29, 30). The V γ 9V δ 2 T-cell subset recognizes phosphoantigens such as isopentenyl pyrophosphate (IPP). IPP is produced in all higher eukaryotic cells including human cancer cells by the mevalonate pathway. In contrast, many bacteria such as *Mycobacterium tuberculosis* and protozoa such as *Malaria* parasites use the non-mevalonate (1-deoxy-D-xylulose-5-phosphate; DOXP) pathway for the phosphoantigenic biosynthesis. (31). These antigens are presented to human V γ 9V δ 2 T-cells bound to the intracellular B30.2 domain of butyrophilin 3A1 (32). Antigens recognized by other human $\gamma\delta$ T-cell subsets remain poorly defined. It has been suggested that V δ 1 recognize MHC class I related molecules MICA, MICB, and ULBPs (21, 33). Infiltration by $\gamma\delta$ T-cells in human breast carcinomas and a potential role of cytotoxic V γ 9 δ 2 T-cells against breast cancer cells has been initially described by Bank et al. in 1993 (34).

Here, we analyzed the presence of $\gamma\delta$ T-cells in the human TIL immune microenvironment of 26 TNBCs comparing triple-negative IDC and triple-negative MBC specimens. Since the amounts of TILs in primary TNBCs appear to be associated with prognosis (35), we studied these tumors, focusing on the possibility that immunohistochemistry (IHC) of $\gamma\delta$ T-cell infiltration may help our understanding of the substantial prognostic difference between IDCs and MBCs.

MATERIALS AND METHODS

TISSUE SPECIMENS

We analyzed 30 formalin-fixed, paraffin-embedded (FFPE) specimens from patients with TNBCs that were obtained between 2003 and 2011 and preserved in the archives of the Institute of Clinical Pathology of the Freiburg University Medical Center. From these, we selected 14 IDC and 12 MBC samples with an equivalent lymphocytic infiltration of at least 50% of the sample area in HE staining (Table 1). All specimens in this study were obtained before the patients were treated with chemotherapy or radiotherapy. In addition, we analyzed for comparison non-malignant breast tissues (11 normal breast tissues and 7 fibroadenomas). Controls included sections from two TCR $\gamma\delta$ lymphomas (kindly provided by Prof. Müller-Hermelink, Würzburg). Negative controls included TCR $\alpha\beta$ lymphomas and isotype controls. The age of the patients ranged between 43 and 82 years (median 57 years). Type of tumor and staging were performed according to the classification of the Union for International Cancer Control (UICC). All tumors included in this study were grade III according to the modified Bloom–Richardson classification (36). MBC was diagnosed using the Ridolfi criteria (7). The Ethics Committee of the University of Freiburg Medical Center approved the use of the patient materials in this study for morphologic analyses.

IMMUNOHISTOCHEMISTRY

Sections (2 μ m) were mounted on Superfrost plus® Adhesion glass slides (R. Langenbrinck, Emmendingen, Germany. Code 03-0060) after dewaxing and rehydration. Antigen retrieval was performed

Table 1 | Characteristics of patients with grade 3 triple negative tumors.

| Diagnosis | Age (mean) | Tumor size mean (cm) | Stage (TNM) ^a | | |
|---------------------|--------------------|----------------------|--------------------------|----|----|
| | | | N0 | N1 | N2 |
| IDC (<i>n</i> =14) | 57.5 (\pm 11.7) | 2.7 (\pm 1.93) | 10 ^b | 1 | 3 |
| MBC (<i>n</i> =12) | 59.1 (\pm 13.3) | 2.2 (\pm 1.28) | | 7 | 4 |

^aAll patients were M0.

^bNumbers of patients.

Table 2 | Antibodies used in immunohistochemistry.

| Antibody | Dilution | Retrieval buffer | Incubation time (min) |
|------------------------------------|-----------|------------------|-----------------------|
| Anti-T $\text{CR}\gamma\delta$ mAb | 1:40 | Citrate pH 6 | 30 |
| CD3 antiserum | Undiluted | Dako pH 9 | 30 |
| Caspase-3 c antiserum | 1:700 | Dako pH 6.1 | 45 |

using the buffers as detailed in Table 2. Endogenous peroxidase activity was blocked by the peroxidase blocking reagent (EnVision™ FLEX Systems FLEX, Dako, Carpinteria, CA, USA. Code S2023) for the rabbit CD3 antiserum that was detected by the peroxidase based detection system. For the alkaline phosphatase based detection method [anti-T $\text{CR}\gamma\delta$ monoclonal antibody (mAb) and caspase-3 polyclonal antiserum], non-specific protein binding was blocked using 3% BSA in PBS. Antigen retrieval was performed in citrate buffer at pH 6 in a pressure cooker (anti-T $\text{CR}\gamma\delta$ mAb), in Dako pH 6.1 buffer (anti-caspase-3 antiserum), or in Dako pH 9 EDTA buffer (anti-CD3 antiserum) in a steam cooking machine (Table 2). The use of a microwave oven did not produce good results. Sections were incubated with primary antibodies that were rabbit-anti-human CD3 polyclonal antiserum (EnVision™ FLEX Systems Dako. Code IS503, undiluted), mouse anti-human T $\text{CR}\gamma\delta$ mAb (clone γ 3.20, Thermo Scientific, Germany. Code 10772535, 1:40) and rabbit-anti-human cleaved-caspase-3 antiserum (Cell Signall Corp., Danvers, MA, USA, Code 9662S, 1:700). Horseradish peroxidase-conjugated secondary antibodies (EnVision™ FLEX Systems Dako, Code 5007) and alkaline phosphatase-conjugated secondary antibodies (Dako REAL™ Detection System, Alkaline Phosphatase/RED, rabbit/mouse, Code K5005) were employed for detection of the primary antibodies, a blue chromogen was used to detect the cleaved-caspase-3 antibody (Dako BCIP/NBT Substrate System Code K0598) and hematoxylin was used as a counterstain.

MICROSCOPY

Immunohistochemistry was analyzed using an Axioplan® microscope (Carl Zeiss, Jena, Germany), equipped with a AxioCam® MRc (Carl Zeiss), digital camera. Twenty randomly selected high-power fields (HPF) of each sample were photographed (10 from the tumor parenchyma and 10 from the stroma). A HPF 400 \times was defined using a 40 \times objective and a 10 \times ocular magnification equipped with a 26-mm ocular reticule (Carl Zeiss). For the caspase-3 analysis, 20 HPF were considered from tumor areas.

The cells were counted manually in all sections by two different investigators.

STATISTICAL ANALYSIS

The unpaired *t*-test using the GraphPad Prism Software (GraphPad Inc., San Diego, CA, USA. Version 6) was used for statistical analysis.

RESULTS

DISTRIBUTION OF $\text{TCR}\gamma\delta^+$ T-CELLS IN NORMAL BREAST TISSUES

We used the mAb γ 3.20 that is able to detect $\gamma\delta$ T-cells in paraffin-embedded material (37) for IHC studies of normal breast tissues ($n = 11$). There were only few CD3^+ cells in normal breast sections (Figure 1A) and very few if any expressed the $\text{TCR}\gamma\delta$ (Figure 1B). In contrast, infiltrations by $\gamma\delta$ T-cells in FFPE samples of $\text{TCR}\gamma\delta$ lymphomas (Figure 1C) stained positive by the anti- $\text{TCR}\gamma\delta$ mAb. $\gamma\delta$ T-cells were also detectable in tonsils and other normal human tissues (data not shown).

$\text{TCR}\gamma\delta^+$ CELLS IN IDCs AND MBCs

Next, we examined triple-negative IDCs ($n = 14$) and MBCs ($n = 12$) for the presence of $\gamma\delta$ T-cells since these tumors are frequently infiltrated by lymphocytes (38). Indeed, the lymphocytic infiltrates in IDCs and MBCs contained many $\gamma\delta$ T-cells (Figure 2). Although both types of TNBCs, contained conspicuous numbers of $\gamma\delta$ T-cells (Table 3), the $\text{TCR}\gamma\delta^+$ cells within the TILs were more frequently located in the stroma of the IDC sections (Figures 2A,B), while in the MBC sections $\text{TCR}\gamma\delta^+$ cells were typically located in the tumor parenchyma (Figure 2C) and at the invasive tumor cell border (Figure 2D). Nevertheless, this distinction was not absolute since individual IDC cases contained many $\gamma\delta$ T-cells both in the tumor stroma and parenchyma (Figure 3). However, there were significantly more $\text{TCR}\gamma\delta^+$ cells within the tumor parenchyma in MBCs than in IDCs (Table 3).

$\text{TCR}\gamma\delta^+$ CELLS IN FIBROCYSTIC BREASTS

For comparison with the malignant tumors IDC and MBC, we analyzed $\text{TCR}\gamma\delta^+$ cells in fibroadenomas ($n = 7$) that are benign breast lumps composed of two elements, epithelium and stroma. Some $\text{TCR}\gamma\delta^+$ T-cells were present in the lymphocytic infiltrates in fibrocytic breasts (Figures 4A,B), rarely at the border or within the epithelial component, but the amount of $\text{TCR}\gamma\delta^+$ cells was much lower than in the TNBC.

ACTIVATED CASPASE-3⁺ TUMOR CELLS IN TRIPLE-NEGATIVE IDCs AND MBCs

Cells positive for cleaved-caspase-3 (CC3) are undergoing apoptosis that could be induced by interaction with cytotoxic T-cells (39). We stained our FFPE tumor sections by IHC for the presence of activated caspase-3. Apoptotic tumor cells were detectable in both types of TNBC. There were significantly more CC3⁺ cells in the MBC-type than in the IDC type of TNBC (Figure 5; Table 3).

DISCUSSION

The functional importance of TILs in breast cancer is controversial. Most studies show that tumor-infiltrating CD8⁺ lymphocytes

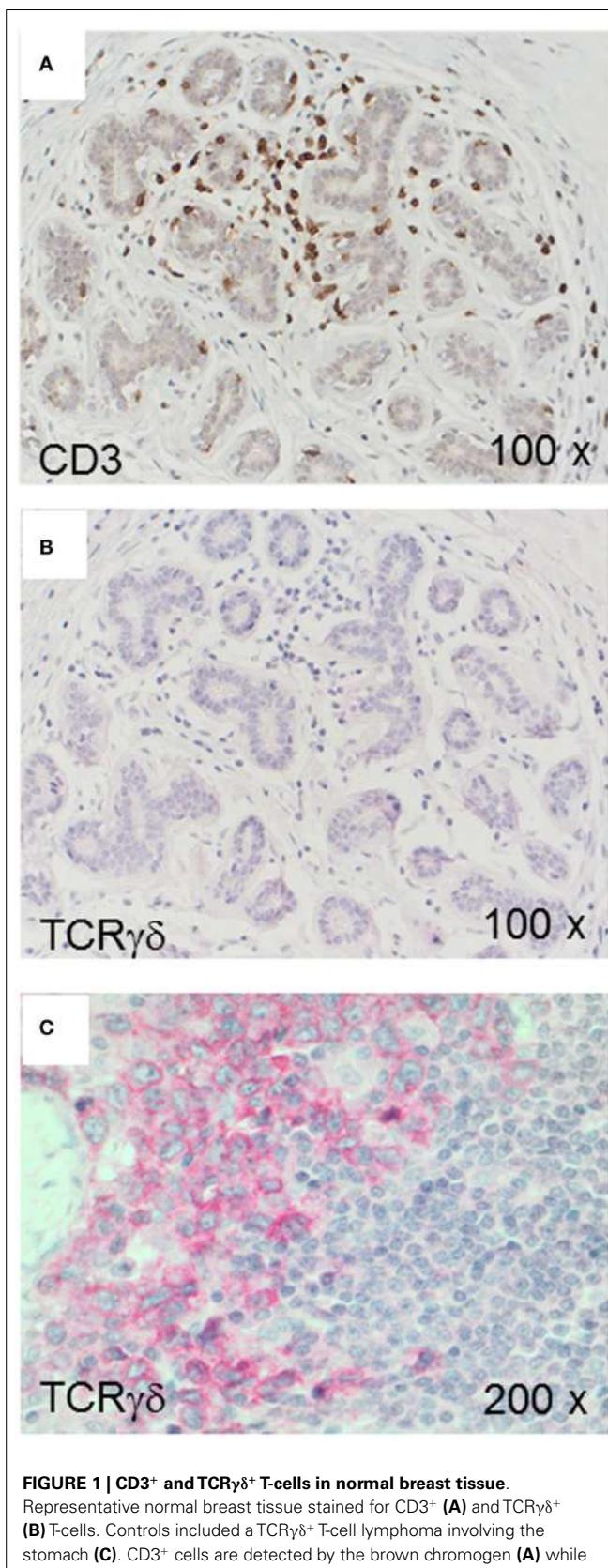


FIGURE 1 | **CD3⁺ and TCR $\gamma\delta^+$ T-cells in normal breast tissue.**

Representative normal breast tissue stained for CD3^+ (A) and $\text{TCR}\gamma\delta^+$ (B) T-cells. Controls included a $\text{TCR}\gamma\delta^+$ T-cell lymphoma involving the stomach (C). CD3^+ cells are detected by the brown chromogen (A) while $\text{TCR}\gamma\delta^+$ cells are stained red (B,C).

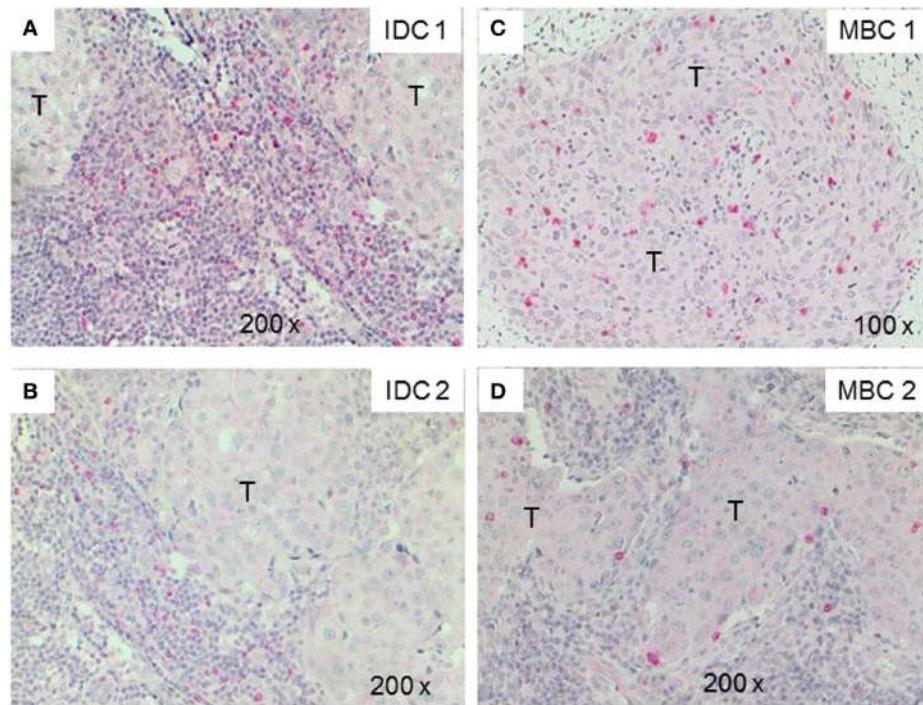


FIGURE 2 | TCR $\gamma\delta^+$ T-cells in representative triple negative invasive ductal carcinomas (IDCs) and medullary breast carcinomas (MBCs). Two representative cases of IDC (A,B) and MBC (C,D) were

stained by IHC for TCR $\gamma\delta^+$ T-cells. The tumor area is marked with T, TCR $\gamma\delta^+$ T-cells are detected by the red chromogen. The tumor area is marked ("T").

in breast cancer show a positive correlation with patient survival (15–18, 40). FOXP3 $^+$ regulatory TILs were a favorable prognostic factor in the HER2 $^+$ /ER $^-$ breast cancers, but an adverse prognostic indicator in ER $^+$ breast cancer (19, 20, 41). In this study, we investigated by IHC the presence of $\gamma\delta$ T-cells in human TNBCs comparing the IDC- and MBC-type tumors. TNBCs have attracted much attention in recent years because there are no targeted therapies for this group of breast cancers and because their profile overlaps with that of “basal-like carcinomas” (42). Histologically, TNBCs are more often the IDC type than the MBC one and are frequently displaying prominent lymphocyte infiltrates. To our knowledge, this is the first IHC analysis of $\gamma\delta$ T-cells within TILs in TNBCs and the first study using FFPE material. Previous studies detected $\gamma\delta$ T-cells in human breast carcinomas by IHC in frozen sections (34, 43). We found significant numbers of $\gamma\delta$ T-cells as constituents of TILs in both the IDC- and the MBC-type of TNBC. In most IDCs, the $\gamma\delta$ T-cells were preferentially located in the stroma and to a lesser degree in the tumor parenchyma. In MBCs, the $\gamma\delta$ T-cells were mainly present within the tumor epithelium or at its invasive border (Figure 2; Table 3). The intratumoral infiltration by $\gamma\delta$ T-cells in IDCs was heterogeneous. Most IDC specimens showed relatively few $\gamma\delta$ T-cells in the tumor parenchyma in comparison with MBCs. However, in some IDCs, a manifest intratumoral $\gamma\delta$ T-cell infiltrate was present (Figure 3). This may be related to the fact that TNBCs themselves constitute a heterogeneous subgroup, with some tumors conceivably having an intraductal and a medullary component and thus in some cases,

distinguishing between the IDC- and the MBC-type may be difficult (44). What could be the reason for the differences that we observed in the $\gamma\delta$ T-cell infiltration patterns between most IDCs and the MBCs? Potential explanations include different antigens for $\gamma\delta$ T-cells expressed by the tumor cells or different galectins (45) or chemokines such as CCL2 (46) present in the particular tumor microenvironment. The apoptotic tumor cells as detected by CC3 expression (Figure 5) might reflect the intratumoral infiltration by cytotoxic T-cells, such as $\gamma\delta$ T-cells that were in direct contact with the tumor cells (Figures 2 and 3). This is compatible with previous findings showing that there are more apoptotic tumor cells in MBC than in IDC (47, 48) and could be linked to the overall better prognosis of MBC.

We found that $\gamma\delta$ T-cells are rare in normal breast tissues (Figure 1) and scarce within the lymphocytic infiltrates in fibroadenomas (Figure 4) suggesting that $\gamma\delta$ T-cells are actively infiltrating TNBCs. The inflammatory immune responses or soluble factors secreted by the tumor cells might induce infiltration by $\gamma\delta$ T-cells in breast carcinomas. For instance, it is possible that some TNBCs or “basal-like” breast carcinomas (49) secrete soluble chemokines attracting $\gamma\delta$ T-cells. Also, it is conceivable that TNBCs may express $\gamma\delta$ T-cell-recognizable antigens that are absent in other breast carcinomas and normal breast tissues. $\gamma\delta$ T-cells in breast carcinomas could play a protective role as observed for CD8 $^+$ T-cells. However, one study by Ma et al. performed on frozen sections from a heterogeneous group of breast cancers suggested that intratumoral $\gamma\delta$ T-cells correlated

with the HER2 expression status, breast cancer progression and poor patient survival rates (43). These findings are compatible with the observation that breast cancer-derived $\gamma\delta$ regulatory T-cells induce immunosenescence, resulting in suppression of innate

and adaptive immunity (50). In the study by Ma et al. (43), the patients' tumors were heterogeneous and the $\gamma\delta$ T-cell numbers correlated with Tregs, therefore, it is not possible to exclude that other variables than $\gamma\delta$ T-cells were involved in tumor progression.

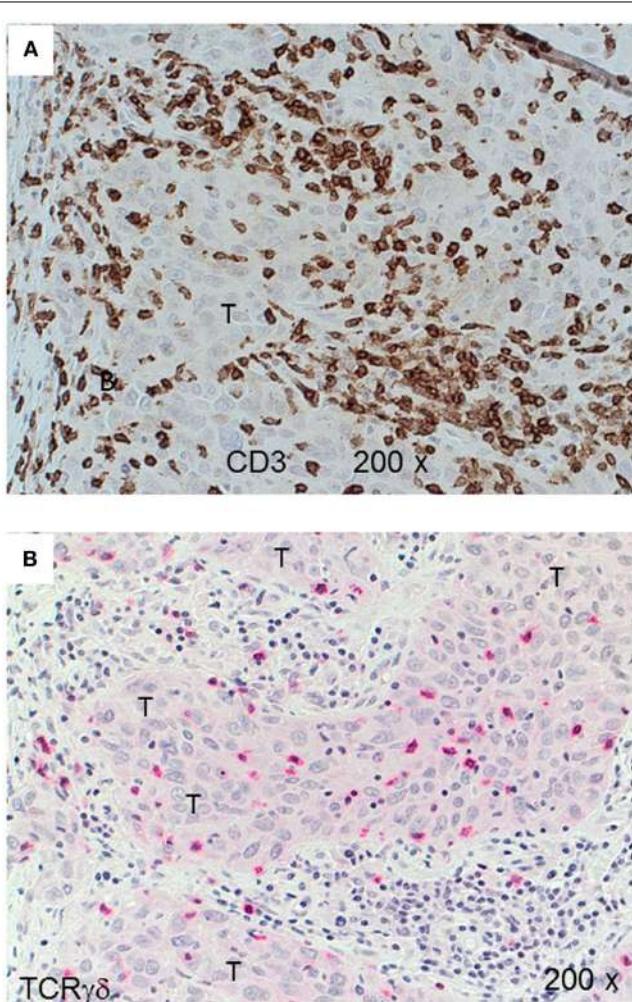


FIGURE 3 | CD3⁺ and TCR $\gamma\delta$ ⁺ T-cells in an invasive ductal carcinoma (IDC). IHC of an IDC with a rich lymphocytic infiltration that extends into the tumor parenchyma (T). CD3⁺ T-cells are detected by the brown chromogen (A) while TCR $\gamma\delta$ ⁺ cells are stained red (B). The tumor area is marked ("T").

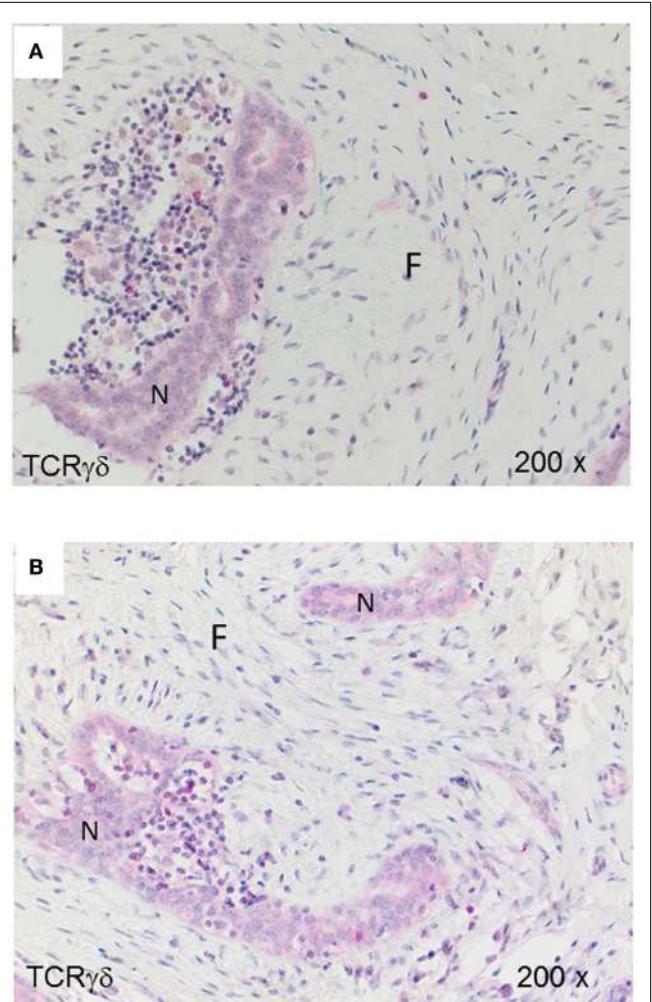


FIGURE 4 | TCR $\gamma\delta$ ⁺ T-cells in benign proliferative breast disease. TCR $\gamma\delta$ ⁺ IHC in two representative fibroadenomas (from seven cases analyzed). Fibrotic tissue ("F") and normal breast tissue ("N") are marked. There is a small lymphocytic infiltrate with very few TCR $\gamma\delta$ ⁺ cells.

Table 3 | CD3⁺ and TCR $\gamma\delta$ ⁺ T-cells in the stroma and parenchyma of IDC and MBC.

| | CD3 ⁺ cells | | TCR $\gamma\delta$ ⁺ cells | | Cleaved-caspase-3 ⁺ cells |
|----------------------|-----------------------------|-----------------------------|---------------------------------------|-----------------------------|--------------------------------------|
| | Stroma | Parenchyma | Stroma | Parenchyma | Tumor |
| IDC (<i>n</i> = 14) | 27 (± 9) ^a | 16 (± 7) [*] | 6 (± 4) | 2 (± 2) ^{**} | 4 (± 2) [*] |
| MBC (<i>n</i> = 12) | 24 (± 2) | 24 (± 9) | 4 (± 2) | 8 (± 4) | 7 (± 3) |

^aThe numbers reflect the mean positive cells per HPF (determined from the means of a total of 20 HPF counted for each patient) of IDC (*n* = 14) and MBC patients (*n* = 12). SD reflects the standard variation within the cohorts of IDC and MBC patients.

*The difference between the CD3⁺ cells within stroma and parenchyma was statistically significant in IDC ($p < 0.05$) but not in MBC ($p = 0.901$). In addition, apoptotic (CC3⁺) cells were significantly higher in MBC than in IDC ($p < 0.05$).

**The difference in the higher amount of TCR $\gamma\delta$ T-cells in the tumor parenchyma in MBC than in IDC was statistically highly significant ($p < 0.001$).

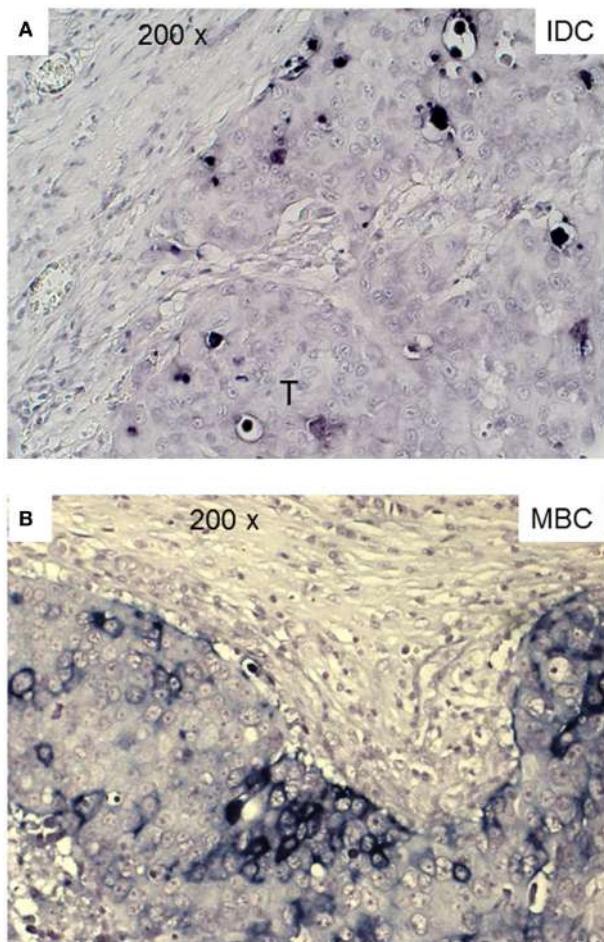


FIGURE 5 | Apoptotic tumor cells in triple negative IDC and MBC.

Cleaved-caspase-3⁺ tumor cells are detected in a representative case of IDC and MBC by a dark blue chromogen within the tumor area.

It might be interesting to investigate the TNBC infiltrating $\gamma\delta$ T-cells at the molecular level to define their variable gene expression and whether they can recognize breast cancer cell lines. A previous study in colon cancer suggested that intratumoral V δ 1⁺ T-cells were cytotoxic and secreted interferon- γ toward epithelial tumor cells. Our preliminary results where we isolated $\gamma\delta$ T-cells from TNBCs by microdissection followed by single-cell PCR suggest that the $\gamma\delta$ T-cells in these tumors do not represent the V γ 9V δ 2 population in the blood, but that they express the V δ 1, V δ 3, and V δ 4 genes (data not shown).

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Tumor-infiltrating $\gamma\delta$ T lymphocytes: pathogenic role, clinical significance, and differential programming in the tumor microenvironment

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There is increasing clinical evidence indicating that the immune system may either promote or inhibit tumor progression. Several studies have demonstrated that tumors undergoing remission are largely infiltrated by T lymphocytes [tumor-infiltrating lymphocytes (TILs)], but on the other hand, several studies have shown that tumors may be infiltrated by TILs endowed with suppressive features, suggesting that TILs are rather associated with tumor progression and unfavorable prognosis. $\gamma\delta$ T lymphocytes are an important component of TILs that may contribute to tumor immunosurveillance, as also suggested by promising reports from several small phase-I clinical trials. Typically, $\gamma\delta$ T lymphocytes perform effector functions involved in anti-tumor immune responses (cytotoxicity, production of IFN- γ and TNF- α , and dendritic cell maturation), but under appropriate conditions they may divert from the typical Th1-like phenotype and polarize to Th2, Th17, and Treg cells thus acquiring the capability to inhibit anti-tumor immune responses and promote tumor growth. Recent studies have shown a high frequency of $\gamma\delta$ T lymphocytes infiltrating different types of cancer, but the nature of this association and the exact mechanisms underlying it remain uncertain and whether or not the presence of tumor-infiltrating $\gamma\delta$ T lymphocytes is a definite prognostic factor remains controversial. In this paper, we will review studies of tumor-infiltrating $\gamma\delta$ T lymphocytes from patients with different types of cancer, and we will discuss their clinical relevance. Moreover, we will also discuss on the complex interplay between cancer, tumor stroma, and $\gamma\delta$ T lymphocytes as a major determinant of the final outcome of the $\gamma\delta$ T lymphocyte response. Finally, we propose that targeting $\gamma\delta$ T lymphocyte polarization and skewing their phenotype to adapt to the microenvironment might hold great promise for the treatment of cancer.

Keywords: $\gamma\delta$ T cells, TIL, IL-17, immunosuppression, tumor microenvironment

$\gamma\delta$ T LYMPHOCYTES: ANTIGEN RECOGNITION AND EFFECTOR FUNCTIONS

$\gamma\delta$ T cells carrying the $\gamma\delta$ T cell receptor (TCR) are important effector cells that may play a role in the anti-tumor immune response. $\gamma\delta$ Cells are not a homogeneous population of cells with a single physiological role. Instead, ever increasing complexity in both phenotype and function is being ascribed to $\gamma\delta$ cell subsets from various tissues and locations, both in mice and humans.

$\gamma\delta$ T cells account for 1–5% of CD3 $^{+}$ T cells in the peripheral blood, but constitute a major subset in other anatomic sites, such as the intestine or the skin [here, however, only in the murine but not in human skin (1)]. In the blood of most healthy individuals, T cells expressing the V δ 2 gene paired with one particular V γ 9 chain (referred to as V γ 9V δ 2 T cells) account for 50 to >90% of the $\gamma\delta$ T cell population. In contrast, intestinal intraepithelial $\gamma\delta$ T cells frequently express the V δ 1 gene, which can associate with different V γ elements (1, 2). V δ 1 $\gamma\delta$ T cells recognize the MHC class I-related molecules MICA, MICB, and ULBPs, which are expressed on epithelial cells by heat shock or oxidative stress and

are constitutively expressed to variable levels on many epithelial and hematopoietic tumor cells (3, 4). It has been debated whether MICA/MICB and ULBPs are directly recognized by the V δ 1 TCR or, indirectly activate V δ 1 T cells upon binding to the stimulatory natural killer (NK) receptor, NKG2D, which is also expressed by the vast majority of $\gamma\delta$ T cells.

V γ 9V δ 2 T cells recognize phosphoantigens (PAGs) without requirement for antigen processing and presentation, and MHC restriction. PAGs are pyrophosphates derived from the microbial non-mevalonate isoprenoid biosynthesis pathway (5, 6). Structurally related pyrophosphates are generated in eukaryotic cells through the mevalonate pathway. Micromolar concentrations of endogenous pyrophosphates are required for V γ 9V δ 2 T cell activation and such concentrations are achieved after cellular stress and transformation (7). Given the cross-reactivity between microbial and self PAGs, there is a great interest in elucidating how TCR signaling can be induced by such small molecules. PAGs can directly activate V γ 9V δ 2 T cells, but such activation is greatly enhanced by monocytes and/or dendritic cells (DCs). Hence, either PAGs

are presented as cargo to the reactive $\gamma\delta$ TCR or their cellular processing somehow sensitizes cell recognition through the engagement of the V γ 9V δ 2 TCR by stabilizing surface expression of a TCR-binding molecules (8). A candidate molecule involved in intracellular PAg processing is the F1-ATPase, which directly binds the V γ 9V δ 2 TCR and interacts with ApppI, an adenosine derivative of IPP (9). Moreover, it has been recently found that PAg-induced V γ 9V δ 2 T cell activation requires butyrophilin 3A1 (BTN3A1) (10–12). Therefore, production of exogenous PAg or up-regulation of endogenous PAGs in human cells in response to either infections or tumor transformation provokes V γ 9V δ 2 T cell reactivity, albeit at substantially different sensitivity.

Intracellular levels of PAGs can be manipulated by drugs. Aminobisphosphonates, such as zoledronic acid, which are in clinical use for the treatment of osteoporosis and bone metastasis, are potent inhibitors of the downstream enzyme of the mevalonate pathway farnesyl pyrophosphate synthase, thereby leading to the intracellular accumulation of upstream metabolites as IPP and in consequence to V γ 9V δ 2 T cell activation (13, 14). On the contrary, statins block the upstream enzyme hydroxymethylglutaryl-CoA reductase and inhibits IPP production, inhibiting V γ 9V δ 2 T cell activation (15).

V γ 9V δ 2 T cells express numerous molecules potentially associated with different stages of differentiation, migration, and functions. V γ 9V δ 2 T cells include “naive” and “central memory” phenotypes (T_{Naive}, CD45RA $^+$ CD27 $^+$; T_{CM}, CD45RA $^-$ CD27 $^+$) that home to secondary lymphoid organs, but lack immediate effector function, and “effector/memory” (T_{EM}, CD45RA $^-$ CD27 $^-$) and “terminally differentiated” (T_{EMRA}, CD45RA $^+$ CD27 $^-$) phenotypes that home to sites of inflammation and display immediate effector function (16).

While T_{Naive} and T_{CM} cells readily respond to PAGs stimulation, T cells with effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) expand in response to homeostatic cytokines as IL-15 (17) and differentiate in the presence of polarizing cytokines (18). On activation, V γ 9V δ 2 cells can be skewed toward distinct effector functions depending on polarizing cytokines, in analogy to CD4 helper T cells. Typically, human V γ 9V δ 2 T cells default toward type 1 cytokine production ($\gamma\delta$ 1), but under appropriate culture conditions they divert from the typical $\gamma\delta$ 1 phenotype and polarize to $\gamma\delta$ 2 (19, 20), $\gamma\delta$ 17 (21–23), $\gamma\delta$ FH (24, 25), and $\gamma\delta$ reg cells (26). Such a broad plasticity emphasizes the capacity of V γ 9V δ 2 T cells to influence the nature of immune response to different challenges.

$\gamma\delta$ T CELLS FOR TUMOR IMMUNOTHERAPY

The major goal of tumor immunotherapy is the induction of adaptive responses of B cells and MHC-restricted $\alpha\beta$ T cells, particularly CD8 cytotoxic T cells. Nonetheless, despite major advances in this area, durable responses are rare and immunotherapy is not yet an established modality to treat tumors. Furthermore, tumors frequently develop strategies to escape immune responses (27, 28). In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells have unique features (see Table 1), which makes them good candidates for effective tumor immunotherapy. For instance, they lack MHC restriction, and do not require co-stimulation. Therefore, common tumor antigens without MHC restriction provide broader applicability of $\gamma\delta$ T

Table 1 | Advantages of using $\gamma\delta$ T cells for tumor immunotherapy.

The frequency of $\gamma\delta$ T cells is very high (1–5/10²), compared to that of Ag-specific $\alpha\beta$ T cells (1/10⁵–10⁶)

$\gamma\delta$ T cells can recognize and lyse a broad range of tumor cells and there is no need to target tumor-specific Ags

$\gamma\delta$ T cells lack MHC restriction in antigen recognition

$\gamma\delta$ T cell activation does not require co-stimulatory signals (e.g., CD28)

mAb can be used *in vivo* to enhance $\gamma\delta$ T cell cytotoxicity (ADCC)

$\gamma\delta$ T cells seem to be devoid of GVH activity

FDA-approved drugs (Zoledronate, IL-2) available for $\gamma\delta$ T cell expansion *in vivo*

Large scale *ex vivo* expansion of $\gamma\delta$ T cells and clinical grade sets for purification

cells across a wide range of tumors and patients with diverse MHC alleles. Moreover, $\gamma\delta$ T cells display potent cytotoxic and anti-tumor activity *in vitro* (29–33) and in xenograft models *in vivo* (34, 35). Cytotoxicity of $\gamma\delta$ T cells against tumor cells is associated with increased production of PAGs (36), which is, at least, partly due to the increased expression of hydroxymethylglutaryl-CoA reductase, the rate limiting enzyme of the mevalonate pathway (36). Moreover, intracellular levels of IPP can be manipulated by aminobisphosphonates (13–15, 37–39), thereby leading to the intracellular accumulation of IPP and in consequence to activation of V γ 9V δ 2 T cells (36). As above discussed, in addition to the binding of the antigenic molecules to the reactive TCR, V γ 9V δ 2 T cells express NK cell activating receptors such as NKG2D, which recognizes target cells expressing MICA, MICB, and ULBPs (3, 4, 40, 41). These interactions may prove crucial in V γ 9V δ 2 T cell recognition and killing of tumors of hematopoietic origin. In fact, the expression levels of ULBP1 determine lymphoma susceptibility to V γ 9V δ 2 T cell-mediated cytolysis, highlighting a thus far unique physiologic relevance for tumor recognition by V γ 9V δ 2 T cells (42, 43).

After recognizing target cells via the TCR, or NKG2D, or both, V γ 9V δ 2 T cells preferentially use the perforin/granzyme (44) and/or TRAIL (45) pathways, as well as the Fas/FasL killing signal (46), for cytotoxicity against target cells like tumor cells. In addition, activated V γ 9V δ 2 T cells secrete IFN- γ and TNF- α , which have cytotoxic activity against tumor cells directly and indirectly *via* stimulating macrophages and DCs (47–49).

Overall, the potent anti-tumor activity of V γ 9V δ 2 T cells and their wide reactivity to several tumor cell types has led to the exploration of their therapeutic potential. Two strategies have been developed to apply the anti-tumor activities of V γ 9V δ 2 T cells to cancer immunotherapy: (1) *in vivo* administration of compounds that activate V γ 9V δ 2 T cells and (2) adoptive transfer of *ex vivo*-expanded V γ 9V δ 2 T cells. Several small-sized clinical trials have tested the efficacy of any of these two strategies in patients with various tumor types and a recent meta-analysis based on data from 13 clinical trials including a total of 204 patients has demonstrated that V γ 9V δ 2 T cell-based immunotherapy improves overall survival and, in view of its low toxicity grade (50), provides a proof of principle for its utilization as adjuvant to conventional therapies.

TUMOR-INFILTRATING $\gamma\delta$ T CELLS AND THEIR CORRELATION TO CANCER OUTCOME

Tumor-infiltrating leukocytes are an heterogeneous population of immune cells that have been found in a wide variety of solid tumors (51) and the extent of leukocyte infiltration has been often associated with improved prognosis (52). However, there is a limited number of studies regarding the contribution of individual leukocyte subsets to survival. Tumor-infiltrating leukocytes include cells of the myeloid lineage (granulocytes, macrophages, and myeloid-derived suppressor cells) and several different lymphocyte subsets (T, B, and NK), each with different impact on tumor progression. Results of mouse tumor models and human cohort studies have suggested that any individual leukocyte population may correlate with poor or better prognostic factors, such as tumor stage/grade, presence of metastasis, and disease-free/overall survival. In general, infiltration by myeloid cells has been associated with tumor progression, while the presence of abundant T cells (particularly of the CD8 subset) is associated with tumor regression and improved prognosis. However, the limits of the immunohistochemical techniques largely used in retrospective clinical studies, have so far prevented a detailed descriptions of different tumor-infiltrating leukocyte populations as well as evaluation of their functional properties in the tumor microenvironment. For instance, tumor-infiltrating T lymphocytes may be endowed with regulatory function and hence promote tumor progression.

Several studies have shown that $\gamma\delta$ T cells are present among tumor-infiltrating lymphocytes (TILs) from patients affected by different types of cancer, but their clinical relevance remains still obscure because of conflicting results obtained.

In detail, there have been five relatively recent large studies, which have correlated tumor-infiltrating $\gamma\delta$ T cells with several different clinical features:

Bialasiewicz et al. (53) evaluated by immunohistochemical analysis TILs in 113 specimens from patients with necrotizing choroidal melanoma. They detected TILs in 76% of samples and $\gamma\delta$ T cells, mainly of the V δ 1 subset were present in 52% of samples. Most notably, the presence of $\gamma\delta$ T cells in tumors positively correlated with patient's survival, indicating that tumor-infiltrating $\gamma\delta$ T cells are a prognostically favorable factor.

Inman et al. (54) assessed by immunohistochemical analysis total $\gamma\delta$ T cells in 248 renal cancer specimens and correlated these values with clinicopathologic prognostic factors and cancer outcome. They found that percentages of intratumoral $\gamma\delta$ T cells were usually very low (<1% of the CD3 $^{+}$ population) in nearly all tested tumor specimens and did not correlate with any examined prognostic factor or even with survival. Authors concluded that the role of $\gamma\delta$ T cells in renal cancer is questionable.

Ma et al. (55) examined by immunohistochemistry total $\gamma\delta$ T cells infiltrating breast cancer in specimens of 46 patients. $\gamma\delta$ T cells were detected in nearly all cancer patients (93%), but only in 3% of normal breast specimen. Authors did not quantify the percentages of intratumoral $\gamma\delta$ T cells, but when an arbitrary cut-off of nine $\gamma\delta$ T cells per high magnification microscopic field was used to define TIL-high (>9) and TIL-low (<9) groups, authors found that $\gamma\delta$ T cell numbers were positively correlated with advanced tumor stages, HER2 expression status, and high

lymph node metastasis, but inversely correlated with relapse-free survival and overall survival of patients. Multivariate and univariate analysis of tumor-infiltrating $\gamma\delta$ T cells and other prognostic factors further suggested that intratumoral $\gamma\delta$ T cells represented the most significant independent prognostic factor for assessing severity of breast cancer compared with the other known factors. Authors concluded that tumor-infiltrating $\gamma\delta$ T cells play a crucial role in breast cancer progression and pathogenesis and may serve as a valuable and independent prognostic biomarker for human breast cancer.

Cordova et al. (56) studied the representation of tumor-infiltrating $\gamma\delta$ T cells from 74 patients with primary melanoma. $\gamma\delta$ T cells were the major subset among CD3 $^{+}$ T lymphocytes and comprised equal percentages of V δ 1 and V δ 2 TEM or TEMRA phenotypes. In this study, the presence of $\gamma\delta$ T cells, and in particular the V δ 2 subset, among TILs significantly correlated with early stage melanoma, while percentages of infiltrating V δ 1 T cells did not correlate with any examined prognostic factor of melanoma.

Finally, Wu et al. (57) demonstrated that $\gamma\delta$ T cells ($\gamma\delta$ 17) are the major source of IL-17 in human colon cancer, with the majority (80%) of the IL-17 $^{+}$ $\gamma\delta$ T cells expressing V δ 1 and 20% expressing V δ 2. Importantly, analyzing 117 colon cancer samples, authors found that $\gamma\delta$ 17 cell infiltration positively correlated with tumor stages and other clinicopathological factors (tumor size, tumor invasion, lymphatic and vascular invasion, lymph node metastasis, and serum CEA levels), indicating that tumor-infiltrating $\gamma\delta$ 17 T cells are associated with tumor invasiveness and progression and may thus represent a prognostic factor in human colon cancer.

TUMOR-INFILTRATING $\gamma\delta$ T CELLS: WHAT ARE THEY AND WHAT DO THEY DO?

The above discussed findings that tumor-infiltrating $\gamma\delta$ T cells correlate with tumor remission, or with tumor progression or even fail to correlate with any prognostic feature strongly suggest that $\gamma\delta$ T cells in the tumor microenvironment may play substantially different functions; hence positive or negative correlation with prognosis may depend on the specific $\gamma\delta$ T cell subset/function recruited at the tumor site. Furthermore, the net biologic effects of $\gamma\delta$ T cells may depend on the tumor type and the tumor site, perhaps reflecting microenvironmental differences: for instance TGF- β , which is abundantly secreted at the tumor site by tumor-infiltrating macrophages or by tumor cells themselves, may favor the differentiation of $\gamma\delta$ cells with Treg-like properties, which in turn inhibit anti-tumor immune responses.

Initial studies on the functional properties of tumor-infiltrating $\gamma\delta$ T cells were performed using polyclonal $\gamma\delta$ T cell lines generated *in vitro* upon long term culture with mitogen/antigen and IL-2: this approach was mainly due to the very low number of $\gamma\delta$ T cells recovered from tumor specimen and to the lack of suitable techniques, which allowed precise detection of functional markers. These studies have unequivocally demonstrated that *ex vivo*-expanded $\gamma\delta$ T cell lines and clones from renal, breast, lung, ovary, colon, and pancreatic cancer efficiently kill stabilized tumor cell lines and freshly isolated tumor cells and generally V δ 1 T cell lines had the higher cytotoxic activity compared to V δ 2 T cell lines (58–63). Accordingly, Cordova et al. (56) confirmed these results using polyclonal $\gamma\delta$ T cell lines derived from melanoma;

both V δ 1 and V δ 2 T cell lines produced equal amounts of TNF- α and IFN- γ , but while the majority (75%) of V δ 1 T cell lines exerted potent cytotoxic activity against melanoma cell line *in vitro*, only 25% of the V δ 2 T cell lines showed appreciable lytic activity. Therefore, based on their cytolytic activity and production of cytokines with proven anti-tumor effect, tumor-infiltrating $\gamma\delta$ T cells have been long regarded as important players of the anti-tumor immune response. However, both the failure to consistently detect a positive correlation between the presence of $\gamma\delta$ T cells in the tumor microenvironment and the patient's prognosis, as well as the improvement of immunological techniques to detect functional signatures even in very small tissue samples have subverted the concept that $\gamma\delta$ T cells are simply an important component of resistance to cancer and suggested that their function may be extremely pleiotropic and including either effector or suppressive potential.

In 2007, Peng and colleagues (64) unexpectedly identified a V δ 1 $^+$ population (which comprised over 95% of the total $\gamma\delta$ T cells population) among breast cancer-infiltrating lymphocytes capable to suppress immune responses. In particular, V δ 1 cells inhibited CD4 and CD8 T cell activation and impaired DC maturation and function. Although the mechanisms responsible for the regulatory activity of tumor-infiltrating V δ 1 cells was not investigated in that paper, it seems to involve TLR8 signaling pathway, as suppression was reversed by TLR8 ligands. Later on, the same group reported (65) that breast cancer-infiltrating V δ 1 cells induced both T cell and DC senescence and the senescent T cells and DCs in turn became regulatory cells, thus determining amplification of the immunosuppressive process. Interestingly, and surprisingly, accumulation of regulatory V δ 1 cells in the context of breast cancer (where they account for approximately 30% of the total lymphocyte population) is not due to proliferation of resident V δ 1 cells but to their recruitment mediated by IP-10 secreted by breast cancer cells.

In addition to the above quoted studies on tumor-infiltrating human regulatory $\gamma\delta$ T cells, four recent reports, three in mice and one in humans, have shed light on the regulatory role played by IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ 17) and have also defined the underlying mechanisms.

Using a transplantable tumor mouse model, Wakita et al. (66) observed that $\gamma\delta$ T cells accounted for 25% of all TILs and selectively produced IL-17 but not IFN- γ . Importantly, absence of IL-17 caused inhibition of tumor growth, which correlated with a reduced number of blood vessels within the tumor and reduced expression levels of VEGF and Ang-2 in tumor cells. This indicates that tumor-infiltrating $\gamma\delta$ 17 T cells promote angiogenesis, and thus tumor growth.

A similar detrimental effect of IL-17 has been reported by Ma and colleagues (67) in an hepatocellular carcinoma mouse model. Similarly to the findings of Wakita et al., $\gamma\delta$ T cells were the major source of IL-17 amongst lymphocytes infiltrating hepatocellular carcinoma. In this model, absence of IL-17 reduced tumor growth, while its administration promoted the growth of hepatocellular carcinoma. However, the mechanism responsible for the anti-tumor activity of IL-17 was different from that reported by Wakita and involved a reciprocal activatory interaction between the $\gamma\delta$ 17 cells and MDSC, which was mediated by cancer cells:

in detail, $\gamma\delta$ T cell-derived IL-17 induced CXCL5 production by tumor cells, which in turn recruited MDSC to the tumor sites *via* CXCL5/CXCR2-interaction. Once at the tumor site, IL-17 induced production of IL-1 β and IL-23 in MDSC, which amplify differentiation of $\gamma\delta$ 17 cells. This positive feedback between $\gamma\delta$ 17 cells and MDSC sustains immunosuppression and promotes tumor growth.

The third mouse study by Silva Santos and colleagues (68) used a transplantable peritoneal/ovarian cancer, and confirmed the crucial role of $\gamma\delta$ 17 in promoting cancer growth. $\gamma\delta$ 17 accumulated in the peritoneal cavity and were the main source of IL-17 also in this model. $\gamma\delta$ 17 caused the recruitment at the tumor site of an unconventional population of small macrophages that expressed IL-17 receptor and a number of pro-tumor and pro-angiogenic molecules amongst which VEGF and TGF- β , which promoted cancer cell proliferation and tumor growth.

The fourth study on the participation of $\gamma\delta$ 17 cells in cancer was performed by Wu et al. (57) in human colorectal cancer. In that study, tumor-infiltrating $\gamma\delta$ T cell was the main source of IL-17 and 80% of the $\gamma\delta$ 17 cells expressed V δ 1. Of note however, $\gamma\delta$ 17 constituted approximately 25% of all tumor-infiltrating V δ 1 cells and co-produced TNF- α , IL-8, and GM-CSF. All these cytokines, in different combinations, caused recruitment (IL-8 and GM-CSF) and survival, activation, and proliferation (TNF- α , IL-8, and IL-17) of MDSC that in turn mediate immunosuppression and promote tumor growth.

Altogether these results clearly demonstrate that $\gamma\delta$ 17 cells are key mediators of tumor-associated immunosuppression thereby influencing tumor progression.

HYPOTHESIS: TUMOR MICROENVIRONMENT AS THE CRITICAL DETERMINANT OF TUMOR-INFILTRATING $\gamma\delta$ T CELL FATE

The conditions under which $\gamma\delta$ T cells can contribute to tumor control versus immune suppression need to be defined. There are several theoretical possibilities to answer the fundamental question of the molecular mechanisms that explain these two $\gamma\delta$ T cell phenotypes.

First, it is possible that genetic differences in tumor cells influence the host response, through the involvement of different pathways that are mutated or activated in a heterogeneous fashion and that regulate the expression of immune system regulatory genes. For instance, tumor cells with STAT3 activation show impaired production of chemokines and cytokines, but increased production of immunosuppressive factors and thus escape immune recognition (69).

Second, it is possible that polymorphism of regulatory genes might influence lymphocyte activation at the tumor site. For instance, IRF5 polymorphism is associated with clinical response to adoptively transferred TILs in melanoma patients (70).

Third, it is likely that exposure to certain pathogens or even the intestinal microbiome could change the frequency, phenotype, and functions of TILs. For instance, Wu et al. (57) showed that in colon cancer patients, destruction of the epithelial barrier caused by tumor development results in tumor invasion by commensal bacteria (*E. coli*) and release of bacterial product, which promote IL-23 production by DCs and $\gamma\delta$ 17 cell polarization *in situ*.

Fourth, it is likely (and this is the possibility we favor) that tumor microenvironment plays a key role. By definition, tumor microenvironment is a complex network of different cell types, soluble factors, signaling molecules, and extracellular matrix components, which orchestrate the fate of tumor progression (71). In fact, in addition to the tumor cells and to the several lymphoid and myeloid cell types that infiltrate tumors, classical cellular components of the solid tumor stromal microenvironment also influence the host immune response. The tumor stroma consists of fibroblasts, macrophages, and vascular endothelial cells, with variable amounts of extracellular matrix, all of which contribute not only as a support structure for tumor growth, but can also impair host immune responses and likely contribute to the quality of immune cell infiltration (71). We hypothesize (**Figure 1**) that, at early stages of tumor development $\gamma\delta$ T cells of the $\gamma\delta 1$ type producing cytokines with proven anti-tumor activity (IFN- γ and TNF- α) and equipped with cytotoxic potential either expand locally (V $\delta 1$) or are recruited at the tumor site from peripheral blood (V $\delta 2$) and may exert anti-tumor activity; however, with tumor progression, factors produced in the microenvironment cause polarization of $\gamma\delta$ cells from $\gamma\delta 1$ to $\gamma\delta 17$ and $\gamma\delta\text{reg}$, which instead promote tumor progression. A plethora of cell types present in the tumor microenvironment may actually provide the source of such $\gamma\delta$ cells polarizing factors.

For instance, colon cancer stem cells and tumor-associated macrophages and fibroblasts produce huge amounts of TGF- β (72) which, in combination with other cytokines present in the microenvironment, contributes to polarization of $\gamma\delta$ T cells to $\gamma\delta 17$ and $\gamma\delta\text{reg}$.

Macrophages, DCs, and other myeloid cells, which are typically found in the solid tumor microenvironment, produce IL-15, which in combination with TGF- β determines $\gamma\delta\text{reg}$ polarization (26), and IL-1 β , IL-6, IL-23, and TGF- β , which in different combination promote $\gamma\delta 17$ Polarization (22).

Finally, it is also possible that those $\gamma\delta$ T cells within TILs equipped with anti-tumor activities die after antigenic activation and the frequencies of $\gamma\delta\text{reg}$, which would be less tumor reactive or resistant to cell death than increase. Moreover, nitric oxide, which is largely produced by MDSC in the tumor microenvironment may contribute to apoptosis of $\gamma\delta$ T cells induced by antigen activation (73).

Once activated, $\gamma\delta\text{reg}$ and $\gamma\delta 17$ amplify the immunoregulatory process in different ways (**Figure 2**): IL-17 promotes VEGF production by cancer cells and macrophages, and CXCL5 production by tumor cells which, in turn, recruited MDSC. An activatory cross-talk is then established at the tumor site between MDSC and $\gamma\delta 17$, by which IL-17 induces IL-1 β and IL-23 production by MDSC and these cytokines promote further differentiation and activation of $\gamma\delta 17$ T cells. Finally, $\gamma\delta\text{reg}$ produce IL-10 and TGF- β , which act on several cellular targets to promote immunosuppression at the tumor site and favor tumor progression.

Several recent discoveries have been made toward understanding the biological effects of cytokines, particularly TGF- β , produced in the tumor microenvironment that can polarize many arms of the immune system. Thus, and similarly to what we propose to occur for $\gamma\delta$ cells, cytokines present in the tumor microenvironment induce DCs to acquire a tolerogenic

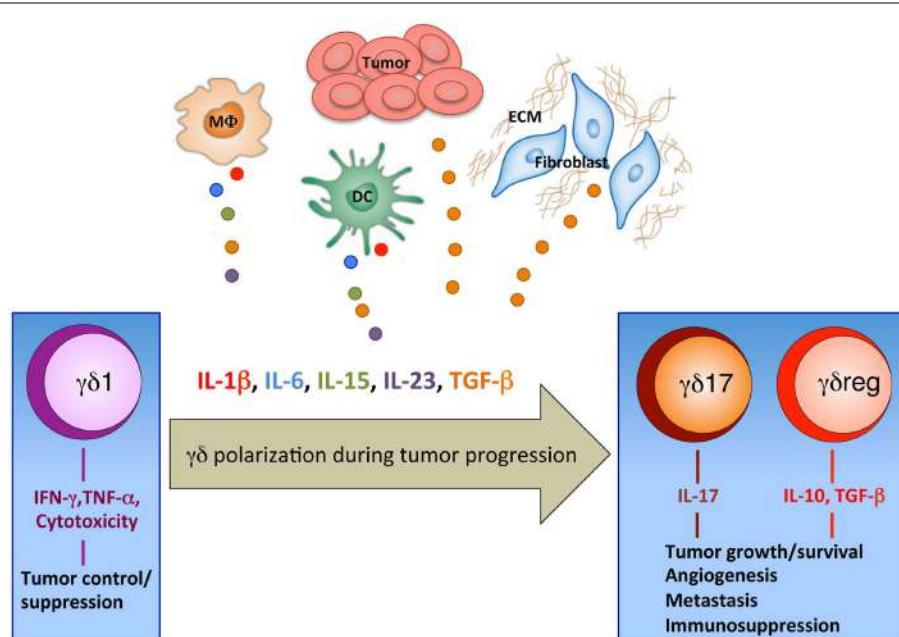
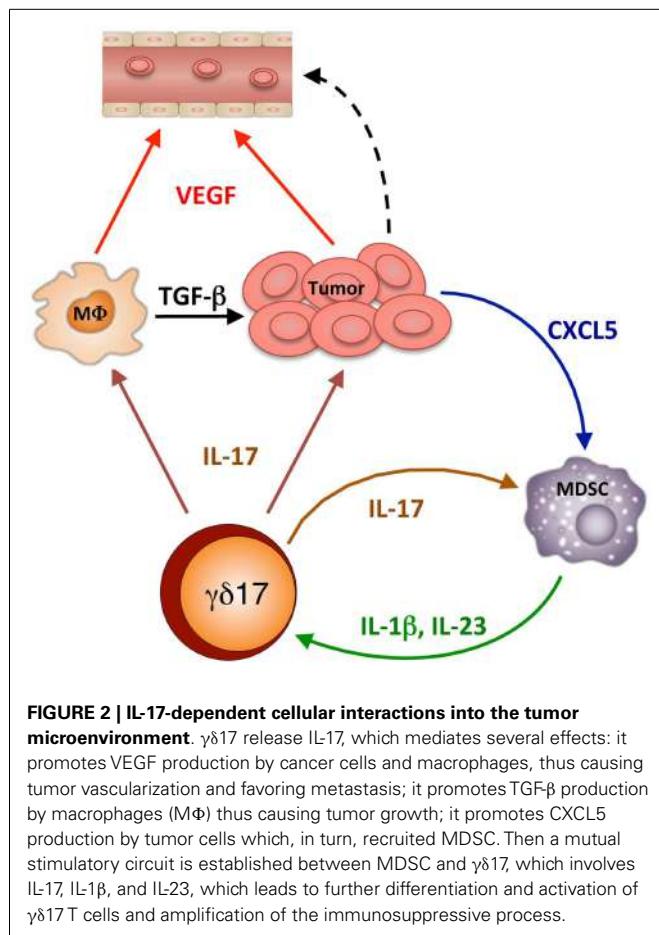


FIGURE 1 | Polarization of $\gamma\delta$ T cells in the tumor microenvironment. Our working hypothesis for the recruitment of $\gamma\delta$ T cells with different phenotypes and functions into the tumor site. At early stages of tumor development tumor cells produce chemokines, which recruit $\gamma\delta$ T cells of the $\gamma\delta 1$ type equipped with anti-tumor activities (IFN- γ and TNF- α production and cytotoxic

potential). It is speculated that during progression, tumors have denser stroma (ECM, extracellular matrix) and alternative DCs, myeloid, or macrophage (M Φ) populations, which produce cytokines that accumulate in the tumor microenvironment and cause polarization of $\gamma\delta$ cells from $\gamma\delta 1$ to $\gamma\delta 17$ and $\gamma\delta\text{reg}$.



phenotype, convert N1 neutrophils to a N2 phenotype and promote the recruitment of M2 over M1 macrophages. Within the tumor microenvironment, cytokines also inhibit Th1 and CD8 CTL functions and probably promote a shift toward Th2, Th17, and Tc17 differentiation and convert CD4 effector T cells to induced Treg (iTReg) cells. For a systematic review on the polarization of immune cells in the tumor microenvironment see in Ref. (74).

CONCLUSION AND PERSPECTIVES

The mutual and interdependent interaction between tumor and its microenvironment is a crucial topic in cancer research and therapy, as recently demonstrated by the finding that targeting stromal factors could improve efficacies of current therapeutics and prevent metastasis. For instance, combinatorial therapy with an agonistic mAb against CD40 and standard gemcitabine chemotherapy, proved unexpectedly efficient in pancreatic cancer (75). In this system, the anti-CD40 mAb caused massive recruitment of macrophages at the tumor site, which caused severe disruption of the tumor stroma thus allowing increased concentrations of gemcitabine to accumulate to the tumor site (75). Similarly, the identification of defined immunosuppressive pathways in the tumor microenvironment has pointed toward therapeutic targets that are amenable to clinical intervention: these include, for instance, mAbs to PD-1 or PD-L1, CTLA-4, CD25,

and small-molecule inhibitors that block IDO enzymatic activity [reviewed in Ref. (76)]. These novel strategies must be kept in mind when designing $\gamma\delta$ T cell-based therapy. Moreover radiation therapy, low dose traditional chemotherapeutic drugs and aminobisphosphonates not only sensitize tumor cells to immune recognition and killing, but also modulate the tumor microenvironment and contribute to the therapeutic effect (77–80): for instance, zoledronic acid at clinically achievable doses repolarizes tumor-associated macrophages to a M1 phenotype and reduces the number of MDSC (81).

$\gamma\delta$ T cell-based immunotherapy is emerging to be a powerful treatment option for patients with different types of tumors. It includes the *in vivo* activation of $\gamma\delta$ cells or the adoptive transfer of *ex vivo*-expanded $\gamma\delta$ cells. Although we have no evidence of the fate of the activated $\gamma\delta$ cells and the long term effects of the $\gamma\delta$ cell-based therapies in preclinical and clinical studies, it is reasonable to predict that the tumor microenvironment plays an indispensable role in limiting the effectiveness of $\gamma\delta$ T cell-based immunotherapies. Additionally, the local expansion of adoptively transferred $\gamma\delta$ T cells has to be increased to achieve higher T cell numbers at the tumor site.

Therefore, combination regimens consisting of $\gamma\delta$ T cell-based therapies and strategies aimed to circumvent the negative impact of the tumor microenvironment onto $\gamma\delta$ T cells and induce $\gamma\delta$ T cell repolarization, may prove efficacious and achieve clinical benefit in patients with different types of tumor.

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Human gamma delta T regulatory cells in cancer: fact or fiction?

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While gamma delta T cell ($\gamma\delta$ Tc) anticancer immunotherapies are being developed, recent reports suggest a regulatory role for $\gamma\delta$ Tc tumor-infiltrating lymphocytes. This mini-review surveys available evidence, determines strengths and weaknesses thereof and suggest directions for further exploration. We focus on human $\gamma\delta$ Tc, as mouse and human $\gamma\delta$ Tc repertoires differ. Regulatory $\gamma\delta$ Tc are defined and compared to conventional Tregs and their roles in health and disease (focusing in on cancer) are discussed. We contrast the suggested regulatory roles for $\gamma\delta$ Tc in breast and colorectal cancer with their cytotoxic capabilities in other malignancies, emphasizing the context dependence of $\gamma\delta$ Tc functional plasticity. Since $\gamma\delta$ Tc can be induced to exhibit regulatory properties (in some cases reversible), we carefully scrutinize experimental procedures in published reports. As $\gamma\delta$ Tc garner increasing interest for their therapeutic potential, it is critical that we appreciate the full extent of their role(s) and interactions with other cell types in both the circulation and the tumor microenvironment. A comprehensive understanding will enable manipulation of $\gamma\delta$ Tc to improve anti-tumor efficacy and patient outcomes.

Keywords: gamma delta T cells, cancer immunotherapy, regulatory T cells, human cancer, gamma delta T cell functional plasticity

INTRODUCTION

While those of us in the immunotherapy world tend to focus on the anti-infection and anti-tumor properties of $\gamma\delta$ Tc, we are now beginning to appreciate that, under certain conditions, these remarkable cells can inhibit or suppress the maturation and/or activation of immune cells around them, leading to beneficial or potentially pathological consequences.

A suppressor function of human $\gamma\delta$ Tc was first described in 1989 by Patel and colleagues; upon *in vitro* stimulation with pokeweed mitogen, most $\gamma\delta$ Tc clones could suppress the generation of Immunoglobulin(Ig)-secreting B cells by CD4 $^{+}$ T helper cells treated with mitomycin C (**Figure 1A**) (1). Since then, regulatory roles for $\gamma\delta$ Tc have been described in several contexts. Both V δ 1 and V δ 2 T cell subsets (V δ 1Tc and V δ 2Tc, respectively) may exhibit regulatory properties, albeit in different settings.

Human peripheral blood-derived $\gamma\delta$ Tc displaying regulatory properties are phenotypically different from conventional regulatory CD4 $^{+}$ $\alpha\beta$ T cells (Treg). In contrast to Treg, freshly isolated $\gamma\delta$ Tc express only low levels of CD25 and cytotoxic T lymphocyte-associated antigen (CTLA)-4, and do not express the transcription factor forkhead box P3 (FoxP3) (2–4). Similar to conventional $\alpha\beta$ T cells ($\alpha\beta$ Tc), CD25 is up-regulated on $\gamma\delta$ Tc after initial phytohemagglutinin (PHA) or anti- $\gamma\delta$ TCR monoclonal antibody (mAb) stimulation (5). Additionally, CD25 is also up-regulated on V δ 2Tc after stimulation with pyrophosphates (phosphorylated antigens), which are intermediates of the isoprenoid pathway and induce selective expansion of V δ 2Tc within peripheral blood mononuclear cells (PBMC) 7–10 days after initial stimulation (6, 7). Furthermore, FoxP3 expression can be

detected with PCH101 mAb but not with the more Treg-specific 259D mAb, in $\gamma\delta$ Tc as well as in Treg-depleted $\alpha\beta$ Tc after activation (4). FoxP3 expression as identified by PCH101 mAb does not correlate with suppressive function (8, 9). In addition, the transcription factor Helios, which is highly expressed by Treg, is constitutively expressed in roughly one-third of freshly isolated $\gamma\delta$ Tc (4). While Helios seems to be involved in the differentiation of (regulatory) $\gamma\delta$ Tc, it is not a specific marker for suppressive $\gamma\delta$ Tc (4, 10). Thus, while freshly isolated $\gamma\delta$ Tc do not express characteristic Treg markers, the literature provides evidence that $\gamma\delta$ Tc may nevertheless exhibit regulatory activity, which will be further described below.

REGULATORY ROLES FOR $\gamma\delta$ Tc IN NON-CANCER CONTEXTS

Before focusing in on the potential regulatory role of $\gamma\delta$ Tc in cancer, it is worthwhile to consider some other contexts in which these cells have displayed suppressive properties. For a more comprehensive description of regulatory roles of $\gamma\delta$ Tc outside of cancer, we recommend a recent review (10).

Immunosuppression via $\gamma\delta$ Tc plays a protective role in several contexts. For example, in pregnancy, decidual $\gamma\delta$ Tc contribute to an immunosuppressive milieu enabling successful implantation and protecting the growing fetus from attack by the mother's immune system (11–14). In celiac disease, patients on a gluten-free diet have enhanced suppressor intestinal intraepithelial $\gamma\delta$ Tc that protect the small intestine from attack by CD8 $^{+}$ TCR $\alpha\beta$ $^{+}$ intraepithelial lymphocytes (IEL) via secretion of transforming growth factor-beta one (TGF- β 1); patients with active disease have lower frequencies of these suppressor $\gamma\delta$ Tc IEL (15). Lower

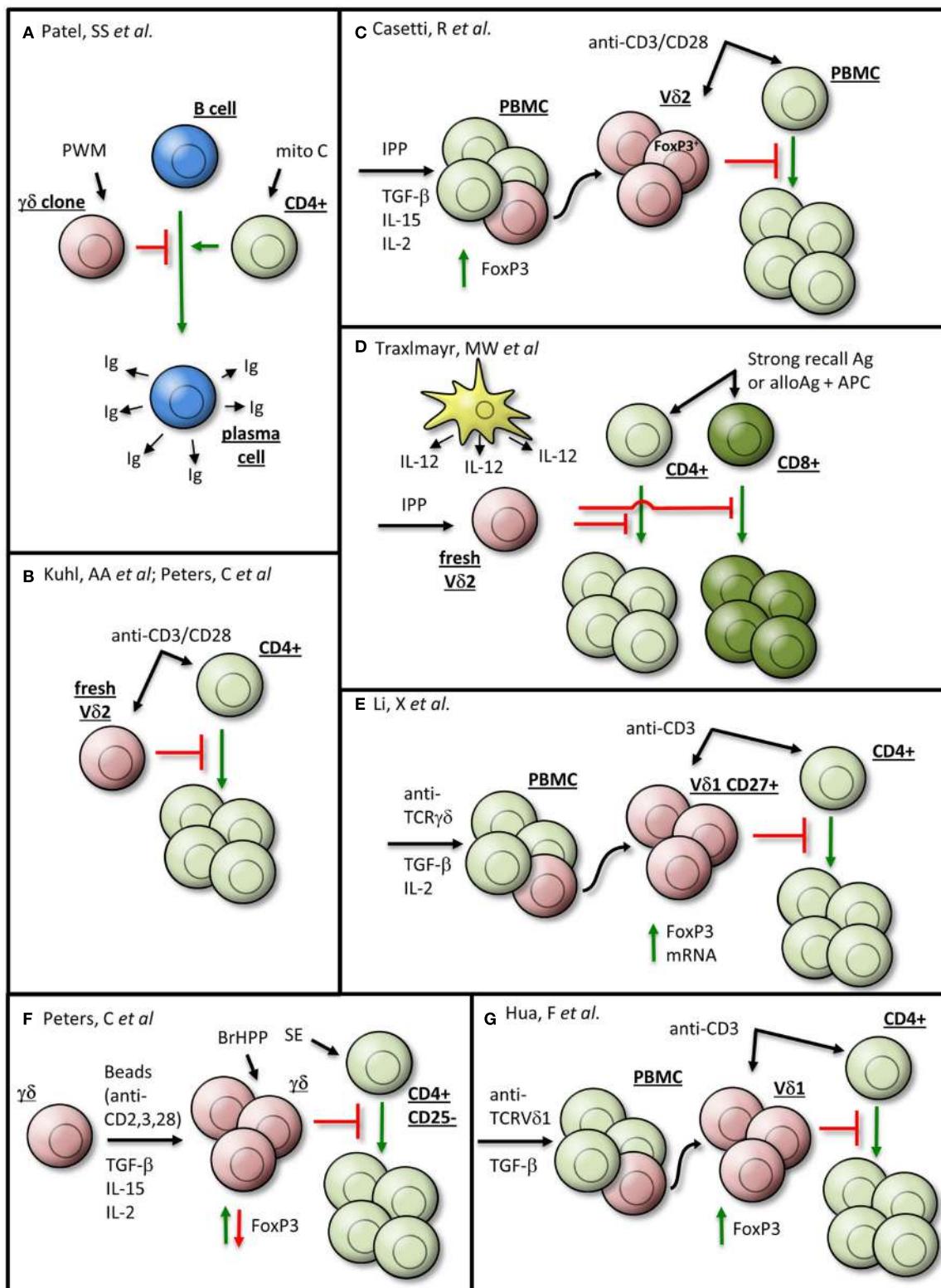


FIGURE 1 | $\gamma\delta$ Tc exhibiting regulatory properties may be generated *in vitro* by various means. Details are given in the text and the indicated references. The $\gamma\delta$ Tc are depicted in red, $\alpha\beta$ Tc in green, B cells in blue, dendritic cells in yellow, and senescent cells in gray. Ag, antigen; APC, antigen-presenting cell; BrHPP, bromohydrin pyrophosphate; fresh, freshly isolated; Ig, immunoglobulin; IPP, isopentenyl pyrophosphate; Mito C, mitomycin C; PBMC, peripheral blood mononuclear cell; PWM, pokeweed mitogen; SE, *Staphylococcus aureus* enterotoxins. **(A)** Patel et al., **(B)** Kuhl et al., Peters et al., **(C)** Casetti et al., **(D)** Traxlmayr et al., **(E)** Li et al., **(F)** Peters et al., and **(G)** Hua et al.

isolated; Ig, immunoglobulin; IPP, isopentenyl pyrophosphate; Mito C, mitomycin C; PBMC, peripheral blood mononuclear cell; PWM, pokeweed mitogen; SE, *Staphylococcus aureus* enterotoxins. **(A)** Patel et al., **(B)** Kuhl et al., Peters et al., **(C)** Casetti et al., **(D)** Traxlmayr et al., **(E)** Li et al., **(F)** Peters et al., and **(G)** Hua et al.

peripheral blood $\gamma\delta$ Tc numbers, more specifically a decreased proportion of central memory $\gamma\delta$ Tc, are correlated with systemic lupus erythematosus pathogenesis, suggesting a protective role for regulatory $\gamma\delta$ Tc in this autoimmune disease as well (16). Of note, V δ 1Tc/V δ 2Tc subset ratios are inverted in patients compared to healthy controls (i.e., V δ 1Tc predominate in blood) (16). Similarly, a higher V δ 1Tc/V δ 2Tc ratio may contribute to the achievement of operational tolerance in pediatric liver transplant recipients (17).

HOW TO MAKE REGULATORY $\gamma\delta$ Tc

So far, it is unknown whether specific subsets, e.g., CD27 $^+$ Helios-expressing $\gamma\delta$ Tc, are innately suppressive or whether their broad range of functional plasticity enables suppressive activity under certain stimulatory conditions (Figure 1). An observation common to all studies on suppressive V δ 2Tc is that they realize their immunosuppressive potential only in the presence of antigen-presenting cells (APC) or after co-stimulation with anti-CD28 mAb (Figures 1B,C) (2–4). CD28 and CTLA-4 are critical regulators of immunosuppressive T cells, whereby CD28 plays a dual role in both the generation and the termination of an immune response (18).

Freshly isolated isopentenyl pyrophosphate (IPP)-stimulated V δ 2Tc can inhibit the proliferation of CD4 $^+$ and CD8 $^+$ $\alpha\beta$ Tc in response to strong recall antigens such as Tetanus toxoid, superantigens such as *Staphylococcus aureus* enterotoxins (SE) or alloantigens in the presence of APCs (Figure 1D) (19). However, the authors could not completely rule out low frequency activation of $\alpha\beta$ Tc by antigen-specific (e.g., Tetanus toxoid) stimulation. Nevertheless, peripheral blood V δ 2Tc also suppress proliferation of co-cultured CD4 $^+$ $\alpha\beta$ Tc after polyclonal stimulation by anti-CD3/CD28 mAb, which simultaneously activates $\alpha\beta$ Tc (Figure 1B) (3, 4). All in all, the presence and strength of a co-stimulatory APC-signal seem to play an important role in the induction of V δ 2Tc suppressive capacity (4).

While TGF- β 1 alone does not induce the generation of regulatory V δ 2Tc, this switch can occur in the presence of additional cytokines (Figures 1B,C,E,F) (2, 4, 9, 16). Up to 30% of V δ 2Tc within IPP-stimulated PBMC cultivated in the presence of TGF- β 1 and interleukin (IL)-15 expressed FoxP3 (clone 259D); after subsequent cell sorting, these FoxP3 $^+$ enriched V δ 2Tc suppressed the proliferation of anti-CD3/CD28 mAb-stimulated PBMC (Figure 1C) (2). Peters and colleagues have since demonstrated that the observed FoxP3 expression was transient, with a steady increase in FoxP3 over 8 days of cell culture followed by a decrease to nearly undetectable protein levels after 16 days (4).

In contrast to the work of Casetti and colleagues, in the study of Peters et al. TGF- β 1 and IL-15 did not induce regulatory functions in bromohydron pyrophosphate (BrHPP)-expanded $\gamma\delta$ Tc. Only anti-CD3/CD28 mAb-stimulated $\gamma\delta$ Tc expanded in the presence of TGF- β 1 and IL-15 were able to suppress the proliferation of $\alpha\beta$ Tc induced by a mixture of SE (Figure 1F) (4). The observed suppressive activity was not dependent on FoxP3 expression but was rather dependent on the presence of initial CD28-co-stimulation. The discrepancy between these two studies might be explained by differences in $\gamma\delta$ Tc expansion as well

as stimulatory conditions in the suppression assays. Casetti et al. used IPP-stimulated PBMC from which V δ 2Tc were sorted after expansion, whereas Peters et al. expanded magnetically isolated, highly purified $\gamma\delta$ Tc (20). In their suppression assay, Casetti et al. analyzed the V δ 2Tc suppression of PBMC stimulated by anti-CD3/CD28 mAb, which could potentially activate other suppressive T cell subsets within the PBMC. In contrast, Peters and colleagues used CD25-depleted CD4 $^+$ T cells as responder cells, which were stimulated by a mixture of SE and BrHPP-restimulation for the co-cultured $\gamma\delta$ Tc. Common to both studies is a correlation between CD28-co-stimulation (although at different time points) and the suppressive effect. This suggests that CD28 signaling in $\gamma\delta$ Tc-mediated suppression should be examined in more detail.

While FoxP3 and $\gamma\delta$ Tc regulatory activity are not strictly connected, it is worthwhile to note that FoxP3 expression can be induced in both V δ 1Tc and V δ 2Tc subsets. Similar to V δ 2Tc, FoxP3 was prominently induced in V δ 1Tc in the presence of TGF- β 1 and additional cytokines such as IL-2 after stimulating PBMC with anti- $\gamma\delta$ TCR for 10 days (16). Additionally, there was an increased expression of both TGF- β 1 and its receptor (CD105) on V δ 1Tc compared to V δ 2Tc; upon activation, TGF- β 1 decreased and CD105 increased on V δ 1Tc. The authors assumed a regulatory role for the V δ 1 CD45 $^-$ CD27 $^+$ $\gamma\delta$ Tc subset due to its increased FoxP3 expression. While they demonstrated inhibition of CD4 $^+$ T cell proliferation by CD27 $^+$ V δ 1Tc, the authors unfortunately did not directly compare the suppressive activity of CD27 $^+$ versus CD27 $^-$ V δ 1Tc (Figure 1E) (16). In this context, the analysis of FoxP3 expression in purified V δ 2Tc versus V δ 1Tc under different culture conditions would be interesting.

Finally, Hua and colleagues induced regulatory V δ 1Tc *in vitro*, upon stimulation of PBMC with plate-bound anti-TCRV δ 1 mAb, that expressed FoxP3 (identified by mAb clone 259D/C7) and suppressed CD4 $^+$ T cell proliferation (Figure 1G) (21). The authors suggested that V δ 1Tc FoxP3 expression was sustained by a positive feedback loop instigated by V δ 1Tc producing TGF- β 1; in addition, V δ 1Tc secreted IL-10 (21).

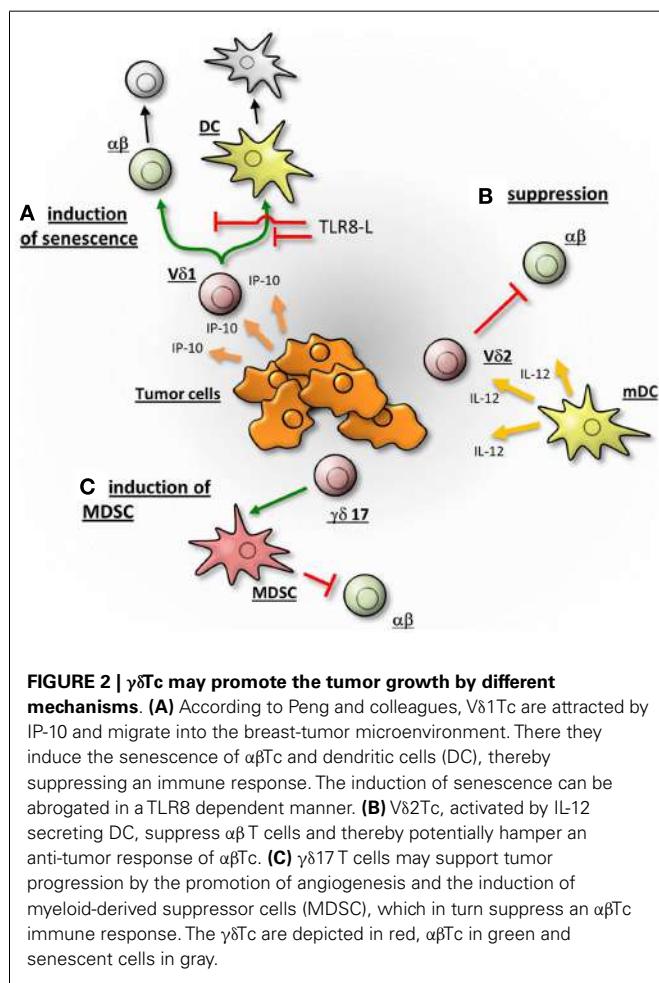
In summary, it is difficult to compare these studies, as their inherent differences in experimental design (cell source/subset/milieu/stimuli) are further confounded by the lack of a defined regulatory $\gamma\delta$ Tc marker. However, it is clear that $\gamma\delta$ Tc can be induced to exhibit regulatory properties.

HOW DO $\gamma\delta$ Tc SUPPRESS OTHER CELLS?

There are, however, some controversial data regarding mechanism(s) of suppression by $\gamma\delta$ Tc. Kuhl and colleagues assumed mediation by the immunosuppressive cytokines TGF- β 1 and IL-10, which were secreted by $\gamma\delta$ Tc after anti-CD3/CD28 mAb stimulation (Figure 1B). After 48 h stimulation, $\gamma\delta$ Tc secreted significantly more TGF- β 1 than conventional CD4 $^+$ CD25 $^+$ Tregs (3). Unfortunately, their ELISAs did not distinguish between TGF- β 1 secretion by V δ 1Tc and V δ 2Tc; however, higher TGF- β 1 mRNA levels after 3 day Concanavalin A treatment would suggest that V δ 1Tc have a greater suppressive capacity than V δ 2Tc or $\alpha\beta$ Tc (3).

Peters and co-workers demonstrated that co-culture with responder cells (CD25-depleted CD4 $^+$ $\alpha\beta$ Tc) induced the

upregulation of CD80 and CD86 as well as programmed death-ligand (PDL)-1 on stimulated V δ 2Tc, which could then interact with CTLA-4 or PD-1 on responder cells, leading to their suppression (4). Furthermore, transwell experiments suggested cell-contact-dependence, as this process was inhibited by mAb disrupting CD86:CTLA-4 or PDL-1:PD-1 interactions between anti-CD3/CD28 mAb-activated V δ 2Tc and activated $\alpha\beta$ Tc (4). Interestingly, the immunosuppressive capacity of V δ 2Tc was abrogated by Toll-like-receptor (TLR) 2 ligands as well as by activating $\alpha\beta$ Tc with a mixture of five SE (in contrast to the publication of Traxlmayr where only one SE was applied), which both induce a strong Th1-response [(4, 19); Peters and Wesch, unpublished data]. Abrogated suppression correlated with increased phosphorylation of Akt and NF κ B in $\alpha\beta$ Tc and down-regulation of inhibitory molecules such as PD-1 and CTLA-4 (4). Similarly, Peng and colleagues found that the regulatory $\gamma\delta$ Tc phenotype could be reversed through administration of TLR8 ligand Poly-G (Figure 2A) (22, 23). Only ligands to TLR8 (and not TLRs 2, 3, 4, 5, 7, or 9) blocked induction of senescence observed in T cells responding to suppression via regulatory $\gamma\delta$ Tc (Figure 2A) (23). These observations exemplify the functional plasticity of $\gamma\delta$ Tc that are influenced by the nature of a stimulus and the surrounding cytokine milieu.



An important question is how TGF- β 1 induction of conventional Tregs compares to that of regulatory $\gamma\delta$ Tc. Li and colleagues provided evidence that TGF- β 1-stimulated CD25 $+$ CD27 $+$ V δ 1Tc exert a suppressive effect on naïve CD4 $+$ T cells similar to classical Tregs, and that this mechanism was cell-cell contact dependent (16) as described for V δ 2Tc above (4).

REPORTS OF REGULATORY $\gamma\delta$ Tc IN CANCER

While several studies have proven the cytotoxic capabilities of circulating $\gamma\delta$ Tc and *in vitro*-expanded $\gamma\delta$ Tc derived thereof [reviewed in Ref. (24–29)], $\gamma\delta$ Tc tumor-infiltrating lymphocytes (TIL) may have very different functional properties (Figure 2). The tumor microenvironment (TME) is characterized in part by the presence of immunosuppressive cytokines such as TGF- β 1 and IL-10 that prevent immune attack against the growing malignancy. Thus, one might assume that this environment would support the generation of regulatory $\gamma\delta$ Tc; however, to date only very few reports support this assumption.

In a study looking at T cells from blood and TIL from lung cancer patients, freshly isolated $\gamma\delta$ Tc only slightly expressed FoxP3 compared to CD4 $+$ T cell TIL, of which almost half were positive for this regulatory marker (9). Blood-derived $\gamma\delta$ Tc stimulated with anti- $\gamma\delta$ TCR mAb for 14 days *in vitro* expressed only low levels of FoxP3, regardless of whether from healthy donors or lung cancer patients. Somewhat higher FoxP3 expression was evident in TIL-derived $\gamma\delta$ Tc from renal cell carcinoma, chromaffin tumor and especially gastric cancer, with the latter comprising 21% of expanded $\gamma\delta$ Tc in the given example. Furthermore, V δ 1Tc FoxP3 expression was greater than that of V δ 2Tc in expanded TILs from renal cell carcinoma (9). However, the authors admitted the inherent drawback that induction was detected by FoxP3 mAb clone PCH101, which is sensitive to cell activation (unlike clone 259D) (9); this has since been further corroborated (4, 9). While researchers attempting to characterize $\gamma\delta$ Tc TIL in various cancer contexts have investigated expression of FoxP3, they have failed to consistently correlate its expression to regulatory function. Thus, we conclude that FoxP3 expression is an inappropriate proxy for $\gamma\delta$ Tc regulatory potential and thus should be regarded with caution.

After vaccination, increased *in vitro* proliferation of V δ 2Tc from bone and connective tissue sarcoma patients undergoing immune therapy with autologous IL-12 secreting dendritic cells (DC; initially treated with tumor-derived soluble antigen plus lipopolysaccharide (LPS) and interferon (IFN)- γ : Trivax) was observed. Gene expression profiling experiments indicated an over-expression of hydroxy-methylglutaryl-CoA reductase (HMGR) in LPS/IFN- γ -stimulated- compared to unstimulated DC. HMGR is the rate-limiting enzyme of the mevalonate pathway that enhanced IPP levels leading to V δ 2Tc activation. Further *in vitro* studies revealed a suppressive potential of V δ 2Tc expanded by phosphoantigens (IPP) in the presence of IL-12 secreting DC (Figure 2B) (19).

While *in vitro*-expanded peripheral blood-derived $\gamma\delta$ Tc kill human breast cancer cells (30) and *in vivo* methods to expand $\gamma\delta$ Tc targeting breast cancer have already been employed in clinical trials (31, 32), a recent study of TIL in human breast tumors deemed $\gamma\delta$ Tc the most significant predictor of negative outcome (33). $\gamma\delta$ Tc frequency was correlated with negative factors such as

advanced tumor stage, positive lymph node status, and human epidermal growth factor receptor 2 (HER2) expression. Exhaustive statistical analysis correlated $\gamma\delta$ Tc with FoxP3⁺ cells (identified with clone 236A/E7) and inversely with CD8⁺ cytotoxic Tc, suggesting a negative role for $\gamma\delta$ Tc (33). However, double staining of $\gamma\delta$ Tc and FoxP3 was not done, leaving the identity of FoxP3⁺ cells ambiguous, and there was no indication as to whether staining was performed on serial sections. Furthermore, $\gamma\delta$ Tc subsets were not specified, likely due to a dearth of subset-specific antibodies suitable for their detection via immunohistochemistry (33). Finally, while $\gamma\delta$ Tc frequency in breast tumors may prove to be a valuable prognostic marker, their role in disease pathogenesis was not determined.

This same group, however, had previously suggested regulatory properties for V81Tc TIL in breast tumors (22). $\gamma\delta$ Tc TIL were extracted from a digested human breast tumor, expanded *in vitro* for 1 week in 1000 IU/ml IL-2, after which bulk TILs were maintained at 50 IU/ml IL-2. Tumor-reactive clones were then generated and both the bulk population and selected clones derived thereof suppressed naïve T cell proliferation, IL-2 secretion, and DC maturation (22). This may not reflect the case *in situ*. While this study proves that V81Tc can assume a regulatory phenotype, several caveats demand attention:

Firstly, the subset prevalence of $\gamma\delta$ Tc in the original tumor was not reported and thus (regulatory) V82Tc may have comprised the majority of tumor-derived cell suspensions at the outset but may have been subsequently eliminated by high levels of IL-2 in the culturing process, since V82Tc are known to be susceptible to activation-induced cell death (34–36). Broad ranges of V81Tc levels were only determined after culturing, while V82Tc percentages were not reported (22). In a follow-up paper, recruitment of $\gamma\delta$ Tc with a regulatory phenotype was linked to high levels of IFN- γ inducible protein 10 (IP-10) in the TME (Figure 2A); however, V81Tc and V82Tc were unfortunately not distinguished (37). Secondly, the high level of IL-2 used to culture TILs may in itself have supported expansion of a regulatory phenotype not truly reflective of the original functional orientation of these cells. Thirdly, most experiments were carried out with one cell line and clones derived from a single tumor, thus cannot represent a universal truth. It is also not clear whether the same V81Tc lines were used in subsequent publications. While valuable insight into the plasticity and regulatory potential of V81Tc can be gleaned from these studies, further investigation of $\gamma\delta$ Tc TIL *in situ* are required to substantiate claims of regulatory function contributing to poor patient prognosis.

While breast-tumor TIL-derived V81Tc can exhibit regulatory properties *in vitro*, V81Tc TIL from other cancers have been reported to be cytotoxic (38, 39). Polyclonal $\gamma\delta$ Tc TIL lines kill melanoma cell lines, and secrete tumor necrosis factor alpha (TNF α) and IFN- γ (38). This functional diversity could well be context-dependent or perhaps, as Donia and colleagues suggest, clones with various V γ pairings are differentially activated (39). It is also possible that these cytotoxic $\gamma\delta$ Tc TIL are simultaneously capable of as-of-yet unnoticed regulatory functions.

Finally, an indirect regulatory role for $\gamma\delta$ Tc has been reported in colorectal cancer (CRC), whereby IL-17 secreting $\gamma\delta$ Tc ($\gamma\delta$ 17)

in the TME may attract and help support immunosuppressive myeloid-derived suppressor cells (MDSC) (Figure 2C). *In vitro* experiments showed that activated inflammatory DC secrete IL-23 facilitating the generation of $\gamma\delta$ 17. DC activation is thought to be caused by release of bacterial products through the compromised epithelial barrier characterizing CRC. Of note, $\gamma\delta$ 17 isolated from CRC tumors were predominantly V81Tc, secreted higher levels of IL-17 compared to normal tissue controls and did not secrete IL-4, IL-22 or immunosuppressive IL-10 (40).

AVENUES TO EXPLORE

If $\gamma\delta$ Tc TIL are indeed regulatory, it is crucial to determine whether they are inherently so or whether factors in the TME induce this function. If the former is true, then presumably infusion of large numbers of cytotoxic $\gamma\delta$ Tc into patients should cause no safety concern (with respect to the further promotion of tumor growth). However, if the latter is true, we need to find a way to target the TME to prevent a potentially detrimental shift to a regulatory phenotype. Better models mimicking the human TME could help us address this question.

Since $\gamma\delta$ Tc can be induced to realize regulatory potential in various ways, including those involving cytokines typically present in the TME, some degree of regulatory function is plausible. However, so far the evidence is scant, limited to *in vitro* experiments with *ex vivo* expanded $\gamma\delta$ Tc. Admittedly, there is an inherent difficulty in assessing the regulatory capacity of $\gamma\delta$ Tc TIL *in situ*, as they are only present in relatively low abundance. Ye and colleagues attempted to address this by performing experiments with freshly purified $\gamma\delta$ Tc from tumor tissues; however, depending on the nature of the antibodies used for purification, $\gamma\delta$ Tc function may already have been altered (23). Finally, as discussed above, assessment using markers such as FoxP3 should be considered carefully because not every mAb clone detecting FoxP3 expression denotes regulatory function.

CONCLUDING REMARKS

Clearly, a more reliable panel of markers or epigenetic signature correlated to the regulatory phenotype of $\gamma\delta$ Tc will be required for us to assess their true function(s) *in situ*. Furthermore, a clear distinction should be made between V81Tc and V82Tc, which may differ dramatically in terms of plasticity and function depending on their localization and exposure to various stimuli/cytokine milieus. $\gamma\delta$ Tc can be both cytotoxic and/or regulatory; therein lies their incredible therapeutic potential in the contexts of autoimmune diseases and cancer. A fuller understanding of these processes should enable us to manipulate $\gamma\delta$ Tc plasticity to ensure optimal efficacy and ultimately improve patient outcomes.

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Plasticity of $\gamma\delta$ T cells: impact on the anti-tumor response

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The tumor immune microenvironment contributes to tumor initiation, progression, and response to therapy. Among the immune cell subsets that play a role in the tumor microenvironment, innate-like T cells that express T cell receptors composed of γ and δ chains ($\gamma\delta$ T cells) are of particular interest. $\gamma\delta$ T cells can contribute to the immune response against many tumor types (lymphoma, myeloma, melanoma, breast, colon, lung, ovary, and prostate cancer) directly through their cytotoxic activity and indirectly by stimulating or regulating the biological functions of other cell types required for the initiation and establishment of the anti-tumor immune response, such as dendritic cells and cytotoxic CD8+ T cells. However, the notion that tumor-infiltrating $\gamma\delta$ T cells are a good prognostic marker in cancer was recently challenged by studies showing that the presence of these cells in the tumor microenvironment was associated with poor prognosis in both breast and colon cancer. These findings suggest that $\gamma\delta$ T cells may also display pro-tumor activities. Indeed, breast tumor-infiltrating $\gamma\delta$ T cells could exert an immunosuppressive activity by negatively regulating dendritic cell maturation. Furthermore, recent studies demonstrated that signals from the microenvironment, particularly cytokines, can confer some plasticity to $\gamma\delta$ T cells and promote their differentiation into $\gamma\delta$ T cells with regulatory functions. This review focuses on the current knowledge on the functional plasticity of $\gamma\delta$ T cells and its effect on their anti-tumor activities. It also discusses the putative mechanisms underlying $\gamma\delta$ T cell expansion, differentiation, and recruitment in the tumor microenvironment.

Keywords: plasticity, $\gamma\delta$ T cells, cytokines, anti-tumor response, pro-tumor response

INTRODUCTION

Cancer initiation, progression, and invasion rely on the active communication between cancer cells and the different cell types in the tumor microenvironment, such as fibroblasts, endothelial cells, and immune cells. It is now well established that the immune contexture of the tumor microenvironment can influence cancer progression and outcome (1). All subsets of immune cells can be found within tumors, but their density, functionality, and organization vary according to the tumor type and stage and also from patient to patient. Within the tumor microenvironment, several sub-populations of effector cells participate in controlling and eliminating cancer cells. Among them, innate-like T cells that express T cell receptors (TCR) composed of γ and δ chains actively contribute to the anti-tumor immune response in many tumors (lymphoma, myeloma, melanoma, breast, colon, lung, ovary, and prostate cancer) (2–12). They can do this directly through their cytotoxic activity against tumor cells, or indirectly by stimulating and regulating the biological functions of other immune cell types, such as dendritic cells (DC) or interferon γ (IFN- γ)-producing CD8+ T cells, required for the initiation and establishment of an efficient anti-tumor immune response.

$\gamma\delta$ T cells belong to the non-conventional or innate lymphocyte family. They differ from conventional $\alpha\beta$ T cells, since most of $\gamma\delta$ T cells do not express the CD4 and CD8 co-receptors and, as a consequence, antigen recognition by $\gamma\delta$ TCR is not restricted

to major histo-compatibility complex (MHC) molecules (13, 14). Thus, while $\alpha\beta$ TCR interact with peptides bound to MHC class I or class II molecules, $\gamma\delta$ TCR recognize a diverse array of self and non-self antigens, such as small peptides, soluble or membrane proteins, phospholipids, prenyl pyrophosphates, and sulfatides. Because of this antigenic diversity, a single mechanism might not explain all observed TCR-dependent $\gamma\delta$ T cell responses (15). Moreover as $\gamma\delta$ T cell activation does not require antigen processing and presentation by antigen-presenting cells (APC), $\gamma\delta$ T cells can be rapidly activated and act during the early phase of the immune response. Like natural killer (NK) cells, $\gamma\delta$ T cells also respond to stimulation by stress- and/or infection-induced ligands, such as the MHC class I-related molecules H60, RAE1, and MULT-1 in mice (16), or MICA/B and ULBP in humans (17). Normally, these ligands are weakly or not expressed, they are up-regulated only in the presence of stress (DNA damage, heat stress) or infection and activate $\gamma\delta$ T cells by binding to the activating NKG2D receptor expressed on these cells (18–21) and, in some cases, through direct recognition by human $\gamma\delta$ TCR (22, 23). Moreover, human $\gamma\delta$ T cells also express pattern recognition receptors (PRR), such as Toll-like receptors (TLR), which modulate their activation (24).

In humans, $\gamma\delta$ T cells represent 0.5–16% (on average: 4%) of all CD3+ cells in adult peripheral blood, in organized lymphoid tissues (thymus, tonsil, lymph nodes, and spleen), <5% in tongue and reproductive tract and 10–30% in intestine (25, 26). In adult

mice, 1–4% of all T cells in thymus, secondary lymphoid organs and lung are $\gamma\delta$ T cells. $\gamma\delta$ T cells are more abundant in other mucosal sites. Indeed, they constitute 10–20% of all T cells in female reproductive organs (27), 20–40% of the intestinal intraepithelial T cells (28) and 50–70% of skin dermal T cells (29, 30). Moreover $\gamma\delta$ TCR repertoire is restricted and depends on the tissue type and their localization. Specifically, V γ 9V δ 2 TCR are expressed by 50–95% of $\gamma\delta$ T cells from human peripheral blood (31), whereas, TCR including other V δ elements are predominantly found in intestinal (V δ 1 and V δ 3) or skin (V δ 1) $\gamma\delta$ T cells (32, 33). In mice, $\gamma\delta$ T cells with distinct V γ /V δ usage are present in spleen (V γ 1 and V γ 4), skin and intestine (V γ 7V δ 4, V γ 7V δ 5, and V γ 7V δ 6), lung (V γ 4 and V γ 6), and reproductive organs (V γ 6V δ 1) (33, 34). While both $\alpha\beta$ and $\gamma\delta$ T cell subsets are found in human skin (35), $\gamma\delta$ T cells expressing the invariant V γ 5V δ 1 are the major population found in mice skin. They form a dense network of dendritic-like cells that are called dendritic epidermal T cells (DETCs) (36).

$\gamma\delta$ T cells share many functional characteristics with conventional effector $\alpha\beta$ T cells, for instance human V γ 9V δ 2 T cells can display cytotoxic activity against infected or transformed cells and produce pro-inflammatory cytokines, such as tumor necrosis factor α (TNF- α), IL-17, and IFN- γ (33, 34, 37). A unique feature of human V γ 9V δ 2 T cells is the TCR-dependent recognition of non-peptidic phosphorylated antigens, called phosphoantigens. Natural phosphoantigens, such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) are produced by many bacteria through the prokaryotic isoprenoid pathway (also called non-mevalonate isoprenoid pathway or Rohmer pathway) and are extremely potent activators of human V γ 9V δ 2 T cells (38, 39). On the other hand, eukaryotic cells use the mevalonate isoprenoid pathway to produce phosphoantigens, such as isopentenyl pyrophosphate (40), which are much less active than the natural phosphoantigens produced by bacteria. As the mevalonate pathway plays a key role in multiple cellular processes, the increased metabolism of tumor cells stimulates the over-production and secretion of endogenous phosphoantigens that are sensed by human $\gamma\delta$ T cells as tumor-related antigens (40). Through their unique capacity to recognize phosphoantigens, V γ 9V δ 2 T cells play an essential role in anti-infection immunity and also in tumor immune surveillance (41, 42).

V γ 9V δ 2 T cells have rapidly emerged as an attractive therapeutic target for anti-tumor therapies. Indeed, they display a very efficient, non-MHC restricted lytic activity against a broad panel of tumors, they abundantly produce IFN- γ and can be easily expanded from peripheral blood with agonist molecules. Many clinical trials have been carried out based on the adoptive transfer of *in vitro* stimulated V γ 9V δ 2 T cells or on the *in vivo* stimulation of their activity using clinical-grade agonists (43, 44). So far, no concluding result has been obtained from clinical trials based on the adoptive transfer of expanded autologous V γ 9V δ 2 T cells; however, *in vivo* stimulation of $\gamma\delta$ T cells showed objective responses in 10–33% of patients (45). Although, the lack of response to therapy could be attributed, in some cases, to deficient expansion of effector V γ 9V δ 2 T cells (5, 10, 12), many patients who did not respond to the treatment exhibited significant and sustained V γ 9V δ 2 T cell activation and proliferation. These results suggest that the current

$\gamma\delta$ T cell-based treatments are feasible and safe, but have some obvious limitations. Thus, a better understanding of effector $\gamma\delta$ T cell regulation is required to improve their efficacy (45). Interestingly, recent *in vitro* and *in vivo* data highlighted that $\gamma\delta$ T cells show some degree of plasticity driven by environmental signals that can affect and modify their anti-tumor functions and limit their efficacy. Therefore, much research effort is currently focused on precisely understanding the molecular mechanisms that govern the functional plasticity of V γ 9V δ 2 T cells and other $\gamma\delta$ T sub-populations and the role of cancer cells and of the tumor microenvironment on the recruitment, polarization, and biological functions of such cells. This knowledge is required to develop optimal strategies for the expansion of $\gamma\delta$ T cells with anti- rather than pro-tumor activity.

Here, we provide an overview of the current knowledge on $\gamma\delta$ T cell functional plasticity and its effect on their tumor activities. We also discuss the putative mechanisms that underlie $\gamma\delta$ T cell expansion, differentiation, and recruitment in the tumor microenvironment.

FUNCTIONAL PLASTICITY OF $\gamma\delta$ CELLS

The differentiation of conventional $\alpha\beta$ T cells into effector cells is driven by TCR engagement and specific environmental signals. For example, naive $\alpha\beta$ CD4 T cells can differentiate into Th1 or Th2 cells following priming by viruses or extracellular parasites, respectively (46–49). This polarization is stably imprinted by lineage-specific transcription factors to allow the generation of memory T cells with appropriate functions to rapidly eliminate the infectious agents after new exposure. However, recent studies demonstrated considerable flexibility, or plasticity, in T cell fate, unraveling the complex relationships among effector and regulatory $\alpha\beta$ T cell sub-populations. Similarly, $\gamma\delta$ T cells also present some plasticity that contributes to their functional specialization.

PLASTICITY OF HUMAN V γ 9V δ 2 T CELLS

Several studies showed that after phosphoantigen activation, peripheral human V γ 9V δ 2 T cells promote a Th1 immune response (50–52) characterized by potent TNF- α and IFN- γ production and cytotoxic responses (53, 54). This Th1 cell-like polarization of V γ 9V δ 2 T cells is probably acquired during their postnatal peripheral expansion upon exposure to environmental microbial antigens. Gibbons and collaborators reported that neonatal $\gamma\delta$ T cells can produce IFN- γ and that they acquire the ability to produce TNF- α after 1 month of post-partum environmental exposure (55). However *in vitro*, depending on the cytokines and the $\gamma\delta$ TCR stimulus provided, adult V γ 9V δ 2 T cells can be polarized into cells with features associated with Th2 cells, Th17 cells, follicular T helper cells (Tfh), or regulatory T cells (Treg) (56–60) (see Table 1).

It has been first demonstrated that, V γ 9V δ 2 T cells can be polarized toward IFN- γ -secreting Th1-like $\gamma\delta$ T cells upon activation by IPP in the presence of IL-12 and an anti-IL-4 antibody, or toward IL-4-producing Th2-like $\gamma\delta$ T cells upon stimulation by IPP in the presence of IL-4 and an anti-IL-12 antibody (56).

Interestingly, Thedrez et al. demonstrated that expansion of phosphoantigen-activated V γ 9V δ 2 T cells from peripheral blood mononuclear cells (PBMCs) in the presence of IL-21 and IL-2

Table 1 | $\gamma\delta$ T cell functional plasticity.

| $\gamma\delta$ T cell subsets | TCR activation | Cytokines | Polarization Transcription factors | Effector molecules | Reference |
|--|----------------|--|---|-----------------------------------|-----------|
| Adult blood V γ 9V δ 2 T cells | + | IL-12 or IL-18 | Th1-like <i>T-bet, eomesodermin</i> | IFN- γ , TNF- α | (56) |
| | + | IL-4 | Th2-like <i>GATA-3</i> | IL-4 | (56) |
| | + | IL-15 + TGF- β | Treg-like <i>Foxp3</i> | IL-10, TGF- β | (60) |
| | + | IL-6 + IL-23 + IL-1 β + TGF- β + Ahr ^a agonists | Th17-like <i>RORγt</i> | IL-17 | (61) |
| | + | IL-23 + IL-1 β + TGF- β | Th17-like, <i>RORγt</i> Th1/17 like, <i>RORγt, T-bet</i> Th22, <i>FOXO4</i> | IL-17 IFN- γ , IL-17 IL-22 | (62) |
| | + | IL-2 | APC functions ND | MHC I and II | (63, 64) |
| | + | IL-21 | Tfh-like <i>Bcl6</i> | IL-4, IL-10, CXCL13 | (58, 59) |
| Th1 V γ 9V δ 2 T cells | - | IFN type I | Th1-like ND | IFN- γ | (65) |
| Cord blood V γ 9V δ 2 T cells | + | IL-6 + IL-1 β + TGF- β | Th17-like, <i>RORγt</i> Th22-like, <i>FOXO4</i> | IL-17 IL-22 | (62) |
| | + | IL-6 + IL-1 β + TGF- β + IL-23 | Th1/17 like <i>RORγt, T-bet</i> | IFN- γ , IL-17 | (62) |
| Human V γ 1+ and V γ 2+ thymocytes | - | IL-2 or IL-15 | Th1 like <i>T-bet, eomesodermin</i> | IFN- γ , TNF- α | (66) |
| Murine $\gamma\delta$ T cells | - | IL-23 + IL-1 β | Th17 <i>RORγt</i> | IL-17, IL-21, IL-22 | (67) |

^aAryl hydrocarbon receptor.

promotes their cytolytic function (Th1 function), with increased expression of CD56 and several lytic molecules and also higher tumor-induced degranulation capacity (68). However, IL-21 can also promote differentiation of V γ 9V δ 2 T cells toward a Tfh-like phenotype. Indeed, activation of purified V γ 9V δ 2 T cells with phosphoantigens in the presence of IL-21 induces Tfh-associated features, as indicated by the expression of the BCL-6 transcription factor, ICOS, CD40-L, and CXCR5 as well as IL-21R, CD244, CXCL10, and CXCL13 and their trafficking to lymph node germinal centers (59). Both soluble and contact-dependent mechanisms seem to be involved in the B cell helper activity of Tfh-like V γ 9V δ 2 T cells. Indeed, Ig production is consistently impaired by inhibition of CD40-L and ICOS interaction with their respective receptor and ligand or by neutralization of IL-4 and IL-10 (58). It would be interesting to determine whether the interaction between Tfh-like V γ 9V δ 2 T cells and B cells in reactive tumor-associated lymphoid tissues might positively affect the production of high affinity antibodies against tumor antigens, thus favoring antibody-dependent cell cytotoxicity (ADCC) mechanisms (**Figure 1E**).

Besides these effects on the cytotoxic activity and B cell helper functions of V γ 9V δ 2 T cells, our preliminary data suggest that IL-21 might also confer some regulatory functions to $\gamma\delta$ T cells. Overall these data suggest that IL-21 together with environmental signals can strongly influence V γ 9V δ 2 T cell functions by polarizing them toward Th1-, Tfh-, or Th1/Treg-like T cells.

Other co-signals can induce the polarization of V γ 9V δ 2 T cells into Treg cells. Particularly, when they are activated by IPP in the presence of IL-15 and TGF- β , V γ 9V δ 2 T cells express the FOXP3 transcription factor and display regulatory/immunosuppressive activity as demonstrated by their capacity to suppress the proliferation of anti-CD3/anti-CD28-stimulated PBMCs (60). However, they do not simultaneously display regulatory and Th1-like

effector functions, differently from regulatory $\gamma\delta$ T cells developed in the presence of IL-21. Interestingly, treatment with decitabine (a DNA hypomethylating agent) and IL-15/IL-2/transforming growth factor- β (TGF- β) associated with phosphoantigen activation facilitates the induction of the immunosuppressive functions of V γ 9V δ 2 T cells derived from human PBMCs and favors the regulatory activity of V γ 9V δ 2 T cells (69).

First established for murine $\gamma\delta$ T cells (67), the production of IL-17 by human $\gamma\delta$ T cells was also recently demonstrated (70). In both mouse and human, IL-7 promotes substantially an expansion of IL-17-producing $\gamma\delta$ T cells (71). Moreover, several studies have shown that when cultured in the presence of various combinations of cytokines, naive V γ 9V δ 2 T cells acquire an IL-17-secreting Th17-like phenotype or a mixed Th1/Th17 phenotype and produce both IFN- γ and IL-17 (61–63). Human cord blood-derived V γ 9V δ 2 T cells stimulated with HMBPP require IL-6, IL-1 β , and TGF- β to differentiate into $\gamma\delta$ Th17 cells, whereas, differentiation into $\gamma\delta$ Th1/Th17 cells needs also IL-23 (62, 63). In adults, differentiation of naive $\gamma\delta$ T cells into memory $\gamma\delta$ Th1/Th17 T cells and $\gamma\delta$ Th17 T cells requires IL-23, IL-1 β , and TGF- β , but not IL-6. $\gamma\delta$ Th17 cells can also produce IL-22 (especially cells in the cord blood) (62, 63). Recently, Wu et al. demonstrated that, in a colorectal cancer model, activated inflammatory DCs polarize V γ 9V δ 2 cells into $\gamma\delta$ Th17 cells that secrete high amount of IL-17, but also IL-8, TNF- α , and granulocyte macrophage colony-stimulating factor (GM-CSF) in an IL-23-dependent manner (64).

Besides their T cell effector functions, phosphoantigen-activated V γ 9V δ 2 T cells can express lymph node migration receptors (e.g., CXCR5) and display several hallmarks of professional APCs, such as up-regulation of MHC class I and II molecules and of the co-stimulatory molecules CD40 and CD83 and also the ability to phagocytose and process antigens and to activate naive $\alpha\beta$ T

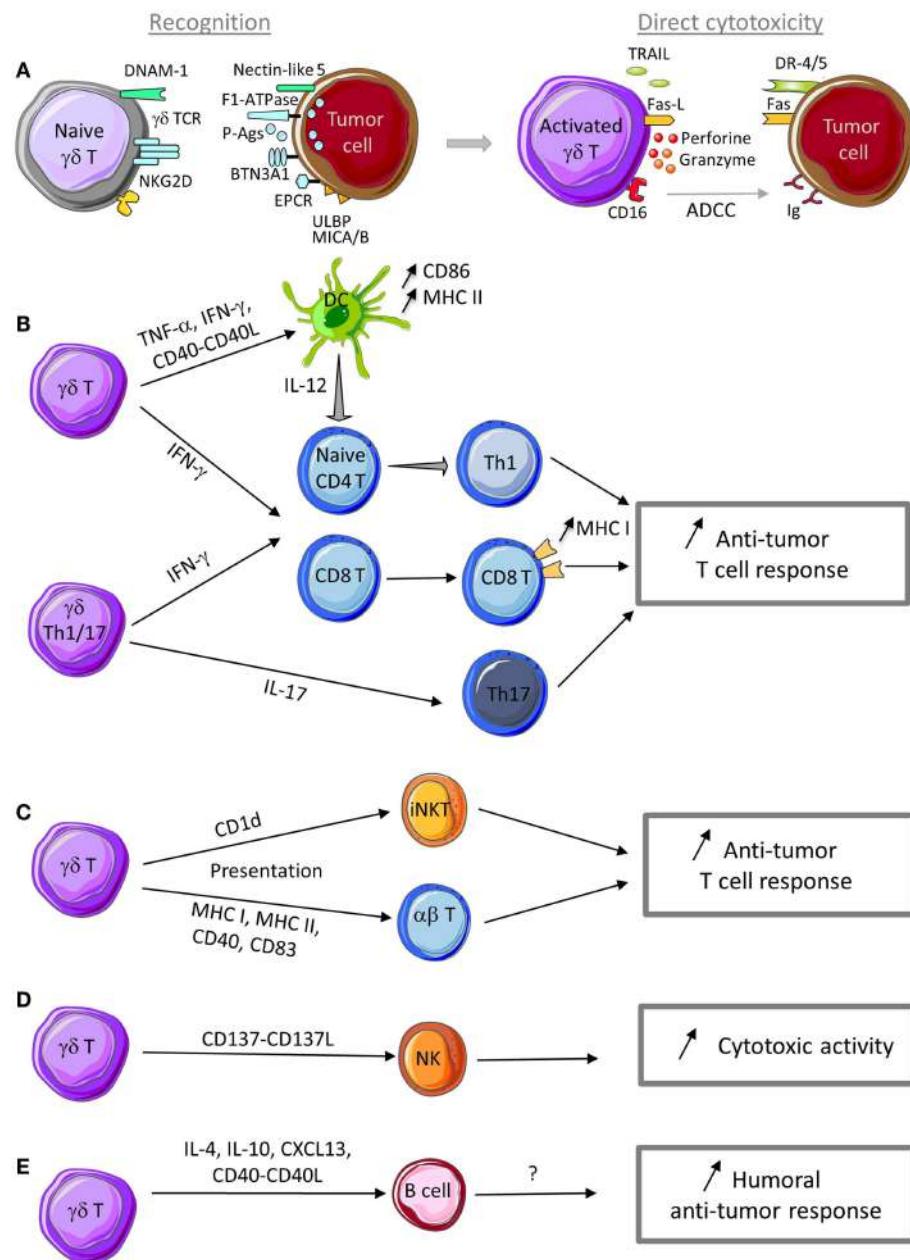


FIGURE 1 | Anti-tumor functions of $\gamma\delta$ T cells. (A) $\gamma\delta$ T cells can recognize tumor cells through interaction with (i) TCR ligands, such as phosphoantigens (P-Ags), F1-ATPase, BTN3A1, EPCR, . . . , and (ii) innate receptor ligands, such as ULBP MICA/B, and nectin-like 5. Following sensing of tumor antigens or stress signals, $\gamma\delta$ T cells are activated and can kill tumor cells through cytotoxic mechanisms that rely on the perforin/granzyme pathway, the death receptor pathway in response to TRAIL or Fas-L expression, and ADCC in the presence of tumor-specific antibodies. **(B)** $\gamma\delta$ T cell activation leads to TNF- α and IFN- γ production and CD40-L expression that promote DC maturation and T cell differentiation into Th1 cells. IL-17-producing $\gamma\delta$ Th17 cells favor Th17 effector cell development. Th1 and Th17 effector T cells display anti-tumor

functions to control tumor development. **(C)** Through a trogocytosis mechanism, activated $\gamma\delta$ T cells can capture and express CD1d molecules and then promote iNKT cell activation. Activated $\gamma\delta$ T cells can also display APC functions (MHC I and II, CD40, CD83, and CD86 expression) and activate both naive and effector T cells with cytotoxic activity against tumor cells. **(D)** Activated $\gamma\delta$ T cells can provide a co-stimulatory signal to NK cells through CD137L expression to promote their anti-tumor activity. **(E)** In the presence of specific signals, activated $\gamma\delta$ T cells can display a Thf profile (i.e., IL-4, IL-10, and CXCL13 production and CD40-L expression) to help B cell antibody production. Although not yet demonstrated, production of antibodies against specific tumor antigens could be involved in the humoral anti-tumor response.

cells (72–74) (Figure 1E). These observations are based on results obtained *in vitro*. The APC functions of $\gamma\delta$ T cells *in vivo* have not been evaluated and remain to be demonstrated.

Moreover, similarly to $\alpha\beta$ T cells, the differential induction of specific effector functions may also depend on the innate immunity receptor class that is engaged and the nature of the cytokine

stimuli. For example, NKG2D engagement triggers the induction of human V γ 9V δ 2 T cell cytotoxic functions, thereby influencing the fate of target cells (lysis or survival), but has limited effects on cytokine production (21). Similarly, type I IFN-released by stimulated myeloid and plasmacytoid DCs induces exclusively IFN- γ , but no TNF- α , production by human V γ 9V δ 2 T cells (65).

In conclusion, V γ 9V δ 2 T cells display a surprisingly broad array of functional activities. One essential question is to determine whether such functional plasticity is an intrinsic feature of the whole V γ 9V δ 2 T cell population or whether it is restricted to specific V γ 9V δ 2 T cell subsets. This is an important issue, because it could directly affect $\gamma\delta$ T cell-based therapeutic strategies. Indeed, boosting $\gamma\delta$ T cell regulatory activity is suitable in some instances (i.e., autoimmune disease), conversely optimizing, for example, their APC or cytotoxic functions could be more important for the treatment of tumors or infections. In terms of cytokine production and cytotoxic activity, V γ 9V δ 2 T cells can be divided in different subsets based on the expression of cell surface markers. Upon *in vitro* activation and extended culture in the presence of IL-2, naive V γ 9V δ 2 T cells (CD27+CD45RA+) can sequentially differentiate into TCM (CD27+CD45RA-), TEM (CD27-CD45RA-), and TEMRA (CD27-CD45RA+) cells. CD45RA-CD27- TEM cells show the highest IFN- γ secretion, while CD45RA+CD27- TEMRA cells are characterized by a strong cytotoxic activity. In contrast, naive CD45RA+CD27+ V γ 9V δ 2 T cells display very low, if any, functional activity (75). Studies using cell sorter-purified V γ 9V δ 2 T cell subsets have determined that only naive CD45RA+CD27+ V γ 9V δ 2 T cells can differentiate into IL-17-producing cells when exposed to IL-1 β , IL-6, IL-23, and TGF- β (61). IL-17-producing V γ 9V δ 2 T cells display a TEMRA phenotype, promote neutrophil migration through production of CXCL8 and up-regulate β -defensin production in epithelial cells (61). Similarly, V γ 9V δ 2 T cell cytotoxic activity can be assigned to specific subsets, especially to (CD45RA+CD27-) TEMRA and (CD56+CD16+) cells (75–77), but their clonal plasticity remains uncertain.

In addition, whether a given V γ 9V δ 2 T cell phenotype induced by specific environmental stimuli, such as cytokines, is stable or reversible, remains to be investigated. Although the expression of lineage-associated transcription factors in V γ 9V δ 2 T cells has been assessed in some studies, so far no clear correlation between the expression of transcription factors and a specific stable cytokine profile has been reported.

Finally, most of these studies concerned the V γ 9V δ 2 T cell subset thus raising the question of whether other human or mouse $\gamma\delta$ T cell populations display similar plasticity. Ribot and collaborators have reported that also human V γ 1 and V γ 2 thymocytes show functional phenotypic plasticity and can differentiate into cytotoxic type 1 effector cells following IL-2 or IL-15 stimulation (66) but no investigation was reported on other human $\gamma\delta$ T cell subsets.

PLASTICITY OF MOUSE $\gamma\delta$ T CELLS

In mice, several studies demonstrated that $\gamma\delta$ thymocytes are functionally pre-committed and polarized in term of cytokine production (78–80). During fetal development, $\gamma\delta$ T cells are generated from two waves of thymocytes that express invariant TCR.

The first group migrates into the skin (V γ 5V δ 1 DETC) and is programmed to produce IFN- γ ; the second group migrates into the vaginal epithelium and the peritoneal cavity (V γ 6V δ 1 subset) and is programmed to produce IL-17 (33, 81). Other $\gamma\delta$ T cell subsets appear postnatally in the thymus and express TCR with various V δ and V γ combinations. In adult mice, these cells are found in all lymphoid organs and below the epithelium or mucosal surfaces of many tissues, including the small intestine and lung. Most of them display a programmed polarization acquired during thymic selection (33, 81) through a process regulated by TCR (78–80) and co-receptor signaling (81). Thus, $\gamma\delta$ T cell differentiation into IFN- γ -producing cells require TCR and CD27 signals (78–80). CD27, a member of the tumor necrosis factor receptor family, regulates the balance between IFN- γ and IL-17 producing $\gamma\delta$ T cell subsets (82). CD27+ $\gamma\delta$ T cells are committed to express IFN- γ genes, whereas, CD27- $\gamma\delta$ T cells display a permissive chromatin configuration at loci encoding IFN- γ and IL-17 as well as their regulatory transcription factors. They can thus differentiate into both IFN- γ - and IL-17-producing cells (82). It has also been shown that IL-23 in combination with IL-1 β promotes IL-17, IL-21, and IL-22 expression by mouse $\gamma\delta$ T cells in the absence of additional signals; however, the authors did not investigate CD27 expression in this setting (67).

Altogether, these results suggest that mouse $\gamma\delta$ T cells have a low plasticity compared to human $\gamma\delta$ T cells. Nevertheless further investigation on mouse and human $\gamma\delta$ T cell functional plasticity are required to better characterize the molecular mechanisms and the precise role of each $\gamma\delta$ T cell subset in the immune response and in pathologic conditions in order to improve $\gamma\delta$ T cell-based therapies.

IMPACT OF $\gamma\delta$ T CELLS ON THE TUMOR IMMUNE RESPONSE

$\gamma\delta$ T cells can: (i) detect and sense any type of stress through a MHC-independent mechanism, (ii) produce huge quantities of pro-inflammatory cytokines, and (iii) exert potent cytotoxic activity against a broad panel of tumors. For these reasons, $\gamma\delta$ T lymphocytes are key players in the tumor immune response. Like other cytotoxic effectors, $\gamma\delta$ T cells directly participate in the elimination of tumor cells, but they also control indirectly the tumor immune response by modulating the activity and functions of other immune cells. In this section, we will summarize both pro- and anti-tumor activities of $\gamma\delta$ T cells by focusing mainly on their tumor recognition mechanisms and the triggered biological responses.

ANTI-TUMOR ACTIVITY OF $\gamma\delta$ T CELLS

Mechanisms of tumor cell recognition

Similarly to any other T cell population, $\gamma\delta$ T cell activation and acquisition of effector functions are triggered by TCR engagement (**Figure 1A**). Specifically, $\gamma\delta$ TCR recognize molecules that are over-expressed in stress conditions. In normal cells, the concentration of metabolites of the isoprenoid pathway, such as IPP, is too low to be sensed as a danger signal by V γ 9V δ 2 T cells. Deregulation of the isoprenoid pathway in some tumors leads to IPP over-production that is detected and considered as a tumor antigen by V γ 9V δ 2 TCR (40, 83). Similarly, incubation of tumor cells with bisphosphonates that inhibit the farnesyl pyrophosphate

synthase enzyme in the isoprenoid pathway leads to IPP accumulation and makes tumor cells more sensitive to V γ 9V δ 2 T cell cytotoxicity (84–86). Several reports have shown that phosphoantigens need to interact with specific proteins to be recognized by TCR and to activate V γ 9V δ 2 T cells. First, Mookerjee-Basu et al. showed that F1-ATPase, which is expressed on the surface of some tumor cells, binds to the adenylated derivative of IPP and is involved in triggering V γ 9V δ 2 T cell activation and anti-tumoral activity (87, 88). More recently, it was reported that butyrophilin 3 A1 (BTN3A1) can contribute to $\gamma\delta$ T cell activation by sensing changes in phosphoantigen concentration within tumor cells. Specifically, phosphoantigen binding to the intracellular domain of BTN3A1 could initiate a cascade of events that result in extracellular changes or cell surface rearrangements (including immobilization of BTN3A extracellular domain) and lead to V γ 9V δ 2 T cell activation (89, 90). Dechanet-Merville and collaborators found that a human δ 2 negative T cell subset recognizes both CMV-infected and transformed cells through the interaction between the endothelial protein C receptor (EPCR) and the TCR (91). EPCR is over-expressed in CMV-infected endothelial cells and transformed cells and it is conceivable that it might act as a determinant of stress surveillance during epithelial cell transformation to communicate a state of “dysregulated self” to $\gamma\delta$ T cells.

In addition to TCR engagement, stimulation of NKR expressed by $\gamma\delta$ T cells and particularly engagement of NKG2D receptor can also efficiently trigger the anti-tumor functions of $\gamma\delta$ T cells. NKG2D is expressed by V γ 9V δ 2 T cells and binds to non-classical MHC molecules of the MIC and ULBP families that are expressed by tumor cells (18, 20, 21). Ligand binding to NKG2D induces the release of IFN- γ and TNF- α , increases the expression of CD25, the α chain of the IL-2 receptor and promotes $\gamma\delta$ T cell cytolytic activity (21). In particular, ULBP molecules have been involved in the recognition by V γ 9V δ 2 T cells of leukemia and lymphoma (92) and also of solid tumors, such as ovarian and colon carcinomas (93, 94). For instance, ULBP1 expression level determines lymphoma susceptibility to $\gamma\delta$ T cell-mediated cytolysis upon NKG2D binding (92). ULBP4 also can bind to V γ 9V δ 2 TCR and thus induce the cytotoxic activity of V γ 9V δ 2 T cells toward tumor cells through both TCR and NKG2D engagement (22). More recently, Lamb and collaborators have shown that temozolomide (TMZ), the main chemotherapeutic agent used to treat glioblastoma multiforme (GMB), increases the expression of stress-associated NKG2D ligands on TMZ-resistant glioma cells, potentially making them more susceptible to $\gamma\delta$ T cell recognition and lysis (95). Furthermore, as described for V γ 9V δ 2 T cells, recognition of MICA, MICB, or ULBP expressed on cancer cells by human V γ 181 T lymphocytes can trigger or increase their cytolytic activity against tumor cells that express NKG2D ligands (23, 96). Indeed, ULBP and MICA interact with NKG2D or TCR on V δ 1 $\gamma\delta$ T cells and induce their activation. However, MICA binds in mutually exclusive manner to NKG2D and TCR, suggesting that the two receptors might be sequentially engaged following recognition of target tumor cells (97).

DNAM-1 (also called CD226) is another NKR involved in the regulation of the cytotoxic activity of $\gamma\delta$ T cells. It is expressed on the surface of both V γ 9V δ 2 and V γ 1 T cell populations and its

ligand nectin-like-5 has been detected on certain tumors. DNAM-1 cooperates with TCR and NKG2D signaling in $\gamma\delta$ T cells to positively regulate their IFN- γ production and cytotoxic activity against tumor cells (98, 99).

Like NK cells, human $\gamma\delta$ T cells also express the CD16 (Fc γ RIII) receptor that binds to the Fc portion of immunoglobulin G (IgG). CD16 expression on V γ 9V δ 2 T cells can be up-regulated following stimulation with phosphoantigens (100). Its engagement leads to ADCC (101), a process that can result in lysis of tumor cells bound by specific antibodies. Indeed, several *in vitro* studies have clearly shown that $\gamma\delta$ T cells are activated through CD16 and mediate ADCC of tumor cells in the presence of therapeutic anti-tumor monoclonal antibodies, such as rituximab, trastuzumab, atumumab, and alemtuzumab (102–105). Reinforcing the relevance of such *in vitro* data, it has been shown that stimulated $\gamma\delta$ T cells increase the efficacy of Trastuzumab in Her2+ breast cancer patients (105).

Impact on immune cell activity

In addition to these direct effects against tumor cells, $\gamma\delta$ T cells can also control indirectly the anti-tumor immune response by promoting the recruitment and modulating the activation of other cell types in the tumor microenvironment, such as DCs, NK cells, and effector T cells (**Figures 1B–D**).

In the presence of tumor cells, or following stimulation with TCR agonists, NKG2D ligands, cytokines (such as IL-12 and IL-18), or DNAM-1 engagement, human $\gamma\delta$ T cells produce IFN- γ and TNF- α (21, 56, 94, 106–108). These two cytokines can inhibit tumor growth through several mechanisms, but especially by enhancing CD8 T cell anti-tumor activity (**Figure 1B**) and by inhibiting tumor angiogenesis (109–111). Mouse $\gamma\delta$ T cells also are an important and early source of IFN- γ within the tumor microenvironment where IFN- γ enhances MHC class I expression on tumor cells and CD8+ T cell responses (112–114). Altogether these findings suggest that both human and mouse $\gamma\delta$ T cells positively influence the anti-tumor immune response by increasing the adaptive anti-tumor immunity (115) (**Figure 1B**).

As previously mentioned, both mouse and human $\gamma\delta$ T cells could be an important source of IL-17. This cytokine plays an essential role in the host defense against microbial infections, but also in autoimmune disorders and cancer (116). IL-17 contribution to the tumor immune surveillance is still controversial. Indeed, IL-17 has often been described as a cytokine with pro-tumor properties, but several studies highlighted that it can also display anti-tumor functions (117). Therefore, IL-17 heterogeneous sources and, perhaps, targets in the tumor microenvironment may determine whether it will negatively or positively affect tumor growth. In human, the majority of $\alpha\beta$ and $\gamma\delta$ Th17 cell populations that produce IL-17 also concomitantly produce IFN- γ (63) and the anti-tumor functions of IL-17-producing $\alpha\beta$ T cells strongly depend on IFN- γ (118). Moreover, IL-17-producing $\alpha\beta$ T cells stimulate the release of several cytokines (such as IL-6, IL-12, CXCL9, and CXCL10) by immune or cancer cells, leading to DC maturation or effector T cell recruitment to the tumor, and as a consequence, to an increase of the anti-tumor immunity (119, 120) (**Figure 1B**). It is likely that $\gamma\delta$ Th17 cells might do the same, but this remains to be formally demonstrated.

Importantly, in mice, IL-17-producing $\gamma\delta$ T cells ($V\gamma 4+$ and $V\gamma 6+$) contribute to chemotherapy efficacy because they are required for the priming of IFN- γ -secreting tumor-specific T cells. In this context, $\gamma\delta$ T cells are considered as part of the innate immune response that is involved in the subsequent specific anti-tumor T cell response following treatment with chemotherapeutic agents (121, 122). Nevertheless, it is not known whether human IL-17 $\gamma\delta$ T cells also contribute to the efficacy of anti-cancer chemotherapy and whether combination treatments with $\gamma\delta$ T cell agonists and anthracyclines could improve the patient outcome.

Dendritic cells are potent inducers of $\gamma\delta$ T cell effector functions through their ability to express $\gamma\delta$ TCR ligands and to provide co-stimulation signals (123, 124). Inversely, interactions between activated $\gamma\delta$ T cells and DCs were shown to induce DC activation and maturation, thus facilitating the establishment of the T cell response (125, 126). Indeed, activated human $V\gamma 9V\delta 2$ T cells enhance IL-12 production by monocyte-derived DCs through an IFN- γ - and IL-12-mediated positive feedback loop that can then promote naive $\alpha\beta$ T cell activation and differentiation into Th1-type cells (127), an effect that may positively influence the anti-tumor immunity (Figure 1B).

As already mentioned, when activated by phosphoantigens, $V\gamma 9V\delta 2$ T cells can display APC features and acquire the ability to activate naive and effector T cells (72, 73) (Figure 1C). Similarly, $V\gamma 9V\delta 2$ T cells can also present antigens to invariant NKT cells (iNKT). Schneiders et al. demonstrated that, when co-cultured with CD1d-positive cells, activated $V\gamma 9V\delta 2$ T cells uptake CD1d on their membrane through trogocytosis and acquire the capacity to present glycolipid antigens to iNKT cells and activate them (128) (Figure 1C). iNKT cell activation triggers the production of large amounts of cytokines that play an important role in initiating and orchestrating anti-tumor immune responses, such as Th1-biased pro-inflammatory responses.

Natural killer cells also have a role in anti-tumor responses and their activity can be regulated by $\gamma\delta$ T cells. When co-localized within tumors, human $\gamma\delta$ T cells can provide co-stimulatory signals to NK cells and induce NK cell-mediated killing of tumor cells (129). Indeed, CD137L is expressed on activated $\gamma\delta$ T cells and interacts with the cognate receptor CD137 on NK cells, leading to the up-regulation of the activation markers CD25, CD54, CD69, and NKG2D on the surface of NK cells and to the increase of their cytotoxic function, particularly against solid tumors that are usually resistant to NK cytolysis (129) (Figure 1D).

PRO-TUMOR ACTIVITY OF $\gamma\delta$ T CELLS

In some conditions, $\gamma\delta$ T cells can also promote tumor growth via regulatory functions that impair the anti-tumor immune responses (Figure 2).

Human $V\gamma 9V\delta 2$ T cells

$V\gamma 9V\delta 2$ T cells with immunosuppressive functions may play an important role in human cancers. Upon activation, human peripheral $V\gamma 9V\delta 2$ T cells also can express IL-4, IL-10, and TGF- β and inhibit T cell proliferation, thus developing a regulatory profile that may play a role in the suppression of anti-tumor responses (130). Indeed, depending on the context, $V\gamma 9V\delta 2$ T cells may display a

Th1-, Th2-, Th17-, or Th1/reg-like profile and synthesize IFN- γ , IL-4, IL-17 or IL-10, and TGF- β , respectively.

While IL-4 is a cytokine involved in Th2 responses (which are not appropriate for anti-tumor immunity), IL-10 and TGF- β are cytokines with immunosuppressive functions and thus could be involved in the pro-tumor activities of $\gamma\delta$ T cells. TGF- β has a crucial role in tumor development because it can promote tumor cell invasiveness and metastasis formation mainly by modulating the immune system and the tumor microenvironment (Figure 2A). The most important mechanisms of tumor progression linked to TGF- β activities are the epithelial-to-mesenchymal transition (EMT), immune system evasion, and promotion of cancer cell proliferation by modulation of the tumor microenvironment (131). The expression of IL-10 and TGF- β is frequently increased in various cancer types. IL-10 directly affects APC function by inhibiting the expression of MHC and co-stimulatory molecules, which induces immune suppression or tolerance (Figure 2B). Additionally, IL-10 down-regulates the expression of Th1 cytokines and induces T-regulatory responses.

IL-17 plays a dual role by promoting both tumor growth and anti-tumor immunity, depending on the tumor type, stage, and target cells present in tumor microenvironment. The number of IL-17-producing cells is increased in cancer and this is associated with poor prognosis (117, 132, 133). Several IL-17 activities contribute to tumor progression. In breast cancer, IL-17 can directly promote tumor cell proliferation and dissemination (119) and favor the development of cancer resistance to conventional chemotherapeutic agents, such as docetaxel (133) (Figure 2A). IL-17 can also act on cells in the tumor microenvironment. For instance, IL-17 up-regulates the secretion of pro-angiogenic and pro-tumor factors (e.g., VEGF, IL-6, and IL-8) by stromal cells and fibroblasts, thus promoting angiogenesis and sustained chronic inflammation (119, 120). In colorectal cancer, $V\gamma 9V\delta 2$ T cells can differentiate into Th17 cells that secrete IL-17 and also IL-8, TNF- α , and GM-CSF and thus contribute to the accumulation of immunosuppressive polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs) within the tumor microenvironment and influence the anti-tumor immune response (64) (Figure 2C).

Human $V\delta 1$ T cells

Besides $V\gamma 9V\delta 2$ T cells, other human $\gamma\delta$ T cell subsets can display immunosuppressive functions. First, Peng et al. demonstrated that $V\delta 1$ $\gamma\delta$ T cells infiltrating human breast cancer suppress DC maturation and T cell effector functions both *in vitro* and *in vivo*. When stimulated by tumor cells and an anti-CD3 antibody, $V\delta 1$ T cells express IFN- γ and GM-CSF, but not IL-1 β , TNF- α , IL-12, IL-2, IL-4, IL-10, or TGF- β (134). Thus neither IL-10 nor TGF- β seems to play a role in this immunosuppressive activity. Although, the involved factor(s) remain to be identified, these authors found that the suppressive activity was in the soluble fraction with a molecular mass higher than 100 kDa and could be inactivated by heat, but not by DNase or RNase treatments (134) (Figure 2D). These $V\delta 1$ $\gamma\delta$ T cells represent a large percentage of tumor-infiltrating lymphocytes in breast and also in prostate cancer, suggesting that they may play an important role in promoting an immunosuppressive tumor microenvironment. Interestingly, stimulation of

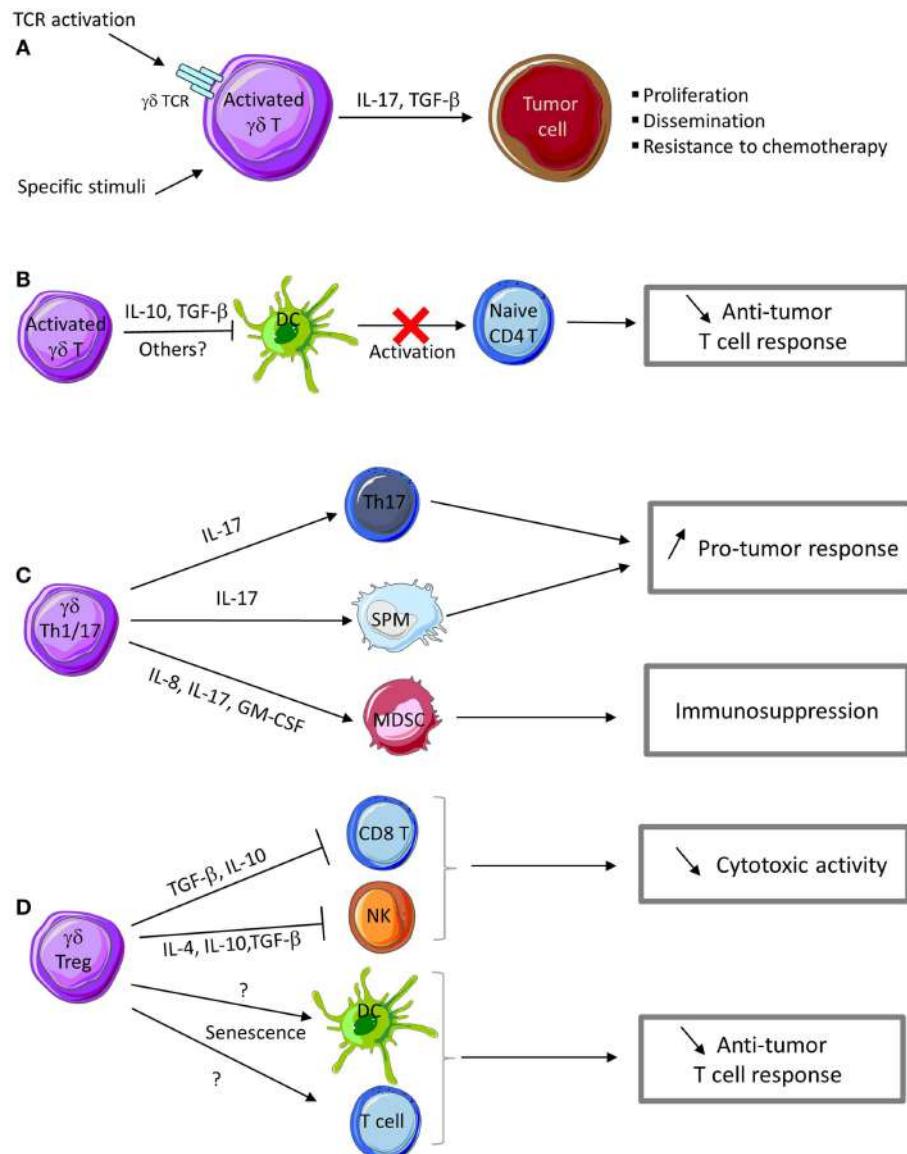


FIGURE 2 | Pro-tumor functions of $\gamma\delta$ T cells. (A) Activation of $\gamma\delta$ T cells in the presence of specific stimuli can promote their polarization into Th17- or Treg-like cells that produce IL-17 and TGF- β , thus favoring tumor cell proliferation and dissemination. IL-17 produced by $\gamma\delta$ T cells confers chemotherapy resistance to tumor cells. **(B)** Activated $\gamma\delta$ T cells can inhibit DC maturation and their APC functions, thus impairing naive T cell activation and differentiation into effector T cells. **(C)** IL-17 produced by $\gamma\delta$ Th17 cells promotes the development of Th17 cells with pro-tumor

functions. $\gamma\delta$ Th17 cells also produce a cocktail of cytokines and chemokines involved in the recruitment of myeloid-derived suppressive cells (MDSC) and small peritoneal macrophages (SPM) with immunosuppressive and pro-tumor functions. **(D)** $\gamma\delta$ Treg cells produce cytokines (IL-4, IL-10, and TGF- β) and other immunosuppressive factors that impair CD8 T and NK cell cytotoxic activity. $\gamma\delta$ Treg cells can also promote senescence of DC or $\alpha\beta$ T cells and consequently favor tumor growth.

suppressive V δ 1 $\gamma\delta$ T cells in breast cancer by using a TLR8 agonist reversed the anti-tumor response inhibition (134). More recently, the same group demonstrated that regulatory $\gamma\delta$ T cells can induce both T cell and DC senescence. Specifically, regulatory $\gamma\delta$ T cells induce senescence of both naive and effector T cells, as indicated by the impaired expression of the co-stimulatory molecules CD27 and CD28 and the low proliferative capacities of both Th1 and Th17 T cell subsets. Senescent T cells and DCs become suppressive

cells, further amplifying the immunosuppression mediated by $\gamma\delta$ Treg cells (135). Furthermore, Ma and collaborators found that high $\gamma\delta$ T cell level in breast cancer tissues is correlated with poor survival and high risk of relapse (136). Similarly, in colon adenocarcinoma, a significant correlation has been observed between presence of γ TCR cells and disease stage. These two reports suggest that $\gamma\delta$ T cells may have a key prognostic role in colon adenocarcinoma and breast cancers (137).

Mouse $\gamma\delta$ T cells

$\gamma\delta$ T cells with immunosuppressive functions have also been observed in mouse tumor models (138, 139). Seo et al. found that murine $\gamma\delta$ T cells that infiltrate tumors arising from B16 melanoma cells produce large amounts of IL-4 and IL-10 and inhibit NK and iNKT cell activity (138) (Figure 2D). They demonstrated that supernatants from these $\gamma\delta$ T cells did not affect NK and iNKT cell cytotoxicity, but reduced their proliferation, suggesting that soluble IL-4 and IL-10 could contribute to the inhibition of NK and iNKT cell activity by $\gamma\delta$ T cells in this model (138). Additional studies from this group showed that $\gamma\delta$ T cells that infiltrate MM2 mammary tumors in mice express IL-10 and TGF- β , but not IFN- γ or IL-4. $\gamma\delta$ T cells isolated from these tumors and from the spleen hindered the cytotoxic activity of NK and CD8 T cells. IL-10 and TGF- β neutralization inhibited some of the immunosuppressive effects of these $\gamma\delta$ T cells, suggesting the involvement of these cytokines (Figure 2D). Moreover, depletion of IL-10- and TGF- β -secreting $\gamma\delta$ T cells by using a specific antibody enhanced the anti-tumor immunity and reduced tumor growth in xenografted mice (139). More recently, Hao et al. using the B16 melanoma model, showed that mouse V γ 1 T cells suppress the anti-tumor functions of the V γ 4 T cell subset, thus promoting tumor growth. Specifically, V γ 1 $\gamma\delta$ T cells reduced IFN- γ , perforin, and NKG2D expression in V γ 4 $\gamma\delta$ T cells through contact-independent mechanisms involving IL-4 (140). Collectively, these data strongly suggest that within the tumor microenvironment, some mouse $\gamma\delta$ T cell populations express IL-4, IL-10, and TGF- β and inhibit the anti-tumor immune response. IL-17-secreting $\gamma\delta$ T cells show pro-tumor activity also in mouse models. Recently, Rei et al. demonstrated that murine CD27-V γ 6 T cells that produce IL-17 promote ovarian cancer growth via mobilization of small peritoneal macrophages (141) (Figure 2C).

Overall, these findings support the idea that $\gamma\delta$ T cells, at least in some cancers, can behave as Tregs or Th17 T cells that impair the anti-tumor immune response and promote tumor growth, through the secretion of different cytokines with regulatory functions or the recruitment of immunosuppressive cells within the tumor microenvironment.

CONCLUSION

During the last decade, our knowledge on the role of $\gamma\delta$ T cells in the tumor microenvironment has hugely improved. Plasticity of $\gamma\delta$ T cells increases the range of their biological responses as different $\gamma\delta$ T cell sub-populations can regulate different aspects of the tumor immunity. Functional plasticity also can explain the heterogeneous responses and contradictory functions of this unconventional T cell population in the context of cancer immune surveillance. As discussed in this review, due to the TCR-mediated recognition and activation mechanisms and the fine regulation of their activation through innate and cytokine receptors, $\gamma\delta$ T lymphocytes are attractive targets for immunotherapeutic protocols with the final objective of boosting the anti-tumor immune response. Several clinical trials have already assessed $\gamma\delta$ T cell-based immunotherapy in patients with advanced hematological malignancies and solid cancers with encouraging results. However, high density of $\gamma\delta$ T cells in the breast and colon tumor microenvironment has been associated with poor clinical outcome. We

are convinced that a better characterization of the mechanisms regulating their polarization should allow the development of optimal therapeutic strategies to favor the expansion of $\gamma\delta$ T cell populations with anti-tumor rather than pro-tumor functions.

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$\gamma\delta$ T cell-mediated antibody-dependent cellular cytotoxicity with CD19 antibodies assessed by an impedance-based label-free real-time cytotoxicity assay

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$\gamma\delta$ T cells are not MHC restricted, elicit cytotoxicity against various malignancies, are present in early post-transplant phases in novel stem cell transplantation strategies and have been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) with monoclonal antibodies (mAbs). These features make $\gamma\delta$ T cells promising effector cells for antibody-based immunotherapy in pediatric patients with B-lineage acute lymphoblastic leukemia (ALL). To evaluate combination of human $\gamma\delta$ T cells with CD19 antibodies for immunotherapy of B-lineage ALL, $\gamma\delta$ T cells were expanded after a GMP-compliant protocol and ADCC of both primary and expanded $\gamma\delta$ T cells with an Fc-optimized CD19 antibody (4G7SDIE) and a bi-specific antibody with the specificities CD19 and CD16 (N19-C16) was evaluated in CD107a-degranulation assays and intracellular cytokine staining. CD107a, TNF α , and IFN γ expression of primary $\gamma\delta$ T cells were significantly increased and correlated with CD16-expression of $\gamma\delta$ T cells. $\gamma\delta$ T cells highly expressed CD107a after expansion and no further increased expression by 4G7SDIE and N19-C16 was measured. Cytotoxicity of purified expanded $\gamma\delta$ T cells targeting CD19-expressing cells was assessed in both europium-TDA release and in an impedance-based label-free method (using the xCELLigence system) measuring $\gamma\delta$ T cell lysis in real-time. Albeit in the 2 h end-point europium-TDA release assay no increased lysis was observed, in real-time xCELLigence assays both significant antibody-independent cytotoxicity and ADCC of $\gamma\delta$ T cells were observed. The xCELLigence system outperformed the end-point europium-TDA release assay in sensitivity and allows drawing of conclusions to lysis kinetics of $\gamma\delta$ T cells over prolonged periods of time periods. Combination of CD19 antibodies with primary as well as expanded $\gamma\delta$ T cells exhibits a promising approach, which may enhance clinical outcome of patients with pediatric B-lineage ALL and requires clinical evaluation.

Keywords: $\gamma\delta$ T cell expansion, ADCC, tumor immunotherapy, therapeutic antibodies, xCELLigence system

INTRODUCTION

Pediatric B-lineage acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and the leading cause of cancer-related death during childhood (1). Improvement of chemotherapeutic protocols as well as application of allogeneic stem cell transplantation (SCT) in relapsed or refractory patients has improved outcome tremendously. Graft manipulation strategies have evolved from CD34-positive selection over CD3/CD19 depletion to TCR $\alpha\beta$ /CD19 depletion, leaving $\gamma\delta$ T cells in the graft and which is currently applied in phase I/II clinical studies (2–4). In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells are not restricted by MHC molecules, which makes them unlikely to elicit graft versus host disease (GvHD) based on HLA alloreactivity (5). This feature makes $\gamma\delta$ T cells potent effector cells not only during early lymphopenic post-transplant phase but also in cell-based immunotherapy after SCT.

Immunotherapy with CD20-specific monoclonal antibodies (mAbs) Rituximab and Ofatumumab has shown promising clinical results in B-cell malignancies. However, CD20 is expressed in <50% of pediatric B-lineage ALLs, thus, limiting the potential use of CD20 antibodies to a minor patient cohort (6, 7). CD19, another signature B-cell antigen, is expressed from early B-cell development onward and, thus, expressed on all B-lineage ALLs making it a well suited target antigen (8, 9). Several CD19 antibodies are currently in pre-clinical and early clinical evaluation (7–12). Main effector function of these mAbs is antibody-dependent cellular cytotoxicity (ADCC), which is mediated by the activating low-affinity receptor Fc γ RIIIa (type III receptor for IgG; CD16), which binds the Fc portion of human antibodies of the subclasses IgG1 and IgG3. Engagement of CD16 induces a potent activating signal, which overcomes inhibitory signals and results in one or more of the effector functions ADCC, cytokine response, and

phagocytosis (13). ADCC is mediated by the release of cytotoxic granules containing perforin and granzyme leading to the lysis of target cells. The relevance of ADCC *in vivo* has been underlined by a recent study showing improved clinical response in patients showing higher capacity for ADCC *in vitro* (14). CD16 is highly expressed by natural killer (NK) cells and by other hematopoietic cells including macrophages and granulocytes.

$\gamma\delta$ T cells share several surface antigens with NK cells, including NKG2D, ULBP, CD56, and CD16 (15). CD16-expression of circulating V γ 9V δ 2 T lymphocytes may be induced by activating $\gamma\delta$ T cells with phosphoantigens and this distinct subset of effector cells has been shown to be highly cytolytic against tumor cells upon activation via CD16 (16, 17). ADCC induced by CD16-expressing $\gamma\delta$ T cells has been shown for therapeutic antibodies as Rituximab and Trastuzumab (18, 19).

Besides second generation mAbs as chimerized antibody Rituximab and humanized antibody Trastuzumab, several third-generation antibodies have been developed in order to further enhance ADCC *in vivo* and, thus, improving clinical efficacy (20). The main approaches to optimize Fc γ RIIIa binding by enhancing the affinity of mAbs developed in recent years, were molecular modifications in the Fc domain of mAbs leading to amino acid substitutions (21–23), modifying Fc-linked glycosylation (24–26) and replacement of the reactive Fc portion by a binding domain for CD16 (27). For treatment of acute myeloid leukemia (AML) several of these third-generation constructs are currently under pre-clinical and early clinical investigation and have been shown to mediate higher ADCC than their unmodified counterparts (28–30).

The standard techniques to determine the antibody-independent cytotoxicity (AIC) and ADCC *in vitro* include 51 chromium release assays, Europium-TDA assays, [(3) H] thymidine incorporation assays, MTT assays, and flow cytometry-based CD107a-degranulation assays (31–35). However, those methods share various limitations including the labeling of cells and that they can only be readily performed as end-point assays, thereby lacking the information required for kinetic studies (36). Recent studies reported on the deployment of a novel label-free electrical impedance-based assay allowing the dynamic detection of AIC and ADCC and suggest several advantages compared to other established killing assays. This technique, based on the continuous assessment of electrical impedance, has been validated for the assessment of NK cell AIC and ADCC and antigen-specific T-cell-mediated cytotoxicity and deployed for the assessment of $\gamma\delta$ T cell-mediated cytotoxicity with bi-specific antibodies binding CD3 and V γ 9 on $\gamma\delta$ T cells, respectively (36–38). Impedance to an electric current is increased by the isolating properties of the cell body, when adherent tumor cells attach to electrodes on the bottom of multi-well plates. Killing of these tumor cells results in detachment or disintegration, reducing the electrical impedance that can be measured by the xCELLigence system (36).

Here, we not only show that primary as well as expanded $\gamma\delta$ T cells mediate ADCC with an Fc-optimized CD19 antibody and a CD19–CD16 bi-specific construct but present a label-free impedance-based method, facilitating the detection of $\gamma\delta$ T cell lysis kinetics over prolonged periods of time.

MATERIAL AND METHODS

CELLS AND CULTURE CONDITIONS

PBMC from leukocytes of thrombaphereses of healthy blood donors and leukemic blasts were isolated by density gradient centrifugation using Biocoll Separating Solution (Biochrom, Berlin, Germany). Healthy donor samples were kindly provided by the Institute for Clinical and Experimental Transfusion Medicine at Tübingen University after obtaining written informed consent. Primary leukemic blasts were obtained from a patient with common-ALL. Over 90% of bone marrow cells were positive for CD10/CD34/CD19 as determined by flow cytometry. PBMC, leukemic blasts, and pediatric B-lineage ALL cell line SEM (ACC 546, DSMZ, Braunschweig, Germany) were cultured in IMDM (Lonza, Basel, Switzerland), breast adenocarcinoma cell line MCF-7 (ACC 115), and B-lineage ALL cell line NALM-6 (ACC 128) were kept in EMEM and RPMI 1640 (Biochrom), respectively. All media were supplemented with 10% fetal calf serum or pooled human AB serum (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine (all reagents Biochrom).

MCF-7 TRANSFECTANT

Full-length cDNA of human CD19 (GenBank no. BC006338.2) was purchased from source Bioscience (Berlin, Germany) and cloned into the expression vector pGH-1. MCF-7 cells were transfected by electroporation (230 V, 975 μ F) and CD19 expressing clones were selected and sorted by flow cytometric analysis. Stable CD19 expression of transfected MCF-7 (termed MCF-7-CD19tm) was verified over course of culture. Medium of selected clones was supplemented with 1 mg/ml G418-BC sulfate (Biochrom).

ANTIBODIES AND FLOW CYTOMETRY

Initial chimerization and Fc-optimization of χ 4G7 and 4G7SDIE, respectively, was described previously (28). For a good manufacturing practice (GMP)-compliant production, antibody genes, codon-usage optimized for CHO expression, were inserted into the expression vector pGH-1 and used for serum-free transfection of CHO cells. The antibody 4G7SDIE was then produced by transfected CHO cells and purified in GMP-compliant clean rooms using disposable technology including a 100-l biowave reactor (Sartorius, Goettingen, Germany) for fermentation and an AKTA ready system for purification by protein A, ion exchange, and hydrophobic interaction chromatography (MabSelect SuRe and Capto-Adhere columns, GE Healthcare, Munich, Germany). N19-C16 (CD19–CD16) and N19-CU (CD19–CD3) were generated in a bi-specific format, termed Fabsc. This bi-specific format contains the CD19 antibody (clone 4G7) as a Fab fragment, which is linked by an Fc-attenuated CH2 domain to a C-terminal single-chain Fv fragments derived from the CD16 antibody (clone 3G8) and the CD3 antibody (clone UCHT1), respectively (Durben et al., manuscript submitted). The constructs were produced by transfected Sp2/0 cells and purified by affinity chromatography with κ -select (GE Healthcare).

CD107a-APC, CD56-PE/Cy7, CD45-APC/Cy7, CD3-PerCP, IFN γ -BV711, TNF α -PB, CD16-AF700, CD4-BV421, CD8-APC/Cy7, and isotype control antibodies were purchased from

Biolegend (San Diego, CA, USA). TCR $\gamma\delta$ -FITC, CD19-PE, CD56-PE, and anti-human CD19 antibody (clone 4G7) were purchased from BD Biosciences (Heidelberg, Germany) and LIVE/DEAD Fixable Aqua and Yellow Dead Stain Kits from Invitrogen. All antibodies were incubated with cells for 20 min at 4°C. Quantitative analysis was performed with QIFIKIT (Dako, Hamburg, Germany) according to the manufacturers' recommendations. Cells were analyzed with a FACSCalibur or a LSRII and sorted with a FACSJazz (BD Biosciences).

CD107a-DEGRANULATION ASSAY

Percentage of $\gamma\delta$ T cells (CD3 $^+$, TCR $\gamma\delta$ $^+$) was determined by flow cytometry and donors with $\gamma\delta$ T cells $>1.5\%$ were selected for CD107a assays. Equal cell numbers of PBMC and NALM-6 or SEM were incubated with 1 μ g/ml 4G7SDIE or N19-C16, 2 μ M GolgiStop (BD Biosciences), 10 μ g/ml Brefeldin A (Sigma, Steinheim, Germany) and CD107a-APC overnight at 37°C, 5% CO₂ in supplemented IMDM. Subsequently, PBMC were stained for surface and intracellular markers and analyzed by flow cytometry.

EXPANSION OF $\gamma\delta$ T CELLS

PBMC were seeded at 1.5×10^6 per well in 24-well plates and cultured in supplemented IMDM containing 100 IU/ml of recombinant human IL-2 (rhIL-2) (Novartis, Basel, Switzerland) and 400 nM zoledronate (Hexal, Holzkirchen, Germany). Every 2–3 days medium containing 100 IU/ml rhIL-2 and 400 nM zoledronate was added. After 12–14 days of culture expanded populations were positively selected using a Hapten-modified anti TCR- $\gamma\delta$ antibody and FITC-conjugated anti-Hapten MicroBeads with the autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated populations was determined by flow cytometric analysis and isolated cells were incubated with 400 IU/ml rhIL-2 overnight prior to functional assays. After 24 h isolated $\gamma\delta$ T cells had lost their FITC-labeling and restored TCR $\gamma\delta$ surface expression (data not shown).

REAL-TIME CYTOTOXICITY ASSAY (xCELLIGENCE ASSAY)

The cytolytic potential of expanded and isolated $\gamma\delta$ T cells was analyzed in a real-time cytotoxicity assay with an xCELLigence RTCA SP instrument (ACEA Biosciences, San Diego, CA, USA). In each well 5×10^3 MCF-7-CD19tm cells were seeded. After 20–24 h expanded $\gamma\delta$ T cells and 1 μ g/ml 4G7SDIE and N19-C16 were added, respectively. Cell viability was monitored every 15 min for 48 h. Cell indexes (CIs) were normalized to CI of the time-point when $\gamma\delta$ T cells were added and specific lysis was calculated in relation to the control cells lacking any effector $\gamma\delta$ T cells.

EUROPIUM-TDA CYTOTOXICITY ASSAY

The cytolytic activity of expanded and isolated $\gamma\delta$ T cells was analyzed in a 2 h-DELFIA EuTDA cytotoxicity assay (PerkinElmer, Waltham, MA, USA) according to the manufacturers recommendations and as described previously (39). Briefly, cryopreserved primary B-lineage ALL blasts were labeled with the fluorescence enhancing ligand BATDA for 60 min at 37°C. After five wash cycles 5×10^3 target cells per well were seeded and $\gamma\delta$ T cells and 1 μ g/ml 4G7SDIE and N19-C16 were added, respectively. After co-culture of 2 h, 20 μ l of supernatant was mixed with 200 μ l DELFIA

Europium Solution and after 15 min fluorescence of Europium-TDA chelates was quantified using a VICTOR multi label reader (Wallac, Turku, Finland). Specific lysis was calculated as follows: % specific lysis = (experimental TDA release – spontaneous TDA release)/(maximum TDA release – spontaneous TDA release) $\times 100$.

GRAPHICAL AND STATISTICAL ANALYSIS

Flow cytometry data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA) and xCELLigence data were analyzed with RTCA Software 1.2 (ACEA Biosciences). Other analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Statistical significance was accepted at $p < 0.05$ and is indicated by *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$).

RESULTS

EXPANSION OF HUMAN CD16 $^+$ $\gamma\delta$ T CELLS

Human $\gamma\delta$ T cells, obtained from leukapheresis products from six healthy volunteers, were expanded after a GMP-compliant protocol using 400 nM zoledronate and 100 IU/ml rhIL-2. After 12–14 days of culture $\gamma\delta$ T cells were enriched from $2.87\% \pm 1.48$ of PBMC prior to expansion to $42.35\% \pm 10.85$ of cultured cells. A $1.27\text{-fold} \pm 0.49$ expansion of total cells and a $22.55\text{-fold} \pm 9.91$ expansion of $\gamma\delta$ T cells were observed. Percentage of CD16 $^+$ $\gamma\delta$ T cells reached $26.55\% \pm 6.02$ after expansion (Table 1). As described previously, expanded $\gamma\delta$ T cells exhibited an continuum of CD16-expression (data not shown) (17, 18).

INDIRECT DETECTION OF CYTOTOXICITY OF PRIMARY $\gamma\delta$ T CELLS AND CD19-SPECIFIC ANTIBODY CONSTRUCTS AGAINST CD19 $^+$ LEUKEMIC CELL LINES

Due to low percentages of primary $\gamma\delta$ T cells in PBMC samples (Table 1), isolation of sufficient numbers of $\gamma\delta$ T cells in order to perform cytotoxicity assays may be challenging. Hence, an assay detecting the activation of $\gamma\delta$ T cells in PBMC samples employing the degranulation marker CD107a as well as simultaneously measuring cytokine production by intracellular cytokine staining (ICS) for IFN γ and TNF α was established. PBMC samples were incubated with pediatric B-cell precursor leukemia cell lines NALM-6 or SEM and CD19 antibody 4G7SDIE or N19-C16 constructs. CD107a, IFN γ , and TNF α expression of CD3 $^+$ TCR $\gamma\delta$ $^+$ $\gamma\delta$ T cells were determined using flow cytometry. $\gamma\delta$ T cells incubated with NALM-6 or SEM and $\gamma\delta$ T cells incubated with irrelevant control antibody and NALM-6 or SEM, respectively, displayed low expression of CD107a, IFN γ , and TNF α (Figure 1). However, Fc-optimized CD19 antibody 4G7SDIE enhanced CD107a and TNF α expression of $\gamma\delta$ T cells, when incubated with target cell lines, significantly (Figure 1A). IFN γ expression was significantly enhanced as well, though percentages of positive cells remained low. Without adding target cell lines, $\gamma\delta$ T cells incubated with 4G7SDIE did not express significantly enhanced levels of CD107a, IFN γ , and TNF α (Figure 1A). The chimerized, unmodified counterpart of 4G7SDIE, χ 4G7, did not increase CD107a, IFN γ , and TNF α expression of $\gamma\delta$ T cells when incubated with target cell lines NALM-6 (Figure 1B). Bi-specific CD19–CD16 antibody construct N19-C16 induced a significantly enhanced CD107a, IFN γ as well

Table 1 | Expansion of human $\gamma\delta$ T cells.

| Donor | $\gamma\delta$ T cells prior to expansion (%) | Days of expansion | $\gamma\delta$ T cells after expansion (%) | CD16 ⁺ $\gamma\delta$ T cells after expansion (%) | Fold-expansion of total cells | Fold-expansion of $\gamma\delta$ T cells cells |
|-----------|---|-------------------|--|--|-------------------------------|--|
| 1 | 2.24 | 13 | 35.9 | 22.70 | 0.93 | 14.84 |
| 2 | 1.60 | 13 | 43.8 | 30.26 | 1.12 | 30.66 |
| 3 | 4.51 | 14 | 60.6 | 35.10 | 2.13 | 28.59 |
| 4 | 1.20 | 13 | 33.8 | 28.90 | 0.88 | 24.84 |
| 5 | 5.18 | 13 | 32.0 | 24.00 | 1.01 | 6.22 |
| 6 | 2.50 | 12 | 48.0 | 18.32 | 1.57 | 30.13 |
| Mean (SD) | 2.87 (\pm 1.61) | 13 (\pm 0.63) | 42.35 (\pm 10.85) | 26.55 (\pm 6.02) | 1.27 (\pm 0.49) | 22.55 (\pm 9.91) |

as TNF α expression of $\gamma\delta$ T cells, when incubated with target cell lines, as well (Figure 1C).

INDIRECT DETECTION OF CYTOTOXICITY OF EXPANDED $\gamma\delta$ T CELLS AND CD19-SPECIFIC ANTIBODY CONSTRUCTS AGAINST CD19 $^{+}$ LEUKEMIC CELL LINES

$\gamma\delta$ T cells were expanded as described and the TCR $\gamma\delta^{+}$ -cell population was isolated. After cell recovery of 24 h, TCR $\gamma\delta$ expression was restored and CD107a assays, in order to determine AIC and ADCC, were performed. When analyzing expanded $\gamma\delta$ T cells in CD107a-degranulation assays a high baseline-expression of CD107a of 30–50% was observed (Figure 1D). Addition of CD19-specific constructs did not enhance expression of CD107a. TNF α expression of $\gamma\delta$ T cells was marginally enhanced by N19-C16 and 4G7SDIE when incubated with target cell line NALM-6. IFN γ expression was slightly enhanced by N19-C16 when incubated with target cell line NALM-6.

CORRELATION OF CD16-EXPRESSION BY PRIMARY $\gamma\delta$ T CELLS WITH ADCC BY CD19-SPECIFIC ANTIBODY CONSTRUCTS

CD16-expression of $\gamma\delta$ T cells was not detected simultaneously in CD107a-degranulation assays due to technical limitations, which prohibited staining of CD16 when analyzing the CD19-specific antibody constructs. Hence, PBMC samples were stained for $\gamma\delta$ T cell markers CD3 and TCR $\gamma\delta$ as well as CD16 prior to CD107a-degranulation assays with 4G7SDIE and N19-C16 and cell line NALM-6 as described above (Figure 2A). Mean percentage of CD16-positive $\gamma\delta$ T cells was $38.93\% \pm 20.43$. Expression of CD107a, TNF α , and IFN γ induced by target cell line only was deducted from expression levels reached by incubation with NALM-6 and CD19-specific antibody constructs. A positive correlation between CD107a-, TNF α -, and IFN γ -expression by 4G7SDIE (Figure 2B) or N19-C16 (Figure 2C) stimulated $\gamma\delta$ T cells and CD16-positive $\gamma\delta$ T cells was observed.

ASSESSMENT OF CYTOTOXICITY OF EXPANDED $\gamma\delta$ T CELLS IN 2 H-EUROPIUM-TDA RELEASE ASSAYS

After expansion of $\gamma\delta$ T cells the TCR $\gamma\delta^{+}$ -cell population was isolated (purity $99.5\% \pm 0.62$) and after cell recovery of 24 h cytotoxicity assays in order to determine AIC and ADCC were performed. First, cytotoxicity of isolated, expanded, and recovered $\gamma\delta$ T cells was assessed in 2 h-europium-TDA release assays with pediatric B-lineage ALL blasts. No significant lysis of leukemic blasts by expanded $\gamma\delta$ T cells and $\gamma\delta$ T cells with CD19-specific

antibody constructs 4G7SDIE and N19-C16 was observed in this end-point assay, respectively (Figures 3A,B). As positive control, 2 h-europium-TDA release assays with pediatric B-lineage ALL blasts, $\gamma\delta$ T cells and a CD19-CD3 bi-specific antibody construct (N19-CU) were performed (Figure 3C). Albeit $\gamma\delta$ T cells alone did not lyse pediatric B-lineage ALL blasts, combination of $\gamma\delta$ T cells with N19-CU did greatly enhance lysis.

ASSESSMENT OF CYTOTOXICITY OF EXPANDED $\gamma\delta$ T CELLS IN A LABEL-FREE REAL-TIME ASSAY (XCELLIGENCE)

In order to test the suitability of impedance-based measurement of cell viability for monitoring $\gamma\delta$ T cell-mediated AIC and ADCC by CD19-specific antibody constructs, CD19-expressing, adherent cells were required. Adherent breast adenocarcinoma cell line MCF-7 was stably transfected with transmembrane CD19. A CD19 $^{+}$ clone, expressing CD19 comparable to CD19 surface expression levels displayed by pediatric B-lineage ALL blasts, was selected by cell sorting (Figure S1 in Supplementary Material).

MCF-7-CD19 transfected cells of the selected clone (MCF-7-CD19tm) were seeded on 96-well E-plates and after growing overnight expanded isolated and recovered $\gamma\delta$ T cells were added in various effector to target ratios (E:T). Medium, control antibody, CD19-specific antibody constructs 4G7SDIE and N19-C16 were added at the same time-point, respectively. An E:T ratio-dependent cytolysis of target cells could be reproducibly monitored as decreasing impedance values in real-time, whereas medium controls were not affected in their growing displayed by continuously increasing normalized CI values (Figures 4A,B). Depending on E:T ratios, normalized CI values of MCF-7-CD19tm incubated with expanded $\gamma\delta$ T cells reached baseline, displaying a target cell lysis of 100%, after 24–48 h (Figures 4A,B). Addition of CD19-specific antibody constructs 4G7SDIE and N19-C16 greatly enhanced the reduction of normalized CIs of MCF-7-CD19tm cells. Specific lysis of target cells was calculated for different time-points (Figures 5A,B). At E:T 20:1 specific lysis was increasing rapidly over time and reached 79% after 12 h whereas lysis with E:T 10:1 and E:T 5:1 was increasing less rapidly over time, reaching 44 and 17% after 12 h, respectively. Addition of CD19-specific antibody constructs 4G7SDIE and N19-C16 greatly enhanced the specific lysis of MCF-7-CD19tm cells. Depending on the time-point to observe AIC and ADCC, differences between $\gamma\delta$ T cells alone (AIC) and $\gamma\delta$ T cells with CD19-specific antibody constructs (ADCC), were more or less pronounced (Figures 5A,B). Greatest differences of AIC and ADCC were at 4 h (20:1) and 8 h

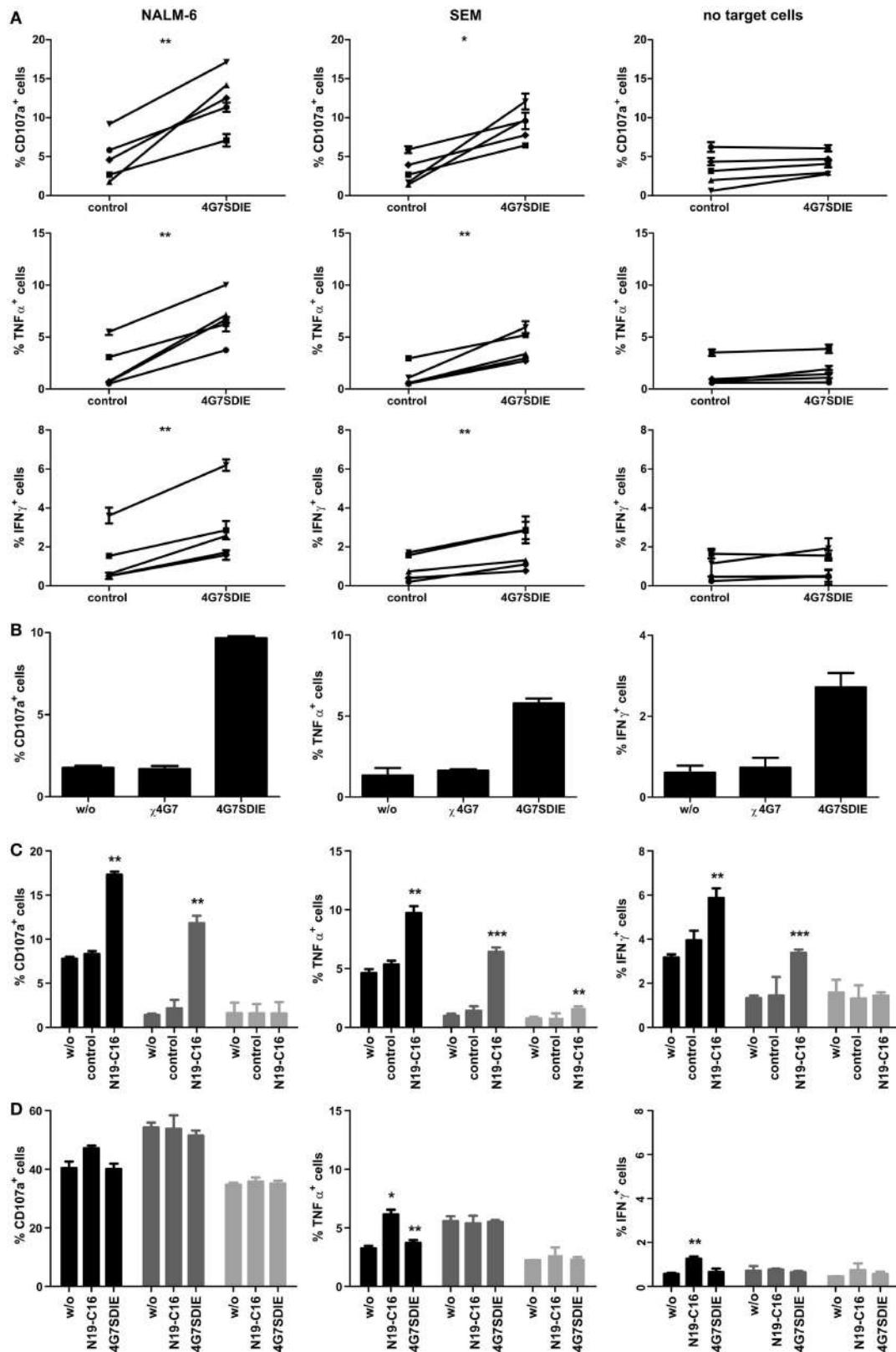


FIGURE 1 | Indirect assessment of primary and expanded $\gamma\delta$ T cell-mediated AIC and ADCC in CD107a-degranulation assays and intracellular cytokine stainings (ICS).

(Continued)

FIGURE 1 | Continued

PBMC (**A–C**) or expanded, isolated, and recovered $\gamma\delta$ T cells (**D**) were incubated with equal cell numbers of target cell lines NALM-6 or SEM and antibodies for 16 h. $\gamma\delta$ T cell degranulation (CD107a) and cytokine production (TNF α and IFN γ) were detected on viable, single CD45 $^+$ CD3 $^+$ TCR $\gamma\delta^+$ lymphocytes by extracellular and intracellular staining and flow cytometric analysis. (**A**) PBMC were incubated with or without target cell lines NALM-6 or SEM and 1 μ g/ml control antibody or 1 μ g/ml 4G7SDIE. Five different donors of 5 independent experiments are shown. (**B**) PBMC were incubated

with target cell line NALM-6 and without antibody, 1 μ g/ml χ 4G7 or 1 μ g/ml 4G7SDIE. One representative experiment, of three independent experiments performed, is shown. (**C**) PBMC were incubated with or without (light gray bars) target cell lines NALM-6 (black bars) or SEM (gray bars) and without, 1 μ g/ml control antibody or 1 μ g/ml N19-C16. Experiments were performed in triplicates. (**D**) Expanded, isolated, and recovered $\gamma\delta$ T cells were incubated with or without (light gray bars) target cell lines NALM-6 (black bars) or SEM (gray bars) and without, 1 μ g/ml N19-C16 or 1 μ g/ml 4G7SDIE. Experiments were performed in triplicates. Error bars represent the standard deviation (SD).

(10:1 and 5:1) for 4G7SDIE and 4 h (20:1), 6 h (10:1) and 8 h (5:1) for N19-C16, respectively.

DISCUSSION

Albeit tremendous improve in outcome of pediatric patients with B-lineage ALL over the last decades, prognosis for primary refractory or relapsed patients remains poor. Since immunotherapeutic therapy with chimeric antigen receptor-modified T cells with specificity for CD19 have been successful in pediatric patients with B-lineage ALL, further immunotherapeutic strategies are emerging (40, 41).

Due to their features including non-MHC restriction, cytotoxicity against hematological malignancies and capacity for ADCC, $\gamma\delta$ T cells are promising effector cells for immunotherapy of pediatric B-lineage ALL during early post-transplant phase and phase and cell-based immunotherapy after SCT.

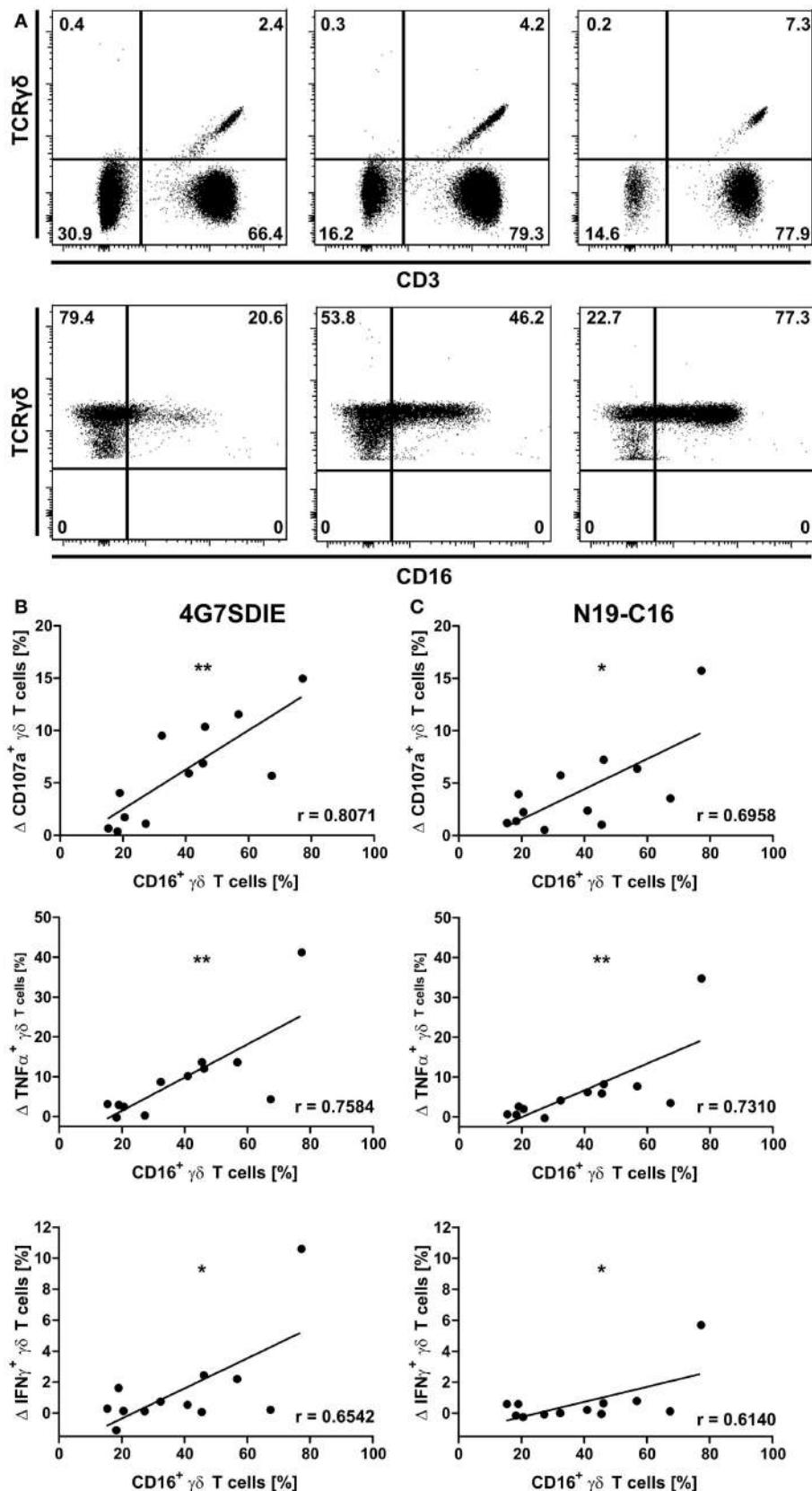
In this study, we evaluated the combination of primary and expanded human $\gamma\delta$ T cells with CD19 antibodies for immunotherapy of pediatric B-lineage ALL and established an label-free method, facilitating the long-term and real-time monitoring of $\gamma\delta$ T cell-mediated antibody-independent (AIC) and ADCC.

Indirect cytotoxicity and cytokine production of primary $\gamma\delta$ T cells was analyzed in CD107a-degranulation assays and ICS. A significantly increased expression of degranulation marker CD107a and cytokine production by $\gamma\delta$ T cells was observed when target cells were incubated with PBMC and CD19 antibody constructs. However, expanded, isolated, and recovered $\gamma\delta$ T cells were highly CD107a-positive and CD107a expression was not further enhanced by CD19 antibody constructs. In contrast to Fc-optimized mAb 4G7SDIE, no activation of $\gamma\delta$ T cells was induced by parental non-optimized antibody χ 4G7. This indicates that a strong activation may be needed for $\gamma\delta$ T cell-mediated ADCC. Measurement of CD107a-degranulation combined with ICS is a suitable method for analyzing ADCC capacity of untouched, primary $\gamma\delta$ T cells without the need to isolate $\gamma\delta$ T cells. For expanded $\gamma\delta$ T cells direct cytotoxicity assays seem favorable. For Rituximab it has been shown that $\gamma\delta$ T cell ADCC is driven by CD16 (42). We observed a positive correlation between percentages of CD16 $^+$ positive $\gamma\delta$ T cells and degranulation and cytokine production upon CD19-specific antibody stimulation. This finding indicates that CD107a-, TNF α -, and IFN γ -positive $\gamma\delta$ T cells belong to the CD16 $^+$ subset of the $\gamma\delta$ T cell pool. Inhibiting CD16-mediated signaling by using a blocking antibody or removing CD16 $^+$ $\gamma\delta$ T cells from the $\gamma\delta$ T cell population may be possible approaches to further underline this hypothesis. It has been shown, that the percentage of CD16 $^+$ cells in the $\gamma\delta$ T cell pool can be elevated

in response to pro-inflammatory cytokines IL-2, IL-15, and IL-21 (43, 44). Enhancing CD16-expression of $\gamma\delta$ T cells *in vivo* and in clinical large-scale expansion protocols, respectively, may be approaches to advance combined immunotherapy with therapeutic antibodies. Examining the effect of these cytokines on $\gamma\delta$ T cell-mediated ADCC would be particularly interesting as it has been shown that NK cell-mediated ADCC is enhanced by IL-2, IL-15, and IL-21 as well (7, 45, 46).

CD107a-degranulation assays measure the cytolytic capacity of effector cells but lack detection of direct cytotoxicity of target cells. However, considering the low frequencies of primary $\gamma\delta$ T cells, isolation of sufficient numbers of $\gamma\delta$ T cells for direct cytotoxicity assays may be impracticable. Furthermore, analysis of ADCC of untouched primary $\gamma\delta$ T cells in direct cytotoxicity assays, requiring isolation of cells, is currently challenging as, to our knowledge, no cell isolation kits are commercially available, which allow isolation of $\gamma\delta$ T cells without depleting CD16 $^+$ $\gamma\delta$ T cells. $\gamma\delta$ T cells were expanded after a protocol, using reagents in pharmaceutical quality, making a possible clinical translation achievable. AIC and ADCC of expanded, isolated and recovered $\gamma\delta$ T cells was analyzed in 2 h-europium-TDA assays. No lysis of leukemic blasts by expanded $\gamma\delta$ T cells alone and with 4G7SDIE and N19-C16 was measured, respectively. When expanded $\gamma\delta$ T cells were strongly activated by a CD19-CD3-recruiting bi-specific antibody construct (N19-CU), strongly enhanced lysis was observed. Unlike CD16, which was expressed by 26.55% \pm 6.02 of the expanded $\gamma\delta$ T cells, CD3 is expressed by every $\gamma\delta$ T cell. Hence, activation of a significantly greater number of $\gamma\delta$ T cell can be achieved with antibodies recruiting CD3-positive cells rather than CD16-positive cells. This end-point assay is feasible for detecting cytotoxicity of expanded $\gamma\delta$ T cells against leukemic blasts. However, potent and rapid activation of expanded $\gamma\delta$ T cells seems to be required. AIC and ADCC of expanded $\gamma\delta$ T cells may be obliterated due to the short end-point of the assay, which is limited by time-dependent increase of spontaneous release of TDA by labeled target cells. Furthermore, no conclusions to lysis kinetics can be drawn.

Due to the mentioned limitations of the end-point assay, AIC and ADCC of $\gamma\delta$ T cells were detected in real-time cytotoxicity assays (xCELLigence). One drawback of this method is the requirement of adherent target cells. Since primary leukemic blasts as well as common leukemic cell lines are suspension cells, a CD19-expressing target cell line was generated. In consideration of that the generated cell line MCF-7-CD19tm expressed comparable CD19 surface levels to leukemic blasts and commonly used cell lines, we hypothesize that results obtained in this assay allow conclusions regarding ADCC by CD19 antibody constructs

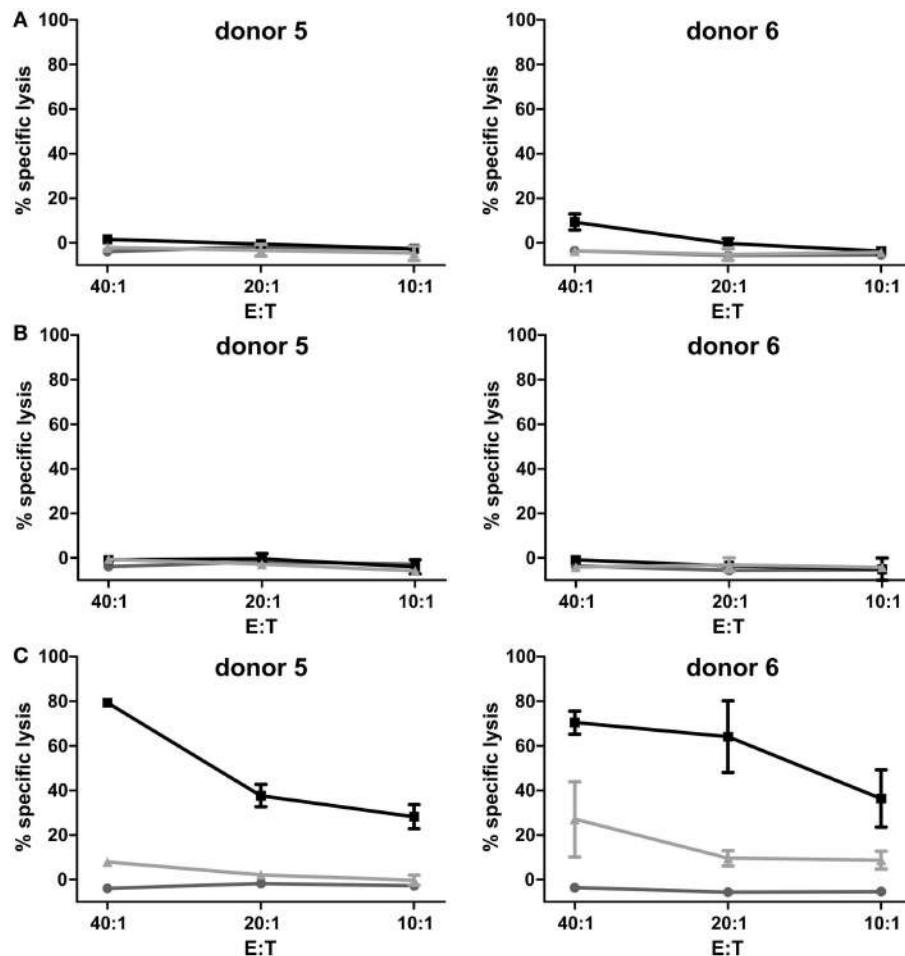
FIGURE 2 | Correlation of CD16-expression by $\gamma\delta$ T cells and degranulation and cytokine production of antibody-stimulated primary $\gamma\delta$ T cells.

(Continued)

FIGURE 2 | Continued

CD16-expression of $\gamma\delta$ T cells was assessed by flow cytometric analyses prior to functional assays. Gating hierarchy was: lymphocytes, single cells, viable cells, CD3 $^+$ TCR $\gamma\delta^+$, TCR $\gamma\delta^+$ CD16 $^+$. Gates were defined based on marker-negative populations within the viable cells. Three representative donors are shown. Same gating strategy was used for all samples (A). PBMC were incubated with equal cell numbers of target cell lines NALM-6 and antibodies for 16 h. $\gamma\delta$ T cell degranulation (CD107a) and cytokine production

(TNF α and IFN γ) were detected on viable, single CD3 $^+$ TCR $\gamma\delta^+$ lymphocytes by extracellular and intracellular staining and flow cytometric analysis. Expression of CD107a, TNF α , and IFN γ induced by NALM-6 only was deducted from expression levels reached by incubation with NALM-6 and 4G7SDIE or N19-C16. PBMC were incubated with target cell line NALM-6 and medium, 1 μ g/ml 4G7SDIE (B) and 1 μ g/ml N19-C16 (C), respectively. Statistical significance was assessed using Pearson's correlation. Experiments were performed in triplicates.

**FIGURE 3 | Assessment of $\gamma\delta$ T cell-mediated AIC and ADCC in**

2 h-europium-TDA release assays. Percentage of specific lysis was detected in europium-TDA release assays after co-incubation of 5000 labeled pediatric B-lineage ALL blasts per well with expanded, isolated and recovered $\gamma\delta$ T cells at different E:T ratios (20:1–5:1) and antibodies for 2 h. The cytolytic activity after 2 h was calculated as the percentage of specific lysis [= (experimental TDA release – spontaneous TDA release)/(maximum TDA release – spontaneous TDA release) \times 100]. Two representative donors of four are shown. (A) $\gamma\delta$ T cells (gray line), $\gamma\delta$ T cells with 1 μ g/ml control

antibody (light gray line), and $\gamma\delta$ T cells with 1 μ g/ml CD19-specific Fc-optimized mAb 4G7SDIE (black line) were added, respectively. (B) $\gamma\delta$ T cells (gray line), $\gamma\delta$ T cells with 1 μ g/ml control antibody (light gray line) and $\gamma\delta$ T cells with 1 μ g/ml CD19–CD16-bi-specific antibody construct N19-C16 (black line) were added, respectively. (C) $\gamma\delta$ T cells (gray line), $\gamma\delta$ T cells with 100 ng/ml control antibody (light gray line) and $\gamma\delta$ T cells with 100 ng/ml CD19–CD3-bi-specific antibody construct N19-CU (black line) were added, respectively. Experiments were performed in triplicates. Error bars represent the standard deviation (SD).

and $\gamma\delta$ T cells against CD19-expressing leukemic blasts. Significant lysis of target cell line MCF-7-CD19tm by expanded $\gamma\delta$ T cells was observed and increased over time. CD19 antibody constructs 4G7SDIE and N19-C16 greatly enhanced the specific lysis of MCF-7-CD19tm cells by expanded $\gamma\delta$ T cells. Notably, maximal

cytotoxicity of expanded $\gamma\delta$ T cells was delayed beyond 24 h and time-points of reaching maximal cytotoxicity varied for expanded $\gamma\delta$ T cells alone (AIC) and expanded $\gamma\delta$ T cells with CD19 antibody constructs (ADCC). Depending on the time-point considering AIC and ADCC, differences between $\gamma\delta$ T cells alone (AIC) and

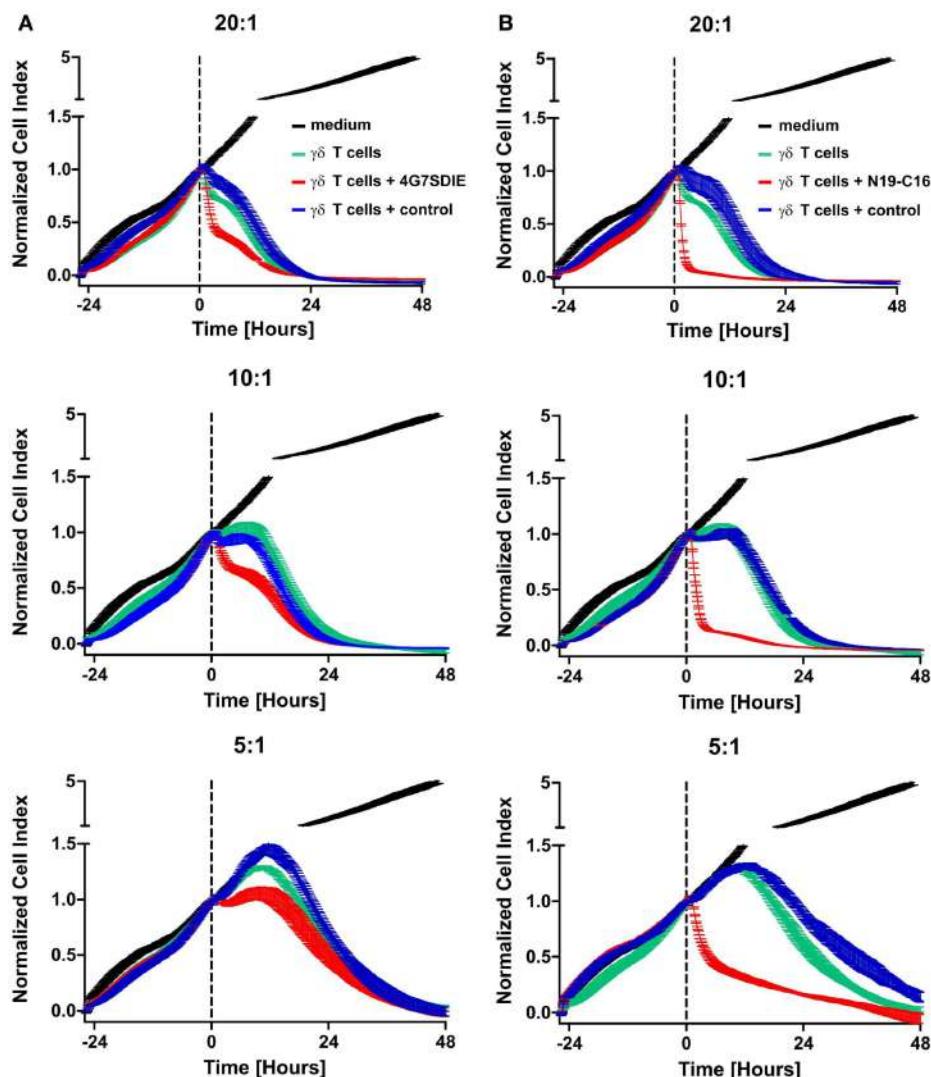


FIGURE 4 | Dynamic monitoring of expanded $\gamma\delta$ T cell-mediated AIC and ADCC (xCELLigence assay). (A,B) MCF-7-CD19tm were seeded into 96-well E-plates at equal densities of 5000 cells per well. After cell attachment and expansion, expanded, isolated, and recovered $\gamma\delta$ T cells at different E:T ratios (20:1–5:1) and antibodies were added at time-point t_0 (indicated by the dashed line) Impedance at well bottoms was measured every 15 min for >48 h and normalized to baseline impedance values with medium only. Changes in impedance normalized to t_0 are given as dimensionless

normalized cell index (CI). One representative experiment, of four independent experiments performed, is shown. (A) Medium without effector cells (black line), $\gamma\delta$ T cells (green line), $\gamma\delta$ T cells with 1 μ g/ml control antibody (blue line), and $\gamma\delta$ T cells with 1 μ g/ml CD19-specific Fc-optimized mAb 4G7SDIE (red line) were added, respectively. (B) Medium without effector cells (black line), $\gamma\delta$ T cells (green line), $\gamma\delta$ T cells with 1 μ g/ml control antibody (blue line), and $\gamma\delta$ T cells with 1 μ g/ml CD19-CD16 bi-specific antibody construct N19-C16 (red line) were added, respectively.

$\gamma\delta$ T cells with CD19 antibody constructs (ADCC), were more or less pronounced. These observations underline the importance of prolonged incubation times in cytotoxicity assays with expanded $\gamma\delta$ T cells and monitoring lysis kinetics by real-time measurement of AIC and ADCC.

In summary, we show that untouched primary as well as expanded $\gamma\delta$ T cells mediate ADCC with CD19 antibody constructs against various targets. Thus, combination of the presented antibody constructs with primary as well as expanded $\gamma\delta$ T cells exhibit promising immunotherapeutic approaches that require clinical evaluation. Notably, assays assessing AIC and ADCC of

$\gamma\delta$ T cells have some limitations and should be chosen deliberately. The assessment of CD107a and cytokine expression is a feasible method for assessing cytotoxicity of untouched primary $\gamma\delta$ T cells rather than expanded $\gamma\delta$ T cells. Europium-TDA release assays are feasible for expanded $\gamma\delta$ T cells but AIC and ADCC of $\gamma\delta$ T cells can be obliterated due to the limited end-point of the assay and no firm conclusions to lysis kinetics may be drawn. The xCELLigence system allows to measure lysis kinetics of $\gamma\delta$ T cells over prolonged periods of time and enables the detection of both AIC and ADCC of expanded $\gamma\delta$ T cells against adherent target cells.

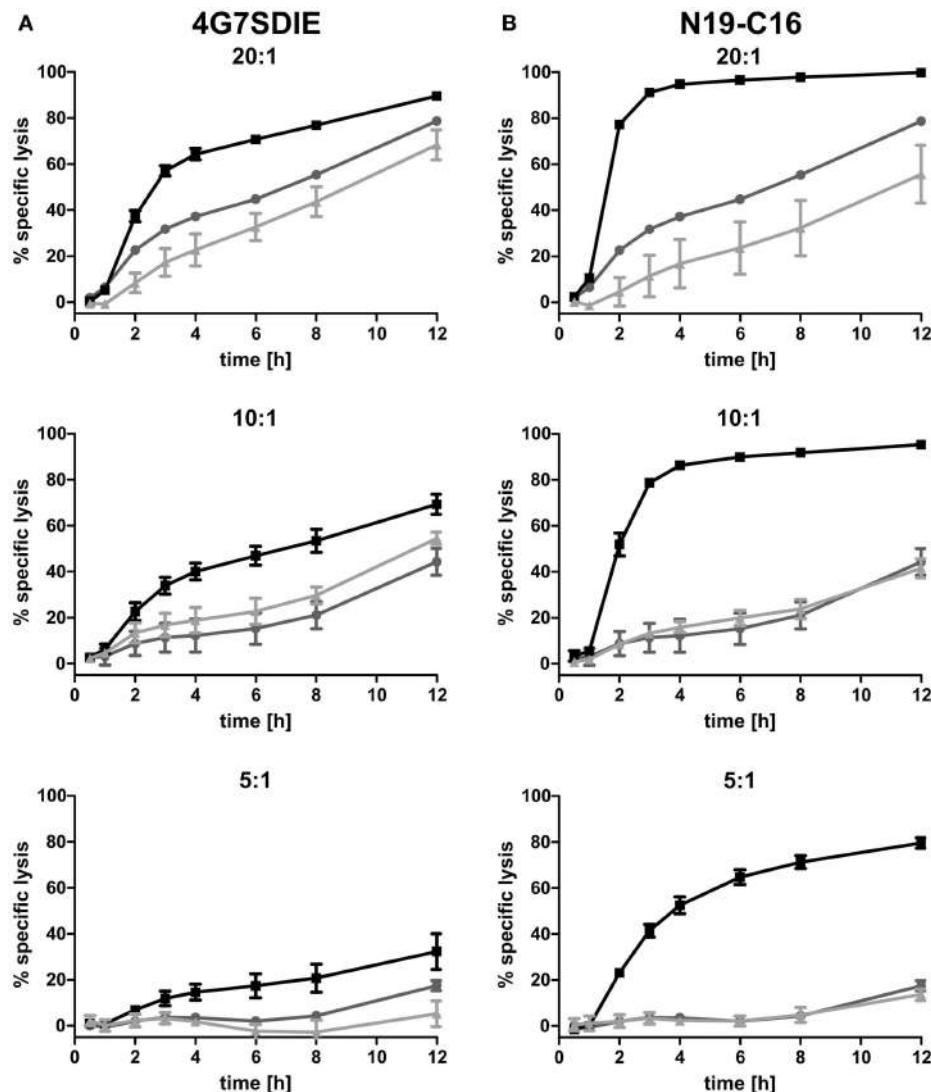


FIGURE 5 | Time-dependent cytolytic activity of expanded $\gamma\delta$ T cells in xCELLigence assays. (A,B) MCF-7-CD19tm were seeded into 96-well E-plates at equal densities of 5000 cells per well. After cell attachment and expansion, expanded, isolated, and recovered $\gamma\delta$ T cells at different E:T ratios (20:1–5:1) and antibodies were added at time-point t_0 . Impedance at well bottoms was measured every 15 min for >12 h and normalized to baseline impedance values with medium only. The cytolytic activity at different time-points was calculated as the percentage of specific lysis (= [normalized

$Cl_{no\ effector\ cells} - normalized\ Cl_{\gamma\delta\ T\ cells\ (with\ antibody)}]/normalized\ Cl_{no\ effector\ cells} \times 100$). One representative experiment, of four independent experiments performed, is shown. (A) $\gamma\delta$ T cells (gray line), $\gamma\delta$ T cells with 1 μ g/ml control antibody (light gray line) and $\gamma\delta$ T cells with 1 μ g/ml CD19-specific Fc-optimized mAb 4G7SDIE (black line) were added, respectively. (B) $\gamma\delta$ T cells (gray line), $\gamma\delta$ T cells with 1 μ g/ml control antibody (light gray line), and $\gamma\delta$ T cells with 1 μ g/ml CD19-CD16-bi-specific antibody construct N19-C16 (black line) were added, respectively.

AUTHOR CONTRIBUTIONS

Ursula Jördis Eva Seidel contributed to the conception and design of the work, development of methodology, acquisition, analysis and interpretation of data, and to the drafting of this article. Fabian Vogt contributed to the development of methodology and interpretation of data and critical revision of this article. Ludger Grosse-Hovest contributed to the development of methodology, acquisition, analysis and interpretation of data, and critical revision of this article. Gundram Jung contributed to the interpretation of data and critical revision of this article. Rupert Handgretinger contributed to the conception and design of the work,

interpretation of data, and critically revised this article for the final approval of the submitted version. Peter Lang contributed to the conception and design of the work, interpretation of data, and critical revision of this article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00618/abstract>

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Potential use of $\gamma\delta$ T cell-based vaccines in cancer immunotherapy

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Immunotherapy is a fast advancing methodology involving one of two approaches: (1) compounds targeting immune checkpoints and (2) cellular immunomodulators. The latter approach is still largely experimental and features *in vitro* generated, live immune effector cells, or antigen-presenting cells. $\gamma\delta$ T cells are known for their efficient *in vitro* tumor killing activities. Consequently, many laboratories worldwide are currently testing the tumor killing function of $\gamma\delta$ T cells in clinical trials. Reported benefits are modest; however, these studies have demonstrated that large $\gamma\delta$ T-cell infusions were well tolerated. Here, we discuss the potential of using human $\gamma\delta$ T cells not as effector cells but as a novel cellular vaccine for treatment of cancer patients. Antigen-presenting $\gamma\delta$ T cells do not require to home to tumor tissues but, instead, need to interact with endogenous, tumor-specific $\alpha\beta$ T cells in secondary lymphoid tissues. Newly mobilized effector $\alpha\beta$ T cells are then thought to overcome the immune blockade by creating proinflammatory conditions fit for effector T-cell homing to and killing of tumor cells. Immunotherapy may include tumor antigen-loaded $\gamma\delta$ T cells alone or in combination with immune checkpoint inhibitors.

Keywords: immunotherapy, $\gamma\delta$ T cells, cancer, antigen-presenting cells, vaccine

HUMAN BLOOD $\gamma\delta$ T CELLS

Human blood $\gamma\delta$ T cells constitute a minor subset (1–5%) of total T cells that is characterized by their T-cell antigen receptors (TCR) encoded by rearranged V-gamma and V-delta genes. Among blood $\gamma\delta$ T cells, those expressing V γ 9V δ 2–TCR greatly outnumber other $\gamma\delta$ T-cell subsets whereas in peripheral tissues V γ 9V δ 2+ T cells are relatively scarce. Their numbers are substantially increased in blood of patients suffering from various infectious diseases, indicating an involvement of V γ 9V δ 2+ T cells in antimicrobial immunity. Indeed, early work revealed that V γ 9V δ 2+ T cells are selective for a microbial non-peptide metabolite, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), found in numerous commensal and pathogenic microbes, including bacteria, *Plasmodium* and *Toxoplasma* species but not in man (1, 2). The same type of $\gamma\delta$ T cells also responds (albeit at >10⁴-fold higher concentrations) to the structurally related compound isopentenyl pyrophosphate (IPP), a metabolite of the mevalonate pathway in eukaryotes including man. Of interest, IPP was suggested to be increased in stressed cells, such as tumor cells, and its interaction with a specific IPP- (and HMBPP-) binding protein (BTN3A/CD277) leads to V γ 9V δ 2+ T-cell activation (reviewed by the groups of E. Adams, E. Scotet, and T. Hermann in this Research Topic). IPP levels can be elevated artificially by addition of amino-bisphosphonates (such as zoledronate; see below) that inhibit an intracellular IPP-metabolizing enzyme. Consequently, treatment of PBMC with zoledronate results in the selective activation and outgrowth of V γ 9V δ 2+ T cells. The exquisite selectivity for HMBPP/IPP distinguishes V γ 9V δ 2+ T cells from $\alpha\beta$ T cells that recognize a myriad of short peptides under MHC restriction. In fact, the V γ 9V δ 2–TCR endows a sizable army of blood $\gamma\delta$ T cells to become immediately mobilized in response to a single

class (“phosphoantigens”) of danger signals produced by microbes and possibly tumor cells (3). Relevant to the discussion below, this “mono-selectivity” allowed us to study the TCR-mediated functionality and migration properties of the entire V γ 9V δ 2+ T-cell population.

DISCOVERY OF $\gamma\delta$ T-APC: $\gamma\delta$ T CELLS WITH ANTIGEN-PRESENTATION FUNCTION

The myriad of effector and memory T cells present in our body can be distinguished based on their functional and migratory profiles. In fact, the characteristic functions of individual T cells are intimately related to their migratory properties, as exemplified by the distinct chemokine receptor profiles decorating individual T helper subsets (4, 5). For example, the chemokine receptor CXCR5 identifies follicular B helper T (T_{FH}) cells that are specialized in orchestrating T cell-dependent antibody responses within the follicular compartments of secondary lymphoid tissues (6, 7). Our research on human blood $\gamma\delta$ T cells (V γ 9V δ 2-TCR+ $\gamma\delta$ T cells) began with the realization that treatment of $\gamma\delta$ T cells with phosphoantigens induced the rapid and transient expression of CCR7 (8), the chemokine receptor enabling the rendezvous between naïve/central memory T cells and mature DC within lymph nodes (9). Immunological analyses revealed their presence in the T-cell zone but also B-cell follicles, the latter location suggesting that $\gamma\delta$ T cells affect humoral responses. Indeed, and similar to T_{FH} cells, co-culture of $\gamma\delta$ T cells with tonsillar B cells resulted in massive production of antibodies (8). In support, another lab identified CXCR5 on activated $\gamma\delta$ T cells (10) and, recently, we and others reported the expression of the IL-21 receptor, linking follicular $\gamma\delta$ T cells with T_{FH} cells and their B-cell targets (11, 12).

CCR7 expression is also in line with the view that activated $\gamma\delta$ T cells team up with $\alpha\beta$ T cells and/or DC in the T-cell compartment of secondary lymphoid tissues. In fact, short-term (1–3 days) activation of $\gamma\delta$ T cells with the phosphoantigens IPP or HMBPP resulted in *de novo* expression (or up-regulation) of cell surface proteins normally associated with DC, including antigen presentation (MHC class I and II), co-stimulatory (CD40, CD80, CD86), and adhesion (CD11a, CD11b, CD11c, CD18, CD54) receptors. This observation led to detailed investigations into the possibility that activated ($CCR7^+$) $\gamma\delta$ T cells behaved like *bona fide* antigen-presenting cells (APC). Indeed, short-term activated $\gamma\delta$ T cells were capable of processing simple (tetanus toxoid) and complex (*M. tuberculosis* PPD) protein antigens and inducing antigen-specific immune responses in primary, autologous $\alpha\beta$ T cells (13). Activated $\gamma\delta$ T cells did this equally well as donor matched monocyte-derived DC (moDC). Of note, short-term activated $\alpha\beta$ T cells failed to express DC markers and were unable to present peptide antigens to $\alpha\beta$ T cells, although under special conditions cloned T-cell lines were reported to induce responses in antigen-specific responder T-cell lines (14). Subsequent studies revealed that short-term activated $\gamma\delta$ T cells were also capable of antigen cross-presentation, a process describing the induction of CD8 $^+$ $\alpha\beta$ T-cell responses to extracellular antigens. The precise mechanisms underlying antigen cross-presentation require further clarification, although it is generally assumed that extracellular antigens need to overcome intracellular membrane barriers in order to access the proteasome in the cytoplasm, which produces the peptide substrates for loading onto nascent MHC class I molecules (15). This process is in clear contrast to the classical MHC I pathway involving the processing of endogenous self- or microbe-derived antigens. Obviously, CD8 $^+$ T cells-mediated killing of tumor and infected cells depends on cross-presentation capabilities of DC involved in processing corresponding cell debris. Activated $\gamma\delta$ T cells are excellent cross-presenting APC, as shown by us (16, 17) and another laboratory (18). In fact, they seem to be able to do so more consistently than donor matched moDC, which may be explained by the reduced lysosomal activity in $\gamma\delta$ T cells (17). After all, being a subtype of phagocytes, DC are very efficient in antigen uptake and intracellular degradation. Of note, $\gamma\delta$ T cells were shown to phagocytose opsonized particles (*E. coli*, cell debris, microbeads), suggesting that phagocytosis, in addition to macropinocytosis, can contribute to antigen processing in this novel type of APC (18, 19). Induction of CD8 $^+$ T-cell responses by peptide-pulsed $\gamma\delta$ T cells was also demonstrated by another group (20, 21). Finally, activated $\gamma\delta$ T cells resemble professional APC in their ability to trigger antigen-specific responses in naïve (antigen inexperienced) T cells. In summary, all these findings document unexpected properties that are normally associated DC and prompted us to designate activated (but not freshly isolated, resting) V γ 9V δ 2 $^+$ T cells as $\gamma\delta$ T-APC (1).

In the past, $\gamma\delta$ T cells have been portrayed as uniquely situated at the cross-road between innate and adaptive immunity, bridging both worlds by means of expressing recombined TCR involved in $\gamma\delta$ T-cell activation and target cell lysis (hallmarks of adaptive immunity) while at the same time recognizing non-peptide antigens (phosphoantigens) in an MHC-unrestricted fashion similar to pattern recognition receptors (hallmarks of innate cells) (22,

23). Our findings with $\gamma\delta$ T-APC add to this functional dichotomy, although proof of APC functions under *in vivo* conditions is still missing. Because $\gamma\delta$ T cells respond to stress, e.g., elevated levels of IPP in tumor cells and microbes harboring HMBPP, one could envisage that the phosphoantigen-induced killing of target cells by mobilized $\gamma\delta$ T cells is accompanied by the processing of tumor/microbial antigens and the subsequent induction of $\alpha\beta$ T-cell responses. Activated $\gamma\delta$ T cells are known for their abundant secretion of proinflammatory cytokines (TNF α , IFN γ) and, accordingly, $\gamma\delta$ T-APC-mediated differentiation of CD4 $^+$ $\alpha\beta$ T cells resulted in effector cells dominated by a Th1-type cytokine profile (13). It will be important to see if under appropriate co-stimulatory conditions, $\gamma\delta$ T-APC are also capable of generating alternative Th subsets. It is worth mentioning, however, that $\gamma\delta$ T-APC are not equipped with the plethora of receptors sensing environmental cues as DC and, thus, are unlikely to compete with the numerous highly specialized DC whose inherent function it is to control T-cell functions in all aspects of immunity (health and disease). Instead, it appears that the APC function of $\gamma\delta$ T cells is limited to situations where they become activated by their TCR ligands (phosphoantigens).

METHODS FOR THE GENERATION OF ANTIGEN-PRESENTING $\gamma\delta$ T-APC

Autologous moDC have been widely used in clinical trials as cellular vaccine with limited success. In fact, despite >10 years of translational research, clinically approved, moDC-based therapies still do not exist. Bottlenecks are manifold, including the difficulty of obtaining large numbers of moDC with robust APC function. Our recent findings suggest that $\gamma\delta$ T-APC represent a promising alternative to moDC. Many laboratories have already demonstrated how easy it is to expand $\gamma\delta$ T cells to very large numbers during *in vitro* culture (24–35). Based on these findings, we have developed a method for the generation of *in vitro* expanded $\gamma\delta$ T-APC that may provide the basis for their large-scale manufacture under clinical-grade conditions (36). In brief, best results, both in terms of yield and quality, are achieved when PBMC samples isolated from fresh blood are stimulated once with zoledronate and then cultured in the presence of the growth factors IL-2 and IL-15. Zoledronate is a bisphosphonate drug known for its ability to increase intracellular IPP in target cells to levels sufficient for the selective activation of V γ 9V δ 2 $^+$ $\gamma\delta$ T cells (37). After 14 days of culture, $\gamma\delta$ Tells have expanded >1000-fold and >800-fold with PBMC of healthy individuals and melanoma patients, respectively, yielding 10–50 million $\gamma\delta$ T cells per milliliter of whole blood. Day 14 $\gamma\delta$ T cells retained their functionality as assessed by cytokine secretion and proliferation in response to HMBPP. Secreted cytokines included IFN γ and TNF α , which are typically produced by primary $\gamma\delta$ T cells in response to phosphoantigens, as well as several inflammatory chemokines whereas immune inhibitory cytokines (TGF β , IL-10) were not detected. Day 14 $\gamma\delta$ T cells also killed tumor cells during *in vitro* culture. Importantly, and similar to short-term activated $\gamma\delta$ T cells, expanded $\gamma\delta$ T cells largely retained many cell surface receptors normally associated with APC, including antigen presentation (MHC I and II), co-stimulation (CD80, CD86), and adhesion (CD11a, CD54) molecules. Some of these were further elevated or re-expressed

transiently (CD40 and CCR7) in response to re-stimulation with HMBPP. APC marker expression was in line with their functionality. Without need for re-stimulation, day 14 $\gamma\delta$ T cells were able to process simple (influenza M1) and complex (PPD) antigens and to induce antigen-specific $\alpha\beta$ T-cell responses in both cultured responder cell lines and primary responder cells present in unfractionated PBMC. Stimulation with HMBPP did not further enhance their APC function although, as discussed earlier, expanded $\gamma\delta$ T cells still largely maintained their responsiveness (proliferation, cytokine secretion) to phosphoantigens. In summary, the current state of our research documents the feasibility of transforming peripheral blood $\gamma\delta$ T cells into almost unlimited numbers of antigen-presenting $\gamma\delta$ T-APC. We now need to investigate the ability of day 14 $\gamma\delta$ T cells to process tumor antigens, either in purified form or as tumor cell extracts. We believe that these APC are able to do so since we have already shown that activated $\gamma\delta$ T cells efficiently processed extracts of influenza-infected epithelial cells and induced M1-specific $\alpha\beta$ T-cell responses (17). It is further possible that interaction of $\gamma\delta$ T cells with tumor cells not only leads to tumor cell killing but also to tumor antigen-presenting $\gamma\delta$ T-APC able to induce $\alpha\beta$ T-cell responses locally (tumor tissue) or at distal sites (lymph nodes).

A PROTOCOL FOR A FIRST-IN-MAN CLINICAL TRIAL WITH TUMOR ANTIGEN-PRESENTING $\gamma\delta$ T-APC

Current state of research supports the following protocol for a first-in-man clinical trial with tumor antigen-presenting $\gamma\delta$ T-APC (**Figure 1**). Preliminary data indicate anticoagulant-treated whole blood survives a 24 h shipment period in that PBMC samples retain responsiveness to zoledronate and IL-1/IL-15; similarly, we know that tumor antigen-loaded $\gamma\delta$ T-APC can be frozen for storage and/or shipment to cancer clinics. Therefore, it appears that cancer patients will not need to visit a centralized $\gamma\delta$ T-cell culture facility for donating blood samples and receiving $\gamma\delta$ T cell-based vaccine infusions. A simplified procedure of $\gamma\delta$ T-APC handling and distribution will facilitate clinical trials involving multiple centers. Following 2 weeks on *in vitro* culture as described above, tumor antigen-loaded $\gamma\delta$ T-APC will be prepared by culturing expanded $\gamma\delta$ T cells for 24 h in the presence of defined tumor antigen(s) or tumor cell extracts. A personalized immunotherapy protocol will involve the treatment autologous $\gamma\delta$ T-cell preparations with extracts from the patient's own tumor cells. After washing and reformulation, tumor antigen-loaded $\gamma\delta$ T-APC will be divided into individual bolus samples and then frozen in liquid nitrogen for shipment to corresponding cancer clinics. The frozen tumor antigen-loaded $\gamma\delta$ T-APC samples will then be prepared locally for i.v. infusion into cancer patients according to treatment regimens that need to be defined during clinical trials. Ideally, a single round of cell culture will provide enough tumor antigen-loaded $\gamma\delta$ T-APC for carrying out the entire round of treatment, which may follow a prime-boost protocol. Obviously, effective cell doses need to be established before being able to apply arithmetic for the generation of expanded $\gamma\delta$ T cells. A small sample of tumor antigen-loaded $\gamma\delta$ T-APC will be retained for quality control that will include sterility, purity, and APC phenotype assessments. Frequently, PBMC from cancer patients yield reduced numbers of expanded $\gamma\delta$ T cells or cell preparations containing large (>50%)

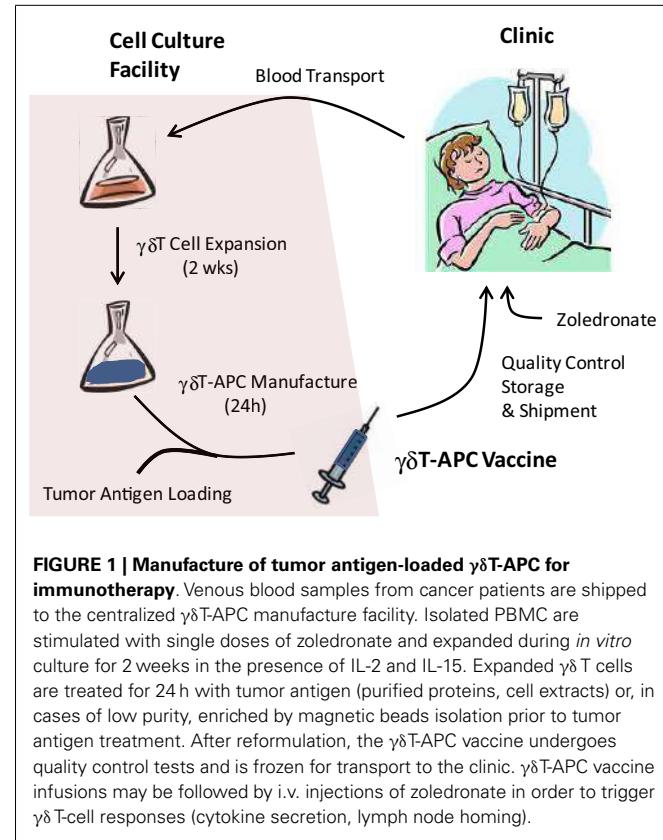


FIGURE 1 | Manufacture of tumor antigen-loaded $\gamma\delta$ T-APC for immunotherapy. Venous blood samples from cancer patients are shipped to the centralized $\gamma\delta$ T-APC manufacture facility. Isolated PBMC are stimulated with single doses of zoledronate and expanded during *in vitro* culture for 2 weeks in the presence of IL-2 and IL-15. Expanded $\gamma\delta$ T cells are treated for 24 h with tumor antigen (purified proteins, cell extracts) or, in cases of low purity, enriched by magnetic beads isolation prior to tumor antigen treatment. After reformulation, the $\gamma\delta$ T-APC vaccine undergoes quality control tests and is frozen for transport to the clinic. $\gamma\delta$ T-APC vaccine infusions may be followed by i.v. injections of zoledronate in order to trigger $\gamma\delta$ T-cell responses (cytokine secretion, lymph node homing).

numbers of non- $\gamma\delta$ T cells (mostly $\alpha\beta$ T cells and/or NK cells) (36), suggesting that a single magnetic beads purification step could improve the vaccine function of antigen-loaded $\gamma\delta$ T-APC. Last but not least, outcome measures for the $\gamma\delta$ T-APC-based vaccine treatment need to be defined but will include immunological parameters determined in blood samples taken at intervals after each infusion step as well as clinical parameters (e.g., see details in EMA document)¹. The above treatment protocol may be extended to include a single i.v. infusion of zoledronate 24 h after administration of each $\gamma\delta$ T-APC vaccine infusion. We know that expanded $\gamma\delta$ T cells retain full functionality (36). Equally important, $\gamma\delta$ T cells in cancer patients who have received $\gamma\delta$ T-cell infusions and even endogenous $\gamma\delta$ T cells of cancer patients who did not receive $\gamma\delta$ T-cell infusions were reported to respond to i.v. injections with zoledronate (32, 33, 38–43). Zoledronate is a safe medication that is frequently used to treat patients with bone disorders (44). Therefore, zoledronate may induce proinflammatory cytokine (TNF α , IFN γ) production and lymph node homing receptor (CCR7) expression and, thus, enhance the vaccine effect in infused $\gamma\delta$ T-APC.

It is important to emphasize that $\gamma\delta$ T-APC-based vaccines target the immune system in cancer patients and beneficial effects could include tumor control through immediate mobilization of endogenous, tumor-specific effector T cells, or establishing

¹http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003875.pdf

tumor immunosurveillance involving the generation of long-lived, tumor-specific memory T cells (or a combination of both processes). In this regard, the here described method involving tumor antigen-loaded $\gamma\delta$ T-APC differs fundamentally from current clinical trials exploiting the tumor killing properties of expanded $\gamma\delta$ T cells (24–35).

CONCLUDING REMARKS

Many questions remain, including the one related to the type of patients selected for a first-in-man clinical trial. As mentioned above, $\gamma\delta$ T-APC-based vaccines target the patients' own immune system, indicating that patients suffering from tumors with well described immunogenicity profiles would benefit the most (45). Of course, tumor-specific immunity is a subject of intensive investigations and numerous tumor antigens have been associated with certain types of tumors. However, we are still far from understanding the complex adaptive immune processes involved in steady-state tumor control (immunosurveillance) and in tumor progression (disease). Perhaps, the most promising indication relates to conditions with prominent involvement of inhibitory immune cells (Treg cells, myeloid-derived suppressor cells), and current success with immune checkpoint inhibitors [anti-CTLA-4 Abs (Ipilimumab), anti-PD-1 Abs (Nivolumab)] may point us in the right direction (46). In fact, combination therapy with $\gamma\delta$ T-APC-based vaccines and immune checkpoint inhibitors may even result in synergistic outcomes, i.e., blockade of inhibitory immune cells by immune checkpoint inhibitors may facilitate the stimulatory effect of $\gamma\delta$ T-APC-based vaccines leading to enhanced tumor-specific effector T-cell responses and long-lived immunosurveillance T-cell formation. T-cell responses against melanoma are well described (47), suggesting that melanoma patients are promising candidates for a first-in-man clinical trial with $\gamma\delta$ T-APC-based vaccines although other cancers with less well studied immunogenicity should not be excluded. We envisage that the proposed $\gamma\delta$ T-APC-based immunotherapy will not be restricted to a single type of cancer since the majority of human cancers are considered to evoke T-cell responses (45).

At present, a single cellular vaccine product (sipuleucel-T) showing limited clinical benefits for prostate cancer patients has been approved by the FDA. Thus far, DC-based vaccines have not developed beyond the experimental stage. We believe that our proposed $\gamma\delta$ T-APC-based therapy combines several features, including ease of manipulation and functional robustness that will make it a serious contender in the race to the first successful vaccine for use in immunotherapy of patients suffering from a broad range of cancers.

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Clinical applications of gamma delta T cells with multivalent immunity

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$\gamma\delta$ T cells hold promise for adoptive immunotherapy because of their reactivity to bacteria, viruses, and tumors. However, these cells represent a small fraction (1–5%) of the peripheral T-cell pool and require activation and propagation to achieve clinical benefit. Aminobisphosphonates specifically expand the V γ 9V δ 2 subset of $\gamma\delta$ T cells and have been used in clinical trials of cancer where objective responses were detected. The V γ 9V δ 2 T cell receptor (TCR) heterodimer binds multiple ligands and results in a multivalent attack by a monoclonal T cell population. Alternatively, populations of $\gamma\delta$ T cells with oligoclonal or polyclonal TCR repertoire could be infused for broad-range specificity. However, this goal has been restricted by a lack of applicable expansion protocols for non-V γ 9V δ 2 cells. Recent advances using immobilized antigens, agonistic monoclonal antibodies (mAbs), tumor-derived artificial antigen presenting cells (aAPC), or combinations of activating mAbs and aAPC have been successful in expanding gamma delta T cells with oligoclonal or polyclonal TCR repertoires. Immobilized major histocompatibility complex Class-I chain-related A was a stimulus for $\gamma\delta$ T cells expressing TCR δ 1 isotypes, and plate-bound activating antibodies have expanded V δ 1 and V δ 2 cells *ex vivo*. Clinically sufficient quantities of TCR δ 1, TCR δ 2, and TCR δ 1^{neg}TCR δ 2^{neg} have been produced following co-culture on aAPC, and these subsets displayed differences in memory phenotype and reactivity to tumors *in vitro* and *in vivo*. Gamma delta T cells are also amenable to genetic modification as evidenced by introduction of $\alpha\beta$ TCRs, chimeric antigen receptors, and drug-resistance genes. This represents a promising future for the clinical application of oligoclonal or polyclonal $\gamma\delta$ T cells in autologous and allogeneic settings that builds on current trials testing the safety and efficacy of V γ 9V δ 2 T cells.

Keywords: cancer, immunotherapy, $\gamma\delta$ T cells, adoptive T-cell therapy, T-cell receptor, allogeneic transplantation, chimeric antigen receptors, artificial APC

INTRODUCTION

$\gamma\delta$ T cells possess a combination of innate and adaptive immune cell qualities rendering them attractive for immunotherapy (1–3). They can produce inflammatory cytokines, directly lyse infected or malignant cells, and establish a memory response to attack pathogens upon re-exposure. $\gamma\delta$ T cells are defined by expression of γ and δ heterodimer of T cell receptor (TCR) chains (TCR γ /TCR δ) that directs intracellular signaling through associated CD3 complexes (4). The $\gamma\delta$ T-cell lineage (1–5% of circulating T cells) can be contrasted to the more prevalent $\alpha\beta$ T cell lineage (~90%) in peripheral blood, which expresses TCR α /TCR β

heterodimers and also signals through associated CD3 complexes (5, 6). CD4 and CD8 co-receptors on $\alpha\beta$ T cells assist binding of TCR $\alpha\beta$ chains to the major histocompatibility complex (MHC) presenting processed peptides (7–9). In contrast, TCR γ directly binds to an antigen's superstructure independent of the MHC/peptide complexes and, as a result, CD4 and CD8 are uncommon on $\gamma\delta$ T cells (10, 11). Given that antigen recognition is achieved outside of MHC/peptide-restriction, $\gamma\delta$ T cells have predictable immune effector functions mediated through their TCR and have potential use as universal ("off-the-shelf") allogeneic T-cell therapies (12).

Functional responses by $\gamma\delta$ T cells can be stratified by the variable (V) region of the TCR δ chain. In humans, the TCR δ locus (*TRD*) lies within the TCR α locus (*TRA*). Three unique V δ alleles, *TRDV1*, *TRDV2*, and *TRDV3*, code for TCR δ 1, TCR δ 2, and TCR δ 3, respectively. Additionally, shared V δ and V α variable regions exist in *TRDV4/TRA14*, *TRDV5/TRA29*, *TRDV6/TRA23*, *TRDV7/TRA36*, and *TRDV8/TRA38-2* loci. Recombination of these shared V alleles with a *TRA* junction region (*TRAJ*) results in TCR α 14, TCR α 29, TCR α 23, TCR α 36,

Abbreviations: 2M3B1PP, 2-methyl-3-butenyl-1-pyrophosphate; AML, acute myeloid leukemia; BrHPP, bromohydrin pyrophosphate; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; EOC, epithelial ovarian cancer; FCL, follicle center lymphoma; GI-cancer, cancers from the gastrointestinal tract; HIV, human immunodeficiency virus; HRPC, hormone-refractory prostate cancer; IC, immunocytoma; MM, multiple myeloma; MZL, mantle zone lymphoma; N/D, not determined; NHL, T-cell non-Hodgkin lymphoma; NSCLC, non-small-cell lung cancer; RCC, renal cell carcinoma; TBI, total body irradiation; T-SPL, secondary plasma cell leukemia; Zoledronic acid.

and TCR α 38-2, respectively, but recombination of these shared V alleles with *TRD* junction (*TRDJ*) and diversity (*TRDD*) regions results in TCR84, TCR85, TCR86, TCR87, and TCR88, respectively (13). Expression of TCR $\gamma\delta$ heterodimers on the T-cell surface in the thymus inhibits recombination of TCR β -chain locus during the CD4^{neg}CD8^{neg} stage thereby committing the T cell to the $\gamma\delta$ T-cell lineage (14). This double negative status is typically maintained upon exit from the thymus, most likely because co-receptors are dispensable for functional TCR $\gamma\delta$ binding to antigens (15). However, the thymus is not required to complete all $\gamma\delta$ T-cell development, as many $\gamma\delta$ T cells directly take up residence in peripheral tissues following exit from the bone marrow and exhibit immediate effector functions against pathogens (16). Thymus-independent “resident” $\gamma\delta$ T cells can be found in the mucosa, tongue, vagina, intestine, lung, liver, and skin and can comprise up to 50% of the T-cell populations in intestinal epithelial lymphocytes (17, 18). In contrast, circulating $\gamma\delta$ T cells can be found in the blood and lymphoid organs, and are dominated by $\gamma\delta$ T cells preferentially expressing TCR82 isotype (commonly referred to as V δ 2 cells). Indeed, $\gamma\delta$ T cells expressing the TCR81 isotype (commonly referred to as V δ 1 cells) are frequently found within tissues (19, 20). V δ 2 cells have preferred pairing with TCR $\gamma 9$ (V $\gamma 9$ V δ 2 cells), but broad γ -chain pairing is observed in V δ 1 cells and V δ 1^{neg}V δ 2^{neg} cells, a generic grouping of all other non-V δ 1/V δ 2 T cells (12, 19). Therefore, $\gamma\delta$ T cells are distributed across an array of anatomical locations with a range of TCR $\gamma\delta$ variable region expression.

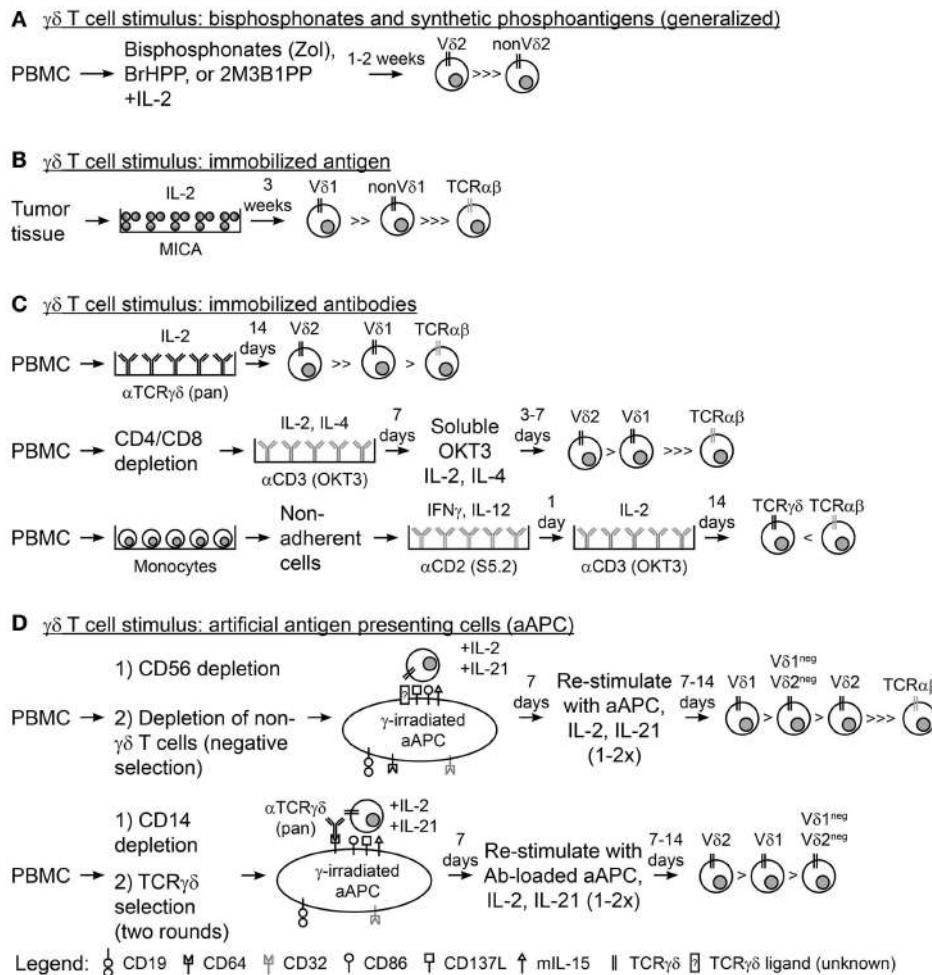
Human TCR $\gamma\delta$ ligands are MHC/peptide complex-independent and are therefore conserved amongst unrelated individuals. Most of the known human ligands are specific for TCR81 or TCR82. TCR $\gamma 1$ /TCR81 (alternatively termed V $\gamma 1$ V δ 1) heterodimers have specificity for MHC Class-I chain-related A (MICA) (21, 22), a molecule participating in evasion of immune surveillance following viral infection and expressed on tumor cells as it is involved in the cellular stress response (23). MICA is also one of the ligands for NKG2D, which is expressed on $\gamma\delta$ T cells, $\alpha\beta$ T cells, and natural killer (NK) cells (23, 24). Both V $\gamma 1$ V δ 1 and V $\gamma 2$ V δ 1 recognize non-polymorphic MHC molecule CD1c (25), and V $\gamma 5$ V δ 1 is a receptor for α -galactosylceramide-CD1d complexes commonly described in the activation of natural killer T (NKT) cells which, like $\gamma\delta$ T cells, have both innate and adaptive immune functions and recognize conserved ligands amongst unrelated individuals (26, 27). $\gamma\delta$ T cells can have specificity for virus as cytomegalovirus (CMV)-reactive V $\gamma 8$ V δ 1 cells have been isolated from umbilical cord blood from infected newborns (28). V δ 1 cells have also been associated with immunity to human immunodeficiency virus (HIV), but the precise HIV ligands for TCR81 have not been determined (29). Bacterial alkylamines and *Listeria monocytogenes* are recognized by V δ 2 cells when paired with V $\gamma 2$ (30–32). V $\gamma 9$ V δ 2 cells are the most extensively studied sub-group of human $\gamma\delta$ T cells and their ligands include phosphoantigens [isopentenyl pyrophosphate (IPP)], F₁-ATPase expressed on the cell surface, apolipoprotein A-I, and *Mycobacterium tuberculosis* (33–37). Moreover, V $\gamma 9$ V δ 2 cells controlled and prevented lethal Epstein–Barr virus (EBV)-transformed leukemia xenografts in immunocompromised mice (4), and *in vitro* and *in vivo* data suggested that V δ 1 cells are also specific for EBV (38, 39). In

contrast to V δ 1 and V δ 2 cells, very little is known about human $\gamma\delta$ T cells expressing other TCR $\gamma\delta$ alleles except for indirect evidence of V δ 3 cell’s immunity against CMV and HIV (40, 41). Given the multivalent nature of $\gamma\delta$ T cells, harnessing $\gamma\delta$ T cells populations with polyclonal TCR repertoire is attractive for adoptive immunotherapy.

$\gamma\delta$ T-CELL CLINICAL EXPERIENCE

Immunotherapy with $\gamma\delta$ T cells requires their activation and expansion as they comprise only a small percentage of circulating T cells. Interleukin-2 (IL-2) and activating CD3 antibody (OKT3), commonly used for the propagation of $\alpha\beta$ T cells directly from peripheral blood mononuclear cells (PBMC), do not reliably expand $\gamma\delta$ T cells without further manipulation and so alternative approaches are needed. Aminobisphosphonates, e.g., Zoledronic Acid (Zol), used in the treatment of bone-related diseases, e.g., osteoporosis, resulted in *in vivo* propagation of $\gamma\delta$ T cells, and the use of aminobisphosphonates has been subsequently translated into laboratory practice to grow $\gamma\delta$ T cells *ex vivo* (Figure 1A) (42, 43). Aminobisphosphonates inhibit cholesterol synthesis and result in the accumulation of phosphoantigen intermediates in the mevalonate–CoA pathway, including IPP, a ligand for V $\gamma 9$ V δ 2 (44). However, only the V $\gamma 9$ V δ 2 T-cell subset is reactive to cells treated with phosphoantigens (45, 46). Synthetic phosphoantigens, e.g., bromohydrin pyrophosphate (BrHPP) (47) and 2-methyl-3-butetyl-1-pyrophosphate (2M3B1PP) (48), can mimic aminobisphosphonates and stimulate V $\gamma 9$ V δ 2 T cells for proliferation.

These reagents have been transitioned to the clinic for investigational treatments of cancer and HIV (Table 1) (49, 50). Six trials have evaluated the ability of aminobisphosphonates or BrHPP to generate *in vivo* expansions of V $\gamma 9$ V δ 2 T cells to fight leukemia/lymphoma (51, 52), melanoma (52), renal cell carcinoma (RCC) (52, 53), hormone-refractory prostate cancer (HRPC) (54), breast cancer (55), and HIV (56). These trials established safety of large V $\gamma 9$ V δ 2 T cell expansions *in vivo* and generated a total of nine objective responses (11.3%; *N* = 80) but no complete responses (CR) as anti-tumor therapies. Six clinical trials have used either Zol, BrHPP, or 2M3B1PP to expand autologous V $\gamma 9$ V δ 2 T cells *ex vivo* and these cells were directly infused (three trials with added IL-2 infusion and three without) for treatment of RCC (57–59), non-small cell lung cancer (NSCLC) (60, 61), and colorectal cancer (CRC) (62). Direct infusion of V $\gamma 9$ V δ 2 T cells was established as a safe regimen and a total of eight objective responses (11.3%; *N* = 71) were detected, including one CR (1.4%; *N* = 71) (62). Three trials have evaluated the combination of adoptive transfer of *ex vivo* expanded V $\gamma 9$ V δ 2 T cells followed by Zol administration to boost their *in vivo* proliferation. Multiple myeloma (63), RCC (64), and multiple metastatic tumors (melanoma, CRC, gastrointestinal tumors, ovarian cancer, breast cancer, cervical cancer, and bone cancer) (65) were treated with this combination, which was established to be safe, and four objective responses (13.8%; *N* = 29) were observed, two of which were CRs (6.9%; *N* = 29) treating intermediate-stage RCC (64) and breast cancer (65). Thus, adoptive transfer and *in vivo* expansions of V $\gamma 9$ V δ 2 T cells are safe therapeutic modalities and can result in objective clinical responses in the treatment of cancer.

**FIGURE 1 | Methodologies for expanding $\gamma\delta$ T cells ex vivo.**

(A) A generalized schematic for the use of aminobisphosphonates (Zol, zoledronic acid) or synthetic phosphoantigens (BrHPP, bromohydrin pyrophosphate; 2M3B1PP, 2-methyl-3-but enyl-1-pyrophosphate) and interleukin-2 (IL-2) to expand $\gamma\delta$ T cells from peripheral blood mononuclear cells (PBMC). (B) Plate-bound MHC class-I chain-related (MICA) and IL-2 were used to expand $\gamma\delta$ T cells from colon and ovarian tumor tissues. (C) Immobilized antibodies (Ab) were used to expand $\gamma\delta$ T cells from PBMC in three scenarios: (top) PBMC directly stimulated with anti-pan-TCR $\gamma\delta$ Ab and IL-2, (middle) PBMC depleted of CD4 and CD8 T cells followed by two rounds of stimulus with anti-CD3 Ab (OKT3), IL-2,

and IL-4, and (bottom) PBMC were depleted of non-adherent cells, stimulated with anti-CD2 Ab (S5.2), interferon- γ (IFN γ), and IL-12, then stimulated with OKT3 and IL-2. (D) Schematic for the use of artificial antigen presenting cells (aAPC) to expand $\gamma\delta$ T cells from PBMC in two scenarios: (top) PBMC was depleted of CD56 $^{+}$ NK cells then of other non- $\gamma\delta$ T cells (TCR $\gamma\delta$ /+ magnetic bead kit) so that $\gamma\delta$ T cell were isolated by “negative selection” and co-cultured recursively with aAPC, IL-2, and IL-21 for 2–3 rounds of stimulation; (bottom) PBMC was depleted of CD14 $^{+}$ monocytes and “positively selected” with TCR $\gamma\delta$ magnetic beads then co-cultured recursively with anti-TCR $\gamma\delta$ Ab-loaded aAPC, IL-2, and IL-21 for 2–3 rounds of stimulation.

Allogeneic $\gamma\delta$ T cells have also been infused but were part of heterogeneous cell populations (Table 1). Patients with acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) were treated with $\alpha\beta$ T cell-depleted hematopoietic stem cell transplant (HSCT), which resulted in 100 objective responses (65%; N = 153) with 36 durable CRs (24%; N = 153) (66–69). These complete remissions could be directly correlated to the elevated persistence of donor-derived V δ 1 cells in the peripheral blood of the patients, suggesting that these cells were involved in long-term clearance of leukemia. Increases in peripheral V δ 1 cells have also been correlated with CMV re-activation in patients with leukemia following allogeneic HSCT (40, 70). Most recently,

haploidentical PBMC were depleted of CD4 $^{+}$ and CD8 $^{+}$ cells using magnetic beads and were administered to patients with refractory hematological malignancies followed by Zol and IL-2 infusions (71). Three of the four patients treated experienced short-lived CRs (2, 5, and 8 months) and the other patient died of infection 6 weeks after treatment. Expansion of $\gamma\delta$ T cells was observed the week after treatment suggesting that they may have directed the anti-tumor response. Currently, clinical trials of direct infusion of activated, homogenous populations of V δ 1 cells, or other non-V δ 9V δ 2 cells have yet to be undertaken but hold promise as future avenues of medical intervention.

Table 1 | Clinical responses from $\gamma\delta$ T cells.

| Year | Treatment | Disease (N) | Total (N) | OR (%) | CR (%) | Reference |
|------|--|--|-----------|---------------|--------------|-----------|
| 1996 | Allogeneic HSCT depleted of $\alpha\beta$ T cells with TBI | ALL AML CLL | 74 | 43/74 (58%) | 25/43 (58%) | (68) |
| 2003 | Pamidronate and IL-2 | MM (8) FCL (4) CLL (4) MZL (2) IC (1) | 19 | 3/19 (16%) | 0/19 (0%) | (51) |
| 2007 | Zol vs. Zol and IL-2 | HRPC (18) | 18 | 3/18 (17%) | 0/18 (0%) | (54) |
| 2007 | 2M3B1PP-expanded autologous V δ 2 T cells and IL-2 | RCC (7) | 7 | 3/7 (43%) | 0/7 (0%) | (57) |
| 2007 | Allogeneic HSCT depleted of $\alpha\beta$ T cells | ALL (77) AML (76) | 153 | 100/153 (65%) | 36/153 (24%) | (66) |
| 2008 | BrHPP-expanded V δ 2 T cells and IL-2 | RCC (10) | 10 | 0/10 (0%) | 0/10 (0%) | (58) |
| 2009 | Zol and IL-2 | HIV (10) | 10 | N/D | N/D | (56) |
| 2009 | Zol-expanded V γ 9V δ 2 T cells, Zol, and IL-2 | MM (6) | 6 | 0/6 (0%) | 0/6 (0%) | (63) |
| 2010 | Zol-expanded V γ 9V δ 2 T cells | NSCLC (10) | 10 | 0/10 (0%) | 0/10 (0%) | (60) |
| 2010 | Zol and IL-2 | Breast cancer (10) | 10 | 1/10 (10%) | 0/10 (0%) | (55) |
| 2010 | BrHPP-expanded V δ 2 T cells and IL-2 | RCC (18) GI-cancer (4) CRC (3) Breast cancer (2) EOC (1) | 28 | 0/28 (0%) | 0/28 (0%) | (59) |
| 2011 | Zol-expanded V γ 9V δ 2 T cells | NSCLC (15) | 15 | 0/10 (0%) | 0/10 (0%) | (61) |
| 2011 | BrHPP-expanded V δ 2 T cells, Zol, and IL-2 | RCC (11) | 11 | 1/11 (9%) | 1/11 (9%) | (64) |
| 2011 | Zol and IL-2 | RCC (12) | 12 | 0/12 (0%) | 0/12 (0%) | (53) |
| 2011 | Zol-expanded V γ 9V δ 2 T cells and Zol | Melanoma (7) CRC (3) GI-cancer (2) EOC (2) Breast cancer (2) Cervical cancer (1) Bone cancer (1) | 18 | 3/12 (25%) | 1/12 (8%) | (65) |
| 2012 | Zol and IL-2 | RCC (7) Melanoma (6) AML (8) | 21 | 2/21 (10%) | 0/21 (0%) | (52) |
| 2013 | Zol-expanded V γ 9V δ 2 T cells | CRC (6) | 6 | 5/6 (83%) | 1/6 (17%) | (62) |
| 2014 | CD4/CD8-depleted haploidentical PBMC, Zol, and IL-2 | T-NHL (1) AML (1) SPL (1) MM (1) | 4 | 3/4 (75%) | 3/4 (75%) | (71) |

A survey was taken of clinical trials that reported the use of aminobisphosphonates, synthetic phosphoantigens, direct infusion of ex vivo expanded $\gamma\delta$ T cells, combinations of aminobisphosphonates/synthetic phosphoantigens/ex vivo expanded $\gamma\delta$ T cells, and allogeneic transplants containing $\gamma\delta$ T cells. The year reported is the year of publication. The total number (N) of each disease treated and overall patients treated with each regimen are reported. Overall responses (OR) and complete responses (CR) from these reports are listed as numbers of patients responding over total patients with frequencies of response below. The OR was pooled partial and complete responses by RECIST (when applicable and reported) or by disease-free progression (when RECIST was not applicable or reported). References to the clinical trials are included in the far right column.

EX VIVO PROPAGATION OF NON-V γ 9V δ 2 $\gamma\delta$ T CELLS

Populations of $\gamma\delta$ T cells outside of the V γ 9V δ 2 subset have been grown with immobilized TCR $\gamma\delta$ agonists. Plate-bound recombinant MICA and IL-2 were used to sustain the proliferation of $\gamma\delta$ T-cell cultures *ex vivo* from epithelial ovarian cancer and CRC tumor infiltrating lymphocytes (TILs) and resulted in high frequencies of V δ 1 cells (**Figure 1B**) (72). In addition, plate-bound pan-TCR $\gamma\delta$ -specific antibody and IL-2 led to proliferation of both V δ 2 and V δ 1 cells (V δ 2 >> V δ 1) from peripheral blood derived from both healthy donors and patients with lung cancer or lymphoma (**Figure 1C, top**) (73, 74). Similarly, OKT3 has been used in combination with IL-2 and IL-4 to stimulate CD4/CD8-depleted T cells from healthy peripheral blood, which resulted in expansion of V δ 2 and V δ 1 cells (V δ 2 > V δ 1), albeit with reduced cell numbers compared to the TCR $\gamma\delta$ monoclonal antibody (mAb)-stimulated cells (**Figure 1C, middle**) (75). A more complex cocktail of cytokines [IL-2, IL-12, and Interferon- γ (IFN γ)] has also been used with OKT3 and CD2-specific antibodies to expand $\gamma\delta$ T cells, but the V δ repertoires were not reported (**Figure 1C, bottom**) (76). Transition of these immobilized antigens and antibodies into clinical manufacture will streamline the application of these expansion strategies for $\gamma\delta$ T cells and could be the source of clinical trials with non-V γ 9V δ 2 cells.

Highly polyclonal $\gamma\delta$ T cells have been generated through co-culture of patient or healthy donor $\gamma\delta$ T cells with irradiated artificial antigen presenting cells (aAPC), IL-2, and IL-21 (77–80). The aAPC (clone#4) are derived from the chronic myelogenous leukemia (CML) cell line K562 following genetic modification with T-cell co-stimulatory molecules (CD86 and CD137L), Fc receptors for antibody loading (introduced CD64 and endogenous CD32), antigens (CD19), and cytokines (a membrane-bound IL-15), and have been produced as a master cell bank (MCB) (81). This MCB is currently used in the production of $\alpha\beta$ T cells for cancer treatments in clinical trials at MD Anderson (NCT01653717, NCT01619761, NCT00968760, and NCT01497184) (79, 82, 83). γ -irradiation of aAPC prior to co-culture with T cells subjects the aAPC to death (typically at or within 3 days) thereby reducing the risk for unintended transfer of this tumor cell line into recipients (83). Deniger et al. demonstrated that circulating $\gamma\delta$ T cells, containing a polyclonal TCR $\gamma\delta$ repertoire, could be isolated from healthy donor venipuncture or umbilical cord blood by “unlabeled/negative” magnetic bead selection and recursively stimulated with irradiated aAPC, IL-2, and IL-21 (**Figure 1D, top**). The aAPC-expanded $\gamma\delta$ T cells proliferated to numbers sufficient for clinical use while maintaining the expression of most TRDV and TRGV alleles and demonstrating TCR δ surface expression of V δ 1 > V δ 1^{neg}V δ 2^{neg} > V δ 2 (77). These polyclonal $\gamma\delta$ T-cell cultures displayed broad tumor reactivity as they were able to lyse leukemia, ovarian cancer, pancreatic cancer, and colon cancer cells. Separation of the polyclonal cultures by TCR δ surface expression showed that each T-cell subset had anti-tumor reactivity and that a polyclonal $\gamma\delta$ T-cell population led to the superior survival of mice with established ovarian cancer xenografts. Propagation of V δ 1^{neg}V δ 2^{neg} cells had not been previously achieved and this was the first evidence of the functional activity of this $\gamma\delta$ T-cell sub-population. In a similar study, Fisher et al. isolated polyclonal $\gamma\delta$ T cells from PBMC of healthy donors or

patients with neuroblastoma by first depleting monocytes followed by “positive/labeled” selection with anti-TCR $\gamma\delta$ -hapten antibody and anti-hapten microbeads (**Figure 1D, bottom**) (79). This study made use of the Fc receptors on the aAPC surface to load anti-TCR $\gamma\delta$ antibody where isolated $\gamma\delta$ T cells were co-cultured with the antibody-loaded aAPC. These expanded $\gamma\delta$ T cells expressed multiple TRDV and TRGV alleles with surface TCR δ expression of V δ 2 > V δ 1 > V δ 1^{neg}V δ 2^{neg}. Using this mode of expansion, V δ 1 and V δ 2 were mediators of antibody-independent (AIC) and antibody-dependent cellular cytotoxicity (ADCC), respectively, to neuroblastoma tumor cells (as predicted by whether or not they expressed Fc receptor CD16). aAPC-expanded polyclonal $\gamma\delta$ T cells could be used for anti-tumor therapies because aAPC are currently available as a clinical reagent. However, human application of aAPC/mAb-expanded $\gamma\delta$ T cells could depend on interest in the use of the current MCB of aAPC, generation of new MCB of aAPC at institutions where there are currently none, and production of $\gamma\delta$ T cell agonistic antibodies in good manufacturing practice (GMP) conditions. Clinical testing of these cells could potentially lead to more widespread acceptance and use of $\gamma\delta$ T cells as adoptive cellular therapies.

Given that the aAPC can sustain the proliferation of non-V γ 9V δ 2 cells to large quantities, there is opportunity for clinical translation, laboratory testing of subsets to elucidate their functions, and correlative studies. A limiting factor in studying $\gamma\delta$ T cells has been the lack of TCR δ and TCR γ isotype-specific antibodies outside of specificity for TCR δ 1, TCR δ 2, TCR γ 9, and TCR γ 3 (where commercially available). Mice can now be immunized to generate mAb specific for desired TCR $\gamma\delta$ isotypes where commercial and academic use of these detection antibodies can have tangible outcomes, including diagnostic and/or prognostic profiling of $\gamma\delta$ T cells resident within tumors. $\gamma\delta$ T-cell clones could be generated through co-culture of single $\gamma\delta$ T cells with aAPC, and this can facilitate studies to determine V δ /V γ pairing, corresponding TCR $\gamma\delta$ ligands, and pathogenic reactivity. The ligands on the K562-derived aAPC that TCR $\gamma\delta$ binds are not currently known. Likely candidates include IPP and MICA/B for TCR δ 2 and TCR δ 1, respectively (22, 35). Elucidation of these interactions could assist attempts to tailor the design of the aAPC for total $\gamma\delta$ T-cell expansion, propagation of a particular $\gamma\delta$ T-cell lineage, or polarization toward a certain $\gamma\delta$ T-cell phenotype (84). As an example, CD27^{neg} and CD27⁺ $\gamma\delta$ T cells are associated with IL17 and IFN γ production, respectively (85–87), leading to the conclusion that expression of CD70, the CD27 ligand, on aAPC could potentially polarize these T cells toward a desired cytokine output. Thus, aAPC could be an excellent source for the study of fundamental $\gamma\delta$ T-cell immunobiology and could yield answers not currently accessible because of limited starting cell numbers and ineffective polyclonal expansion protocols.

GENETIC MODIFICATION OF $\gamma\delta$ T CELLS FOR THERAPEUTIC USE

$\gamma\delta$ T cells are also amenable to genetic modification allowing for the introduction of genes to improve their therapeutic function. For instance, re-directed specificity of T cells can also be accomplished through the introduction of recombinant TCRs with defined antigen specificity. The conventional thought is

that transfer of TCR α /TCR β genes into $\gamma\delta$ T cells or transfer of TCR γ /TCR δ genes into $\alpha\beta$ T cells would not cause mis-pairing with the TCR α /TCR β and TCR γ /TCR δ heterodimers, thereby mitigating the risk of generating inappropriate pairings such as TCR α /TCR δ , TCR α /TCR γ , TCR β /TCR γ , or TCR β /TCR δ heterodimers with unknown specificity (88). This mis-pairing hypothesis was modeled in mice with the ovalbumin-specific $\alpha\beta$ TCR OT-I, which resulted in re-directed specificity of murine $\gamma\delta$ T cells toward ovalbumin peptide, but whether or not the TCRs were actually mis-paired was not reported (89). V γ 2V δ 2 cells have been expanded with 2M3B1PP and infected with γ -retrovirus to transduce TCR $\alpha\beta$ chains with specificity toward MAGE-A4 peptide, but co-transduction with CD8 was required in order to transfer significant MHC Class-I-restricted recognition of MAGE-A4 peptide-pulsed tumor cells (90, 91). Similar studies have transferred $\alpha\beta$ TCRs specific for CMV pp65 peptide or minor histocompatibility antigens into $\gamma\delta$ T cells rendering them reactive to antigen-appropriate tumor cells (92). In contrast to the above reports of introducing $\alpha\beta$ TCRs into $\gamma\delta$ T cells, the V γ 9V δ 2 TCR has been transferred into $\alpha\beta$ T cells and rendered both CD4 $^+$ and CD8 $^+$ T cells reactive to multiple tumor cell lines (93). Chemotherapy (temozolomide)-resistant $\gamma\delta$ T cells have been generated by lentiviral transduction of (6)-alkylguanine DNA alkyltransferase into V γ 9V δ 2 cells expanded on Zol (94). Chimeric antigen receptors (CARs) can be introduced into T cells and re-direct the T cell toward a specific antigen. CARs are formed by fusing a single chain antibody to one or more T-cell intracellular signaling domains, e.g., CD3 ζ , CD28, and/or CD137 (95). The antibody confers specificity through its variable regions toward a particular antigen, e.g., CD19, GD $_2$, HER2, etc., and CAR binding to the antigen transmits intracellular T-cell signals for antigen-dependent proliferation, cytokine production, and cytotoxicity (96, 97). Following expansion on Zol, V γ 9V δ 2 cells were efficiently transduced to express CD19- and GD $_2$ -specific CARs with γ -retroviral vectors and displayed re-directed specificity toward CD19 $^+$ and GD $_2^+$ tumor targets, respectively (98). Zol and γ -retroviruses engineered to transduce CD19- and GD $_2$ -specific CARs are available for human application, but have not been combined in a clinical trial to date. Thus, subsets of $\gamma\delta$ T cells are amenable to viral gene transfer to improve their therapeutic impact.

In contrast to γ -retroviruses and lentiviruses, which require cell division for efficient transduction, non-viral *Sleeping Beauty* (SB) transposition transfers genes into quiescent T cells and allows manipulation of cells that are difficult to culture *ex vivo* (99–102). SB transposase enzyme was originally derived from fish that were undergoing active transposition in their evolutionary maturation and was adapted for human application (103). In short, a DNA transposon with flanking inverted repeats and direct repeats is ligated into the human genome at TA dinucleotide repeats by the SB transposase enzyme (104). TA dinucleotide repeats are widely distributed in the human genome, yielding potential for random integration into the genome, and have been shown to be safe in regards to transgene insertion in pre-clinical studies (99, 101, 105). This is of particular importance in gene therapy as inappropriate integration at gene start sites or promoters, within exons, or even distal to genes within enhancers or repressors may cause cellular

transformation. Lentiviruses and γ -retroviruses have higher efficiency in transgene delivery than SB, but these vectors are known to integrate near genes or within genes (97). Application of SB to human clinical-grade T cells has been reduced to practice as a two DNA plasmid system, where one plasmid contains the SB transposon with the transgene of interest, e.g., CAR, and the other plasmid encodes a hyperactive SB transposase (106). Electro-transfer of the DNA plasmids by nucleofection into circulating (quiescent) PBMC results in transient expression of SB transposase that then ligates the transposon into the genome using a “cut-and-paste” mechanism. As soon as the SB transposase mRNA is degraded translation of SB transposase protein is halted, thereby negating additional transposition events. T cells with stable CAR expression can be encouraged through the co-culture of T cells on irradiated aAPC that express antigen for the CAR (83). This process, originally developed for $\alpha\beta$ T cells, has been adapted for expression of CAR in $\gamma\delta$ T cells (78). Resting PBMCs were electroporated with CD19-specific CAR transposon and SB11 transposase plasmids and sorted the following day to deplete non- $\gamma\delta$ T cells with magnetic beads from the transfected mixture. Isolated $\gamma\delta$ T cells were recursively stimulated with CD19 $^+$ aAPC along with IL-2 and IL-21, which resulted in the outgrowth of CAR $^+$ $\gamma\delta$ T cells with a highly polyclonal TCR $\gamma\delta$ repertoire. Endogenous leukemia reactivity by the aAPC-expanded $\gamma\delta$ T cells was improved through expression of CD19-specific CAR rendering these T cells bi-specific through CAR and TCR $\gamma\delta$. SB transposon and transposase are available as clinical reagents; therefore, clinical trials can test the safety and efficacy of bi-specific CAR $^+$ $\gamma\delta$ T cells.

CONCLUDING REMARKS

Given that $\gamma\delta$ T cells are unlikely to cause graft-versus-host disease (GVHD) because their TCR ligands (IPP, MICA, etc.) are not MHC-restricted, $\gamma\delta$ T cells (with or without genetic modification) could be generated from healthy donors in a third party manufacturing facility and given in the allogeneic setting as an “off-the-shelf” therapeutic. Additionally, a “universal” bank of polyclonal $\gamma\delta$ T cells could be established that was known to have high anti-tumor immunity or contain a particular set frequency of V δ 1, V δ 2, and V δ 1 neg V δ 2 neg populations to achieve superior efficacy (66). This could have specialized application in cases where T cells were difficult to manufacture, e.g., high tumor burden in blood or after extensive systemic (lymphodepleting) chemotherapy. Polyclonal $\gamma\delta$ T cells could also be used as front-line therapy before addition of HSCT, CAR $^+$ T cells, TILs, etc. in order to prime the tumor microenvironment for other adaptive immune cells with broader tumor specificity or to reveal neo-tumor antigens, including somatic non-synonymous mutations expressed only in the tumor (107–109). If immunity is restored in the recipients then the 3rd party $\gamma\delta$ T-cell graft may be rejected, but there may still be a therapeutic window before this occurs. Both pro-tumor and anti-tumor effects of $\gamma\delta$ T cells infiltrating the tumor microenvironment have been described (110, 111), and whether or not these cells could be useful for therapy could be delineated following expansion of $\gamma\delta$ T cells from solid tumors on aAPC, which have been shown to expand TIL ($\alpha\beta$ T cells) from metastatic melanoma (112). Tumor lysis by $\gamma\delta$ T cells could lead to other resident cell types, e.g., NK cells, macrophages, $\alpha\beta$ T cells, etc., to have renewed

reactivity to the malignancy (113). Indeed, B-ALL cell lines coated with mAb were lysed by CD16⁺ V γ 9V δ 2 cells via ADCC, and subsequently the V γ 9V δ 2 had antigen presenting cell function to generate antigen-specific CD8⁺ $\alpha\beta$ T cell responses to known B-ALL peptides, e.g., PAX5 (114, 115). Unknown is whether $\gamma\delta$ T cells will be subjected to inhibition by regulatory T cells or other immunosuppressive forces. Some $\gamma\delta$ T cells have been reported to have immunosuppressive function, and it would be of interest to identify these cells and eliminate them from the adoptive T-cell product prior to infusion (116). In summary, administration of graded doses of autologous and allogeneic, even 3rd party, $\gamma\delta$ T cells in humans have tested and will continue to evaluate the ability of these lymphocytes to home and recycle effector function in the tumor microenvironment. Given the development of aminobisphosphonates, synthetic phosphoantigens, immobilized antigens, antibodies, and designer clinical-grade aAPC, it now appears practical to sculpt and expand $\gamma\delta$ T cells to achieve a therapeutic effect.

AUTHOR CONTRIBUTIONS

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Monitoring circulating $\gamma\delta$ T cells in cancer patients to optimize $\gamma\delta$ T cell-based immunotherapy

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The success of $\gamma\delta$ T cell-based immunotherapy, where the cytotoxic activity of circulating $\gamma\delta$ T lymphocytes is activated by nitrogen-containing bisphosphonates (n-BP), or possibly by bispecific antibodies or the combination of both, requires a profound knowledge of patients' $\gamma\delta$ T cells. A possible influence of radio- or chemotherapy on $\gamma\delta$ T cells as well as their reported exhaustion after repetitive treatment with n-BP or their lack of response to various cancers can be easily determined by the monitoring assays described in this perspective article. Monitoring the absolute cell numbers of circulating $\gamma\delta$ T cell subpopulations in small volumes of whole blood from cancer patients and determining $\gamma\delta$ T cell cytotoxicity using the Real-Time Cell Analyzer can give a more comprehensive assessment of a personalized tumor treatment. Possible future directions such as the combined usage of n-BP or phosphorylated antigens together with bispecific antibodies that selectively target $\gamma\delta$ T cells to tumor-associated antigens, will be discussed. Such strategies induce expansion and enhance $\gamma\delta$ T cell cytotoxicity and might possibly avoid their exhaustion and overcome the immunosuppressive tumor microenvironment.

Keywords: monitoring, human, $\gamma\delta$ T cells, pancreatic ductal adenocarcinoma, bispecific antibodies, phosphorylated antigens, aminobisphosphonate

INTRODUCTION

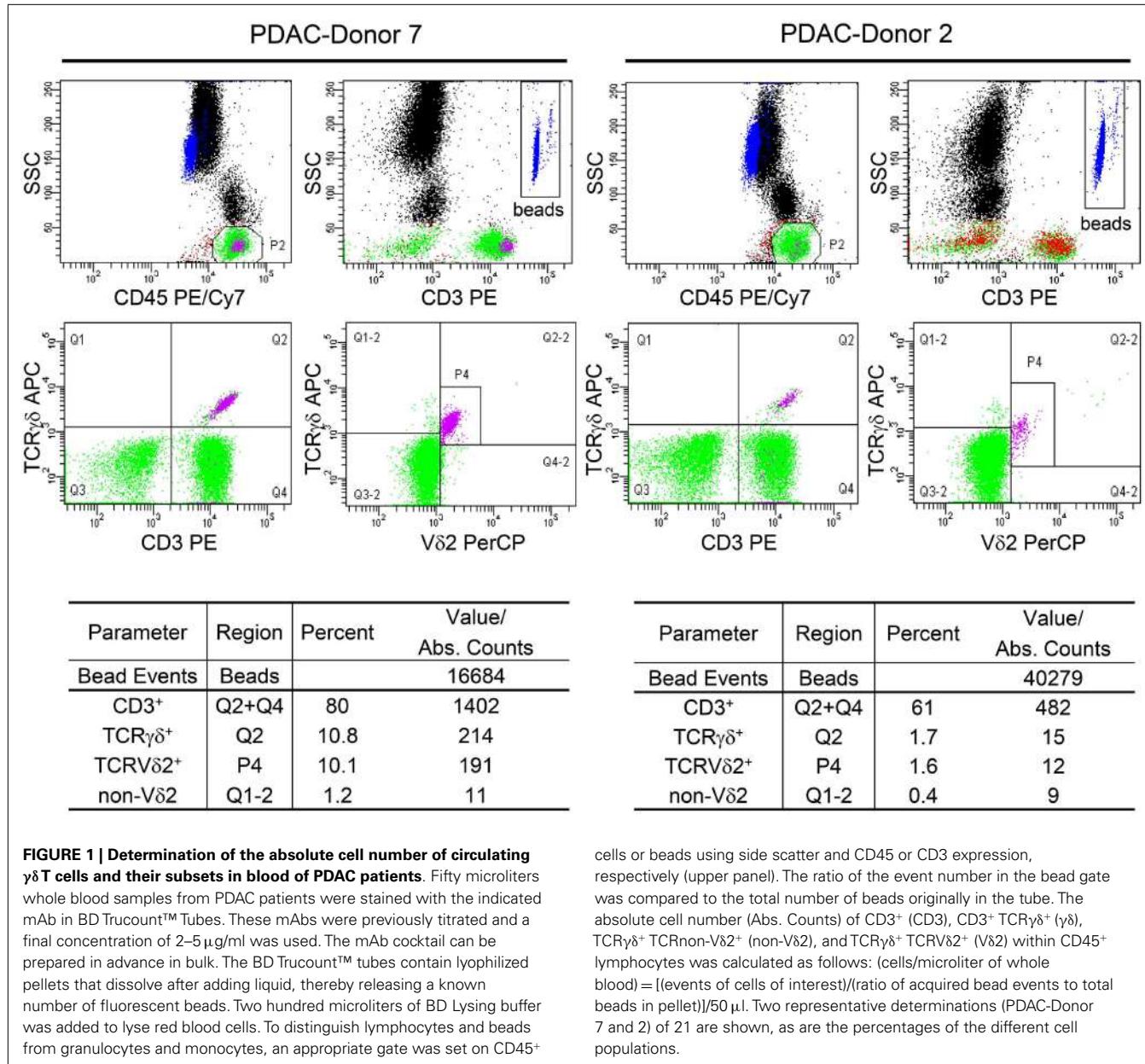
Human $\gamma\delta$ T cells ($\gamma\delta$ Tc) represent a small subset (1–10%) of CD3⁺ T lymphocytes with several unconventional features. Similar to antigen presenting cells (APC), $\gamma\delta$ Tc can phagocytose and present soluble antigens to CD3⁺ $\alpha\beta$ T cells (1, 2). Additionally, $\gamma\delta$ Tc can induce the maturation of dendritic cells (DCs), and kill various tumor cells in a HLA-independent manner (3, 4). Thus, there is a substantial interest in $\gamma\delta$ Tc in the context of T cell-based immunotherapeutic strategies (5, 6). Several pilot studies have described a partial success of $\gamma\delta$ T cell-based immunotherapy in different types of cancer after the application of amino-bisphosphonates (n-BP) or phosphorylated antigens (PAg) plus IL-2 *in vivo* or after repetitive transfer of *in vitro* expanded V82-expressing $\gamma\delta$ Tc (7–10). Although $\gamma\delta$ T cell-based immunotherapy has delivered promising results, sustained stimulation of V82 $\gamma\delta$ Tc by n-BP or PAg often leads to V82 T cell exhaustion (8, 11, 12). Additionally, a low number of functionally unresponsive $\gamma\delta$ Tc has been described in patients with chronic lymphocytic leukemia or multiple myeloma (13–15). Novel bispecific antibodies (with concomitant specificity for epitopes on both $\gamma\delta$ Tc and tumor cells) provide a tool to enhance cytotoxic activity of $\gamma\delta$ Tc against cancer cells by selectively targeting $\gamma\delta$ Tc to antigens expressed by tumor

cells (16). Additionally, independent of previous immunotherapeutic strategies and prior to the application of a $\gamma\delta$ T cell-based immunotherapy, it is mandatory to analyze the number and functional capacity of patients' $\gamma\delta$ Tc in a simple manner. This article demonstrates that the analysis of absolute cell numbers of circulating $\gamma\delta$ Tc from patients as well as the determination of the cytotoxic capacity against tumor cells of interest can give a better assessment of subsequent personalized tumor treatment.

MONITORING OF ABSOLUTE CELL NUMBERS

The monitoring system that uses the BD Multitest 6-color TBNK (M6T) Reagent with BD Trucount™ Beads (www.bd.com/resource.aspx?IDX=17743, BD Biosciences, San Jose, CA, US) allows determination of absolute cell numbers of $\alpha\beta$ T and B lymphocytes and NK cells as well as CD4⁺ and CD8⁺ T cell subsets (17, 18). Since $\gamma\delta$ T lymphocytes and their subpopulations are not detected by the M6T, we adapted $\gamma\delta$ Tc staining from the BD Trucount™ Tube technical data sheet (version 8/2010) as follows: 50 μ l whole blood from cancer patients were stained with anti-CD45-PE/Cy7 (clone HI30), CD3-PE (clone SK7) pan-TCR $\gamma\delta$ -APC (clone 11F2, customized) (all from BD Biosciences, Heidelberg, Germany), and V82-PerCP (clone B6, Biolegend, Fell, Germany) mAbs and occasionally with V81-FITC mAb (clone TS8.2, Thermo Fisher Scientific, Germany) in BD Trucount™ Tubes as described (16). After staining, red blood cells were lysed with 200 μ l BD Lysing buffer and analyzed using the FACS Canto flow cytometer and FACS Diva software (both from BD Biosciences). For two

Abbreviations: BrHPP, bromohydrin-pyrophosphate; $\gamma\delta$ Tc, $\gamma\delta$ T cells; mAb, monoclonal antibody; n-BP, nitrogen-containing bisphosphonate; PAg, phosphorylated antigens; PDAC, pancreatic ductal adenocarcinoma; RTCA, real-time cell analyzer; TCR, T cell-antigen receptor.



representative donors, the absolute numbers of total $\gamma\delta$ Tc as well as V $\delta 2$ and non-V $\delta 2$ subsets are shown (Figure 1). Moreover, cells can be stained with anti-V $\delta 1$ mAb labeled with an additional fluorochrome (data not shown).

Certainly, other bead-based detection systems could be used alternatively to determine absolute cell numbers. Importantly, however, these strategies must allow this determination from a small volume of patient's blood.

In addition, a possible influence of radio- or chemotherapy on circulating immune cell numbers can be easily determined by this monitoring system. For instance, our own data reveal that the absolute number of V $\delta 2$ $\gamma\delta$ Tc in a cohort of 10 breast cancer patients receiving chemotherapy did not differ from age-matched breast cancer patients without treatment (Adam-Klages et al.,

unpublished data). Moreover, in a cohort of 41 patients with pancreatic ductal adenocarcinoma (PDAC, stage pT3–4, pN0–1, L0–1 and V0–1), we recently observed that the decrease in absolute numbers of V $\delta 2$ $\gamma\delta$ Tc did not correlate with cancer stage/progression, but rather with patient age (16).

While determination of the absolute $\gamma\delta$ T cell numbers and that of their subsets provides no information about their cytotoxic capacity, this can be addressed in an additional functional assay.

DETERMINATION OF CYTOTOXIC CAPACITY

We recently examined the functional capacity of $\gamma\delta$ Tc from patients with PDAC (16). PDAC is a highly aggressive gastrointestinal malignancy characterized by the presence of

desmoplastic stromal microenvironment where conventional treatment approaches including surgery, chemotherapy, and/or radiation are often not effective (19). The observed decrease in absolute V82 T cell numbers in untreated patients with advanced PDAC is attributable to age, not disease status, as similar numbers were found in age-matched healthy controls (16). In an attempt to avoid V82 T cell exhaustion through repetitive n-BP stimulation and overcome the immunosuppressive activity of PDAC stromal cells on cytotoxic $\gamma\delta$ T cells, novel bispecific antibodies such as [Her2xCD3] and [(Her2)₂xV γ 9] were designed. [(Her2)₂xV γ 9] is specific for V γ 9 on $\gamma\delta$ Tc (associated with V82) and for human epidermal growth factor receptor HER2/neu overexpressed on PDAC, breast, and prostate cancer cells. The [(Her2)₂xV γ 9] tribody design allows monovalent binding to $\gamma\delta$ Tc and bivalent HER2-targeting, which enhances avidity to the tumor cell and thereby increases cytolytic activity. Both bispecific antibodies selectively target $\gamma\delta$ Tc to tumor antigens, thereby enhancing the cytotoxic activity of $\gamma\delta$ Tc *in vitro* as well as *in vivo* in a PDAC grafted SCID-Beige mouse model (16).

In previous studies, we usually examined the functional capacity of $\gamma\delta$ T cell lines or freshly isolated $\gamma\delta$ Tc. Aiming to simplify handling of cells from patients with a low $\gamma\delta$ T cell number in the following experiments, we investigated the functional capacity of cytotoxic $\gamma\delta$ Tc within PBMC. We observed that the functional cytotoxic activity of circulating $\gamma\delta$ Tc from patients can be determined in as few as of 1–2 $\times 10^6$ PBMC, readily obtainable from 2 to 4 ml of patients' blood. We analyzed blood from 21 patients with PDAC after obtaining their informed consent and relevant institutional review board approvals (code number: D401/14). As a read out system for cytotoxic activity of $\gamma\delta$ Tc within freshly isolated PBMC, the real-time cell analyzer (RTCA) single-plate system (ACEA, San Diego, CA, USA) was used. RTCA measures the impedance of adherent tumor cell monolayers, but not of suspended cells such as PBMCs with electronic sensors. The measurement of impedance in arbitrary cell index units reflects changes in cellular parameters of tumor cells, which allows monitoring of cellular events in real time without the incorporation of labels over time periods of several days. The loss of impedance correlates with the $\gamma\delta$ T cell-mediated lysis of tumor cells (16). A further advantage of measuring impedance over an extended time is that it enables us to observe whether tumor cells can regenerate when lysis is incomplete.

To ensure adherence of tumor cells, PDAC cells were cultured for 24–27 h in RTCA plates before the addition of $\gamma\delta$ Tc alone with or without additional substances. Thereafter, PDAC cells were still cultured alone or together with PDAC patient-derived PBMC in (i) medium, (ii) PAg such as bromohydrin-pyrophosphate (BrHPP), or (iii) [(Her2)₂xV γ 9]. During the extended time course, we observed that $\gamma\delta$ Tc within PBMC required almost 24–36 h after initial stimulation to exert their cytotoxic capacity (**Figure 2A**, red arrow with a star). Moreover, we observed that [(Her2)₂xV γ 9] triggered tumor cell lysis more efficiently than PAg in 30% of PDAC patient samples (**Figure 2A**, responder), while neither substance was effective in 70% of patient samples (**Figure 2A**, non-responder). The unexpected cytotoxicity against PDAC cells in the absence of a stimulus (medium, orange line) is likely due to the reactivity of NK cells in the presence of IL-2 (**Figure 2A**), or

because additional experiments with untouched, freshly isolated $\gamma\delta$ Tc demonstrated that cytotoxic activity of $\gamma\delta$ Tc is not induced by IL-2 alone (16).

Regarding the absolute V82 T cell numbers presented in **Figure 2B** (table), we correlated the unresponsiveness of the majority of the tested patient samples [negative [(Her2)₂xV γ 9] reactivity] with their low initial V82 T cell number. PBMC from patients with more than 30 V82 $^{+}$ $\gamma\delta$ Tc/ μ l blood were responsive (responder in **Figure 2A** and "positive" in **Figure 2B**), whereas in samples with <30 V82 $^{+}$ $\gamma\delta$ Tc/ μ l blood, no induction of cytotoxic activity to PAg or [(Her2)₂xV γ 9] stimulation was observed (non-responder in **Figure 2A** and "negative" in **Figure 2B**).

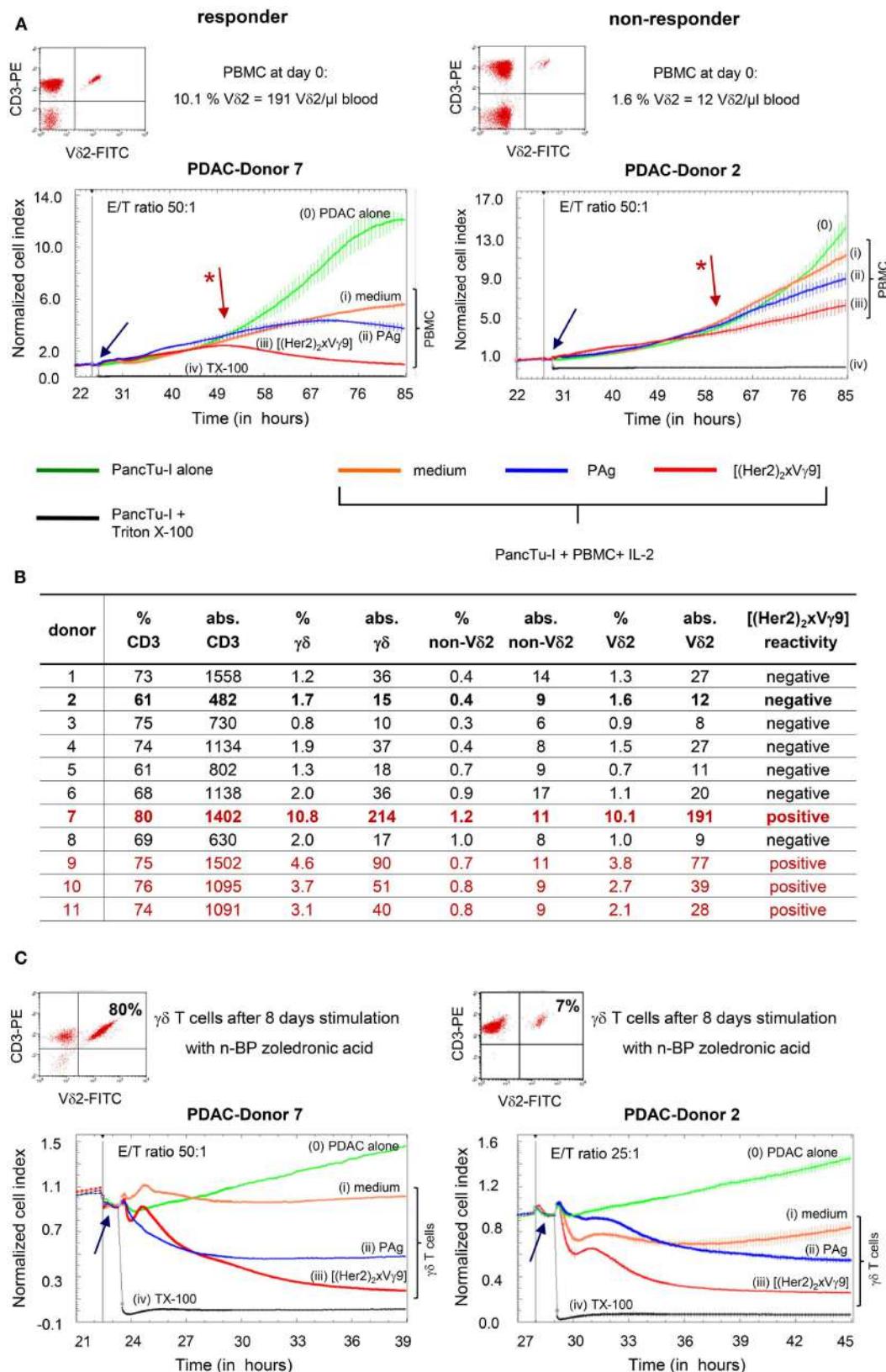
The weak capacity of bispecific antibodies to induce $\gamma\delta$ T cell proliferation could explain the observed unresponsiveness to [(Her2)₂xV γ 9]. Therefore, PBMC from the same patients were stimulated with the PAg BrHPP or, as presented in **Figure 2C**, with n-BP zoledronic acid for 7–14 days. Although the responder cells expanded to 80% $\gamma\delta$ Tc in culture, while non-responders comprised only 7% after n-BP stimulation, this small population of non-responders exhibited nearly the same degree of cytotoxicity as responders after re-stimulation with [(Her2)₂xV γ 9], despite the lower effector/target ratio (**Figure 2C**).

Taken together, our results demonstrate that prior analysis of absolute circulating cell numbers of immune cell subsets as well as determination of their cytotoxic capacity against tumor cells of interest may provide a better assessment of whether a particular personalized tumor treatment will be effective.

WHAT CAN WE LEARN FROM THIS MONITORING SYSTEM?

$\gamma\delta$ T cell monitoring can provide an estimate for a potential treatment of cancer patients. Although knowledge of the functional capacity of $\gamma\delta$ Tc within PBMC does not provide information about their migration and infiltration into the tumor, characterization of these circulating $\gamma\delta$ Tc is useful since they are activated by intravenous n-BP or PAg administration (8, 10). In clinical trials where $\gamma\delta$ Tc were repetitively activated with n-BP or PAg together with low-dose IL-2, effects on tumor growth were observed; however, this was associated with exhaustion, anergy, or depletion of $\gamma\delta$ Tc due to repetitive stimulation (8, 11, 12). In light of these observations, it is necessary to optimize cytotoxic activity, which can be achieved with bispecific antibodies such as the tribody [(Her2)₂xV γ 9]. Adoptive transfer of $\gamma\delta$ Tc with [(Her2)₂xV γ 9] and IL-2 significantly reduced growth of pancreatic tumors grafted into SCID-Beige mice in comparison to adoptively transferred $\gamma\delta$ Tc together with n-BP and IL-2 (16).

V82 $\gamma\delta$ Tc used for adoptive transfer are cells within PBMC that are initially activated with n-BP or PAg plus IL-2 (7, 20). Such initial activation with n-BP or PAg plus IL-2 causes selective V82 T cell-expansion, while [(Her2)₂xV γ 9] does not induce strong proliferation of $\gamma\delta$ Tc (unpublished data). Independently of the proliferative response of $\gamma\delta$ Tc, the cytotoxic activity of PAg or n-BP expanded V82 T cell lines can be significantly enhanced after re-stimulation with [(Her2)₂xV γ 9]. Moreover, the addition of [(Her2)₂xV γ 9] did not induce cell death of V82 T cells, in contrast to restimulation of V82 T cell lines with PAg (unpublished data). Thus, [(Her2)₂xV γ 9] provides a tool to further enhance cytotoxic activity of adoptively transferred $\gamma\delta$ Tc, whereas PAg or

FIGURE 2 | Correlation between absolute cell number and functional capacity of V δ 2 T cells.

(Continued)

FIGURE 2 | Continued

(A) Flow cytometric analysis of CD3 $^{+}$ V82 $^{+}$ $\gamma\delta$ Tc within PBMC, and RTCA of PBMC from two representative donors (Donors 7 and 2) of 21; **(B)** list of the relative and absolute numbers (abs.) of CD3, $\gamma\delta$, V82, and non-V82 T cells in whole blood from 11 representative PDAC patients out of 21 as well as reactivity to the tribody; **(C)** flow cytometric analysis of selective expansion of CD3 $^{+}$ V82 $^{+}$ $\gamma\delta$ Tc after PAg-activation within PBMC for 8 days, and RTCA with these short-term expanded $\gamma\delta$ Tc from Donors 7 and 2. Two representative donors of 21 are shown. **(A,C)** For RTCA, 5 \times 10 3 PDAC cells (PancTu-I) were cultured in 10% FCS RPMI medium for 24–27 h on an E-plate covered at the bottom with electronic sensors that measure the impedance of the cells expressed as an arbitrary unit called cell index (CI). The CI was analyzed every 5 min to determine adherence and thus cell growth. Since the initial adherence in different wells can differ slightly, the CI

was normalized to 1 shortly before the time of addition of suspended cells \pm substances (vertical black line). After 24–27 h, PDAC cells were treated again with medium [green line (0)] or with PBMC **(A)** or short-term expanded $\gamma\delta$ Tc **(C)** together with medium [orange line (i)], 300 nM PAg BrHPP [dark blue line (ii)], or 1 μ g/ml [(Her2) $_{2x}V\gamma9$] [red line (iii)] at the indicated E:T ratio over the indicated time. As a positive control for maximal lysis, PDAC cells were treated with Triton X-100 [TX-100, black line (iv)]. The addition of substances, PBMC or expanded $\gamma\delta$ Tc is indicated by the blue arrow. CI was then measured every minute for analysis of precise cytotoxicity time point for >15 to 55 h as indicated. The loss of tumor cell impedance and thus a decrease of the Normalized CI correlates with $\gamma\delta$ T cell-mediated lysis of PDAC cells. The red arrow with the * points out the initiation of cytotoxicity. The average of triplicates and standard deviation were calculated; one representative experiment is shown.

n-BP failed because they induce cell death in almost half of the activated cells (unpublished data).

The observation that the majority of elderly people has a low frequency of $\gamma\delta$ Tc hampers the expansion of autologous $\gamma\delta$ Tc required for adoptive transfer. Considering these challenges, one might suggest adoptively transferring allogeneic or haploidentical $\gamma\delta$ Tc from (younger) healthy donors or activating $\gamma\delta$ Tc within PBMC *in vivo* with bispecific antibodies (21–23). To investigate the effect of bispecific antibodies on unstimulated $\gamma\delta$ Tc, we monitored whether [(Her2) $_{2x}V\gamma9$] can induce cytotoxic activity in $\gamma\delta$ Tc within PBMC. As described above, no or weak responses to [(Her2) $_{2x}V\gamma9$] were obtained with PBMC from PDAC donors with a lower frequency of V82 $\gamma\delta$ Tc (non-responder), whereas PBMC with a higher V82 $\gamma\delta$ T cell frequency responded to [(Her2) $_{2x}V\gamma9$] resulting in enhanced cytotoxicity (responder) (**Figure 2A**). Interestingly, n-BP- or PAg-mediated enrichment of non-responder $\gamma\delta$ Tc within PBMC for 7–14 days led to enhanced cytotoxic activity after restimulating the cells with [(Her2) $_{2x}V\gamma9$] (**Figure 2C**).

The validity of this monitoring system to determine $\gamma\delta$ T cell-reactivity within PBMC needs to be confirmed in patients undergoing $\gamma\delta$ T cell-targeting therapy. Based on our experience, one might suggest initially administration of n-BP together with IL-2 in cancer patients to induce proliferation of V82 $\gamma\delta$ Tc followed by treatment with bispecific antibodies engaging $\gamma\delta$ Tc plus IL-2 in order to avoid the V82 T cell exhaustion observed in patients mediated by repetitive application of n-BP plus IL-2.

WHAT ARE THE BENEFITS OF COMBINING $\gamma\delta$ T CELL-BASED IMMUNOTHERAPY WITH BISPECIFIC ANTIBODIES?

Therapeutic antibodies such as rituximab (anti-CD20 mAb) and trastuzumab or pertuzumab (both anti-HER2 mAb) as well as different combined therapies have clearly improved the treatment outcome of patients with B-cell lymphoma or breast cancer, respectively (24, 25). Furthermore, combining these therapeutic antibodies with $\gamma\delta$ T cell-based immunotherapy seems very promising. Rituximab enhanced cytotoxic activity of *ex vivo* expanded CD16 $^{+}$ (Fc γ III) $\gamma\delta$ Tc against CD20 $^{+}$ chronic lymphocytic leukemia, while Trastuzumab increased $\gamma\delta$ T cell cytotoxicity against HER2 $^{+}$ breast cancer cells (26).

The success of such therapeutic antibodies has inspired antibody engineers to improve the antibody efficacy. One promising approach to enhance cytotoxicity and selectively target T cells to

tumor-associated antigens is based on the usage of single-chain bispecific antibody constructs. One such construct is Blinatumomab with specificity for CD19 on lymphoma or leukemia and CD3 on T cells, which has proved efficient for the treatment of patients with hematological malignancies (27). The short half-life of only a few hours in serum requires continuous intravenous infusion of Blinatumomab, which induces an almost complete molecular response and prolonged leukemia-free survival in patients with minimal residual B-lineage acute lymphoblastic leukemia (28). The favorable characteristics of bispecific antibodies such as high specificity, high cytotoxic potential, and low immunogenicity, led us to design a bispecific antibody targeted to V γ 9 instead of CD3 and to HER2 expressed on several PDAC as well as on breast and prostate cancer, which could be easily replaced by another tumor target antigen of interest.

Of course, the question arises as to what differentiates bispecific antibodies with specificity for $\gamma\delta$ Tc and those with specificity for CD3 T cells. For instance, a target group could be patients with advanced hematological malignancies (e.g., AML) who require allogeneic stem cell transplantation. A major advantage of $\gamma\delta$ T cell-based immunotherapy is the HLA-independent killing of tumor cells, thereby reducing the risk of graft-versus-host disease often caused by alloreactive CD3 $^{+}$ $\alpha\beta$ T cells (21, 22, 29, 30). A successful anti-tumor activity was described for patients with refractory hematological malignancies after adoptive transfer of haploidentical $\gamma\delta$ Tc (23). Labeling *ex vivo* expanded haploidentical $\gamma\delta$ Tc with bispecific antibodies could perhaps further enhance the cytotoxic capacity of these cells. A further advantage could be envisioned with respect to the innate lymphocyte capacity of $\gamma\delta$ Tc to phagocytose and present antigens to $\alpha\beta$ T cells, an activity that may be enhanced in the presence of a bispecific antibody. In the treatment of solid tumors, the initial administration of n-BP/IL-2 followed by infusion of bispecific antibody together with IL-2 could probably enhance cytotoxic activity of $\gamma\delta$ Tc, which infiltrate several different tumor types at low frequency.

CONCLUDING REMARKS

Bispecific antibodies have been designed in different formats. Clinical trials with bispecific antibodies such as Catumaxomab (TriomAb [EpCAMxCD3]), Ertumaxomab (Triomab [HER2xCD3]), and Blinatumomab (Bispecific T Cell Engager (BiTE) [CD19xCD3]) have delivered impressive therapeutic results. Additional clinical studies are certainly required to deeper

evaluate and improve their therapeutic potential. Bispecific antibodies with specificity for CD3 enhance the cytotoxic potential of $\alpha\beta$ as well $\gamma\delta$ T cells. However, under certain circumstances, it would be desirable to activate only $\gamma\delta$ Tc rather than a polyclonal population of T cells. For instance, CD8 $^+$ $\gamma\delta$ Tc were presented at low frequency but at higher number than CD8 $^+$ $\alpha\beta$ T cells in ductal epithelium and nearby stroma in PDAC tissues. This $\gamma\delta$ Tc accumulation suggests an important role of $\gamma\delta$ Tc in the immune response against PDAC, which is apparently suppressed by the pronounced immunosuppressive PDAC-microenvironment.

Together with the monitoring system described in this article, the tribody [(Her2) $_{2x}$ V γ 9], which selectively targets $\gamma\delta$ Tc and enhances their cytotoxic activity, provides a tool to determine the functional capacity of $\gamma\delta$ Tc within the blood or within tumor-infiltrating T lymphocytes isolated from fresh tumor tissue of tumor patients. Whether bispecific antibodies targeting $\gamma\delta$ Tc have the capacity to overcome the immunosuppressive stroma in PDAC patients, has yet to be investigated in further *in vivo* studies.

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Pathogen-specific immune fingerprints during acute infection: the diagnostic potential of human $\gamma\delta$ T-cells

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APOCALYPSE NOW: THE END OF MODERN MEDICINE AS WE KNOW IT

Gentlemen, it is the microbes who will have the last word. [Messieurs, c'est les microbes qui auront le dernier mot].
– Louis Pasteur, 1822–1895

The last 200 years have seen a dramatic reduction in the prevalence and severity of microbial infections, due to the implementation of groundbreaking measures ranging from improved sanitation and hygiene and the introduction of aseptic techniques to the development of successful vaccines and the discovery of effective antibiotics. Devastating infections that were common until the late nineteenth century such as cholera, diphtheria, plague, syphilis, tuberculosis, and typhoid came into the reach of effective control, at least in developed countries, and with a minimized risk of wound infections surgical procedures began to revolutionize modern medicine. Antibiotics, in particular, radically transformed the treatment and prevention of microbial infections and have saved millions of lives since their introduction (1). However, antibiotic usage is invariably linked to the selective pressure it exerts on the target organism to develop escape strategies (2).

We are at present witnessing how the pendulum begins to swing backwards, with anti-microbial resistances developing on an unprecedented global scale. New classes of Gram-positive and Gram-negative “superbugs” are emerging and spreading at an alarming rate, some of

which are virtually insusceptible to all available drugs (3–5). The once apocalyptic vision of a “post-antibiotic era” where common infections and minor injuries may result untreatable and eventually fatal is rapidly becoming a real possibility (1, 2, 6, 7), heralding what Margaret Chan, Director-General of the WHO, in 2012 called “the end of modern medicine as we know it.” The appearance of multidrug-resistant bacteria has been identified by the WHO, the Centers for Disease Control and Prevention (CDC) in the USA and their European counterpart, the ECDC, as one of the major global health challenges humankind is facing in the twenty-first century (8–10). According to Sally Davies, the UK Chief Medical Officer, “there are few public health issues of greater importance than anti-microbial resistance in terms of impact on society” (11).

There is now an urgent call for anti-microbial stewardship programs that aim to prescribe antibiotics more prudently, and to tailor their use to defined patient groups who will benefit most. The fact that the prevalence of resistance appears to correlate directly with antibiotic consumption across different countries (12) argues in favor of the immediate effectiveness of such tightly controlled programs. As highlighted in a recent Outlook issue in *Nature*, “the potential to save lives with faster and more targeted diagnoses, decrease unnecessary and often incorrect prescriptions, and even help identify early on where bacterial resistance could occur,

will have a drastic effect on the way patients are treated” (13).

MISSION IMPOSSIBLE: THE FUNDAMENTAL FLAWS OF CONVENTIONAL DIAGNOSIS

When it concerns the search for pathogenic organisms suspected in the diseased body, in the first instance bacteria, then during conventional microscopic examination carried out without special preparations and artifices one encounters the most substantial, at times virtually insurmountable, obstacles. [Wenn es sich nun darum handelt, die im erkrankten Körper vermuteten pathogenen Organismen, zunächst Bakterien, aufzusuchen, so begegnet man bei der gewöhnlichen und Kunstgriffe ausgeführten mikroskopischen Untersuchung den erheblichsten, stellenweise geradezu unübersteiglichen Hindernissen]. – Robert Koch, 1843–1910 (14)

More than a century after Robert Koch's landmark discovery of the causative agents of anthrax, cholera, and tuberculosis, the diagnosis of suspected infections still depends largely on the definitive identification of the likely pathogen in biological samples. However, standard microbiological culture is inefficient and slow (typically >1–2 days, for a confirmed diagnosis of tuberculosis >4 weeks), and in many cases no organism can be grown despite

Abbreviation: HMB-PP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.

clinical signs of infection, indicating that conventional diagnostic methods are not specific and/or rapid enough to target therapy (15–17). Early management of patients with acute symptoms who require immediate medical intervention, including virtually all hospital-based infections, thus remains largely empirical. As direct consequence, the fundamental uncertainty about the real cause underlying the clinical signs observed leads to inappropriate and unnecessary treatments exposing patients to drug-related side effects; raising the risk of opportunistic, chronic, or recurrent infections; and contributing to the emergence and spread of multidrug resistance (1–7). This dilemma eventually results in potentially avoidable patient morbidity/mortality, and imposes a considerable burden on health care systems and societies (8–11). There remains an unmet clinical need for rapid and accurate diagnostic tests for patients with acute infections. According to Kessel and Sharland (18), “new technology focusing on rapid diagnosis of specific bacteria and resistance genes, along with combination biomarkers indicating bacterial or viral infections, especially if adapted to near patient testing, could have a major impact on targeting appropriate antibiotic treatment.”

In order to circumvent the almost insurmountable obstacles of a rapid and accurate identification of the causative pathogen by traditional microbiological techniques, efforts are being made to utilize state-of-the-art molecular methods. Approaches based on the detection of microbial nucleic acids, cell wall constituents, or other unique features of distinct pathogens by PCR, chromatography, or mass spectrometry certainly complement culture-based tests and speed up microbial identification, yet they require considerable resources and may not be applicable to primary care or home settings (19–23). Moreover, they do not provide information about the pathogenicity of the identified species and its interaction with the host. Of note, neither microbiological nor molecular methods discriminate between pathogens causing disease, asymptomatic carriage, and sample contaminants, and thus even positive test results require extensive interpretation by the treating physician (24–26).

There is a plethora of disease-related markers that are commonly assessed by clinicians to aid a correct diagnosis, ranging from basic blood and urine parameters to indicators of tissue damage, tumor progression and autoimmunity, among others. However, there is a conspicuous paucity of biomarkers for accurate diagnosis of microbial disease. Current biomarkers of inflammation such as C-reactive protein (CRP) or procalcitonin (PCT) are often not sensitive or specific enough and are only poor surrogates for acute infections (22, 27, 28). The vast majority of research on novel diagnostics has so far focused on identifying individual factors and assessing their performance in isolation. Yet, it may come as no surprise that none of these proposed parameters have reached sufficient discriminatory power on their own, given the complex and multifactorial processes underlying local and systemic inflammatory responses to a broad range of pathogens (29, 30). As a result, neither the direct identification of the causative pathogen nor the measurement of currently used biomarkers of inflammation is sufficiently accurate or rapid for a reliable point-of-care diagnosis of acute microbial infection.

QUANTUM OF SOLACE: EXPLOITATION OF PATHOGEN-SPECIFIC HOST RESPONSES FOR NOVEL DIAGNOSTICS

The immune system appears to have originated as a set of effector cells having multiple distinct receptors that discriminate self from infectious non-self by recognition of patterns found exclusively on microorganisms. – Charles A. Janeway, Jr., 1943–2003 (31)

Key to developing better and stratified approaches to treating infection is a detailed understanding of the intricate host-pathogen relationships in disease, in order to exploit the unique sophistication of the human immune system for diagnostic and therapeutic purposes (32, 33). In a radical departure from current practice, our research is based upon the premise that each type of infection evokes a distinct pathogen-specific host response – what we refer to as “immune fingerprint.” A patient’s early anti-microbial response itself is likely to provide far more detailed

insight into the true cause and severity of acute infections than conventional methods, independently of the subsequent clinical course of the disease (34). The human immune system is a highly complex network of interdependent cellular and humoral players that has evolved over millions of years in order to survey the body for potentially hazardous structures and initiate an appropriate defense. The communication with invading micro-organisms thus occurs at multiple levels, giving rise to a plethora of biomarkers of potential relevance for diagnostic purposes. Different pathogens interact uniquely with different components of the innate immune system due to the efficient self/non-self discrimination based on conserved microbial signals such as non-methylated bacterial DNA, bacterial flagella, and cell wall constituents. These structures are typically recognized by members of the Toll-like receptor family and/or other pattern recognition receptors expressed by sentinel cells (35–37). However, there is also emerging evidence that certain types of innate or “unconventional” T-cells such as $\gamma\delta$ T-cells and mucosal-associated invariant T (MAIT) cells are able to detect common microbial metabolites through their T-cell receptors, by sensing intermediates of the non-mevalonate and riboflavin biosynthesis pathways that are unique to certain types of microorganisms (38, 39).

$V\gamma 9/V82$ T-cells represent a unique subpopulation of human T-cells (40, 41) that appears to have a particularly crucial role in contributing to immune fingerprints of diagnostic relevance (34). This is due to their exquisite responsiveness to the microbial isoprenoid precursor (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) that is produced by the majority of Gram-negative pathogens and a large proportion of Gram-positive species such as *Clostridium difficile*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*, while it is not found in other bacteria including staphylococci and streptococci as well as fungi (42–44). The rapid and sensitive response of $V\gamma 9/V82$ T-cells to a broad range of pathogens evokes Janeway’s criteria for a “pathogen-associated molecular pattern” in that HMB-PP is an invariant metabolite in many different species that is essential in the microbial physiology but absent from the human

host (43, 45). Bacterial extracts prepared from HMB-PP producing species typically activate V γ 9/V δ 2 T-cells much stronger than extracts prepared from HMB-PP deficient micro-organisms (42, 44, 46), and peripheral and/or local V γ 9/V δ 2 T-cell levels are often elevated in patients infected with defined HMB-PP producing pathogens (43, 47). Elegant proof of concept for this responsiveness comes from the demonstration that HMB-PP producing wildtype *L. monocytogenes* activate V γ 9/V δ 2 T-cells far better, both *in vitro* (48) and in primate models *in vivo* (49), than genetically engineered *L. monocytogenes* that are identical to the parental strain except for an inability to produce HMB-PP. Similarly, overexpression of HMB-PP synthase through genetic manipulation increases the stimulatory potential of bacteria such as *E. coli*, *L. monocytogenes*, *M. tuberculosis*, and *Salmonella enterica* on V γ 9/V δ 2 T-cells *in vitro* (42, 46, 48, 50, 51) and *in vivo* (52). Our own data demonstrate that even in heterogeneous patient cohorts infected with a whole spectrum of diverse bacteria,

differences in V γ 9/V δ 2 T-cell frequencies between patients with microbiologically confirmed infections caused by HMB-PP producing and HMB-PP deficient species remain apparent. This is true both for peritoneal dialysis patients with acute peritonitis as an exemplar of localized immune responses restricted to the peritoneal cavity (34, 46, 53), as well as on a systemic level in the peripheral blood of critically ill patients with severe sepsis (54). Most importantly, studies in patients with acute peritonitis suggest that a diagnostic test measuring local V γ 9/V δ 2 T-cells on the first day of presentation with acute symptoms may not only indicate the presence of Gram-negative (predominantly HMB-PP producing) bacteria but also identify patients at an increased risk of inflammation-related downstream complications (34).

The exquisite responsiveness of V γ 9/V δ 2 T-cells and other unconventional T-cells to microbial metabolites shared by certain pathogens but not by others identifies these cell types as key constituent of diagnostically relevant

immune fingerprints at the point of care. This is especially the case when V γ 9/V δ 2 T-cell levels are assessed locally and when they are combined with other powerful discriminators such as peritoneal proportions of neutrophils, monocytes, and CD4 $^{+}$ T-cells in the inflammatory infiltrate as well as intraperitoneal concentrations of certain soluble immune mediators (34) (Figure 1). Such a combination with further parameters provides additional information as to the precise nature of the causative pathogen, for instance to distinguish between immune responses induced by Gram-negative (LPS producing) and Gram-positive (LPS deficient) bacteria, and is also likely to help increase sensitivity owing to the age and gender-dependent variability of V γ 9/V δ 2 T-cell levels (55). Pathogen-specific immune fingerprints that discriminate between certain subgroups of patients (e.g., with Gram-negative vs. Gram-positive bacterial infections) can be determined within hours of presentation with acute symptoms, long before traditional culture results become available, and by guiding early patient

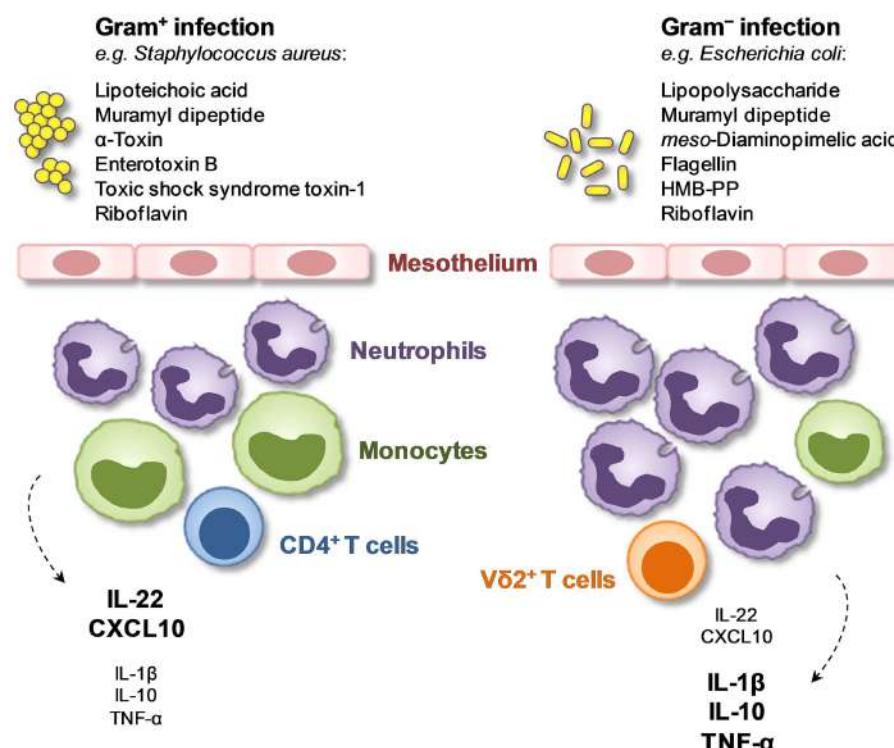


FIGURE 1 | Local immune fingerprints in peritoneal dialysis patients on the day of presentation with acute peritonitis. Shown are cellular and humoral biomarkers that are associated with the presence of Gram-positive or Gram-negative bacteria and that may be exploited for novel diagnostic tests (34).

management and optimizing targeted treatment will contribute to improving outcomes and advancing antibiotic stewardship. It remains to be investigated how much these findings on diagnostic immune fingerprints in peritoneal dialysis patients can be extended to other local or systemic scenarios to diagnose infections at the point of care, and whether they can also be applied to monitoring the course of the disease and the response to treatment.

Applied research on $\gamma\delta$ T-cells has so far focused predominantly on their use for novel immunotherapies against different types of cancers (56–58). Thirty years after the unexpected cloning of the TCR γ chain (59, 60) and 20 years after the first description of microbial “phosphoantigens” as specific activators of human V γ 9/V δ 2 T-cells (61, 62), the diagnostic potential of $\gamma\delta$ T-cells is only beginning to unfold (34, 47, 63, 64).

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$\gamma\delta$ T cells in HIV disease: past, present, and future

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Human immunodeficiency virus (HIV) type 1 dysregulates $\gamma\delta$ T cells as part of an immune evasion mechanism. Nearly three decades of research defined the effects of HIV on $\gamma\delta$ T cells and how this impacts disease. With highly effective antiretroviral therapy providing virus suppression and longer survival, we expected a return to normal for $\gamma\delta$ T cells. This is not the case. Even in patients with CD4 T cell reconstitution, normal $\gamma\delta$ T cell levels and function are not recovered. The durable damage to V δ 2 T cells is paralleled by defects in NK, CD8T cells, and dendritic cells. Whether these consequences of HIV stem from similar or distinct mechanisms are not known and effective means for recovering the full range of cellular immunity have not been discovered. These unanswered questions receive too little attention in the overall program of efforts to cure HIV this disease. Approved drugs capable of increasing V δ 2 T cell function are being tested in clinical trials for cancer and hold promise for restoring normal function in patients with HIV disease. The impetus for conducting clinical trials will come from understanding the significance of $\gamma\delta$ T cells in HIV disease and what might be gained from targeted immunotherapy. This review traces the history and current progress of AIDS-related research on $\gamma\delta$ T cells. We emphasize the damage to $\gamma\delta$ T cells that persists despite effective virus suppression. These chronic immune deficits may be linked to the comorbidities of AIDS (cancer, cardiovascular disease, metabolic disease, and others) and will hinder efforts to eradicate HIV by cytotoxic T or NK cell killing. Here, we focus on one subset of T cells that may be critical in the pathogenesis of HIV and an attractive target for new immune-based therapies.

Keywords: gamma delta T cell, HIV, V δ 1 gamma delta T cells, V γ 9 V δ 2 T cells, immunotherapy

ORIGINAL STUDIES ON HIV AND $\gamma\delta$ T CELLS

Human immunodeficiency virus (HIV) is an aggressive, lymphotropic virus known for CD4 depletion and immune suppression. In addition to killing CD4 T cells, HIV affects several other lymphocyte subsets (1) and impairs both acquired and innate immunity (2). We focus on HIV damage to $\gamma\delta$ T cells and how this is related to acute or chronic disease. The two major subsets of human $\gamma\delta$ T cells (designated V δ 1 or V δ 2) are both altered after HIV infection. Early reports that V δ 1 T cells were increased (3) and that the normal ratio of V δ 2:V δ 1 cells was inverted (4) identified an important and reproducible effect of HIV on these CD4-negative T cells. The increased levels of V δ 1 cells suggested they may be involved in antiviral immunity (5) and parallels were drawn between the expansion of V δ 1 cells and similar increases in CD8+ T cells (5) that contribute to the inverted CD4:CD8 T cell ratio (6). Non-human primate studies showed that V δ 1 cell expansion is an indirect consequence of viral infection and reflects increased translocation of stimulatory bacterial products across the gut epithelium (7). The V δ 1 cells may have antiviral functions through killing of infected cells using the NKP30 (8) or NKG2C recognition receptors (9). Killing of uninfected CD4 T cells by V δ 1 cells may also be a mechanism for HIV disease (10). Whether V δ 1 T cells accelerate or slow HIV progression has not been resolved.

In contrast to V δ 1 cells, the V δ 2 subset is uniformly depleted in HIV disease with the greatest declines seen among patients with high viremia (11). These early studies [before the introduction of

combination antiretroviral therapy (ART)] involved patients who were untreated or received single drug therapy where viremia was reduced 10–50-fold but never suppressed fully. The lowest levels of V δ 2 cells were found in patients with opportunistic infections or CD4 T cells < 200 cell/mm³ of blood (12, 13). These individuals frequently had no detectable cells bearing the phosphoantigen-responsive V γ 9 chain (14). Importantly, decreases in V δ 2 cells and inversion of the V δ 2:V δ 1 cell ratio were early events in HIV disease that occurred while CD4 cell counts and their CD4:CD8 T cell ratio were still in the normal ranges.

Molecular analysis of γ and δ T cell receptor (TCR) chain usage showed that HIV-driven expansion of V δ 1 cells did not select for specific V δ chain rearrangements or individual V γ chains (15). Sequencing studies to describe the TCR repertoire confirmed that the population of V δ 1 chains was similar in donors with or without HIV infection and that HIV-driven V δ 1 cell expansion was not similar to antigen selection in $\alpha\beta$ T cells (16) but more likely was a polyclonal or super-antigen response. In contrast to the case for V δ 1 cells, there was strong evidence for selective V δ 2 cell depletion based on TCR structure. Flow cytometry studies documented the specific loss of V γ 9 (also called V γ 2) expressing cells, a chain more frequently associated with V δ 2 than V δ 1 in healthy controls. Finding that the relative proportion of V γ 9 expressing cells was decreased in HIV patients, being lower in both blood and bronchoalveolar lavage specimens despite having increased V δ 1 cells (17), encouraged a closer look at the V γ 9V δ 2 subset.

The importance of V γ 9V82 T cell depletion and the relationship between depletion and TCR became more clear as other groups defined antigens for these unusual cells.

FINDING THE ANTIGENS FOR V82 T CELLS

Major activities of V γ 9V82 T cells include a strong response to Mycobacterium-infected human PBMC, even though these cells do not react to purified mycobacterial 65-kd heat shock protein (18). The V γ 9V82 T cells respond to Mycobacteria-pulsed cells or antigens found on the human MOLT-4 lymphoma cell line (19), which was confusing in the context of the model for MHC-restriction that explained $\alpha\beta$ T cell recognition of peptide antigens. Recognition of either Mycobacterium-pulsed cells or lymphoma cell lines required a specific rearrangement between the variable γ 9 (V γ 9) and joining P (JP) segments; constant region 1 (C1) was incorporated by mRNA splicing to form V γ 9JPC1 chains that combine with a V82DJC chains to form functional receptors (20, 21). In an alternate nomenclature, the V-J rearrangement is designated V γ 2J γ 1.2; the nomenclature is consistent for δ chains. This combination of specifically rearranged V γ 9 with V82 chains endowed the capacity for recognizing mycobacterial antigens (22, 23). Efforts to characterize stimulatory molecules showed first they were phosphorylated (24) and later that they were prenyl pyrophosphate intermediates of sterol synthesis in mammalian cells (25). A variety of natural and non-natural molecules were tested for V γ 9V82 T cell stimulation to define the essential antigenic structure (26) within a group of chemicals now known as phosphoantigens. The natural abundance of mammalian phosphoantigens, being made in every living cell, in addition to structurally similar but often more potent compounds produced by bacteria, protists, plants, or fungi, serves to inundate human physiology with stimulators of V γ 9V82 T cells. These compounds select for and amplify the V γ 9JPV82 cell subset during early life as was described in a remarkable paper from Michael Brenner's group (27).

In children, a fetal V γ 9 chain repertoire is replaced slowly with one dominated by the V γ 9J γ P rearrangement. Under continuous positive selection, the V γ 9V82 cell count rises and phosphoantigen-responsive cells are increasingly found in blood as central or effector memory types with declining proportions of naïve cells. Healthy adults maintain a diverse but highly redundant repertoire such that 1 in 40 circulating memory T cells is a phosphoantigen-responsive V γ 9V82 T cell. Clearly, the V γ 9JPV82 cell dominates T cell memory in healthy adults. Baseline levels of V γ 9V82 T cells differ by two to fourfold between white European-origin or Asian-origin (high) and African-origin (low) peoples but repertoire complexity is similar among these groups and *in vitro* responses to phosphoantigen are also similar (28). Positive selection and amplification of V γ 9JPV82 T cells is ubiquitous in man and present in most non-human primate species studied so far, but is not present in lower mammals including rodents that lack both a gamma chain gene similar to V γ 9 and butyrophilin 3A1 that is also required for phosphoantigen responses (29–34).

SPECIFIC DESTRUCTION OF ANTIGEN-SPECIFIC V82 T CELLS IN HIV DISEASE

Two important papers in 1996 and 1997 helped to bridge HIV studies with the emerging understanding of phosphoantigens and

their importance to $\gamma\delta$ T cell biology. Gougeon's group confirmed earlier studies on V82 cell depletion in HIV patients and reported a disease-associated "functional anergy" measured by lack of proliferation or cytokine responses after stimulation with mycobacterial antigens (35). These authors studied the junctional diversity of V γ 9V82 TCR chains expressed in HIV+ individuals and reported that the V82 cell chain repertoire remained diverse. They also noted there were no differences in spontaneous apoptosis between HIV patients or uninfected control donors after *in vitro* phosphoantigen stimulation. A second group led by Malkovsky confirmed the functional anergy in V82 T cells from HIV patients by documenting decreased responses to phosphoantigen or to the prototypical cell target Daudi B cell (36). Both groups noted that V82 T cells were reduced but not eliminated in HIV disease, and were substantially deficient in their response to phosphoantigen due to anergy that may have resulted from inappropriate activation *in vivo*. A smaller study of HIV+ individuals noted differences from controls regarding $\gamma\delta$ T cell responses to *Salmonella typhimurium* or *Candida albicans*, and reported that V82 cell responses to Mycobacteria remained intact only in patients with >500 CD4 T cells/mm³ (37). Further, V82 cells were depleted from blood but increased in liver from both HIV patients and HIV-negative patients who had disseminated *Mycobacterium avium* complex (38). V81 cells were increased in tissue sites among HIV patients, notably liver (39) or bone marrow (40). The pattern of changes among $\gamma\delta$ T cells for both V82 and V81 cells was a distinguishing feature of HIV disease.

MILESTONE ACHIEVEMENTS FROM EARLY STUDIES ON $\gamma\delta$ T CELLS IN HIV DISEASE

By 1997, there was a basic understanding of HIV infection and its impact on $\gamma\delta$ T cells. Four major concepts had emerged: (1) Inversion of the V82:V81 cell ratio was an early event, occurring prior to inversion of the CD4:CD8 T cell ratio. (2) V81 cells are increased in patients with HIV. (3) The V82 cell depletion was accompanied by decreased responsiveness to phosphoantigens or tumor cells. (4) Loss of V82 cells was greatest in patients with low CD4+ T cells, high viremia, opportunistic infections and late stage disease (AIDS). Consequently, HIV-mediated changes in $\gamma\delta$ T cells appear to be part of the mechanism for evading antiviral immunity and establishing persistent infection with chronic disease. Persistent infection is essential for viruses like HIV that are transmitted with relatively low efficiency and require direct person-to-person contact. These studies highlighted the need to understand mechanisms for $\gamma\delta$ T cell dysregulation, define impacts of these changes on immunity to HIV and look more broadly at "unintended consequences" of the viral immune evasion strategy.

MECHANISMS FOR DYSREGULATING $\gamma\delta$ T CELLS

Model studies in non-human primates have helped to explain some of the $\gamma\delta$ T cell changes during disease. Because rodents lack the TCR sequences needed for phosphoantigen recognition, studies on V γ 9V82 T cells have been restricted to human beings and non-human primates. A recent genome mining study revealed that functional genes for V γ 9V82 and butyrophilin 3A1 were actually present in a few other placental mammals including alpaca, sloth, bottlenose dolphin, killer whale, horse, and armadillo (41). Most of these species are unfamiliar experimental systems with the

exception of armadillo that has been used for research on *Mycobacterium leprae*, but we can expect humans and non-human primate models to dominate this field for the foreseeable future.

When peripheral blood V γ 9V δ 2 T cells were isolated from uninfected rhesus monkeys (naïve to viral antigens) they were directly cytotoxic for SIV-infected target cells (42). There were rapid increases in blood V γ 9V δ 2 T cell counts and higher expression of activation markers within a few days after SIV infection of rhesus monkeys even though these cells were already showing decreased proliferation responds *in vitro* (43). The V γ 9V δ 2 T cell expansion seen early after SIV infection of macaques was brief and was followed by a rapid decline in cell count and function. This animal model recapitulates the decline in V γ 9V δ 2 T cells and inversion of the V δ 2:V δ 1 cell ratio. Similarly, V γ 9V δ 2 cell lines or clones from HIV-negative donors recognized and killed HIV-infected cells (44). It was also known that healthy V γ 9V δ 2 cells produced large amounts of interferon- γ , tumor necrosis factor- α , and chemokines RANTES or MIP-1 β (45) that were associated with antiviral immunity. Normally, the circulating V γ 9V δ 2 cells (mostly memory phenotype) have preformed cytoplasmic vesicles containing RANTES that are released immediately upon phosphoantigen stimulation or after contact with target cells (46). The release of RANTES and other chemokines suppressed HIV replication *in vitro* by blocking co-receptors for virus entry (47, 48). The CCR5 receptor is highly expressed on V δ 2 cells (49) meaning RANTES release would attract even more V γ 9V δ 2 cells able to release additional chemokine that would block HIV entry, kill already infected cells through direct cytotoxicity, or mediate antibody-dependent cellular cytotoxicity through cell surface Fc γ receptors (50). Circulating V γ 9V δ 2 cells also activate innate and acquired immunity through the release of pro-inflammatory IFN- γ and TNF- α or regulatory cytokines (51).

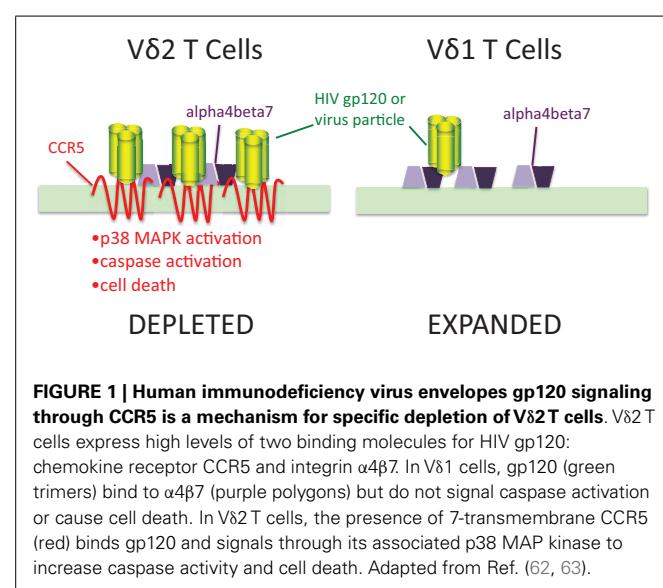
While it seemed clear that $\gamma\delta$ T cell dysregulation is part of HIV immune evasion, the precise impact on pathogen immunity was less clear. Little was known about how V δ 1 expansion or V δ 2 T cell depletion affect normal immunity and whether these are important aspects of viral immune suppression linked to progressing disease. Brenchley's group (7) reported that V δ 1 cell expansion in SIV-infected rhesus monkeys was related to pathologic changes in the intestinal epithelium that increased bacterial translocation causing higher levels of bacterial products in circulation that would stimulate V δ 1 T cells. The V δ 1 cells were also increased by influenza vaccination in HIV patients but only if the vaccine contained MF-59 adjuvant (52). Thus, V δ 1 cells are responsive to stimulation in HIV patients and expansion could be linked to bacterial translocation or reflect the normal responses to *Candida albicans* (53) and other common intercurrent infections in HIV patients.

Like V δ 1, phosphoantigen-responsive V δ 2 cells increase soon after infection as was documented in the SIV infection of macaques (43) but then are depleted and often extinguished. When cloned V δ 2 T cells are stimulated by anti-CD3 monoclonal antibody plus IL-2 they frequently die due to apoptosis, yet anti-CD3 stimulation plus feeder cells in the absence of exogenous IL-2 leads to proliferation (54). Thus, stimulation conditions impact outcome. It remains difficult to extrapolate *in vitro* conditions into explanations for *in vivo* outcomes and the mechanism for HIV-mediated depletion are still unclear. Laboratories working with

V δ 2 T cells, including our groups, know that phosphoantigen plus IL-2 stimulation of human or macaque PBMC results in rapid cell death followed by outgrowth of a surviving population that peaks around 10–14 days later. The repertoire of V γ 9JPC1 chains is essentially unchanged in the expanded subset compared to fresh cells (55), despite the fact that stimulation indices for individual clones vary 10–100-fold (56). Surprisingly, there were no significant differences in apoptosis after *in vitro* stimulation comparing V δ 2 cells from HIV-infected patients or uninfected controls (35) but the preferential loss of phosphoantigen responses in HIV disease still argues for a direct link between antigen specificity and depletion, apparently a different type of activation-induced cell death.

Gene expression array studies provided a surprising insight into infection and $\gamma\delta$ T cell dysregulation that may impact V δ 2 cells. During HIV infection, mRNA for enzymes in the cholesterol biosynthesis pathway is upregulated (57, 58). Higher production of cholesterol is needed to meet the demands for viral membrane synthesis and more of the biosynthetic intermediates are required including prenyl pyrophosphates (phosphoantigens). With more phosphoantigen present, it is reasonable to expect greater activation of V γ 9V δ 2 T cells and this may explain the rapid decreases during acute infection when viremia is highest and the frequency of infected cells is also highest compared to other stages of disease.

We also know (49, 59) that a large proportion of activated $\gamma\delta$ T cells express chemokine receptors including CCR5 and CXCR4 that are major co-receptors for HIV (Figure 1). These receptors bind sequences in the V3 loop of envelope glycoprotein gp120 and are important for viral entry. Both V δ 2 and V δ 1 cells express α 4 β 7 integrin that also binds the V2 loop of gp120 (60, 61). CCR5 expression was only seen in the V δ 2 subset where it was estimated to be present at >50,000 molecules per cell surface (62), or roughly 10-fold higher surface density compared to activated α β CD4 T cells. Treating expanded V δ 2 cells with gp120 lead to induction/activation of caspases followed by apoptosis. Inhibitor studies confirmed that signaling through CCR5 and its associated



p38 MAP kinase, was necessary for this cell death pathway (62). We also detected gp120 binding to V δ 1 cells that could be blocked by ligands for $\alpha 4\beta 7$ but was not affected by the CCR5 antagonistic drug Maraviroc. Flow cytometry showed that V δ 1 cells have no detectable surface CCR5 (63). The presence of high density CCR5 on V δ 2 T cells and its absence on V δ 1 T cells may explain specific cell killing of V δ 2 cells by gp120 that is similar to the pattern seen during HIV infection. Since high density CCR5 expression occurs on phosphoantigen-stimulated cells, this mechanism might account for the connection between antigen specificity and cell loss.

Several other models have been proposed to explain the $\gamma\delta$ T cell defect in HIV disease. We first reported that V γ 9V δ 2 T cells activated *in vitro* were permissive for HIV infection (36) but the number of productively infected cells in patient samples seems to be very low. However, it was reported that human herpes virus 6 elevates CD4 on several cell types including $\gamma\delta$ T cells (64) and could impact susceptibility of these cells *in vivo*. Both direct infection of intrathymic $\gamma\delta$ T cell precursors (65) and inhibition of thymic development by HIV-infected $\gamma\delta$ T cells (66) were proposed as models for specific or general T cell depletion. However, it is important to note that V δ 1 cells are not depleted during HIV disease as would be expected for a mechanism acting at the level of thymopoiesis. In addition, the normal human blood repertoire includes V γ 9V δ 2 cells that use both the JP and other rearrangements even though we emphasize V γ 9JP because of its responses to phosphoantigens. Sequencing studies showed that V γ 9V δ 2 cells using J segments other than JP, remained at normal levels and appeared to be unchanged during HIV disease (14). Further, the rapid depletion of V γ 9V δ 2 T cells before the onset of immunodeficiency and reactivation of pathogens like HHV-6, argues this is not a major mechanism for depletion. The impact of direct infection, despite infrequent CD4 expression or negative effects on thymopoiesis may contribute to V γ 9V δ 2 cell depletion but are not likely to be the major mechanisms.

Based on studies of former plasma donors in southern China who were infected at the same time and with similar strains of HIV, we know that V δ 2 levels correlate with viremia (hence levels of gp120) but not with CD4 T cell count (67). This adds support to a model where viral proteins are directly responsible for V δ 2 cell depletion as was proposed earlier (11). During natural history studies of HIV disease it will be important to identify patients being treated with CCR5 antagonists including Maraviroc, who may have increased V δ 2 T cells compared to patients treated with regimens that do not include this drug class.

In order to understand better the dynamics of V δ 2 T cell depletion and the potential for immune reconstitution during therapy, extensive use was made of TCR repertoire analysis (Figure 2). The focus has been on the V γ 9 chain that is most tightly linked to phosphoantigen responsiveness due to the predominant V γ 9JP rearrangement (also called V γ 2J γ 1.2). Indeed, sequencing studies on the γ chain repertoire established the specificity of HIV-mediated deletion in the V δ 2 cell population (12) and subsequent work [reviewed in Ref. (68)] showed this TCR-specific defect is common to all HIV patients regardless of virus type, geographic distribution, race, or ethnicity. In the era before effective antiretroviral therapy, repertoire sequencing studies showed that patients

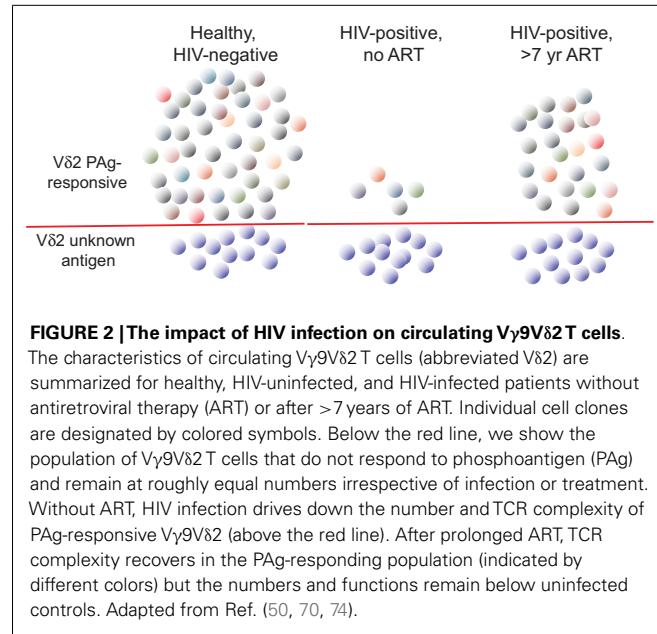


FIGURE 2 | The impact of HIV infection on circulating V γ 9V δ 2 T cells.

The characteristics of circulating V γ 9V δ 2 T cells (abbreviated V δ 2) are summarized for healthy, HIV-uninfected, and HIV-infected patients without antiretroviral therapy (ART) or after >7 years of ART. Individual cell clones are designated by colored symbols. Below the red line, we show the population of V γ 9V δ 2 T cells that do not respond to phosphoantigen (PAg) and remain at roughly equal numbers irrespective of infection or treatment. Without ART, HIV infection drives down the number and TCR complexity of PAg-responsive V γ 9V δ 2 (above the red line). After prolonged ART, TCR complexity recovers in the PAg-responding population (indicated by different colors) but the numbers and functions remain below uninfected controls. Adapted from Ref. (50, 70, 74).

with CD4 < 200 cells/mm³ had no detectable V γ 9JP cells in PBMC (14). When these patients initiated combination antiretroviral therapy and achieved virus suppression, we did not observe rapid rebound of V δ 2 cells in blood despite increases in CD4 cell count during 2–2.5 years of treatment. The lack of rapid V δ 2 cell recovery argues against the idea that depletion in peripheral blood is due to increased tissue compartmentalization, which is an important mechanism for controlling CD4 T cell count early in disease (69). A subsequent study of patients with longer intervals of antiretroviral therapy provided some initial evidence that V γ 9JP cells were reconstituted during virus suppression but the recovery rates were slow (13). More recently, studies with patients after >7 years of antiretroviral therapy revealed extensive reconstitution of the TCR cell repertoire (based on V γ 9 chain sequencing) and a population of V γ 9JP chains having nearly the complexity found in healthy, HIV-negative controls. Considering that some of the treated patients had nadir CD4 counts below 100 cells/mm³ and were expected to have no circulating V γ 9JP cells, reconstitution of this population was a surprising result (70).

The $\gamma\delta$ T cell repertoire is defined by analyzing the number and frequency of clonotypes (predicted peptide sequences in the CDR3 or V-J region of the gamma chain) or nucleotypes (nucleotide sequences for these peptides) within a sample of the total population of TCR sequences in a blood or tissue specimen. These sequences are classified as public (identical amino acid sequences present in unrelated donors) or private (unique to one individual in our population of donors). For healthy, HIV-negative adults most of the clonotypes are public and many of the nucleotypes are also public indicating a high degree of similarity among unrelated individuals. This pattern is consistent with the use of monomorphic antigen-presenting molecules and is probably biased by nucleotide sequences in the germline regions of V γ 9 and JP that provide alternate routes to expressing the “germline” V γ 9JP rearrangement (70) using the

mechanism of convergent recombination (71–73). In long-term treated HIV patients, there were clear differences between uninfected controls or treated patients who reconstituted the V γ 9 repertoire as measured by clonotype and nucleotype abundance (70). These differences prove that the repertoire was reconstituted by new cell synthesis and not by regrowth of a cell population that survived an initial HIV attack. Further, the reconstituted TCR repertoire included many sequences that should respond to phosphoantigen. Despite having a reconstituted repertoire and TCR against phosphoantigen, V δ 2 cells in these patients remained at levels well below matched, HIV-negative controls and had greater proportions of naïve cells indicating a lack of phosphoantigen responses and impaired positive selection *in vivo*. Patients also had lower expression of the CD56 cell surface marker (74) that is associated with cytotoxic effector function (75). V δ 2 cells from these long-term treated patients could be activated by potent *in vitro* stimulation whereupon they regained Fc receptor expression (CD16) and effector function in antibody-dependent cellular cytotoxicity (50). Despite reconstitution of the V δ 2 T cell population by new cell synthesis during prolonged ART, this subset does not recover to normal levels or function after treatment intervals of years to decades. The majority of V δ 2 cells found in treated HIV patients were generated and selected in an environment almost devoid of HIV due to effective therapy. The nature of this long-term defect is an obstacle to immune reconstitution and might contribute to chronic comorbid diseases in HIV patients.

A very small fraction of HIV patients actually have normal V δ 2 cell levels and function; these are elite controllers or natural virus suppressors defined as HIV patients with undetectable viremia except for occasional blips and no history of antiretroviral therapy (76). Among natural virus suppressor patients (approximately 0.5% of all persons with HIV infection), V δ 2 cell levels are equivalent to age, gender, and race-matched uninfected controls but there are significant differences in the V γ 9 chain repertoire (77). These repertoire differences reflect an early impact of HIV before the time when virus replication was controlled by host immunity and in this way, are similar to what we observed in treated patients where viremia was suppressed by chemotherapy. This also tells us that normal function of V γ 9V δ 2 T cells can return in HIV-infected patients but as yet, we have not uncovered the critical mechanisms for recovery that might help us to find new therapeutic targets.

MILESTONE ACHIEVEMENTS FROM THE MIDDLE PERIOD OF STUDYING $\gamma\delta$ T CELLS IN HIV DISEASE

Nearly three decades of research produced many insights into HIV and $\gamma\delta$ T cells. Studying HIV disease proved that V γ 9JPV δ 2 cell depletion explained the defective response to phosphoantigen. Mechanisms were described for specific loss of V γ 9JPV δ 2 cells that account for the relationship between depletion and phosphoantigen responsiveness. The findings showing that therapy reconstituted the V γ 9 repertoire but did not restore normal cell counts or function, were both encouraging and cautionary. Changes in $\gamma\delta$ T cells are clearly related to HIV immune evasion but the long-term defects pose substantial challenges to the clinical management of HIV and efforts to reduce comorbid diseases.

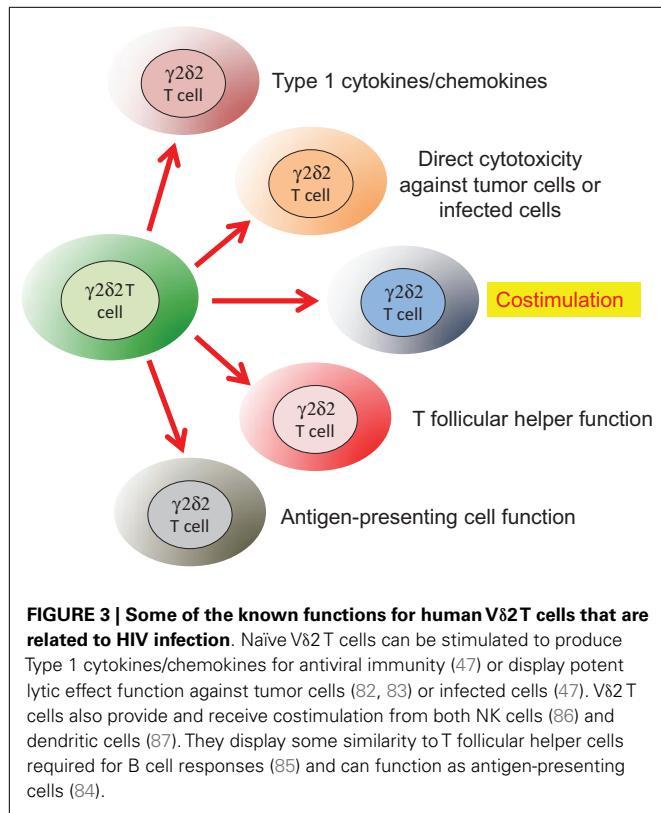
PROBLEM OF DURABLE $\gamma\delta$ T CELL DAMAGE DESPITE CHEMOTHERAPEUTIC SUPPRESSION OF HIV

Natural history studies of HIV infection documented a brief, violent interval of acute infection characterized by extraordinarily high viremia and dramatic changes in immune cell populations, which continued until a stable viral set-point (78) was established. We prefer the term “immune set-point” that better describes the balance between immune system destruction and chronic infection that allows HIV to persist in an individual or in the human population. The immune set-point includes damage to V δ 2 T cell function that goes beyond what is seen in acute, adult malaria (79) for example, where there was a more limited transient loss of V γ 9V δ 2 T cells.

Both viral and immunological set-points designate the phase of disease when acute HIV infection gives way to a stable, persistent infection with slower progression and more time for virus to spread by sexual contact. In some virus examples, the acute to persistent transition reflects changes in viral gene regulation but in HIV disease the most important mechanisms are viral evasion of host immunity. Once persistence is established, chronic disease progression causes death around 11 years later in untreated HIV disease, as declining CD4 T cell counts and collapsing innate immunity increase the incidence and severity of lethal opportunistic infections, cancer, cardiovascular disease, and other terminal conditions. Evolutionary selection of HIV created a pathogen capable of spreading in the human population by relatively inefficient sexual transmission even when the median life expectancy after infection was only 11 years for adults and shorter for infected infants or elderly persons. Now, in the era of effective ART and much longer survival times, we are seeing a new scenario that was not part of the selection pressures driving natural evolution of HIV. Therapy with effective virus suppression repairs some of the earlier immune damage and likely reduces transmission rates (80) but key cell types including $\gamma\delta$ T cells fail to recover normal numbers, phenotypes, or function, even after prolonged treatment that also fails to eradicate the virus. These enduring defects and their impacts on durable virus persistence or comorbidities of HIV are unanticipated – or better said – unselected consequences of HIV biology that are continuing decades after a viral immune evasion mechanism that caused damage to host immunity during the interval of acute infection.

Why these defects linger is a critical and unsolved question. We know that low numbers of viral RNA remain in plasma even during successful antiretroviral therapy (81) but these levels are around 10⁵ vRNA copies per ml of plasma lower than what is found during acute viremia. Accordingly, when the γ chain repertoire is reconstituted by new cell synthesis (70), we are dealing with cells that were birthed in an environment nearly devoid of HIV replication. Reconstitution is indeed slow as was predicted several years ago (13), but the return of function lags behind the gain of TCR repertoire complexity and as of now, there is no reliable estimate for the kinetics of functional recovery.

The durable impact of HIV on $\gamma\delta$ T cells impedes their contributions to key immune effector and regulatory mechanisms. Relating the list of possible $\gamma\delta$ T cell functions to the known outcomes of HIV disease is a challenging undertaking and will trigger much debate. Our list of relevant V δ 2 T cell functions (**Figure 3**)



includes control of antiviral immunity (47), tumor or infected cell killing (82, 83), antigen presentation (84), B helper T cell function (85), and costimulation of NK cells (86, 87) although alternate lists with additional functions can be imagined. In this review, we focus on the impact of losing V γ 82 T cell costimulation and why this loss can be an important underlying factor in HIV-associated comorbid diseases that are causing death and disability in persons despite effective viral suppression by ART.

The consequences of prolonged depression in V γ 82 T cell function may appear in unexpected comorbidities of HIV disease. For example, psoriasis affects 1–3% of patients with HIV (88), which seems paradoxical since this is a T cell-mediated autoimmune disorder (89). Normally, psoriasis is associated with influx of blood V γ 9V γ 82 T cells into the inflamed skin that may be a mechanism for resolving the acute condition. In HIV disease, we can imagine that the absence of V γ 9V γ 82 T cells or their chronically poor responses in patients treated with antiretroviral therapy will reduce their beneficial effect and increase the severity or duration of psoriasis in these patients. The V γ 9V γ 82 T cells are intimately related to both NK and dendritic cells through interactions that control cell activation and inflammation. Psoriasis is an example where the loss of V γ 9V γ 82 function removes a key protective mechanism.

Cell:cell interactions (cross-talk) involving $\gamma\delta$ T cells were implicated in the activation of dendritic cells (90), enhancement of antibody production by $\gamma\delta$ T cells with T follicular helper cell activity (85) and antigen presentation or cross presentation (84). An additional and more specific example of cross-talk involved NK tumor cytotoxicity, which was activated by V γ 82 T cells (86).

The critical role for V γ 82 T cells in NK tumor cytotoxicity was mapped to expression of the 4-1BB ligand (CD137L). Phosphoantigen stimulation of human PBMC expanded the V γ 82 subset and upregulated both 4-1BB (costimulatory receptor) and NKG2D (activating receptor that recognizes stressed-self antigens) on the NK cells (Figure 4). A murine cell line expressing 4-1BBL could be substituted for V γ 82+ T cells indicating that this costimulatory ligand was the major signal for V γ 82 T cell enhancement of NK tumor cytotoxicity (86). This surprising result linked the anti-tumor effector activity of NK cells to the presence of phosphoantigens and activation of V γ 82 T cells.

A second example of V γ 82 T cell costimulation of NK is enhancement of effector activity capable of eliminating immature or mature, antigen-presenting dendritic cells (Figure 5). Again, phosphoantigen-expanded V γ 82 T cells delivered a costimulatory signal to NK that increased the killing of autologous dendritic cells. Along with increased production of Type 1 cytokines and chemokines, the increased levels of ICOS on V γ 82 T cells binding to ICOSL on NK cells was related to improving cytotoxic killing of dendritic cells (87). The V γ 82 T cells are reciprocally activated by the cross-talk with NK and may also be effectors for dendritic cell killing, but these experiments are complicated by the use of anti-TCR or anti-CD3 antibodies to purify V γ 82 cells. When antibodies are used for positive selection, V γ 82 T cells are super-activated and the effects of costimulation are obscured. These studies on costimulation support an earlier model for NK:V γ :DC interactions that focused on $\gamma\delta$ T cell cytokines that regulate NK and DC function (91). In this example, we see a key role for antigen-specific V γ 82 T cells in costimulation of NK cells that will then gain the capacity for killing activated dendritic cells and help to control chronic inflammation.

When V γ 82 T cell costimulation of NK cells is lost we might expect increased cancer risk among HIV patients, despite effective viral suppression by therapy. In HIV clinics of the University of Maryland, Baltimore, more than 15% of all HIV patients will have a cancer diagnosis and in 85% of those cases, cancer will be the cause of death (92). The great variety of cancers that increase in HIV patients suggests that a common mechanism of tumor control is missing or ineffective. Decreased tumor surveillance by NK cells may contribute to this comorbidity of HIV-associated cancer and the loss of V γ 82 T cell costimulation could be a key mechanism for the loss of effector function.

V γ 82 T cell depletion, loss of costimulation, and the consequent reduction in dendritic cell killing may have important effects on several HIV-associated comorbidities. Chronic immune activation with inflammation is a hallmark of HIV disease and a danger to patients even after virus replication is suppressed. The lingering defect in V γ 82 T cells and the linked failure to co-stimulate NK for dendritic cell killing, likely increases the risk for chronic activation/inflammation as potent antigen-presenting cells accumulate in the absence of normal control mechanisms. Indeed, Fauci's group noted the lack of potent dendritic cell editing by NK in HIV-infected and treated patients (93) and defects in plasmacytoid dendritic cell–NK cross-talk in HIV patient specimens that reflected poor activation of NK during innate immune responses (94). V γ 82 T cells are also involved in reciprocal interactions with dendritic cells that affect activation or maturation of both cell

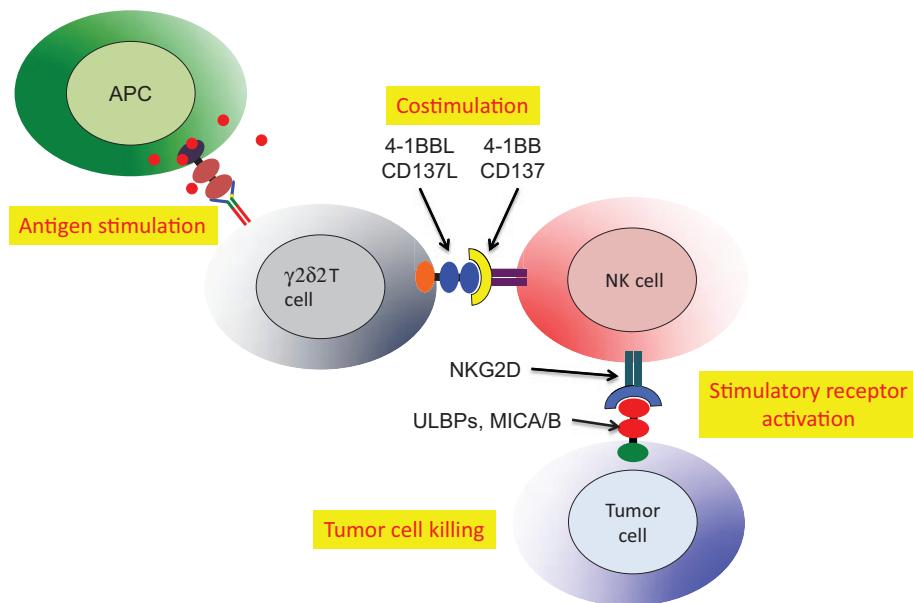


FIGURE 4 | Stimulated V δ 2 T cells co-stimulate NK to increase tumor cell cytosis. Activated V δ 2 T cells express 4-1BBL that binds 4-1BB on NK cells. The NKG2D receptor on NK is upregulated allowing stronger recognition of

tumor cells expressing self-stress ligands ULBP or MIC A/B (86). HIV-mediated destruction of V δ 2 T cells will cripple the important mechanism for NK costimulation and reduce natural tumor surveillance.

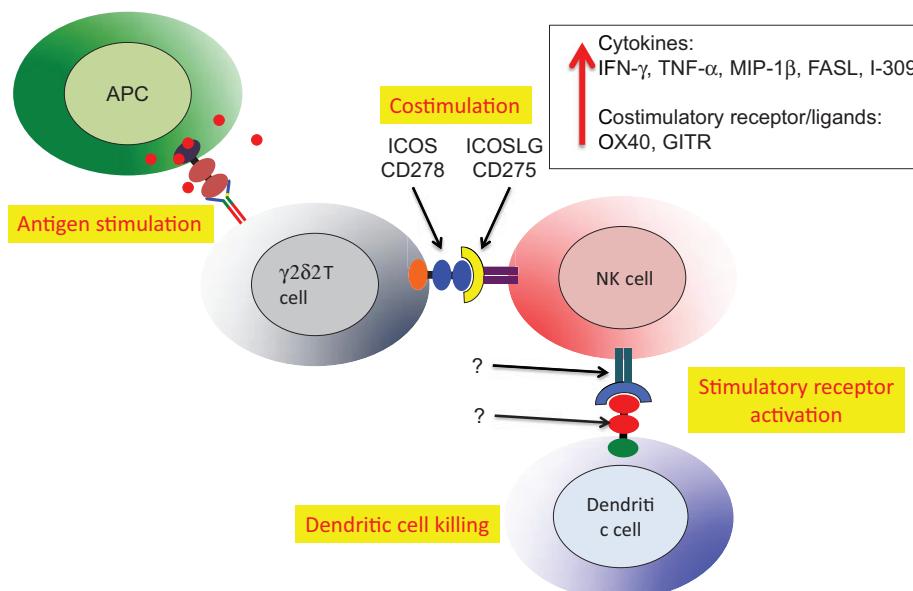


FIGURE 5 | V δ 2 costimulation of NK cells increases lytic effector activity against autologous dendritic cells. The V δ 2 and NK cells interact through ICOS/ICOSL interactions resulting in upregulation of cytokine expression, increased mRNA for several other costimulatory receptors and increase lytic effector activity against dendritic cells (87). The relevant

receptor(s) on NK and recognition molecules on dendritic cells have not been identified. Similar to the example in **Figure 4**, HIV-mediated depletion of V δ 2 is expected to reduce the capacity for NK killing of dendritic cells that will accumulate and promote chronic immune activation/inflammation that is a hallmark of HIV disease.

types (90). Consequently, dysfunction of V δ 2 cells will affect NK and dendritic cell interactions as all three cell types are interdependent. When all three cell types are normal, there is a balance

between cell activation and cell killing that modulates inflammation. When one piece is missing, in this case V δ 2 T cells, the control over inflammation is lost.

The examples for defective V δ 2 T cells in HIV-infected and treated patients have important parallels in the NK literature. There are many reports about the phenotype of circulating NK cells in patients where HIV is controlled by antiretroviral therapy. In these patients, NK have lower density of NKp46, NKp30, and NKp44 receptors (95) and often appear as CD56-/CD16+ cells lacking cytotoxic effector activity (96). Because HIV infection is associated with decreased NKG2D (activating) and increased NKG2A (inhibiting) receptor expression (97), even the increased levels of ULBP target ligands failed to trigger cytosis of HIV-infected target cells. The earliest mark of NK dysfunction seems to be the decreased expression of Siglec-7 (98). When the lack of Siglec-7 is combined with lower expression of CD56 (99), we are beginning to define the phenotype of defective NK cells that is common among HIV patients. Whether these defects could be corrected by costimulation has not yet been tested. Clearly, there is a convergence of functional defects among V δ 2, NK, and dendritic cells in HIV and potent antiretroviral therapy does not restore the normal cell interactions or function of this regulatory triangle. It is worthwhile to look for defects in multiple mechanisms of immunity including those highlighted in **Figure 3**.

With highly effective antiretroviral therapy and increasing interest in eradicating HIV, it is important to look at the future of research on $\gamma\delta$ T cells. Surely, V δ 2 T cells provide an attractive target for immunotherapy, probably using aminobisphosphonate compounds plus IL-2 or IL-15 to increase cell levels and functions. Patterned on a number of ongoing clinical studies in cancer (100–105), aminobisphosphonate drugs may be useful for correcting the HIV-associated V δ 2 T cell defect. A pilot study showed this approach was safe for HIV patients (106) but these studies have not been repeated or expanded to include clinical and immunological endpoints such as NK or dendritic cell phenotype and function. The lack of progress may be due to insufficient justification for the outcome of bisphosphonate/IL-2 therapy, since viremia can be suppressed more easily with once-a-day antiretroviral agents.

PLEA FOR INNOVATIVE CLINICAL TRIALS TARGETING $\gamma\delta$ T CELLS IN HIV DISEASE

Clinical research on therapeutic restoration of $\gamma\delta$ T cells in HIV disease will require innovative clinical trial strategies. It may be difficult to launch therapeutic interventions such as aminobisphosphonate plus IL-2 for 2 or 3 months, then wait 20 years or more to accumulate significant data on changing rates of pulmonary arterial hypertension (found in <5% of HIV patients) or other comorbidities. While each of the comorbid conditions affects only a portion of the HIV population, their combined impact is substantial but clinical trials rarely lump disparate conditions into single endpoints. We might overcome this problem by using immunological endpoints that are related to chronic disease mechanisms. For example, direct immunotherapy targeting $\gamma\delta$ T cells might be tested as a way to activate V δ 2 T cells that would normalize NK cell phenotype and function. Recovering of normal properties in two distinct lymphocyte subsets may be sufficient to document a clinical benefit, even though neither change is likely to result in short term changes to the incidence, prevalence, or severity of comorbid diseases.

SUMMARY

The V γ V δ 2 T cells in HIV-infected individuals contain the historical record of disease, documenting the intensity of initial depletion and reconstitution during therapy by their abundance, activity, and sequences of their TCR. They are barometers for current disease status, reflecting the capacity for resisting opportunistic infections, natural tumor surveillance, the control of immune activation/inflammation, and likely several other conditions critical to the clinical management of HIV disease. In addition, reconstitution of the TCR repertoire may be a measure for treatment success and help to identify patients with the highest potential response to immunotherapies targeted at V δ 2 T cell activation. Their compact TCR repertoire and extraordinarily high proportion of public V γ 9 chains facilitates comparisons among individuals or groups of patients. When combined with the sophisticated knowledge about cell surface marker expression including costimulatory receptors/ligands, and their patterns of cytokine/chemokine expression, these cells become critical tools for the immunology of infectious diseases. Adding to the interest is the availability of approved drugs that activate V γ V δ 2 T cells *in vivo* with high specificity, and a rich pipeline of compounds under development that may do even better. The missing link is a commitment to small interventional clinical trials testing the capacity for $\gamma\delta$ T cell-targeted immunotherapies to alter the profile of immunity and recover normal control in diseases like HIV/AIDS.

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Direct and indirect effects of cytomegalovirus-induced $\gamma\delta$ T cells after kidney transplantation

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Despite effective anti-viral therapies, cytomegalovirus (CMV) is still associated with direct (CMV disease) and indirect effects (rejection and poor graft survival) in kidney transplant recipients. Recently, an unconventional T cell population (collectively designated as V δ 2^{neg} $\gamma\delta$ T cells) has been characterized during the anti-CMV immune response in all solid-organ and bone-marrow transplant recipients, neonates, and healthy people. These CMV-induced V δ 2^{neg} $\gamma\delta$ T cells undergo a dramatic and stable expansion after CMV infection, in a conventional "adaptive" manner. Similarly, as CMV-specific CD8+ $\alpha\beta$ T cells, they exhibit an effector/memory TEMRA phenotype and cytotoxic effector functions. Activation of V δ 2^{neg} $\gamma\delta$ T cells by CMV-infected cells involves the $\gamma\delta$ T cell receptor (TCR) and still ill-defined co-stimulatory molecules such as LFA-1. A multiple of V δ 2^{neg} $\gamma\delta$ TCR ligands are apparently recognized on CMV-infected cells, the first one identified being the major histocompatibility complex-related molecule endothelial protein C receptor. A singularity of CMV-induced V δ 2^{neg} $\gamma\delta$ T cells is to acquire CD16 expression and to exert an antibody-dependent cell-mediated inhibition on CMV replication, which is controlled by a specific cytokine microenvironment. Beyond the well-demonstrated direct anti-CMV effect of V δ 2^{neg} $\gamma\delta$ T cells, unexpected indirect effects of these cells have been also observed in the context of kidney transplantation. CMV-induced V δ 2^{neg} $\gamma\delta$ T cells have been involved in surveillance of malignancy subsequent to long-term immunosuppression. Moreover, CMV-induced CD16+ $\gamma\delta$ T cells are cell effectors of antibody-mediated rejection of kidney transplants, and represent a new physiopathological contribution to the well-known association between CMV infection and poor graft survival. All these basic and clinical studies paved the road to the development of a future $\gamma\delta$ T cell-based immunotherapy. In the meantime, $\gamma\delta$ T cell monitoring should prove a valuable immunological biomarker in the management of CMV infection.

Keywords: antibody-mediated rejection, cancer, cytomegalovirus, gamma-delta T cells, lymphocytes, renal transplantation

INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end-stage renal failure (1, 2). However, transplantation implies long-term chronic immunosuppression to avoid acute rejection and to extend graft survival. Chronic immunosuppression reshapes host-pathogen relationships, by modifying the type or changing the magnitude of immune responses against pathogens and tumor cells. Therefore, the two main complications associated with immunosuppressive therapies are opportunistic infections and cancer.

Cytomegalovirus infection is the most frequent opportunistic infection occurring after kidney transplantation. Human cytomegalovirus (CMV) is an ubiquitous human herpesviridae, with a double-stranded linear DNA genome of 235 kb (3). Primary CMV infection in an immunocompetent host is usually asymptomatic due to the establishment of a robust and specific adaptive

immune response involving CMV-specific CD4+ T cells, CD8+ T cells, and IgG, which persist lifelong. Moreover, after primo-infection, all these actors contribute to inhibit virus reactivation (3). Despite effective anti-viral therapies, CMV is still associated with CMV infection or disease in immunocompromised kidney transplant recipients (4, 5). CMV infection is characterized by CMV DNAemia (CMV DNA in blood or plasma, also called CMV viremia) regardless of symptoms and occurs in about 50% of CMV-seropositive patients (R+, patients with peripheral blood CMV IgG) (6–10), and up to 70% of donor-positive, seronegative-recipients (D+R-) in the absence of anti-viral prophylaxis (11–18). CMV disease can be a viral syndrome (CMV DNAemia with fever, malaise, leukopenia, and/or thrombocytopenia) or a tissue-invasive disease (where CMV is detected in the injured organs, mostly lungs, liver and intestines) (4, 5). It occurs in 15–20% of D+R- patients and 5–10% of R+ patients, with or without

prophylaxis. Infections with high viral load require prolonged anti-viral therapy, which can lead to the emergence of CMV gene mutations associated with anti-viral resistance (mutations in *UL97* or *UL54* genes), a situation associated with high morbidity, graft loss, and death (12, 19–21). Moreover, CMV is also associated with indirect effects after kidney transplantation (22): worse patient and graft survivals (specially late-onset CMV infection or disease) (16, 23–28), more interstitial fibrosis/tubular atrophy (17), more acute rejection (17, 24, 29–31), more other opportunistic infections (32–35), an increased cardiovascular risk (36), more new-onset diabetes after transplantation (37, 38), and more graft artery stenosis (39, 40). Prophylactic anti-CMV immunoglobulin also prevents the development of early post-transplant non-Hodgkin lymphoma in kidney transplant recipients (41).

Cytomegalovirus-specific CD4+ and/or CD8+ T cell responses have been extensively documented after kidney transplantation (42–48). The efficacy of cell therapy protocols using expanded CMV-specific CD8+ T cells has demonstrated the central role played by these cells in the control of the virus (49). Therefore, it has been proposed to monitor these cells before and after transplantation to better use anti-CMV prophylaxis and therapy (50).

In 1999, we observed a massive expansion of a $\gamma\delta$ T cell population after CMV infection in kidney transplant recipients (51, 52). This CMV-induced $\gamma\delta$ T cell expansion did not involve the V82 subset, which is usually the main subset of $\gamma\delta$ T cells observed in the peripheral blood. Surprisingly, this increase can concern any of the V81, V83, and V85 sub-populations (collectively designated as V82^{neg} $\gamma\delta$ T cells) (52). This initial observation, since largely confirmed by others, suggested that a population of V82^{neg} $\gamma\delta$ T cells might play an important role in the immune response to CMV infection, but raised many questions about these cells. At the afferent phase of the CMV immune response, where is their site of priming? When and how are naïve V82^{neg} $\gamma\delta$ T cells activated? At the efferent phase, where is their site of action? What is their function? When and how do they recognize target cells? This review summarizes the recent findings tentatively addressing these points and leading to the conclusion that V82^{neg} $\gamma\delta$ T cells are important actors of the anti-CMV immune response, with direct anti-CMV effects, but also unexpected indirect effects observed in the context of kidney transplantation.

LOCALIZATION OF V82^{neg} $\gamma\delta$ T CELLS

Once established, the expansion of circulating V82^{neg} $\gamma\delta$ T cells following CMV infection in kidney transplant recipients is prominent and stable over time (51–53). This subset, which represents 0.5% on average of the T cell pool in CMV-seronegative patients, reaches an average of 5–10% of the circulating T cell pool in CMV-seropositive patients, and up to 50% in some patients. This phenomenon is not exclusive to the kidney transplant scenario as V82^{neg} $\gamma\delta$ T cell peripheral blood expansion after CMV infection has been shown in other solid-organ transplants (54–56), in recipients of hematopoietic stem cell transplantation (57–59), in immunodeficient children (60, 61), in neonates (62), in pregnant women (63), and in healthy individuals (64). CMV-specific CD4+ and CD8+ $\alpha\beta$ T cells on their own already represent around 5% of the T cell pool in CMV-seropositive healthy individuals (65) and

accumulate in older people (66). V82^{neg} $\gamma\delta$ T cell peripheral blood expansion further strengthens this high magnitude of the anti-CMV immune response. This accumulation of CMV-induced T cells may exert a detrimental effect on host by reducing immunity against other pathogens and could contribute to the CMV-induced immune senescence (67).

One of the most intriguing questions regarding V82^{neg} $\gamma\delta$ T cells is about their localization during the afferent and efferent phases of the immune response against CMV. To date, we still do not know where naïve V82^{neg} $\gamma\delta$ T cells are primed and where they exert their function. In physiological context, V82^{neg} $\gamma\delta$ T cells are the first $\gamma\delta$ T cell subset to emigrate from the thymus where they represent 1–15% of thymic T cells (68–71). Although poorly represented in lymph nodes, they represent 15% of T cells in the spleen where they are located in the marginal zone and red pulp (68, 69, 72). In tissues, V82^{neg} $\gamma\delta$ T cells are occasional in the kidney and the lung (68, 69). However, up to 15% of liver T cells can be $\gamma\delta$ T cells (73–75). They are predominantly found within normal human epithelia, with a selective accumulation in intestinal and skin epithelia (76–78). In the skin, they are mainly located in the basal epithelium of epidermis, where they represent 18–29% of T cells, but they are also present in the dermis (7–9% of T cells) (69, 79–81). They express homing receptors as CCR8 and cutaneous lymphocyte-associated antigen (78, 81). The gut epithelium is where V82^{neg} $\gamma\delta$ T cells are the most abundant. They are located in the epithelium close to the basal membrane where they represent one-third of resident T cells. They are also found within the lamina propria (5% of T cells) (76, 77, 82–84). Both skin and intestinal V81 repertoire are compartmentalized, with no overlap with the circulating V81 repertoire, suggesting these cells are resident cells (85, 86). However, these data are counterbalanced by observations made in cattle and sheep, showing that $\gamma\delta$ T cells could recirculate from the skin and intestinal epithelium, to the blood via afferent lymph and lymph nodes (87). Therefore in the future, the question about the localization of V82^{neg} $\gamma\delta$ T cells during the anti-CMV immune response needs to be addressed to elucidate if their peripheral blood expansion reflects an expansion from CMV-injured tissues or if blood and more probably capillaries are the theater of an immunological function of these cells. Primary CMV infection in healthy individuals initiates with replication in mucosal epithelium, a leading tissue for future V82^{neg} $\gamma\delta$ T cell exploration (3). Alternatively, endothelial cells, which are also the target of CMV express one of the V82^{neg} $\gamma\delta$ T cell receptor (TCR) ligand identified so far, endothelial protein C receptor (EPCR) (see below), and as V82^{neg} $\gamma\delta$ T cells are retrieved in vascular beds during antibody-mediated allograft rejection (see below), microcirculation should not be disregarded in these investigations.

WHEN DO THESE CELLS PARTICIPATE TO THE ANTI-CMV IMMUNE RESPONSE?

The classical pathway for activating adaptive immune response and achieving a broad systemic immune response, starts with immature dendritic cells that capture pathogens and then mature and migrate to lymph nodes where they prime $\alpha\beta$ T cells and B cells, some of which migrating back to infected tissues (88). This specific response is complemented by $\gamma\delta$ T cells, which have the capability to recognize a large spectrum of stress-induced

signals (sometimes considered as pathogen-associated-molecular patterns) and to mount local effector responses at the early stage of the immune response (89, 90). They act in synchrony with the innate immune cells as a sensor of self-dysregulation against infected or tumor cells, a function referred to as “lymphoid-stress surveillance” (89, 90). In accordance with this concept, natural and induced $\gamma\delta$ T cell IL-17 responses occur within 12 and 60 h after stimulation, while naïve $\alpha\beta$ T cells require antigen-specific priming and take at least 5–7 days to acquire effector function (88).

In human, early kinetics of $\gamma\delta$ T cell response to infections are generally difficult to depict because patients present to medical care after symptom occurrence and the time of infection is not known. In this respect, post-transplantation CMV infection is a unique context because patients can be monitored before and very early after infection. In kidney transplant recipients during primo-infection, CMV-specific CD4+ T cells are detectable in the peripheral blood 7–10 days after CMV DNAemia (42, 48). CD4+ T cells are critical to control virus (44, 91). They are followed by the production of CMV IgG and CMV-specific CD8+ T cells 20 days after DNAemia (42). Surprisingly, CMV-induced V δ 2 neg $\gamma\delta$ T cells undergo an expansion kinetic in the peripheral blood similar to that of CMV-specific CD8+ T cells (92). This expansion, defined as the time necessary to reach a “plateau,” although variable between patients, occurs at an average of 50 days after CMV infection (median: 45 days, min–max: 20–240 days) (93). This observation is apparently not consistent with the concept of early “lymphoid-stress surveillance.” To reconcile the late kinetics of CMV-induced V δ 2 neg $\gamma\delta$ T cells with the early action of other $\gamma\delta$ T cell populations, it has been proposed that $\gamma\delta$ T cell populations could be divided at least in two groups: (1) innate-like cells that respond rapidly and at a relatively high frequency in many tissue sites, and (2) lymphoid-homing $\gamma\delta$ T cells that may be primed in the circulation and clonally expanded in a conventional “adaptive” manner (90). Sampling being limited to blood of transplant recipients may have hampered detection of rapidly responding innate-like $\gamma\delta$ T cells in CMV-infected tissues and permitted only the observation of late expanded $\gamma\delta$ T cells in the blood. In the future, studies in animals should analyze concomitantly $\gamma\delta$ T cells in tissues and blood, as well as their recirculation, in order to determine if a bridge exists between innate-like $\gamma\delta$ T cells, which act at an early stage and peripheral blood CMV-induced $\gamma\delta$ T cells, which expand later. What we can detect in blood does not necessarily represent what is going on in tissues or lymphoid organs.

WHAT IS THE FUNCTION OF CMV-INDUCED V δ 2 neg $\gamma\delta$ T CELLS?

Like CD4+ T cells, there are many $\gamma\delta$ T cell subsets with various functionalities. A large literature described their production of Th1 cytokines and their cytotoxic activity against tumor and infected cells (94–99). However, other $\gamma\delta$ T cell sub-populations produce IL-4 and Th2 cytokines (100), are IL-17 natural or induced $\gamma\delta$ T cells (101–103), or have characteristics of regulatory T cells (104, 105). Moreover, some $\gamma\delta$ T cells can also regulate B cells and IgE production (100) or provide the help to rapidly generate from immature dendritic cells a pool of mature dendritic cells early during microbial invasion (106–108). Some $\gamma\delta$ T cells can differentiate into professional antigen presenting cells, capable of inducing

CD4+ T cell responses and cross-presenting soluble microbial and tumor antigens to CD8+ responder cells (109, 110). Human epidermal $\gamma\delta$ T cells are also able to produce insulin-like growth factor 1 upon activation to control neighboring stromal cells and promote wound healing (78, 111). This high level of functional plasticity could explain why $\gamma\delta$ T cells can be found at different locations and at different stages of the immune response.

The function of CMV-induced V δ 2 neg $\gamma\delta$ T cells can be first understood by analyzing their phenotype. Whereas a naive phenotype is observed in V δ 2 neg $\gamma\delta$ T cells of CMV-seronegative patients, peripheral blood CMV-induced V δ 2 neg $\gamma\delta$ T cells exhibit an effector/memory TEMRA phenotype, strikingly similar to and characteristic of that observed in CMV-specific CD8+ $\alpha\beta$ T cells (112, 113). Most of these cells are CD27-, CD28-, CD45RA+, CD45RO-, Perforin ++, Granzyme B++, CCR7-, CD62L-, and have an activated phenotype (CD69+, HLA-DR+, and but CD25-), suggesting a potential cytotoxic function against CMV-infected cells (Figure 1) (52, 64, 92). A central/memory phenotype is observed less frequently than on CMV-specific CD8+ T cells (92, 112, 113). The accumulation of the TEMRA CD45RA+CD27- phenotype on both CMV-specific CD8+ T $\alpha\beta$ cells and V δ 2 neg $\gamma\delta$ T cells, suggests that this phenotype is induced by the virus (92, 114). Like the CD4+ CD28- $\alpha\beta$ T cells and the CD8+ CD45RA+ CD27- $\alpha\beta$ T cells described by van Lier (114), the presence of CD45RA+CD27- V δ 2 neg $\gamma\delta$ T cells can also be considered as a cell signature of a “past contact with CMV” (64). The absence of these cells in the peripheral blood of patients infected with others viruses is the witness of its peculiar CMV specificity, probably under the dependence of a specific CMV-induced stress signature.

Three quarters of CMV-induced V δ 2 neg $\gamma\delta$ T cells also express CD16 (Fc γ RIIIA), which is a low-affinity receptor for Fc portion of immunoglobulin. This feature, shared with NK cells, represents a specificity of V δ 2 neg $\gamma\delta$ T cells when compared to CD8 $\alpha\beta$ T cells responding to CMV. CMV infection has therefore the unique capability to deeply reshape the CD16 compartment, because CD16 is only expressed by 20% of V δ 2 neg $\gamma\delta$ T cells of CMV-seronegative patients (115). As depicted in Figure 2, CMV infection doubles the number of circulating CD16+ lymphocytes, through this

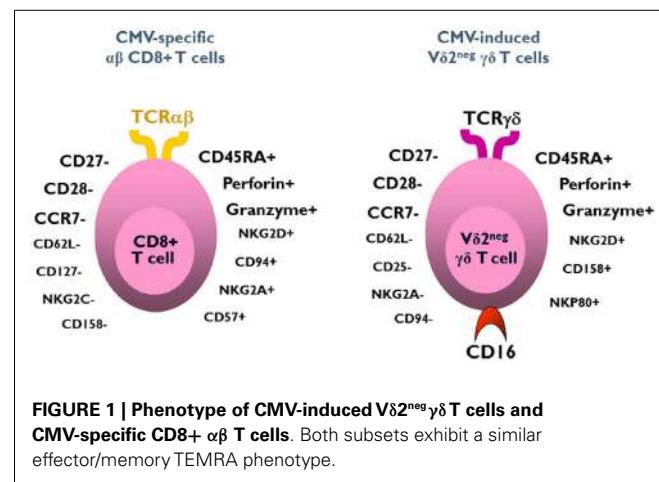


FIGURE 1 | Phenotype of CMV-induced V δ 2 neg $\gamma\delta$ T cells and CMV-specific CD8+ $\alpha\beta$ T cells. Both subsets exhibit a similar effector/memory TEMRA phenotype.

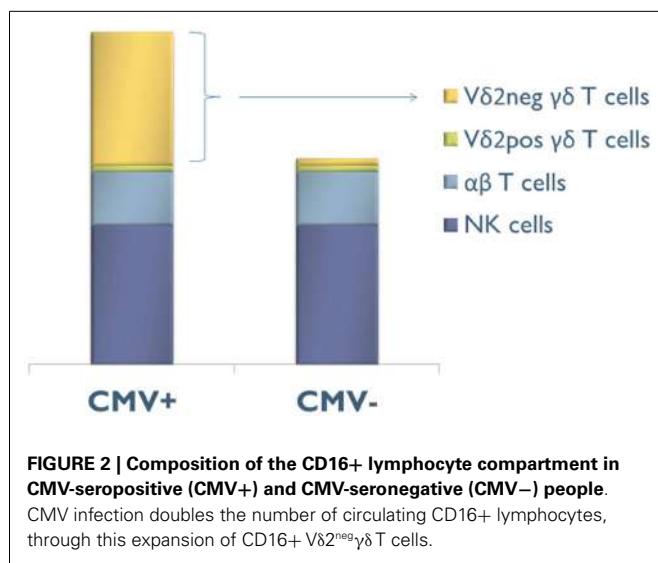


FIGURE 2 | Composition of the CD16+ lymphocyte compartment in CMV-seropositive (CMV+) and CMV-seronegative (CMV-) people. CMV infection doubles the number of circulating CD16+ lymphocytes, through this expansion of CD16+ V δ 2^{neg} $\gamma\delta$ T cells.

expansion of CD16+ V δ 2^{neg} $\gamma\delta$ T cells. A majority of these cells also express NK receptors (NKG2D, CD158b/j, and NKp80), by contrast to CMV-specific CD8+ T $\alpha\beta$ cells (52, 64, 92, 115, 116). This innate-like cell phenotype probably confers to V δ 2^{neg} $\gamma\delta$ T cells a mode of activation and of regulation different from that of $\alpha\beta$ T cells and a non-redundant role in the control of CMV. Moreover, heterogeneity in NK receptor expression can be found within a single clone of V δ 2^{neg} $\gamma\delta$ T cells. Therefore, V δ 2^{neg} $\gamma\delta$ T cell clones can be a mosaic of cells with similar TCR but different activating or inhibiting susceptibility, which could regulate them differently according to the context or tissues (117). In line with this singular phenotype, V δ 2^{neg} $\gamma\delta$ T cells can be considered at the crossroads between T cells and NK cells (118, 119).

In vitro, V δ 2^{neg} $\gamma\delta$ T cells are activated in the presence of free IgG-opsonized CMV or of CMV-infected fibroblast lysates, but not uninfected or other herpes virus-infected fibroblast lysates (HSV or VZV) (52). In culture with CMV-infected cells or IgG-opsonized human CMV, V δ 2^{neg} $\gamma\delta$ T cell lines or clones coming from CMV-infected solid-organ transplant recipients produce large amounts of TNF- α and/or interferon- γ (58, 59, 62, 115, 120). *In vitro*, this CMV-induced interferon- γ production is able to inhibit CMV replication. V δ 2^{neg} $\gamma\delta$ T cells also show perforin/granzyme B dependent cytotoxicity against CMV-infected cells *in vitro* (62, 120). All the data coming from different groups support the concept that most of the V δ 2^{neg} $\gamma\delta$ T cells share the same cytotoxic effector function as CMV-specific CD8+ T $\alpha\beta$ cells (42, 49). However, distinct CMV-induced V δ 2^{neg} $\gamma\delta$ T cell clones can also provide the help to generate from immature dendritic cells a pool of mature dendritic cells (58).

In BALB/c mice and Sprague-Dawley rats, the number of $\gamma\delta$ T cells increase after CMV infection in the draining lymph nodes, liver, peritoneal cavity, and salivary glands (121, 122). $\gamma\delta$ T cell-depleted mice have a significantly higher viral load after CMV infection (123). Using C57BL/6 $\alpha\beta$ and/or $\gamma\delta$ T cell-deficient mice, we recently observed that $\gamma\delta$ T cells were as competent as $\alpha\beta$ T cells to control viral spread and murine CMV-induced disease and to protect mice from death (unpublished data).

All these *in vitro* indications of an anti-viral function of V δ 2^{neg} $\gamma\delta$ T cells are supported *in vivo* by the observation that early expansion of V δ 2^{neg} $\gamma\delta$ T cells correlates with low viral loads, less symptomatic infection, and a rapid viral clearance in renal transplant patients (93).

HOW DO V δ 2^{neg} $\gamma\delta$ T CELLS RECOGNIZE CMV-INFECTED CELLS OR CMV?

Given their large panel of activating receptors, activation of V δ 2^{neg} $\gamma\delta$ T cells during CMV infection may be multifactorial. We will develop here the involvement of the TCR and the CD16 molecule, which could act at different stages of the immune response. While often involved in $\gamma\delta$ T cell activation, NKGD or its ligands (MICA/B and ULBP1-3) do not seem involved in this situation (120), probably because these $\gamma\delta$ T cells are selected by CMV, which is able to inhibit NKG2D-ligands surface expression on infected cells (124). Two other molecules have been shown to co-stimulate activation of CMV-induced V δ 2^{neg} $\gamma\delta$ T cells: CD8 $\alpha\alpha$ (58) and LFA-1, which recognizes up-regulation of ICAM-1 expression by CMV on infected cells (125).

$\gamma\delta$ TCR

T cell receptor involvement in V δ 2^{neg} $\gamma\delta$ T cell reactivity against CMV-infected cells has been demonstrated by inhibition of their activation using blocking anti-TCR antibodies or through transfer of reactivity after transduction of the $\gamma\delta$ TCR in reporter cell lines (120, 125). Analysis of $\gamma\delta$ TCR junctional diversity shows that expansion of V δ 1 and V δ 3 T cells during CMV infection is associated with a restricted repertoire, which is suggestive of an antigens-driven selection (52, 64). This was also observed in neonates infected *in utero* with CMV, who specifically display a preponderant expansion of a particular $\gamma\delta$ T cell population expressing a public invariant V γ 8V δ 1 TCR (62). This population has not been reported in CMV-infected adults, suggesting that it might recognize an antigen specifically induced during *in utero* infection or that this invariant TCR is generated only during fetal life. Recognition of CMV-infected cells by V δ 2^{neg} $\gamma\delta$ T cells is independent of classical major histocompatibility complex (MHC) antigens, by contrast to CMV-specific $\alpha\beta$ T cells. This is consistent with the reported recognition by $\gamma\delta$ T cells of structurally diverse proteins of self and microbial origins (88), and that resembles immunoglobulin-like antigen recognition (126). V δ 1 TCR have also been shown to recognize MHC-like molecules such as MICA/B and CD1. MICA and MICB (MHC class I chain-related proteins A and B) are overexpressed in stressed cells, as in tumor or infected cells. They co-localize with V δ 1 $\gamma\delta$ TCR in some tumors. Both $\gamma\delta$ chains are necessary for the recognition of the MICA/B α 1 and α 2 domains, which is independent of any loaded peptide (94, 127–129). CD1c and CD1d are non-polymorphic molecules, which present lipids and glycolipids to NKT cells (130, 131) and also activate V δ 1 and V δ 3 $\gamma\delta$ T cells (107, 132). Specific interaction between V δ 1 $\gamma\delta$ TCR and CD1c molecule has been demonstrated using TCR transduction in reporter cell line, showing that no glycolipid are involved in this recognition (107). Interaction between V δ 1 $\gamma\delta$ TCR and CD1d has also been demonstrated using tetramers, recombinants TCR, and structural studies (133–135). CD1d can be recognized by V δ 1 $\gamma\delta$ TCR as an “unloaded” form or

when loaded with endogenous glycosphingolipids (133–135) or exogenous phospholipids (108, 136).

MICA/B and CD1d are not expressed on the surface of CMV-infected cells (120) and only 0.3% of CMV-induced V δ 2^{neg} $\gamma\delta$ T cells are stained with CD1d- α GalCer tetramers (our unpublished data), suggesting that CMV does not select for MICA/B or CD1d-specific V δ 2^{neg} $\gamma\delta$ T cells. CMV-infected cells therefore offer the opportunity to discover new V δ 2^{neg} $\gamma\delta$ T cell ligands. Using a strategy based on the generation of monoclonal antibodies with the same antigen specificity as the CMV-induced V δ 2^{neg} $\gamma\delta$ T cells, we identified EPCR as another MHC-like ligand for a V γ 4V δ 5 TCR (125). EPCR is a non-polymorphic protein constitutively expressed on endothelial cells and involved in the regulation of coagulation through the activation of protein C (137). It did not have any described “immunologic” function, although it displays a structural homology with CD1d (125). Recognition of EPCR by V γ 4V δ 5 TCR is independent of glycosylation and has a binding mode that does not involve discrimination of lipid antigens. Cell infection by CMV does not increase EPCR expression and V γ 4V δ 5 T cell clone reactivity requires co-stimulatory molecules, which are over expressed in CMV-infected cells, such as LFA-3 (CD2 ligand) and ICAM-1 (LFA-1 ligand) (Figure 3A) (128, 138–140). This constitutive expression of EPCR opens the possibility of its homeostatic interaction with $\gamma\delta$ TCR, as previously reported for mice skin epithelial $\gamma\delta$ T cells and ligands expressed

on keratinocytes (141). This interaction could serve either to keep tissue $\gamma\delta$ T cells pre-activated and ready to swiftly engage in the immune response or to activate regulatory functions necessary for maintenance of tissue integrity at steady state. Whether such a constitutively expressed TCR ligand needs conformation, topology or molecular interaction changes at the surface of target cells to prime stress surveillance response of $\gamma\delta$ T cells deserves further investigations. Not all V δ 2^{neg} $\gamma\delta$ T cells reactive against CMV-infected cells recognize EPCR, indicating the existence of other TCR ligands. Their characterization will be important to improve our knowledge of how cell stress and self-dysregulation are captured by V δ 2^{neg} $\gamma\delta$ T cells.

CD16

As mentioned above, CMV infection is associated with the expression of CD16 at the cell surface of a large majority of circulating V δ 2^{neg} $\gamma\delta$ T cells. This expression did not allow $\gamma\delta$ T cells to perform antibody-dependent cell-mediated cytotoxicity (ADCC) against CMV-infected cells pre-incubated with CMV hyperimmune IgGs, probably because of the seemingly low rate of IgGs directed against CMV-infected cells in sera of infected people (115). However, even in the absence of TCR stimulation, CD16+ V δ 2^{neg} $\gamma\delta$ T cells produce interferon- γ and inhibit CMV replication when activated by IgG-opsonized free CMV, in presence of IL-12 and interferon- α , two cytokines produced by

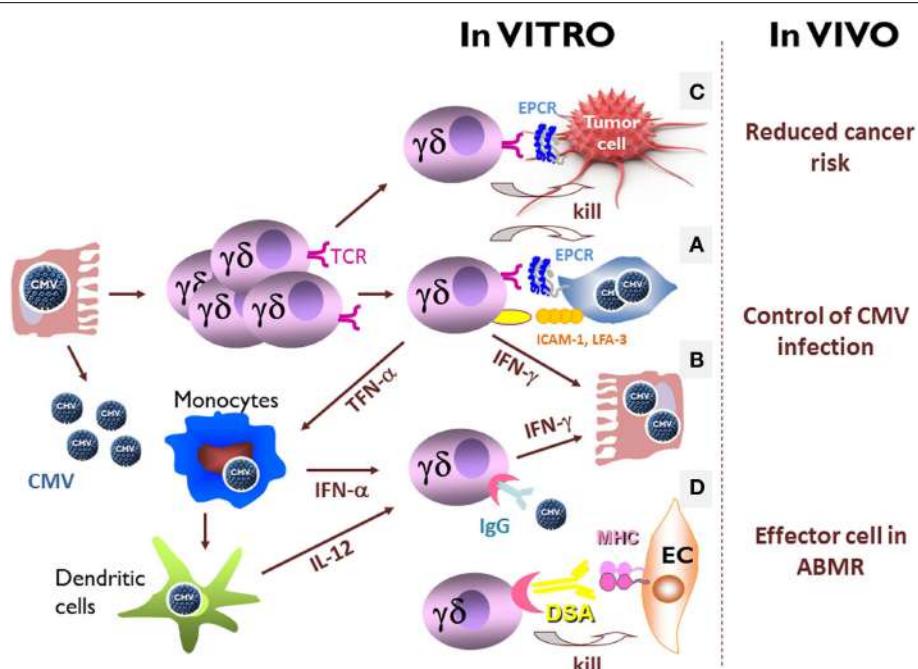


FIGURE 3 | *In vitro* and *in vivo* direct and indirect effects of CMV-induced V δ 2^{neg} $\gamma\delta$ T cells. (A) In culture with CMV-infected cells, V δ 2^{neg} $\gamma\delta$ T cell lines or clones coming from CMV-infected solid-organ transplant recipients produce large amounts of TNF- α and/or interferon- γ , and exert a strong cytotoxicity against CMV-infected cells. V δ 2^{neg} $\gamma\delta$ T cell reactivity requires EPCR expression and co-stimulatory molecules, which are over expressed in CMV-infected cells, as LFA-3 (CD2 ligand) and ICAM-1 (LFA-1 ligand). (B) In the absence of TCR stimulation, CD16+ V δ 2^{neg} $\gamma\delta$ T cells produce interferon- γ and inhibit CMV replication when activated by IgG-opsonized free CMV, in

presence of IL-12 and interferon- α , two cytokines produced by monocytes/macrophages and dendritic cells during CMV infection.

(C) CMV-induced V δ 2^{neg} $\gamma\delta$ T cells have a TCR-dependent cross-reactivity against CMV-infected cells and tumor cells. (D) CMV-induced CD16+ V δ 2^{neg} $\gamma\delta$ T cells are able to perform antibody-dependent cell-mediated cytotoxicity (ADCC) against endothelial cells (EC) coated with donor-specific antibody (DSA). Within the grafts, $\gamma\delta$ T cells are retrieved in close contact with endothelial cells in the peritubular capillaritis and glomerulitis associated with acute antibody-mediated rejection, only in CMV-experienced patients.

monocytes/macrophages and dendritic cells during CMV infection (**Figure 3B**) (115). This antibody-dependent cell-mediated inhibition (ADCI) is a new function of V δ 2^{neg} $\gamma\delta$ T cells in their arsenal to control the virus, where antigen specificity is mediated by the antibody and not by the TCR, and is probably controlled by the cytokine microenvironment. ADCI could be restricted to specific areas, such as CMV-infected tissues or mucosa infiltrated by activated macrophages or dendritic cells, and where V δ 2^{neg} $\gamma\delta$ T cells are homing and suspected to play a pivotal role. In accordance with the late expansion of V δ 2^{neg} $\gamma\delta$ T in the blood during the infection, ADCI could be involved in the prevention of CMV reactivation by V δ 2^{neg} $\gamma\delta$ T cells, when antibodies have been generated (42).

UNEXPECTED ANTI-TUMOR EFFECTS OF CMV-INDUCED V δ 2^{neg} $\gamma\delta$ T CELLS

Because of their immunosuppressed status, the risk of cancer in kidney transplant recipients is between 2.5 and 4 times greater than in the general population, with mainly non-melanoma skin cancer (the most common type of malignancy in kidney transplant recipients), lymphoma, cancer of the lip, vulvovaginal tumors, and kidney cancers (142–145). This is consistent with the concept of cancer immunosurveillance and cancer immunoediting, which has been well characterized in recombinase-activating gene (RAG) knock-out mice (146), as well as in humans (147–150). Among the cells involved in anti-tumor immunity, $\gamma\delta$ T cells are considered to play a key role (95). As a major demonstration, $\gamma\delta$ TCR knock-out mice have been shown to develop more skin cancers than wild-type mice (151). In humans, $\gamma\delta$ T cells infiltrate many carcinomas and exert a strong interferon- γ production and cytotoxicity against carcinoma cells *in vitro* (77, 79, 81, 94–99, 151–157). More recent studies also reported opposite results suggesting pro-tumoral functions of $\gamma\delta$ T cells both in human cancers (158) and in murine models (159–161) making the role played by the different $\gamma\delta$ T cells in tumor surveillance more subtle. Nevertheless, during the past years, $\gamma\delta$ T cells have been targeted in cancer immunotherapy trials showing mitigated but encouraging clinical benefit [reviewed in Ref. (162)]. It is noteworthy that all these trials uniquely targeted V γ 9V δ 2 T cells. Immunity to tumors may be acquired during events that have no clear relationship to cancer, and some infectious diseases have been associated with a reduced risk of cancers (163, 164). In line with these observations, CMV-induced V δ 2^{neg} $\gamma\delta$ T cells have a TCR-dependent cross-reactivity against CMV-infected cells and tumor cells (**Figure 3C**) (58, 120). V δ 2^{neg} $\gamma\delta$ T cell lines or clones kill tumor cells as efficiently as CMV-infected cell *in vitro*. Moreover, using a human tumor xenograft model in immunodeficient mouse, we observed that CMV-induced V δ 2^{neg} $\gamma\delta$ T cells could inhibit tumor growth *in vivo* (165, 166). Finally in kidney transplant recipients, high CMV-induced V δ 2^{neg} $\gamma\delta$ T cell counts as well as a past contact with CMV were associated with reduced cancer occurrence in the upcoming years (167). Taken together, these data reveal a dual role for CMV-induced V δ 2^{neg} $\gamma\delta$ T cells in kidney transplant recipients in viral control and in surveillance of subsequent malignancy. This shared reactivity against CMV-infected and tumor cells has been observed also after allogeneic stem cell transplantation (58), where CMV infection is associated with a decreased risk of acute myeloid

leukemia relapse (168, 169), and where $\gamma\delta$ T cell expansion is associated with a reduced risk of relapse (170). This potential protective role of CMV against cancer in transplant recipients has been challenged by other groups (171), and could be in apparent contrast to the previously reported presence of the CMV genome and antigens in diverse types of carcinomas (172, 173). However, even if CMV has been suggested to play a direct role in carcinogenesis, one cannot exclude that its reactivation in tumors represents an epiphenomenon due for instance to inflammation (174, 175). All of these studies may be consistent with our results if we assume that both CMV-infected cells and tumor cells (infected or not) express the same stress-induced molecules recognized by $\gamma\delta$ TCRs, resulting in the selection of common immune effector cells among which V δ 2^{neg} $\gamma\delta$ T cells take an important part. They also highlight the ambiguous relationships interwoven between a virus, CMV, and its host: Parasitism or symbiosis?

UNEXPECTED INDIRECT EFFECT OF V δ 2^{neg} $\gamma\delta$ T DURING ANTIBODY-MEDIATED REJECTION

The epidemiological link observed between CMV and acute or chronic rejection is still not well understood. Many hypotheses have been proposed. CD4+ T cells of CMV-seropositive patients produce interferon- γ and induce both MHC class II and adhesion molecules overexpression on endothelial cells, which could potentiate *in situ* allogeneic reaction (176, 177). A cross-reactivity of CMV-specific T cells against alloantigens is also discussed (178, 179). A direct CMV effect is also likely because the persistence of the virus in the blood or the kidney leads to aggressive fibrotic lesions (26, 28, 180–182).

Recently, the importance of the recipient's humoral response against the renal allograft has been recognized to play a key role in immunological injuries contributing to graft deterioration (183–191). Nowadays, antibody-mediated rejection is considered as the leading cause of graft loss on the long range (192). From an immunological point of view, donor-specific antibody (DSA)-mediated lesions are considered to rely on complement-fixing DSA-mediated lysis (187), direct DSA-mediated apoptosis (193), and/or ADCC by NK cells (194, 195). Until recently, complement was the most recognized way leading to graft endothelial cell injury, because deposition of C4d, a breakdown product of complement component C4, in peritubular capillaries represented the only specific tool providing the "immunopathological evidence" of DSA interaction with graft tissue (191, 196, 197). However, it does not encompass all DSA-mediated lesions (198). Glomerulitis and peritubular capillaritis, which are defined by an accumulation of polymorphonuclear cells, macrophages, and lymphocytes around capillaries, are associated with DSA, are more predictive of graft loss than C4d deposition (188, 199), and are now recognized as the main lesions of antibody-mediated rejection (200). Among these infiltrates, NK cells have recently been shown to be involved in DSA-mediated lesions of kidney microcirculation, suggesting that ADCC could play a role in DSA-mediated lesions through DSA interaction with the low-affinity Fc receptor for IgG (Fc γ RIIIA-CD16) expressed on NK cells (194, 195, 201). Interestingly, NK cells are not the only candidate as cell mediator of these lesions. As pointed before, CMV infection deeply reshapes the CD16+ lymphocyte compartment composition in CMV+

transplant recipients who exhibits an equal amount of CD16+ NK cells and CD16+ V δ 2 neg $\gamma\delta$ T cells at the periphery (115). We have shown that CMV-induced CD16+ V δ 2 neg $\gamma\delta$ T cells are able to perform ADCC against stromal cells coated with DSA *in vitro* (Figure 3D) (202). Within the grafts, $\gamma\delta$ T cells are found in close contact with endothelial cells in the peritubular capillaritis and glomerulitis associated with acute antibody-mediated rejection, only in CMV-experienced patients. Their localization in antibody-mediated microcirculation injuries is similar to that reported for NK cells (195) and macrophages (203). Finally, an inverse correlation between a persistently increased percentage of circulating CMV-induced $\gamma\delta$ T cells and the 1-year estimated glomerular filtration rate is observed only in kidney recipients with DSA (202). $\gamma\delta$ T cells are usually viewed non-alloreactive because they do not recognize peptides bound to MHC molecules. However, our recent data support the conclusion that CMV-induced CD16+ $\gamma\delta$ T cells are a new player in antibody-mediated lesions of kidney transplants. As for recognition of IgG-opsonized CMV, the antigen specificity of $\gamma\delta$ T cell activation relies on the antibody and not on $\gamma\delta$ TCR. Moreover, these findings suggest that $\gamma\delta$ T cell ADCC could represent a new physiopathological contribution to the well-known but poorly understood association between CMV infection and the increased occurrence of rejection (17, 29), poor long-term graft function (16, 23, 180, 204), and low graft survival (25, 26).

In contrast to these data, two teams have proposed that V δ 1 $\gamma\delta$ T cells play regulatory functions associated with an operational tolerance in liver transplantation (205–209). However, The Spanish team finally showed that alterations in the $\gamma\delta$ T cell compartment were not restricted to tolerant liver recipients and confirmed the association between CMV infection and V δ 1 $\gamma\delta$ T cell expansions (55). Most interestingly, the Japanese team described V δ 1 T cells with a public TCR infiltrating all tested tolerant liver grafts and normal livers and not found in rejected organs (209). Identification of the antigen recognized in healthy liver by this TCR could valuably contribute to decipher the mode of activation of $\gamma\delta$ T cells with regulatory functions involved in preservation of tissue integrity.

Altogether, these data suggest that depending on the presence of CMV and/or DSA, $\gamma\delta$ T cells could play different seemingly opposite functions on transplanted organ, which deserve further investigation in the future.

CONCLUSION AND PERSPECTIVES

In summary, numerous studies have now shown the involvement of V δ 2 neg $\gamma\delta$ T cells within the immune response directed against CMV, with direct anti-viral effects, but also unexpected indirect effects in the context of kidney transplantation. Although most of the literature about $\gamma\delta$ T cells considers them as actors of the innate immune response, the peripheral blood CMV-induced V δ 2 neg $\gamma\delta$ T cells exhibit surprisingly at least three characteristics of the adaptive immunity. First like B cells, and $\alpha\beta$ T cells, they use somatic rearrangement of V, D, and J genes to generate diverse antigen receptors (88). Secondly, they undergo monoclonal to polyclonal expansions, characterized by a variable extent of their repertoire from one patient to the other. Finally, these cells seem to have the ability to mount anamnestic responses, because

they have the phenotype of effector/memory cells, and undergo a more rapid expansion during CMV reactivation than during primo-infection (64).

At the efferent phase of the immune response, their functions, activating pathways and kinetics have been better characterized. Understanding where, when and how naïve V δ 2 neg $\gamma\delta$ T cells are activated at the afferent phase of the CMV immune response is more challenging and will most probably require *in vivo* studies in animal models. The encouraging results obtained by ours and Thomas Winkler's team on the protective role of mouse $\gamma\delta$ T cells against murine CMV, certainly pave the way for addressing these issues (210). Molecular understanding of how CMV-induced V δ 2 neg $\gamma\delta$ T cells recognize CMV-infected cells and tumor cells necessitates the identification of representative antigenic ligands that could reveal valuable tools for vaccination trials targeting $\gamma\delta$ T cells. An alternative is the use of $\gamma\delta$ T cell therapy after *ex vivo* expansion of V δ 2 neg $\gamma\delta$ T cells. Interesting progress has recently been made in this direction by the teams of Laurence Cooper and John Anderson who set up conditions for clinical scale propagation of polyclonal $\gamma\delta$ T cell lines (211, 212).

All these basic and clinical studies are prerequisite to improve $\gamma\delta$ T cell-based immunotherapy, but a shorter term use of V δ 2 neg $\gamma\delta$ T cells in the clinics, will probably come from solid-organ transplantation, in which V δ 2 neg $\gamma\delta$ T cell monitoring could prove a useful immunological biomarker to classify patients at risk to develop CMV infection or cancer.

Moreover, transplant patients are also prone to develop other types of infections, either parasitic (with e.g., *Toxoplasma gondii*) or bacterial (bartonella, atypical mycobacteria), which induce V γ 9V δ 2 T cell expansion due to their production of phospho-antigens. Routine monitoring of V γ 9V δ 2 T cells in our center also allowed us in several cases during the last decade to make differential diagnosis of these infections in kidney transplant recipients.

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$\gamma\delta$ T cell-mediated immune responses in disease and therapy

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The role of $\gamma\delta$ T cells in immunotherapy has gained specific importance in the recent years because of their prominent function involving directly or indirectly in the rehabilitation of the diseases. $\gamma\delta$ T cells represent a minor population of T cells that express a distinct T cell receptor (TCR) composed of $\gamma\delta$ chains instead of $\alpha\beta$ chains. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells display a restricted TCR repertoire and recognize mostly unknown non-peptide antigens. $\gamma\delta$ T cells act as a link between innate and adaptive immunity, because they lack precise major histocompatibility complex (MHC) restriction and seize the ability to recognize ligands that are generated during affliction. Skin epidermal $\gamma\delta$ T cells recognize antigen expressed by damaged or stressed keratinocytes and play an indispensable role in tissue homeostasis and repair through secretion of distinct growth factors. $\gamma\delta$ T cell based immunotherapy strategies possess great prominence in the treatment because of the property of their MHC-independent cytotoxicity, copious amount of cytokine release, and a immediate response in infections. Understanding the role of $\gamma\delta$ T cells in pathogenic infections, wound healing, autoimmune diseases, and cancer might provide knowledge for the successful treatment of these diseases using $\gamma\delta$ T cell based immunotherapy. Enhancing the human $V\gamma9V\delta2$ T cells functions by administration of aminobisphosphonates like zoledronate, pamidronate, and bromohydrin pyrophosphate along with cytokines and monoclonal antibodies shows a hopeful approach for treatment of tumors and infections. The current review summarizes the role of $\gamma\delta$ T cells in various human diseases and immunotherapeutic approaches using $\gamma\delta$ T cells.

Keywords: $\gamma\delta$ T cells, pathogenic infections, cancer, autoimmunity, wound healing, immunotherapy

INTRODUCTION

T cells are the principal lymphocytes that play a vital role in cell-mediated immune responses. Majority of the T cells are $\alpha\beta$ T cells with α and β chains and a minor population of $\gamma\delta$ T cells do exist that play a pivotal role in the host defense system (1). $\gamma\delta$ T cells are located in peripheral blood (PB), intestine, skin, spleen, and lymph nodes where they were found to act as interface for the cross talk between innate and cell-mediated immune cells (2). The functional development of $\gamma\delta$ T cells is initiated much earlier than the development of $\alpha\beta$ T cells in embryogenesis, where $\gamma\delta$ T cell Receptor (TCR) gene rearrangements were detected in the fetal thymus by “day 14” of embryogenesis (E14). The earliest murine $\gamma\delta$ T cell “wave” expresses $V\gamma3V\delta1$ and gives rise to skin dendritic epidermal $\gamma\delta$ T cells (DETC). Later, vaginal and gut intra-epithelial $\gamma\delta$ T lymphocytes (IEL) and immune organ $\gamma\delta$ T cells were developed (3). $V\gamma3V\delta1$ DETCs are exclusively generated in the fetal thymus, migrate to epidermal epithelia, expand locally and are maintained throughout the life of an individual (4). $\gamma\delta$ T cells express TCR molecule on their cell surface, but there are only a few variable genes available to construct $V\gamma/V\delta$ TCR proteins. In

addition, the usage of the $V\gamma/V\delta$ genes is not random but appears to be dictated by the localization of $\gamma\delta$ T cells (5). Hence $\gamma\delta$ T cells are well engaged in newborns to contribute to immune-protection and immune-regulation (6, 7).

$\gamma\delta$ TCR recognize non-peptide antigens like glycerolipids and other small molecules, polypeptides that are soluble or membrane anchored, and cross linked to major histocompatibility complex (MHC) molecules or MHC-like molecules in an antigen-independent manner (8). Human $V\gamma9V\delta2$ (also known as $V\gamma2V\delta2$) T cells can be activated by metabolites from isoprenoid synthetic pathway. These include (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), exogenous prenyl pyrophosphate from bacteria and parasitic protozoa and isopentenyl pyrophosphate (IPP), and endogenous prenyl pyrophosphate deriving from mevalonate pathway that operates in human. Aminobisphosphonates may activate $V\gamma9V\delta2$ T cells by inhibiting the key enzyme farnesyl pyrophosphate synthase of mevalonate pathway in certain tumors leads to upregulating the endogenous pool of IPP (9). Vavassori et al. identified butyrophilin BTN3A1 molecule, which is involved in the presentation of phosphorylated antigens to

$V\gamma 9V\delta 2$ T cells (10). Recently, Sandstrom et al. demonstrated that intracellular B30.2 domain of butyrophilin 3A1 (BTN3 A1) protein binds phosphoantigens (pAg) to mediate activation of human $V\gamma 9V\delta 2$ T cells. This intracellular B30.2 domain of BTN3A1 directly binds pAg through a positively charged surface pocket and charge reversal of pocket residues abrogates binding and activation of $V\gamma 9V\delta 2$ T cells (11). Furthermore, Uldrich et al. reported that CD1d presents lipid-based antigens to human $V\delta 1^+ \gamma\delta$ T cells (12). $\gamma\delta$ T cells use both the TCR and also additional activating receptors like natural killer (NK) cell activating receptor (NKG2D), toll like receptor (TLR), and NOTCH signaling to respond to stress induced ligands and infection (13). Activated $\gamma\delta$ T cells promote the anti-infection capabilities of resident macrophages, NK cells, and also enhance the maturation of dendritic cells (DCs). Besides that, they secrete cytokines and chemokines to recruit pro-inflammatory neutrophils to accelerate the elimination of pathogens and the repair of damaged tissues. Neutrophils in turn suppress the $\gamma\delta$ T cells activation to reduce the inflammation when the infection has been resolved (14). In addition, $\gamma\delta$ T cells in the skin produce keratinocyte growth factor (KGF), important cytokine for wound repair, and epithelial cell regeneration. It has been demonstrated that the human $\gamma\delta$ T cells activation and expansion can be controlled by forkhead box P3 (FOXP3) expressing regulatory T cells (Tregs), programmed death-1 (PD-1), and cytotoxic T lymphocyte antigen (CTLA)-4 both *in vivo* and *in vitro* (15).

$\gamma\delta$ T cells bridge innate and adaptive immunity and play a protective role in immune-surveillance. Effector $\gamma\delta$ T cells produce interferon (IFN)- γ , tumor necrosis factor (TNF)- α , which enhance cell-mediated immune response and interleukin (IL)-17 that plays a vital role in early neutrophil mediated response. In addition, cytotoxic components such as perforin, granzymes secreted by these cells ultimately cause direct or indirect effect of cytotoxicity against infected cells (16). They provide a wide range of defense mechanisms against microorganisms such as viruses, bacteria, protozoa, and diseases like cancer and also in healing of wounds and burns. In addition, $\gamma\delta$ T cells also play a role in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) through their antigen-presenting capacity, release of pro-inflammatory cytokines, immunomodulatory properties, interaction with Tregs, and promotion of antibody production (17). Pantelyushin et al. reported that apart from retinoid-related orphan receptor gamma-t (ROR γ t $^+$) innate lymphocytes, $\gamma\delta$ T cells also produce cytokines like IL-17A, IL-17F, and IL-22 that are essential and enough for psoriatic plaque formation in a disease model that closely resembles human psoriatic plaque formation (18). Current review exclusively focuses on the role $\gamma\delta$ T cells in specific pathogenic infections, anti-tumor activity, healing of wounds and burns, autoimmune diseases, and few insights on their immunotherapy.

PATHOGENIC INFECTIONS

TUBERCULOSIS

Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) is considered to be one of the serious infectious disease worldwide causing 1.7 million deaths every year. Around 30% of the world's population is affected by *M. tuberculosis* and approximately 100

million people died due to tuberculosis (TB) over the last century (19). Hence, there is an urgent need to find out the host factors that delineate the individuals susceptible to TB. pAg such IPP and HMBPP are the key ligands that activate $V\gamma 9V\delta 2$ T cells. HMBPP is nearly 1000-fold more effective than IPP for the *in vitro* activation of $V\gamma 9V\delta 2$ T cells (20). Mtb produces HMBPP, which is recognized by $V\gamma 9V\delta 2$ TCR and drives the activation of $V\gamma 9V\delta 2$ T cells (21). Effector $V\gamma 9V\delta 2$ T cells are shown to participate in the anti-TB immune response by production of various cytokines (Th1, Th2, and Th17) and also activation of other immune cells such as CD4 $^+$ and CD8 $^+$ T cells, B cells, DCs, and macrophages (22). The *in vivo* studies have demonstrated that the major expansion of $V\gamma 9V\delta 2$ T cells in macaques is induced only by HMBPP plus IL-2 co-treatment, but not IL-2 or HMBPP alone (23) although IL-2 treatment of macaques expands CD4 $^+$ CD25 $^+$ Foxp3 $^+$ Treg cells (24). In a primate model for TB, $\gamma\delta$ T cells produce IL-22 initially, which can be down regulated by HMBPP. There are various subsets of $\gamma\delta$ T cells, which are self regulative, and HMBPP treatment during early stages of infection might be helpful in evading Mtb (25). Peng et al. showed that upon stimulation with Mtb heat treated antigen (Mtb-HAg), levels of IFN- γ producing $V\gamma 9V\delta 2$ T cells increased in number and were the main source of IL-17 (26). This led to the increased recruitment of phagocytic cells to the infected site and formation of granulomas in pulmonary TB. This reaction was antigen specific, because immunizing the same host once again with Mtb-HAg has led to faster reactivation of $V\gamma 9V\delta 2$ T cells. Thus, stimulation of $V\gamma 9V\delta 2$ T cells with pyrophosphates like IPP and HMBPP might represent a novel vaccine strategy to identify the key effector pathways of *Mycobacteria* stimulated $V\gamma 9V\delta 2$ T cells that potentially act to inhibit the intracellular growth of *Mycobacteria*. In addition to that, human $V\gamma 9V\delta 2$ T cells can recognize many pathogen antigens and show rapid immune responses during infections including *E. coli* infections, salmonellosis, brucellosis, leprosy, tularemia, legionellosis, and listeriosis (27).

Bovine TB is caused by *Mycobacterium bovis* is a major zoonotic problem in United Kingdom and developing countries. $\gamma\delta$ T cells show a major immunological response against *M. bovis* infection. Workshop cluster 1 (WC1) molecule expressed on $V\gamma 9V\delta 2$ T cells is involved in the antigen recognition including heat-shock proteins, phospholipids derived from *Mycobacteria*, and other non-peptide antigens. WC1 $^+$ $\gamma\delta$ T cells are important in the development of granulomas during *M. bovis* infection by upregulating IFN- γ , IL-12, IL-18, MHC II, CD80/86, CD40, and adhesion molecules (22).

MALARIA

Malaria is a mosquito borne infectious disease caused by parasitic protozoan *Plasmodium falciparum* in humans and other animals. The WHO report 2013, has an estimate of 207 million cases of malaria with approximately 0.627 million deaths and 3.4 billion people prone to the risk of malaria (28). The information about the *in vivo* activation and anti-plasmodial action of the $V\gamma 9V\delta 2$ T cells is indistinct and it is important to understand the mechanisms of early control of the parasite multiplication and parasite density. It has been shown that elevated levels of $\gamma\delta$ T cells in PB and spleen occur during acute plasmodium infection (29). $\gamma\delta$ T

cell-deficient [TCR δ -knockout (KO)] mice were unable to clear the infected red blood cells, showed high parasitemia and eventually died (30). Therefore, $\gamma\delta$ T cells have the potential to react with malaria antigens rather than the $\alpha\beta$ T cells or by NK cells (31). V γ 9V82 TCR recognizes schizont associated antigen (SAA) and HMBPP, are the antigens of *P. falciparum* result in the activation of V γ 9V82 T cells (31, 32). Activated $\gamma\delta$ T cells produce huge amount of IFN- γ in presence of activated monocyte cytokines IL-10, IL-2, and IL-1 β . TLR-sensitized DCs express enhanced co-stimulatory factors on their surface and induce high levels of IFN- γ production by human V γ 9V82 T cells (33). The CD40 ligand on $\gamma\delta$ T cells is ligated with CD40 on DCs and this signaling synergistically enhances the uptake of plasmodium antigens via DCs by increasing the production of IL-12 (30). Previous reports suggest that the number of polyclonally activated V82 $^{+}$ T cells increase in PB during the acute phase of *P. falciparum* in malaria. In addition, *in vitro* activated V γ 9V82 T cells express granzyme-A and B, perforin, Fas/Fas ligand (FasL) and granulysin to kill the asexual stages of *P. falciparum* in the blood as well as inhibit the growth of intra erythrocytic stages (34). The *P. falciparum* was also known to be inhibited by human V γ 9V82 T cells *in vitro*. The targets recognized by V γ 9V82 T cells are extracellular merozoites of the host erythrocytes and this exclusively requires the contact between $\gamma\delta$ T cells and the merozoites (35). Both the blood stages of intra erythrocytic parasite and extracellular merozoites themselves activates the V γ 9V82 T cells resulting in their degranulation by granulysin, but not by perforin (36). Teirlinck et al. reported that V γ 9V82 T cells have the ability to develop effector memory cells after infection with *P. falciparum* (37) and this feature might be helpful in the development of novel cell based malaria vaccine.

ACQUIRED IMMUNODEFICIENCY SYNDROME

Acquired immunodeficiency syndrome is caused by human immunodeficiency virus (HIV) and is one of the greatest health crises ever faced by the global community. It has been demonstrated that circulating V γ 9V82 T cells exhibit anti-HIV role by secreting chemokines for HIV entry co-receptors, producing soluble antiviral factors, and killing the infected cells by the mechanisms similar to cytotoxic T lymphocytes (CTL) and NK cells. However, V γ 9V82 T cells are found to be depleted in the advanced stages of the HIV infection and the insufficient number of V γ 9V82 T cells leads to increased potential for chronic inflammation. The envelope glycoprotein 120 (gp120) of CCR5 tropic strains of HIV could bind with the surface receptors CCR5 and α 4 β 7 expressing on $\gamma\delta$ T cells. This binding activates the P38 MAP kinase, which in turn promotes the Fas dependent caspase activation and induces the cell death (38). Further, when macaques were immunized with proteins like simian immunodeficiency virus (SIV) gp120 and gag p27 induced the production of chemokines CCL-3, CCL-4, CCL-5 (RANTES), which in turn bind with the CCR5 thus inhibiting the entry of HIV into the host (39). The levels of these chemokines were increased by the engagement of the ectopically expressed receptors called Natural Cytotoxic Receptors (NCRs) like NKP30 on the surface of the V81 T cells that induced the production of high levels of significant chemokines and controlled the levels of HIV (40). However, activated V γ 9V82 T cells may directly suppress HIV replication by releasing CC-chemokines, competing

with HIV for CCR5 entry coreceptor, and other soluble antiviral factors (41). Hence, the treatment approaches might include targeting proviral infection using the activation of V γ 9V82 T cells with aminobisphosphonates and IL-2 to improve the anti-viral activity but not on the prolonged virus as the viral load is severe in this condition. $\gamma\delta$ T cells coordinate activated innate immunity with adaptive antibody and T cell responses in preventive vaccination against HIV-1 infection.

Apart from HIV, $\gamma\delta$ T cells were known to be involved in following viral infections. $\gamma\delta$ T cells are highly specific to the micro-environment in which they thrive and said to possess organ specific functions. $\gamma\delta$ T cells in liver produce IL-17 is very essential for meliorating adenovirus mediated hepatitis, neutralizing IL-17 with antibodies aggravated these conditions (42). $\gamma\delta$ T cells can compensate neutrophil IL-17 production. For instance, when mice were infected with *Cryptococcus neoformans* strain-H99 γ , which leads to neutropenia, IL-17 producing $\gamma\delta$ T cells mediated the regulation of innate and adaptive cells to mount a successful immune response (43). $\gamma\delta$ T cells were known to possess memory just like other adaptive immune cells. This character of $\gamma\delta$ T cells was brought into limelight by the association of $\gamma\delta$ T cells response on cytomegalovirus (44). $\gamma\delta$ T cells were reduced in number during sepsis and acute reduction of these cells resulted in mortality of patients suffering with sepsis (45). The protective role of $\gamma\delta$ T cells have also been confirmed in few other infectious diseases caused by viruses including influenza virus, West Nile virus, herpes simplex virus, Epstein–Barr virus, and human hepatitis virus C.

CANCER

$\gamma\delta$ T cells have a unique role in the immune-surveillance against malignancies and also an advantage over $\alpha\beta$ T cells because they can directly recognize molecules that are expressed on cancer cells without need of antigen processing and presentation (46). An important therapeutic feature of $\gamma\delta$ T cells is that these favorably kill cancer cells and show low reactivity toward non-transformed cells (47). Variable region of the V γ 9V82 TCR plays a major role in recognition of the antigen (48). V γ 9V82 TCR recognize increased pool of endogenous IPP, which may only be found in tumor cells but not in healthy tissues. Activated V γ 9V82 TCRs promote $\gamma\delta$ T cell cytotoxicity through increased secretion of perforin/granzymes, IFN- γ , and TNF- α , IL-17, up-regulates expression of FasL and TNF-related apoptosis-inducing ligand (TRAIL) (49). Among the list of mediators promoting anti-cancer cytotoxicity mentioned above, IL-17 in tumor micro-environment remains controversial showing anti-tumor role and pro-tumor role. In several murine transplantable tumor models, anti-cancer drugs (such as oxaliplatin or anthracyclines) that induced immunogenic cell death, triggered the local invasion of IL-17 producing $\gamma\delta$ T cells, which occurred before and was required for the subsequent invasion of tumor-reactive CTL (50, 51). However, it was shown that IL-17 producing $\gamma\delta$ cells acts as tumor promoting cells by inducing angiogenesis (52). In addition, Rei et al. demonstrated that IL-17 producing V γ 6 $^{+}$ $\gamma\delta$ cells promotes tumor growth in the ID8 ovarian cancer model and thus opposes the widely accepted anti-tumor function of $\gamma\delta$ cells (53). In addition to TCR dependent pathways, NKG2D and DNAX accessory molecule-1 (DNAM-1) expressed on V γ 9V82 T cells plays a critical role in anti-tumor

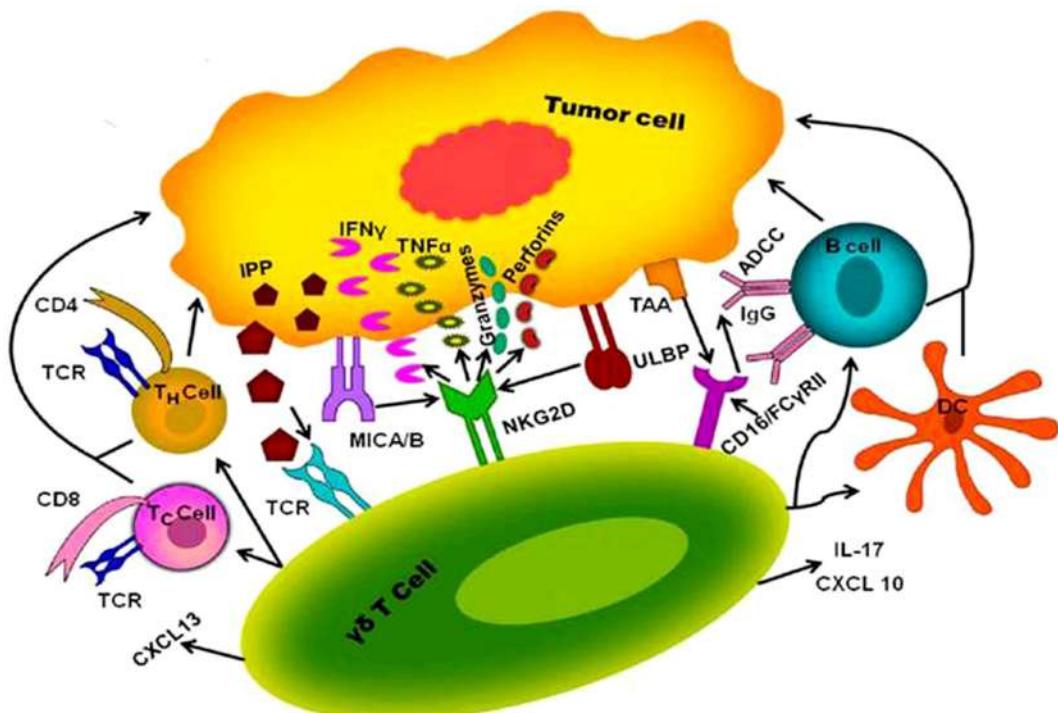


FIGURE 1 | Proposed mechanism of interaction between human $\gamma\delta$ T cell and tumor cell. $\gamma\delta$ T cells activated through interaction of $\gamma\delta$ T cell receptor with IPP produced via mevalonate pathway at higher concentration in tumor cell. Besides that NKG2D provides activation signals upon binding to MICA/B and ULBP of tumor antigens results in the release of cytokines and

chemokines such as IFN- γ , TNF- α , IL-2, IL-17, perforin, granzymes, CXCL-10, and CXCL-13, which can directly lyse the tumor cell and can recruit other immune cells like T cells (T_h cells and T_c cells), B cells, and dendritic cells (DC) to aid killing of tumors. $\gamma\delta$ T cells expressing CD16 receptor (FCyRIII) interacts with TAA mediate ADCC through activation of B cell.

response as shown in the Figure 1. NKG2D provides activation signals upon binding to non-classical MHC molecules of the MHC class-I chain-related molecules (MICa/B) and UL-16 binding protein (ULBP) families expressed on tumor cells (54). Nectin-like-5 expressed in carcinoma cells recognized by DNAX and provides activation signals (50). $\gamma\delta$ T cells also express CD16 (FCyRIII) receptor, upon recognition of tumor associated antigens (TAA), CD16/FCyRIII receptor binds to Fc portion of immunoglobulin G (IgG) and leads to antibody-dependent cellular cytotoxicity (ADCC) (55). In addition to the cytokines, cytotoxic soluble factors, activated V γ 9V δ 2 T cells can produce large amount of chemokines like CXCL-13 and CXCL-10 result in the recruitment of B cells, macrophages, T cells, and NK cells toward the tumor micro-environment (49). It leads to inhibition of tumor growth and reduced survival of autologous tumor cells. $\gamma\delta$ T cells have a unique capacity to present antigens to both CD8 and CD4 T cells and potentially elicit strong adaptive anti-tumor response.

Presently two strategies were applied for $\gamma\delta$ T cell based cancer immunotherapy. One is the adoptive cell transfer of *in vitro* expanded V γ 9V δ 2 T cells and second one is the administration of pAg or aminobisphosphonates along with low-dose recombinant IL-2 to stimulate V γ 9V δ 2 T cells *in vivo*. It has been shown that the two synthetic molecule drugs bromohydrin pyrophosphate (BrHPP) and zoledronate selectively activate human V γ 9V δ 2 T cells in clinical trials. Bioactivity (EC₅₀) of BrHPP (56, 57), and

zoledronate (58) were 24 nM and 1 μ M, respectively to activate TCR V γ 9V δ 2 T cell clones and PB mononuclear cells *in vitro*. Dieli et al. reported that increased survival rates in prostate cancer patients when administered zoledronate (4 mg) in combination with IL-2 (0.6×10^6 IU) was observed (59). In another clinical trial (60), administration of low-dose IL-2 (0.25 to 3×10^6 IU) in combination with pamidronate (90 mg) in multiple myeloma patients showed that five out of nine patients had significant *in vivo* activation and proliferation of V γ 9V δ 2 T cells, indicating that V γ 9V δ 2 T cells might contribute to this anti lymphoma effect. V γ 9V δ 2 T cells from tumor infiltrate lymphocytes (TIL) of colorectal cancer are able to eliminate cancer cells *in vivo* and have been associated with reduced metastasis and longer mean survival (61). BrHPP activated TCR V γ 9 $+$ $\gamma\delta$ T cells obtained from healthy subjects co-incubated *in vitro* with B-lymphoma cell targets coated with rituximab, enhance ADCC of target cells (62). In primates, administration of pAg synthetic analogs and low doses of IL-2 expand V γ 9V δ 2 T cells in lung tissue, which in turn confer activity against human non-small cell lung cancer (NSCLC) cell lines by increasing the secretion of IFN- γ , TNF- α , and TRAIL (63). Phospho Ag-expanded V δ 2 T cells infusion has also been tested in lymphoid neoplasms and renal cancer. In metastatic renal carcinoma, six cycles of adaptive immunotherapy with infusions of phospho Ag-expanded $\gamma\delta$ T cells plus zoledronic acid (4 mg) and low doses of IL-2 (1.4×10^6 IU) caused complete

remission without progression for 2 years (64). *In vitro* studies reported $\gamma\delta$ T cells killing efficacy was increased against three cervical cancer cells pretreated with pamidronate and proved by LDH cytotoxicity test (65). Elevated levels of V γ 9V δ 2 T cells in the liver were found in hepatocellular carcinoma (HCC) patients as well as tumor bearing mice compared to healthy controls, indicating the importance of V γ 9V δ 2 T cell in the anti-tumor immunity (66). Oberg et al. reported increased $\gamma\delta$ T cell cytotoxicity against pancreatic ductal adenocarcinomas (PDAC) *in vitro* and *in vivo* in immunocompromised mice, when administered healthy donors $\gamma\delta$ T cell with Her2/V γ 9 bispecific antibodies (67). Hence, novel regimens combining $\gamma\delta$ T cells with drugs or monoclonal antibodies would be helpful for treatment of solid tumors and hematological malignancies.

BURNS AND WOUNDS

$\gamma\delta$ T cells participate in several aspects of healing from burn injuries and wounds. Healing is a dynamic and complex process requiring constant communication between cells in the form of cytokine release, cell-to-cell contacts, and cell-to-matrix interactions. The epidermis is a barrier tissue that is exposed to the environment and susceptible to injury. $\gamma\delta$ T cells are found in both epidermis and dermis of human skin express V δ 1 chain. These skin-resident T cells involved in reepithelialization of acute and chronic wounds. Human epidermal $\alpha\beta$ and V δ 1 bearing T cells are able to produce insulin like growth factor upon activation and promote wound healing in a skin organ culture model (68). Murine epidermal $\gamma\delta$ T cells are named as DETCs because of their unique dendritic morphology. DETC express an invariant V γ 3V δ 1 $^+$ TCR. Oppeltz et al. reported that epidermal $\gamma\delta$ T cells play a major role in the expression of inducible nitric oxide synthase (iNOS) at the burn wound site and is important in wound closure and collagen deposition (69). Recently, it has been suggested that DETC participate in tissue repair, likely through the production of Th2 and Th17 cytokines (70), chemokines, and growth factors. Hence the immediate goal of DETC is to repair wound and maintain tissue integrity and homeostasis.

Wound occurs as a result of trauma, infection or by the pathological infections. Cellular damage and stressed keratinocytes produce an unknown antigen, which binds with the TCR of DETC, which are proximal to the wound (71). Further, DETC changes its morphology and become rounded with in 4 h post wounding (72). The rounded morphology of DETC correlates with functional activity. The distal DETC to the wound retain dendritic and maintain tissue homeostasis. Activated DETC produce TNF- α , insulin growth factor (IGF)-1, KGF-1, KGF-2, and up regulate the activation markers CD25 and CD69 in response to the epithelial damage (73). Rapamycin, an immunosuppressor that is known to regulate the DETC rounding by targeting the serine threonine kinase, cause delay in cytokine production and wound healing (74). DETCs are shown to produce lymphoid associated thymosin- β 4 variant in contact dermatitis that exhibits the anti-inflammatory role (75). In normal condition, DETCs are slightly activated at the epidermal region by expressing the activation markers CD25, CD69, and also secrete some cytokines in low amount that allows them for quick activation in response to local trauma (76). DETC express the co-stimulatory activating receptors like NKG2D, and H60c,

ligand expressed on wounded epidermis and shown to provide co-stimulatory signals with NKG2D participating in TCR mediated signaling (77). A prominent signaling by co-stimulatory mode of DETC activation is by junctional adhesion molecule-like protein (JAML) that is expressed by the DETC. The recognition of JAML to its ligand coxsackie and adenovirus receptor (CAR) expressed by the keratinocytes results in the recruitment of phosphoinositide 3-kinase (PI3K) (78) and also with the HLA4E10 (79) stimulatory antibody that helps in promoting wound healing as shown in Figure 2. Recently, it was found that plexin B2 expressed on keratinocytes and CD100 an intra cellular signaling domain of DETCs shows much interaction and makes the cellular rounding via signals through ERK kinase and cofilin (80). Rani et al. reported that, $\gamma\delta$ T cells regulate myeloid cell activity, which in turn enhances macrophage influx into the wound site to repair wound (81).

$\gamma\delta$ T cells also participate in the repair of epithelia in other organs, such as intestine, lung, and cornea. $\gamma\delta$ T cells play a key role in the maintaining of intestinal mucosa by producing KGF. Mice lacking $\gamma\delta$ T cells treated with a dextran sodium sulfate (DSS), exhibit severe mucosal injury and decreased epithelial cell proliferation (82). Mice deficient for TCR δ chain (TCR δ $^{-/-}$) exhibit significantly reduced inflammation and epithelial repair in the lung after bleomycin treatment (83) and also in the corneal epithelial abrasion (84). In the lung injury, $\gamma\delta$ T cells are required for the neutrophils influx and also produce IL-17, which are responsible for the inflammatory response and epithelial repair (85). Corneal healing involves both epithelium and sensory nerves that have a trophic effect for the epithelium. Intracellular cell adhesion molecule (ICAM)-1 and CCL20 chemokine are necessary for attracting the $\gamma\delta$ T cells into the healing epithelium (86). $\gamma\delta$ T cells are responsible for the secretion of cytokines such as IL-17, IL-22, and the influx of neutrophils and platelets, which contribute to the epithelium healing. Accumulation of neutrophils and platelets leads to the rise in vascular endothelial growth factor (VEGF), which is required for nerve regeneration (87).

AUTOIMMUNE DISEASES

Autoimmune diseases are characterized by abnormal immune responses to self antigens. These diseases are induced by many environmental factors on a genetically susceptible background, which lead to production of huge number of inflammatory cytokines and auto-antibodies to make path for the outbreak and progression of the disease. The roles of $\gamma\delta$ T cells in autoimmune diseases are not yet very clear. Bendersky et al. showed that elevated number of V γ 9V δ 2 T cells (~35%) in synovial fluid and PB, produced TNF- α and IFN- γ during the pathogenesis of juvenile idiopathic arthritis (JIA). Further, activation of $\gamma\delta$ T cells leads to apoptosis of synovial fibroblasts, the effect of which is manifested in JIA (88). $\gamma\delta$ T cells in intestine were significantly reduced during intestinal inflammation, which leads to the uncontrolled activity of CD4 $^+$ T helper cells to cause autoimmune inflammatory bowel disease called Crohn's disease (89). The role of $\gamma\delta$ T cells in allergic encephalomyelitis and multiple sclerosis is ambiguous. $\gamma\delta$ T cells were certainly abundant in cerebrospinal fluid of the respective mouse models but their precise role is not yet established (90). In non-obese diabetic mouse model, $\gamma\delta$ T

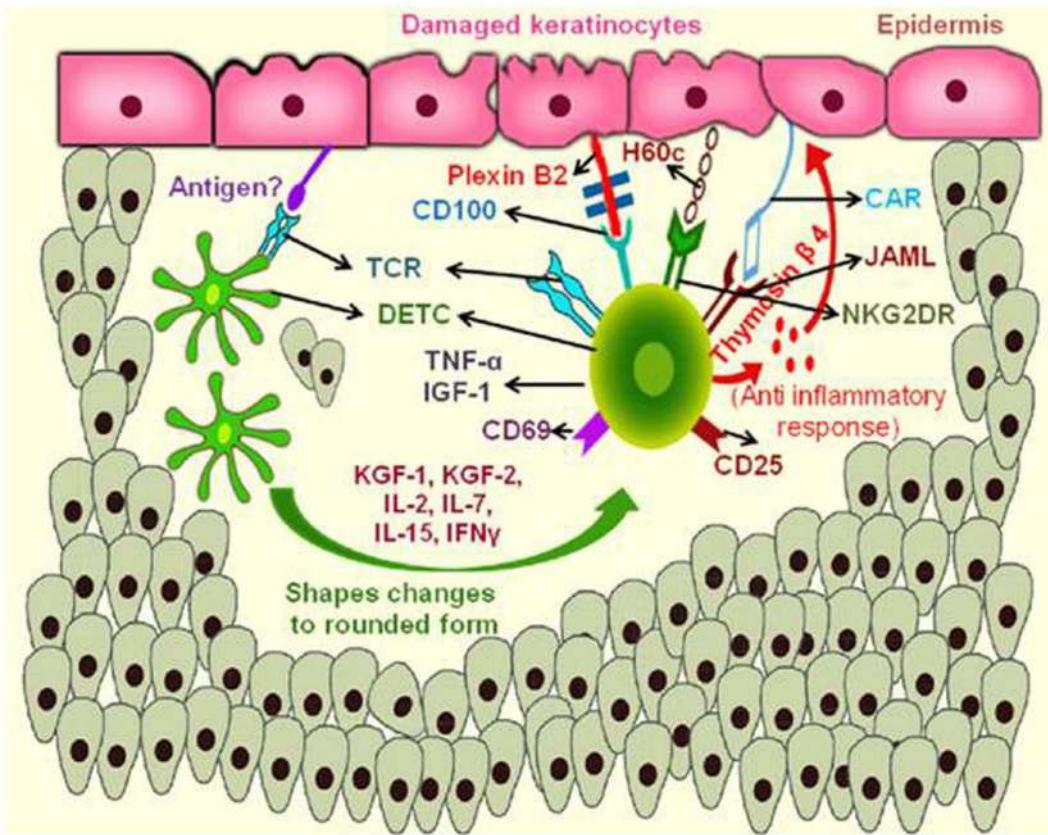


FIGURE 2 | Mechanism of action of murine skin resident $\gamma\delta$ T cells in wound repair. During keratinocyte damage, the epidermal $\gamma\delta$ T cells (DETCs) are activated upon recognition of an unknown antigen, rounding of the DETCs occur and produce thymosin β 4 and cytokines like TNF- α , IFN- γ , and IL-2 required for anti-inflammatory response and wound healing.

Engagement of NKG2DR with H60c and JAML-CAR interactions also enhances the production of these cytokines and provide epidermal repair. (JAML, junction adhesion molecule like protein; CAR, coxsackie and adenovirus receptor; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; NKG2DR, natural killer cell activating receptor).

cells infiltrate into islet cells and secrete IL-17 to cause apoptosis. Antibody mediated blockade of IL-17 stopped the process and rescued mouse from diabetes (91). $\gamma\delta$ T cells cross react with aminoacyl-tRNA synthetases of the muscle cells that lead to the pathogenesis of myositis, an inflammation of muscle tissues caused by autoimmune T cells (92).

Behcet's disease is an immune mediated disorder that is often associated with the symptoms like ulceration of mucous membrane, skin, and ocular problems. In general, normal individuals consist of CD45RA $^-$ CD45RO $^+$ V γ 9 $^+$ V δ 2 $^+$ T cells but as the disease progress abnormal $\gamma\delta$ T cells with CD45RA $^+$ CD45RO $^-$ V γ 9 $^+$ V δ 2 $^+$ phenotype are increased in number and activated, then produced IL-2R in patients suffering with Behcet's disease (88). Infliximab, is a chimeric monoclonal antibody against TNF- α , suppress the V γ 9V δ 2 T cell expansion and activation by blocking the TNF receptor on V γ 9V δ 2 T cells. Moreover, it also reduces the cell-mediated immune responses by reducing the expression TNF RII, perforin, granzyme-A, and IFN- γ production from V γ 9V δ 2 T cell (93).

Psoriasis is speculated as the T cell operated chronic inflammatory skin disease (94), but the mechanisms of pathogenesis

are still poorly understood. Chemokine receptor CCR6 (receptor for a CCL20) is very abundant in psoriatic skin T cells. Chemokine receptors are transmembrane proteins that are activated by chemokines, which play key roles in cell trafficking, cell motility, and survival. CCR6 regulates epidermal trafficking of $\gamma\delta$ T cell subsets in the skin (95). The chemokine receptor CCR6 is expressed on the Th17 cells and $\gamma\delta$ T cells, which produce cytokines like IL-17 and IL-22, TNF- α , and IL-20 (94). IL-22 plays a key role in the activation of immunity and has been implicated in the pathogenesis of psoriasis (96). $\gamma\delta$ T cells from CCR6 KO mice not only failed to accumulate in the epidermis after IL-23 treatment and even though entered into the epidermis produced low amounts of IL-22 compared with wild type $\gamma\delta$ T cells. Hence, this data suggest that not only recruitment but also function of $\gamma\delta$ T cells may be impaired in the absence of CCR6 (97). Laggner et al. showed that, redistribution of V γ 9V δ 2 T cells from PB to skin in psoriasis patients as compared to the healthy control. These skin homing V γ 9V δ 2 T cells produce the psoriasis relevant cytokines IFN- γ , TNF- α , and IL-17A similar to blood derived V γ 9V δ 2 T cells (98, 99). Prominent roles for skin homing V γ 9V δ 2 T cells are influencing the resident immune and epithelial cells by the

Table 1 | Antigen recognition and functions of $\gamma\delta$ T cells in various diseases. $\gamma\delta$ T cells recognizes various antigenic factors and produce respective chemokines and cytokines to protect immune system against from different diseases like pathogenic infections, cancer, wound repair, and autoimmune.

| S. No. | Disease | Stimulator/activator for $\gamma\delta$ T cells | Function of $\gamma\delta$ T cells in immune-protection |
|--------|---|---|---|
| 1. | Pathogenic infections | | |
| a. | Tuberculosis (<i>M. tuberculosis</i>) | HMBPP produced by the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in microorganisms | Produce IL-22, IL-17, and IFN- γ . Regulating both innate and adaptive immunity |
| b. | Malaria (<i>P. falciparum</i>) | Schizont associated antigen (SAA) and HMBPP | IL-10, IL2, IL-1 β , and IFN- γ degranulation of infected RBC and merozoites |
| c. | AIDS (<i>HIV</i>) | Recognize the envelope protein GP 120 by CCR5 receptor on V γ 9V δ 2 T cells | Activates P38 MAP kinase, which promotes the FAS dependent caspase activation and induces the cell death |
| 2. | Cancer | V γ 9V δ 2 TCR recognizes endogenous IPP, produced by mevalonate pathway in tumor cells NKG2D expressed on $\gamma\delta$ T cells recognizes MICA/B and ULBP families expressed on tumor cells | Increased secretion of perforin/granzymes, TNF- α , IFN- γ , and suppress the tumor Produce IFN- γ , IL-17, and chemokines, which recruit the macrophages, NK cells, B cells, and T cells |
| 3. | Wound repair | Non-specific antigen recognition by DETC | IL-2, TNF- α , IFN- γ , KGF-1, and KGF-2 are produced against the damaged keratinocytes result in the healing of wound |
| 4. | Autoimmune diseases | Recognizes self antigens by $\gamma\delta$ TCR | Enhances the production of IL-17, IL-23, and IFN- γ . The exact mechanism of $\gamma\delta$ T cells in these diseases is not yet clear |

HMBPP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; $\gamma\delta$ TCR, $\gamma\delta$ T cell receptor; IFN, interferon; IL, interleukin; GP 120, glycoprotein; CCR, chemokine receptor; MAP, mitogen activated protein; NKG2D, natural killer cell activating receptor; MICA/B, MHC class-I chain-related molecule; ULBP, UL-16 binding protein; TNF, tumor necrosis factor; DETC, dendritic epidermal $\gamma\delta$ T cell; KGF, keratinocyte growth factor.

rapid release of pro-inflammatory cytokines, the recruitment of immune cells from the circulation, and in tissue remodeling by the release of growth factors (99), which make V γ 9V δ 2 T cells as the biomarker of psoriasis.

$\gamma\delta$ T cells show enhanced antigen-presenting cell (APC) functions, which may play a role in the pathogenesis of RA by over activating T and B cells. Abnormal activation of Th1 and Th17 cells result in the production of pro-inflammatory cytokines, which play crucial role in the pathogenesis of RA (100). Peripheral V γ 9V δ 2 T cells after stimulation with IPP *in vitro* are shown to upregulate the expression of APC specific molecules HLA-DR and CD80/86 and presented soluble antigens and synthetic peptides to CD4 $^+$ T cells and B cells thus contributing to activation of CD4 $^+$ T cells and being associated with RA onset and disease progression (100). A subset of CD27 $^+$ CD25 $^{\text{high}}$ V δ 1 T cells expressing FoxP3 were gradually decreased in the PB of systemic lupus erythematosus patients progressing in the pathogenesis of SLE and these regulatory $\gamma\delta$ T cells could be generated *in vitro* when stimulated with anti $\gamma\delta$ -TCR in presence of IL-2 and transforming growth factor-beta (TGF- β) (101). The activating marker CD69 and HLA-DR were up regulated while the expression levels of the inhibiting receptor CD94/NKG2A remained low after the antigen stimulation on these $\gamma\delta$ T cells, upon activation might lead to the over activation of $\gamma\delta$ T cells in patients with SLE (102).

Graft rejection is a serious problem during transplanting of solid organs. Conventionally, the B cells and $\alpha\beta$ T cells of adaptive immune system were considered to be the key in this phenomenon as they have immune memory and can bind numerous antigens. However, there is an evidence that $\gamma\delta$ T cells play a vital role in graft rejection. Gorczynski et al. studied the role of $\gamma\delta$ T cells in

skin graft rejection using mouse model (103). $\gamma\delta$ T cells were not restricted by immunosuppressive drugs during organ transplantation. Some subsets of $\gamma\delta$ T cells expand oligo clonally and the reasons for this expansion are unknown but may be linked to persistent viral infections. It has been suggested that quantification of $\gamma\delta$ T cells in PB may not be essential to decide graft tolerance (104). The known role of $\gamma\delta$ T cells in autoimmune diseases is limited, so an extensive research has to be focused particularly on the effects of these cells in the pathogenesis and development of disease and ultimately for the development of $\gamma\delta$ T cell based therapies.

$\gamma\delta$ T cells functions in various diseases, described in the review are summarized in the Table 1.

IMMUNOTHERAPY

Immunotherapy has become an increasingly attractive option for the treatment of cancer (105). $\gamma\delta$ T cells may be an excellent target for modulation of immune responses in human diseases. Enhancing $\gamma\delta$ T cell functions may open the possibility to formulate new immunotherapeutic regimens, which could impact the improvement of immune control of various diseases. Aminobisphosphonates like zoledronate, pamidronate, and BrHPP were well-studied among the numerous activators of V γ 9V δ 2 T cells. IPP is an intermediate metabolite of mevalonate pathway. Bisphosphonates may activate V γ 9V δ 2 T cells, by inhibiting the key enzyme farnesyl pyrophosphate synthase of mevalonate pathway in certain tumors leads to upregulating the endogenous pool of IPP. Mevalonate is the product of HMG-CoA reductase, a rate limiting enzyme subject to tight regulation. However, high exogenous mevalonate concentration would bypass normal regulation and indirectly

stimulates V γ 9V δ 2 T cells by increasing endogenous IPP levels (106). Taken together, bisphosphonates and mevalonate may activate V γ 9V δ 2 T cells and aid in curing bacterial infections (107). Bisphosphonates were proven to treat gliomas (108). A clinical trial of immunotherapy using a combination of zoledronate, a bisphosphonate, and IL-2 was found to alleviate renal cell carcinoma (109). HMBPP is one of the key ligands that activate V γ 9V δ 2 T cells. HMBPP is produced by the 2-C-methyl-D-erythritol 4-phosphate (MEP) biosynthetic pathway in microorganisms. HMBPP is nearly 1000-fold more effective than IPP for the *in vitro* activation of V γ 9V δ 2 T cells. V γ 9V δ 2 T cells express surface marker CCR5, which is recognized by gp120 of HIV. This leads to apoptosis of HIV infected cells through a caspase mediated pathway. It has been suggested that by triggering rapid proliferation of V γ 9V δ 2 T cells may help in curing HIV infection (110). $\gamma\delta$ T cell based immunotherapy might have a beneficial effect in patients with chronic hepatitis C virus infection (111).

$\alpha\beta$ T cell populations potently attack specific targets but are limited by their specificity, whereas $\gamma\delta$ T cells in combination with tumor-targeting antibodies might provide anti-tumor cytotoxic effects and also long-lasting protection upon antigen presentation. $\gamma\delta$ T cells have dual role of stimulating both $\gamma\delta$ T cell-directed anti-tumor activity and antigen-specific CD4 and CD8 $\alpha\beta$ T cell responses. $\gamma\delta$ T cells are attractive agents for cancer immunotherapy because they are not MHC restricted like conventional T cells. So, a single vaccine can be used in all individuals regardless of MHC haplotype (112). Memory like expansion of V γ 9V δ 2 T cells has been reported in primates after subclinical systemic infection and reinfection with attenuated *Listeria monocytogenes* strains (20). $\gamma\delta$ T cells acquire memory in order to play protective antiviral and anti-tumor roles. The direct or indirect stimulation of $\gamma\delta$ T cells by TLR agonists could be a strategy to optimize Th1 mediated immune responses as adjuvant in clinical trial of cancer immunotherapy (113). Recent studies suggest that, combination of $\gamma\delta$ T cells with therapeutic monoclonal antibodies can efficiently mediate ADCC against tumors (114). Chronic wounds are an increasing clinical problem, understanding advanced mechanisms of DETC during wounds and burns in humans suggesting possible therapeutic targets for tissue repair and skin homeostasis. Disabling $\gamma\delta$ T cells at the specific site by using monoclonal antibodies is a viable therapeutic option for the treatment of autoimmune diseases. Peters et al. reported immunosuppression of $\gamma\delta$ T cells by using anti-CD28 mAb and antagonized by TLR-2 ligands (113). Suppressive $\gamma\delta$ T cells could have major therapeutic potential for the control of autoimmunity or allergic reactions. Enhancing the activity of Tregs, which in turn secrete PD-1 and CTLA-4 to suppress the activity of $\gamma\delta$ T cells, could improve treatment for the autoimmune diseases (115). Modulation of these immunosuppressive check points may be interesting clinical trial in the $\gamma\delta$ T cells based immunotherapy.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The role of $\gamma\delta$ T cells in immunotherapy has gained specific importance due to their prominent function involving directly or indirectly in the rehabilitation of the diseases. $\gamma\delta$ T cells act as connecting bridge between both the innate and adaptive immune responses without the antigen presentation and producing some

important key cytokines like IFN- γ and TNF- α . When stimulated with the combination of aminobisphosphonates and IL-2 activate the V γ 9V δ 2 T cells further and they can also act as the APCs to treat particular infections and tumors. Factors that potentiate the pathogenesis of $\gamma\delta$ T cells in autoimmune diseases are yet to be revealed so as to develop drugs, which can target the $\gamma\delta$ T cell in particular. New strategies and models that target the molecules involved during disease onset and progression need to be developed, as this makes a pavement for $\gamma\delta$ T cell based immunotherapy. New ligands and chemokines responsible for activation and effector functions of $\gamma\delta$ T cells are to be deciphered. Many new molecules and proteins can be designed to trigger the $\gamma\delta$ T cell through a TCR independent pathway. Vaccines have to be developed to enhance the production levels of chemokines and cytokines by these $\gamma\delta$ T cells at the tumor site. For the preparation of large number of cells for adoptive cell transfer, it is necessary to develop better antigens, which stimulate $\gamma\delta$ T cell expansion *in vitro*. Further, clinical trials are required for the $\gamma\delta$ T cell targeted immunotherapy in case of chronic infections and diseases. Further research might shed more light on the in depth understanding of the underlying mechanisms of the antigen recognition and key factors influencing the $\gamma\delta$ T cell production during the disease.

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A novel prothrombotic pathway in systemic sclerosis patients: possible role of bisphosphonate-activated $\gamma\delta$ T cells

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Objectives: Infusions of aminobisphonates (ABP) activate V γ 9 δ 2T cells *in vivo* and induce an acute inflammatory response in 30% of patients treated for osteoporosis. Following the observation of digital thrombosis in a systemic sclerosis (SSc) patient after treatment with an intravenous ABP, zoledronate (Zol), we evaluated whether patient and control peripheral blood (PB) mononuclear cell (MC, PBMC) acquire a prothrombotic phenotype in response to Zol.

Results: V γ 9 δ 2T cells of both patients and healthy donors (HD) upregulated the CD69 activation antigen and secreted tumor necrosis factor (TNF) α in response to Zol *in vitro*. In addition, exposure to either Zol or lipopolysaccharide (LPS), or to both additively, induced expression of the highly procoagulant, tissue factor (TF)-1 on CD14+ monocytes. Importantly, only Zol-induced TF-1 was blocked by a monoclonal antibody to TNF α . Interestingly, we found that SSc, but not HD, V δ 1+T cells were concurrently activated by Zol to produce interleukin (IL)-4. Addition of plasma from the blood of the SSc patient who developed critical digital ischemia after infusion of Zol, but neither plasma from a second patient with no adverse clinical response to Zol infusion nor of a HD, strongly enhanced Zol-induced monocyte TF-1, which could still be blocked by anti-TNF α .

Conclusion: Aminobisphonates induced secretion of TNF α by V γ 9 δ 2+ T cells may lead to TNF α -dependent induction of procoagulant TF-1 induction on monocytes. In certain clinical settings, e.g., SSc, TF-1+ monocytes could play a role in triggering clinically relevant thrombosis.

Keywords: T cells, $\gamma\delta$ T cell, scleroderma, tissue factor, thrombosis, aminobisphosphonate, V γ 9 δ 2T cells

INTRODUCTION

$\gamma\delta$ T cells are a subset of T cells combining innate and adaptive functions (1). In Caucasians, 50% of the circulating $\gamma\delta$ T cells express the $\gamma 9$ and $\delta 2$ genes in the Variable (V) region of the $\gamma\delta$ T cell receptor (TCR) V γ 9 δ 2 T cells (2). V γ 9 δ 2 TCR recognize metabolites produced in the classical (isopentenyl pyrophosphate, IPP) and alternative [(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)] mevalonate metabolic pathways. These antigens are presented for TCR-mediated recognition by CD277, a ubiquitously expressed cell surface membrane antigen presenting molecule (APM) (3). Together with co-stimulatory signals delivered by antigen presenting cells (APC), V γ 9 δ 2 TCR-CD277/IPP cognitive interactions activate the V γ 9 δ 2 T cells to secrete cytokines and exert cytotoxic effects. A second major subset of $\gamma\delta$ T cells expresses the V δ 1 gene in the TCR structure,

among which a major portion recognize phospholipid antigens (e.g., sulfatide) presented by CD1 family molecules (4).

V δ 1 $\gamma\delta$ T cells have been shown to expand oligoclonally in the PB of certain systemic sclerosis (SSc) patients, infiltrate the skin in early phases of the disease, and may secrete factors enhancing collagen production (5). V γ 9 δ 2 T cells are also functional in SSc patients, since their circulating V γ 9 δ 2 T cells secrete tumor necrosis factor (TNF) α and IFN γ and induce fibroblast apoptosis in the presence of exogenously added IPP (6). However, the immunopathogenic significance of these cells concerning the clinical manifestations in SSc patients remains largely unknown.

In this regard, interactions of $\gamma\delta$ T cells with bisphosphonate-activated CD14+ monocytes may play a critical role. Bisphosphonates block farnesylpyrophosphate synthase (FPPS) downstream

of IPP in the mevalonate pathway in circulating CD14+ monocytes, increasing intracellular IPP, which is presented to circulating V γ 9 δ 2 T cells leading to their activation (7, 8). As a consequence, these cells produce TNF α and IFN γ , the central mediators of the acute phase response (APR) following infusion of zoledronate (Zol) to patients (8). Accordingly, upon administration of a bisphosphonate drug for osteoporosis or to decrease bone metastasis in cancer, an APR characterized by fever, chills, and arthralgia occurs in up to 30% of patients (8). Zol also activates dendritic cells and natural killer (NK) cells at least in part dependent upon V γ 9 δ 2 T cell activation (9, 10). CD86 and other stimulatory molecules, which enhance activation of other T cell subsets are also upregulated by Zol on dendritic cells (11). Recently, Zol was also shown to stimulate B cells directly (12). Our recent observation of the rapid onset of gangrene of fingers and toes in a patient with SSc after Zol administration prompted the experiments in this study that were designed to evaluate how bisphosphonate-activated $\gamma\delta$ T cells could play a pathogenic role in SSc.

MATERIALS AND METHODS

PATIENTS

The study was approved by the Institutional Review Board (Helsinki Committee) of the Sheba Medical Center, Ramat Gan, Israel. All patients participating in the study were seen in the Rheumatology and Pulmonary clinics at Sheba or Rambam medical centers. Patients fulfilled the criteria of the American College of Rheumatology for systemic sclerosis (SSc), also named herein scleroderma (SCL) (13). Controls included six healthy donors (HD), two patients with idiopathic pulmonary fibrosis (IPF), and one patient with polymyalgia rheumatica (PMR).

ISOLATION OF PBMC AND CHARACTERIZATION OF CELL SUBSETS

PBMC were isolated by Lymphoprep (AXIS-SHIELD, Oslo, Norway) density centrifugation and cultured in growth medium as previously described (14). Cells were stained with fluorochrome conjugated monoclonal antibodies (mAb) specific to CD4, CD3, CD142, CD14, CD69, or isotype control (BD Biosciences), or to human V γ 9, V δ 2 (Immunotech), and V δ 1 (Endogen, Pierce) and analyzed by flow cytometry (Calibur, Beckton Dickinson, CA, USA).

TISSUE FACTOR INDUCTION AND INHIBITION

PBMC were incubated and stimulated either with 2 μ M of Zol (Novartis) overnight (ON) or with 50 ng/ml of *E. coli* 0111:B4 lipopolysaccharide (LPS) (InvivoGen) for 3 h. For double LPS + Zol stimulation, cells were cultured with Zol ON then LPS was added for an additional 3 h. Cells were harvested, washed, and stained with CD14 and CD142 specific antibodies. For inhibition experiments, PBMC from HD were cultured in medium with increasing dilutions of either anti-TNF α antibody (Infliximab, Janssen Biologics) or control IgG mix (Gammaphlex, Bio Products Laboratory, Herts, UK) prior to stimulation with Zol or/and LPS.

CYTOKINE DETECTION

Intracellular cytokine detection was performed as previously reported (14). IFN γ detection in supernatants was done using

the ELISA max Deluxe Sets (Biolegend, CA, USA) according to the manufacturer's instructions.

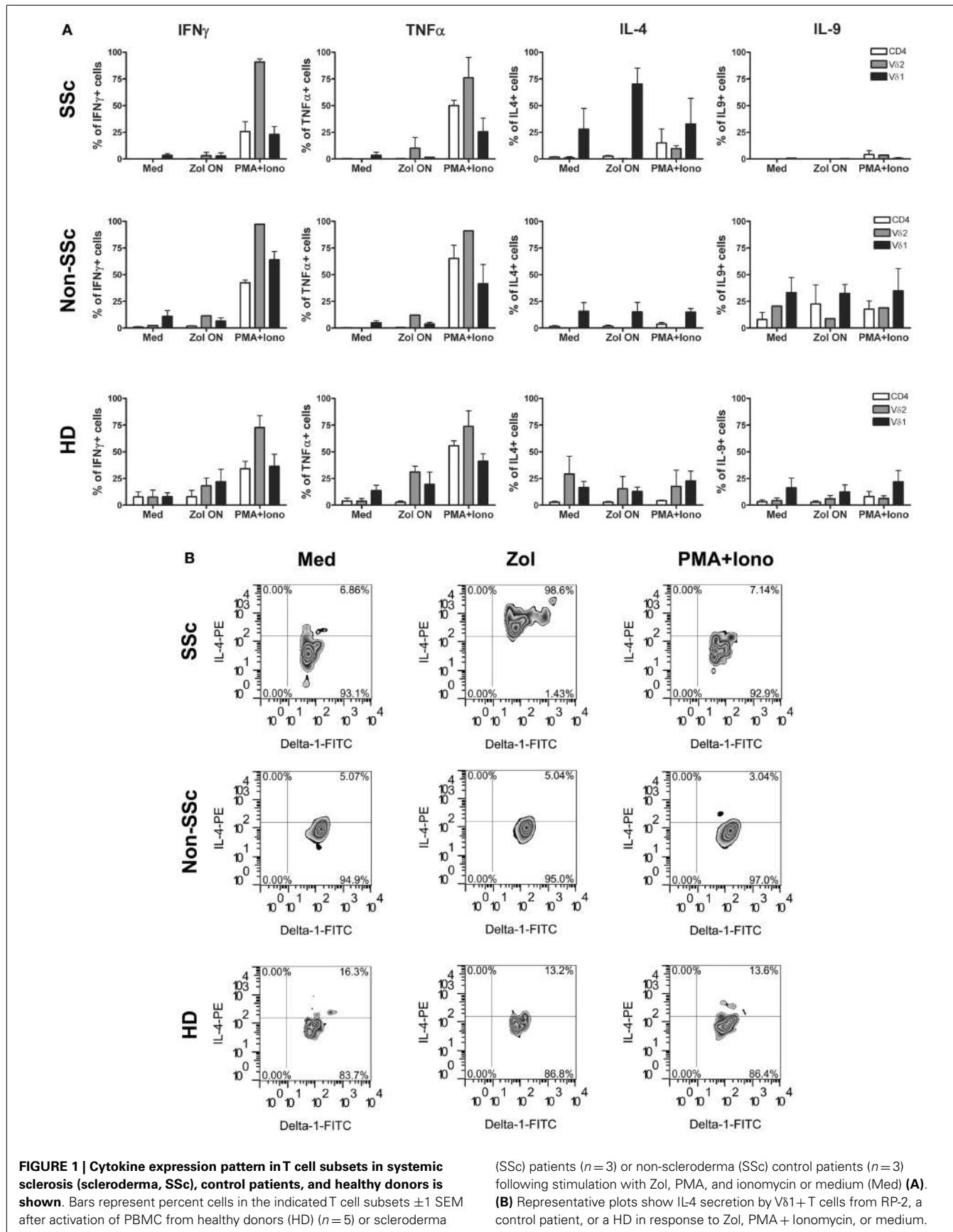
RESULTS

SSc-DISEASE SPECIFIC RESPONSE OF V δ 1+ T CELLS TO ZOL

Activation of $\gamma\delta$ T cells in SSc patients was compared to that of healthy individuals and patients with other chronic inflammatory diseases quantitating percentage of cells producing a panel of relevant intracellular cytokines. Thus, PBMC were incubated ON with Zol and secretion of cytokines was measured by intracellular staining of PB T cell subsets. Percentage of cells among the CD4+, V γ 9 δ 2+, and V δ 1+ T cell subsets in PBMC of 3 SSc patients (RP0-2), 3 non-SSc patients with IPF or PMR (Pt 1–3), and 6 HD that were induced to secrete IFN γ , TNF α , IL-4, or IL-9 after 4.5 h of incubation in medium alone, or with Zol or PMA (20 ng/ml) and ionomycin (0.8 μ M) (P/I) are shown in Figure 1. A significantly higher percentage of IL-4 producing cells was observed among SSc patient's V δ 1+ cells exposed to Zol compared to either non-SSc patients ($p < 0.03$, Student T test) or HD ($p < 0.003$, Student T test). In contrast, there was no significant difference in the mean percentage of cells secreting any of the other cytokines in SSc compared to HD in the remaining T cell subsets. SSc patient IFN γ production was, however, significantly lower than in the non-SSc patients among Zol-activated CD4+ T cells, and P/I-activated V δ 1+ T cells. Among SSc V δ 1+ T cells, the percentage of Zol-activated IL-9 producers were also significantly lower than in non-SSc patients ($p < 0.05$). These results point to a unique pattern of production of cytokines of SSc V δ 1+ $\gamma\delta$ T cells in response to Zol, relative to HD and patients with other chronic inflammatory and fibrotic diseases, characterized by increased production of IL-4 (relative to both HD and disease controls), and decreased production of IL-9 relative to the disease controls.

ACTIVATION OF PATIENT RP2 V γ 9 δ 2+ T CELLS BY ZOL

During the course of these studies, only one of our SSc patients (RP2) developed an unusually dramatic APR after receiving intravenous Zol. Since TNF α and IFN γ produced by V γ 9 δ 2 $\gamma\delta$ T cells are thought to be the mediators of the APR and IFN γ was weakly produced in SSc patients in response to Zol (Figure 1), we examined in further detail how Zol had affected TNF α production by this patient's (RP2) PB T cell subsets. As expected, CD4+, V γ 9 δ 2 as well as V δ 1+ T cells in PBMC of RP2, RP1, and a healthy blood donor all increased their intracellular TNF α in response to P/I, an activating stimulus for T cells that bypasses signals dependent upon cognitive TCR-antigen interactions. Zol potently induced RP2 V γ 9 δ 2 T cells (but not CD4+ or V δ 1+ T cells) to produce TNF α , similar to its effect on a HD PBMC (Figure 2) whereas those of RP1 an SSc patient who had received Zol but no clinical APR did not secrete TNF α in response to Zol application *in vitro*. Production of TNF α was linked to Zol-dependent activation of V γ 9 δ 2+ $\gamma\delta$ T cells. Thus, a markedly increased expression of CD69 on the surface of V γ 9 δ 2 cells (but not V δ 1+ cells or CD4+ T cells) was concomitantly noted in the presence of Zol on RP2 and HD but not on RP1 V γ 9 δ 2+ T cells. In contrast, P/I stimulation increased CD69 expression in all T cell subsets in all individuals tested (except in HD V δ 1+ cells).



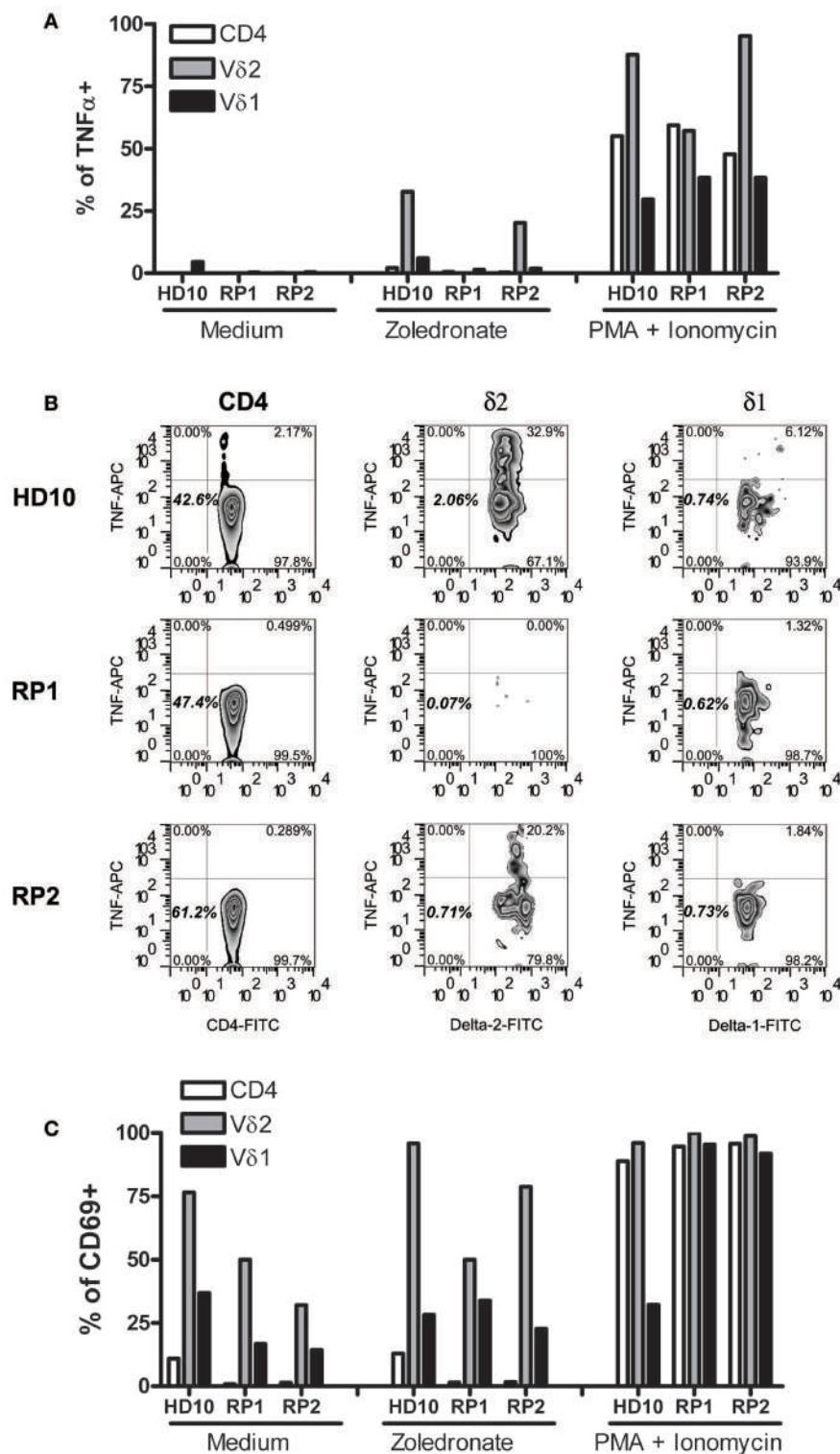


FIGURE 2 | Vy982 T cells of patient RP2 produced TNF α in response to zoledronate. Bars represent percent cells of HD10, and of SSc patients RP1 and RP2, within the indicated T cells subsets expressing intracellular TNF α (A) or surface CD69 (C) after stimulation with medium, Zol or PMA, and

ionomycin. (B) Representative FACS plots of TNF α staining in the corresponding T cells from patients indicated on the left after Zol stimulation. Numbers indicate percentages of the particular cell population in the respective rectangle.

INDUCTION OF TNF α -DEPENDENT TISSUE FACTOR ON CD14+ MONOCYTES BY ZOL

The occurrence of digital ischemia during the APR in RP2 prompted us to examine whether TNF α produced by V γ 982+ cells in response to Zol is sufficient to induce tissue factor (TF)-1, a potent procoagulant factor, on the cell surface membrane of monocytes present in PBMC (15). We utilized HD PBMC to address this issue, because we could obtain only two PB samples from patient RP2 and the patient's response to Zol with respect to TNF α secretion was similar to that of HD (Figure 2). Thus, HD PBMC were incubated ON in medium alone or medium containing Zol. Subsequently, LPS, a known inducer of TF-1 on

monocytes, was added to the medium for an additional 3 h. The expression of the CD142 antigen, which identifies TF-1 on the cell surface, was assessed by FACS analysis after gating on CD14+ monocytes. A marked and significant upregulation of CD142 on HD-derived monocytic CD14+ cells cultured with either Zol alone ON or after the brief 3 h LPS stimulation was observed (Figure 3). There was no upregulation of CD14 in these experiments, indicating that upregulation of CD142 was not due to non-specific elevation of surface membrane molecules on monocytes (data not shown). Furthermore, an additive effect on CD142 levels of expression was noted in PBMC cultured in the presence of both reagents (Figure 3).

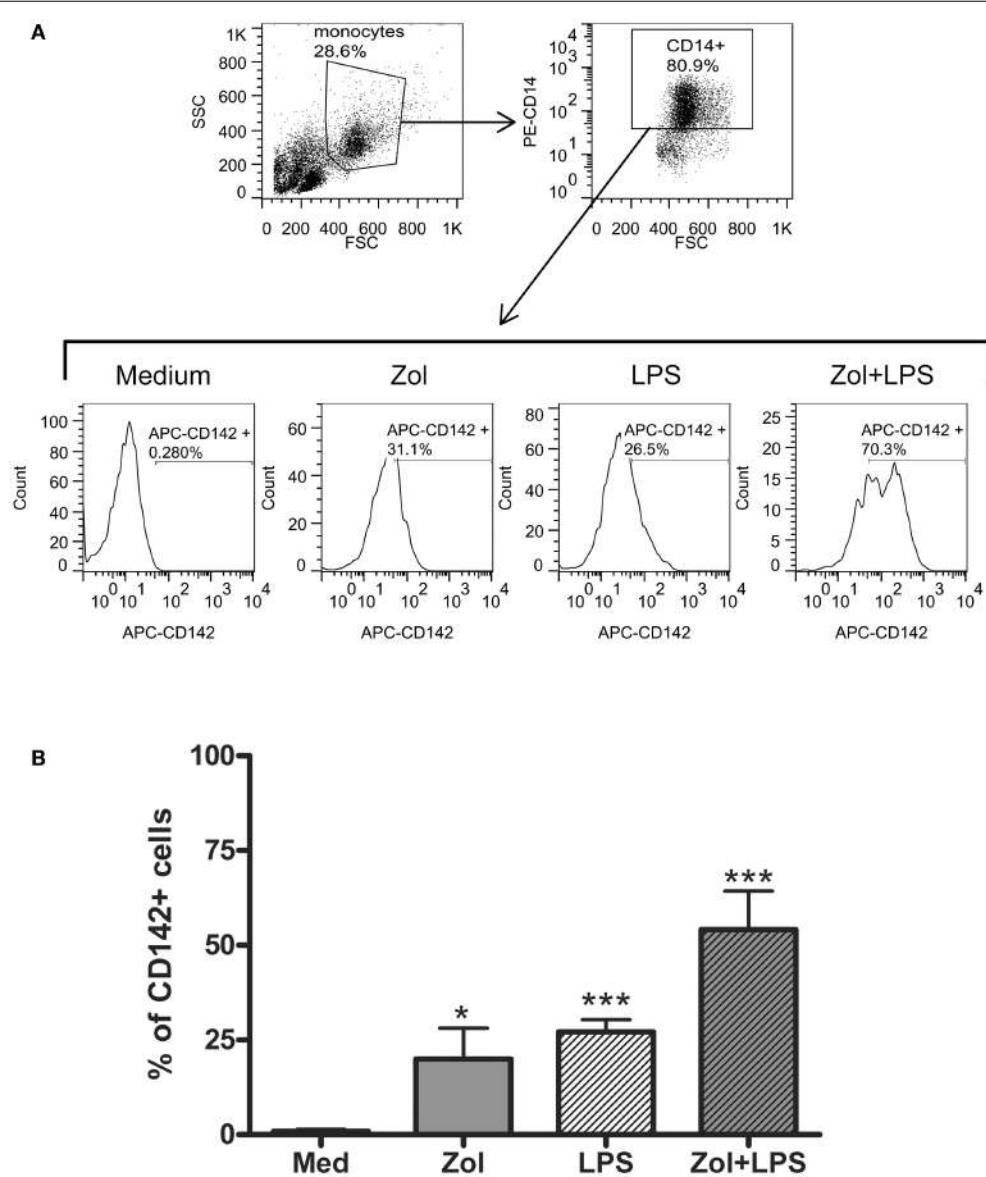


FIGURE 3 | Zoledronate induces tissue factor-1 (TF-1) surface expression on CD14+ monocytes. **(A)** Histograms in the second panels depict TF-1 expression as percent CD142+ among CD14+ monocytes in PBMC cultured in medium or after stimulation with Zol, LPS, or with both, as indicated (see Materials and Methods). The panel above shows

forward (FSC) and side scatter (SSC) dot plots of the PBMC gating used to identify monocytes and expression of CD14 in the gated monocyte subset. **(B)** Summary of five independent experiments showing %CD142+ monocytes in cultures of HD PBMC as above. * $p < 0.05$, *** $p < 0.0001$.

We next performed similar experiments and included infliximab, a TNF α neutralizing humanized mAb, or similar concentrations of non-specific human IgG, in parallel cultures. Infliximab abolished, in a dose-dependent manner, induction of TF-1 on the surface membrane of CD14+ monocytes in response to Zol but not to LPS (**Figure 4**). Although control IgG also slightly reduced TF-1 expression induced by Zol, inhibition was incomplete even at high concentrations of IgG. Together, these results indicate that Zol, when added to PBMC, induces TNF α secretion by the V γ 982+ T cells in the PBMC and TNF α -dependent expression of TF-1 on CD14+ monocytes.

EFFECT OF PATIENT PLASMA ON TF-1 INDUCTION

Zoledronate has not been previously reported to induce digital necrosis despite its widespread use in patients. Furthermore, in our experience, which included four SSc patients, only RP2 developed an APR and critical digital ischemia, in which TF-1 may have contributed. It was thus of interest to determine the role of RP2 patient specific factors in the development of the unusual response to Zol. We found that addition of RP2-plasma (RP2-P) markedly increased TF-1 induction on CD14+ monocytes in response to Zol as well as LPS, whereas control plasma (RP1-P) from SSc patient RP1 who had received Zol with no toxic effect did not enhance TF-1 induction (**Figure 5**). Importantly, addition of anti-TNF α mAb but not of non-specific IgG still completely abolished TF-1 upregulation in response to Zol even in the presence of RP2-P. Anti-TNF α mAb did not reduce TF-1 upregulation in response to LPS or the combination of LPS and Zol in the presence of RP2-P. These results suggest that the patient's plasma specifically contained factor/s that enhance Zol-induced V γ 982+ produced TNF α -dependent TF-1 expression on monocytes *in vitro*, suggesting that a similar effect may have taken place following IV infusion on her circulating monocytes.

DISCUSSION

This paper shows, for the first time, an SSc specific effect of Zol, i.e., the enhancement of IL-4 secretion by PB V δ 1+ T cells, coupled with diminished ability of these cells to secrete IL-9 relative to two

patients with another form of fibrosing disease, IPF. Furthermore, Zol induced secretion of TNF α by V γ 982+ T cells from some SSc patients similar to healthy individuals, which in turn induced TF-1 on CD14+ monocytes. Finally, plasma of an SSc patient who suffered a clinically detrimental inflammatory response strongly augmented this TNF α -dependent TF-1 expression on monocytes. These data suggest that activation of both major $\gamma\delta$ T cell subsets – V δ 1+ and V γ 982+ by Zol could activate pathogenic mechanisms, e.g., fibrosis (via IL-4) and thrombosis (via TF-1) – relevant to clinical manifestations in SSc patients.

The bisphosphonate-induced APR in patients receiving intravenous therapy for osteoporosis or cancer differs from a typical APR. Thus, CD14+ monocytes and macrophages are the primary cytokine producing cells in the latter, whereas bisphosphonates induce rapid and copious production of TNF α , IFN γ , and IL-6 primarily by PB V γ 982 T cells (16). Activation of the V γ 982+ T cells triggered by upregulation of IPP in monocytes in turn enhances CD14, CD40, CD80, and HLA-DR on circulating monocytes (8). Zol also enhances TNF-related apoptosis-inducing ligand (TRAIL) in $\gamma\delta$ T and NK cells, and release of high mobility group box 1 (HMGB1) from $\gamma\delta$ T cells and monocytes (17). Furthermore, soluble factors released by activated V δ 2/monocytes co-cultures induce granulocyte migration and activation (18). Activated V γ 982T cells also trigger granulocyte functions via MCP-2 release during bacterial infection (9).

The novel finding shown here, i.e., induction of TF-1 on monocytes, in a manner dependent upon TNF α produced by V γ 982+ T cells stimulated with Zol, adds an additional dimension to the role of monocyte–V γ 982 interactions, that may play a critical role in clinical medicine, since TF-1 expressing monocytes play an important role in thrombotic diseases (19). For example, patients with cardio- and cerebro-vascular disease have increased TF-1 expression on circulating monocytes and TF-1-positive monocyte-derived circulating microparticles in the blood (20, 21). Furthermore, circulating monocyte-derived microparticles expressing TF-1 are associated with acute recurrent deep venous thrombosis (22). In experimental hypercholesterolemic mice, the associated prothrombotic state is caused by oxidized low density lipoprotein

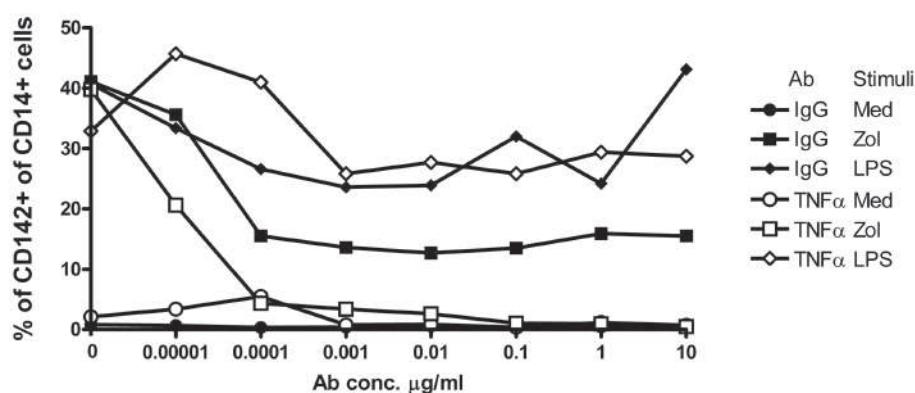


FIGURE 4 | Upregulation of TF-1 in response to Zol is TNF α -dependent.

PBMC from a healthy donor were cultured in medium with graded dilutions (indicated on the x axis) of either anti-TNF α mAb or control IgG. Cells were

stimulated with Zol, with LPS or not stimulated (medium), and TF-1 was measured by flow cytometry as described in **Figure 3**. Results are representative of three experiments.

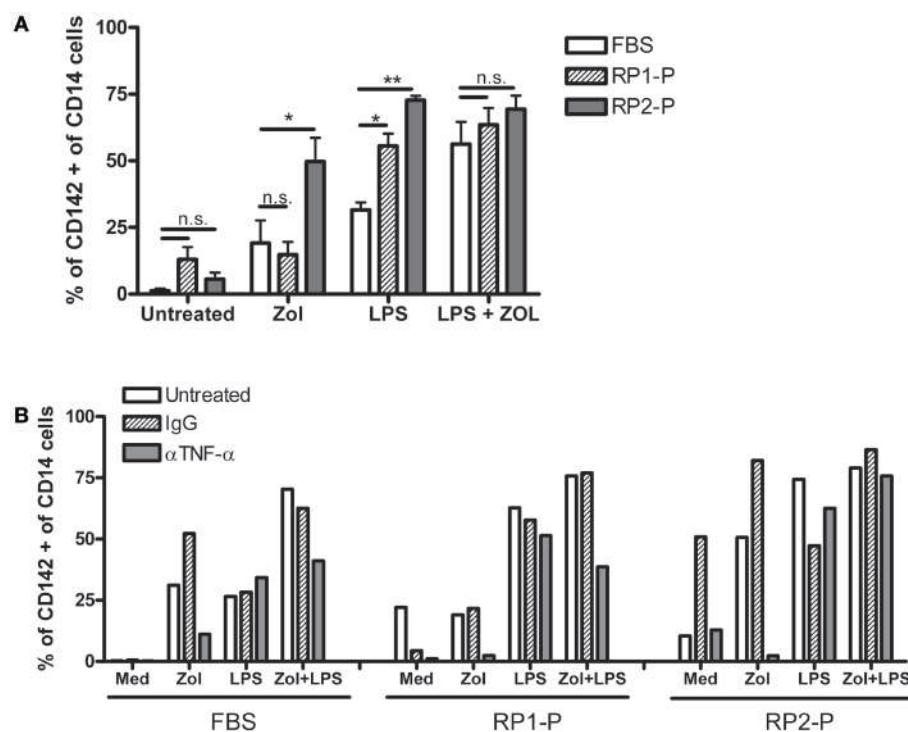


FIGURE 5 | RP2-plasma increases TNF α -dependent upregulation of TF-1 in response to LPS and Zol. (A) Bars represent percent \pm 1 SEM of CD14+ cells expressing TF-1 (CD142) in PBMC cultures from three HDs, cultured with the indicated stimuli on the X axis, in the

presence of medium containing 10% FBS or RP1- or RP2-plasma (RP1-P or RP2-P). **(B)** Shows the same experiment done in the presence of 10 μ g/ml anti-TNF α antibody or control IgG as indicated.

engagement of a toll-like receptor (TLR)4/TLR6 complex, leading to induction of TF-1 in monocytes (23). In addition, in rats, monocytes in blood vessels of kidneys undergoing acute rejection express high levels of TF-1 (24). These data suggest that Zol infusion, which achieves concentrations of zoledronate in the plasma similar to those we have used in our experiments, may lead to induction of TF-1 on circulating monocytes resulting in a pro-thrombotic state, which may have contributed to development of ischemic digits in SSc RP2 patient described here (25). Our data, furthermore, show that Zol-induced TF-1 on monocytes is at least partly dependent upon V γ 9 δ 2 cell produced TNF α (Figures 1 and 5). In contrast, a combination of mAb to TNF α and IL-1 β , but neither alone, was required to inhibit high molecular weight kininogen induced monocyte TF-1 (26). TNF α may also upregulate monocyte TF-1 activity indirectly via its effects on endothelial cells (27, 28).

Although Zol induced increase of TF-1 on monocytes, overt thrombosis in the absence of additional factors is rare. Nevertheless, inclusion of zoledronic acid in the treatment protocols for multiple myeloma (MM) and breast cancer significantly increases venous thrombosis (29–32). In this regard, V γ 9 δ 2+ T cells may be playing a role, since in both MM and breast cancer patients, these cells are known to become activated by Zol (33–36). These data suggest that Zol-induced thrombosis is dependent upon disease and/or patient specific factors, which may include Zol responsive V γ 9 δ 2+ T cells in the PB and additional factors.

Our data suggest that SSc may constitute a risk for severe prothrombotic $\gamma\delta$ T cell-mediated Zol-induced reactions. In this regard, the already diseased endothelium in SSc may play a role, since activated $\gamma\delta$ T cells of SSc patients, in particular, adhere to and damage endothelial cells, creating a substrate for enhancing thrombosis (37). The current data suggest that at least two additional factors could play a role in Zol-induced thrombosis in SSc patients. First, SSc patient's plasma may contain factors that enhance TF-1 expression in response to Zol [Figure 5 and Ref. (16)]. Indeed, SSc plasma has been shown previously to contain increased levels of circulating TNF α , platelet microparticles, and soluble CD40 ligand, which could collaborate in the induction of TF-1 (38). That TF-1 induction in the setting of exposure to Zol can be blocked by an anti-TNF α antibody even in the presence of enhancement by patient's plasma (Figure 5) suggests that TNF α released by Zol-activated V γ 9 δ 2+ T cells plays a major role in induction of TF-1 and that anti-TNF α mAb could be used to prevent thrombosis in high risk SSc patients treated with Zol. In addition, our study is the first, to our knowledge, to describe a disease-specific IL-4 response of SSc patients V δ 1+ T cells to Zol (Figure 1). A bias toward IL-4 secretion by SSc patients CD4+ TCR $\alpha\beta$ T cells in response to non-specific stimulation has already been observed (39). Moreover, V δ 1+ $\gamma\delta$ T cells often predominate in the context of a Th2-biased environment, e.g., in the broncho-alveolar lavage fluid obtained from allergic individuals (40, 41). In addition, the majority of

phosphatidyl-ethanolamine CD1d-restricted $\gamma\delta$ T clones in allergic individuals are V δ 1+ and secrete high levels of IL-4 (42). We hypothesize that Zol stimulation may upregulate co-stimulatory monocytes CD40, CD80, and CD1d molecules, which in turn, enhance IL-4 secretion by CD1d-restricted lipid antigen responsive V δ 1+ T cells in the PB of SSc patients (43, 44). The combined effects of the TNF α secreted by V γ 982 cells, together with the IL-4 produced by V δ 1+ T cells increases vascular cell adhesion molecule 1 (VCAM-1) expression on endothelial cells in digital arteries and VCAM-1-mediated adhesion of TF-1+ monocytes to the endothelium could then activate local thrombosis and gangrene (45).

In summary, the hitherto described disastrous consequences of a seemingly innocuous and highly utilized drug, Zol, in an SSc patient, which prompted these investigations, led to the discovery of a novel prothrombotic pathway involving V γ 982 $\gamma\delta$ T cells and CD14+ monocytes and a disease-specific activation of IL-4 producing V δ 1 $\gamma\delta$ T cells. Future studies into this pathway may lead to new insights into the immunopathogenic mechanisms of thrombotic diathesis in immune-mediated, infectious, and malignant diseases, in which $\gamma\delta$ T cells play a role.

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$\gamma\delta$ T lymphocytes in the diagnosis of human T cell receptor immunodeficiencies

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INTRODUCTION

Human T cell receptor (TCR) immunodeficiencies (TCRID) are rare autosomal recessive disorders caused by mutations affecting TCR, CD3, or CD247 chains, which share developmental, functional, and TCR expression defects (1). Their rapid diagnosis is fundamental for patient survival and early hematopoietic stem cell transplantation. Here, we propose that studying $\gamma\delta$ T cells, which are often neglected, can be helpful for a timely diagnosis. We thus offer a diagnostic flowchart and some lab tricks based on published cases.

$\gamma\delta$ T CELL AND TCR PHYSIOPATHOLOGY

$\gamma\delta$ T lymphocytes are a minor subset (1–10%) of human peripheral blood T cells. Most (>70%) are CD4⁻CD8⁻ [double negative (DN)], some (30%) are CD8⁺CD4⁻ and very few (<1%) are CD4⁺CD8⁻ [CD8⁺ or CD4⁺ single positive (SP), respectively]. Most $\gamma\delta$ T cells in adults express V δ 2/V γ 9 TCR variable regions (65–90%), the rest being mostly V δ 1⁺, some V δ 3⁺ or V δ 5⁺, all with different V γ chains (2). As peripheral blood $\gamma\delta$ T cells are scarce, their over-representation is more conspicuous than their under-representation, which is very rarely reported and normally associated to a single subset, such as V δ 2⁺ in

granulomatosis (3) or aging (4). Indeed, no selective $\gamma\delta$ T cell immunodeficiency (ID) has been reported to date, although absence of $\gamma\delta$ T cells has been described together with other lymphocyte derangements in rare primary ID (5). The clinical significance of increased $\gamma\delta$ T cells, defined as >10% of peripheral blood T lymphocytes (6), requires clarification in several diseases including infection, autoimmunity, cancer, and primary ID.

The human $\gamma\delta$ TCR (Figure 1A inset) is an octameric protein complex composed of three heterodimers (TCR γ /TCR δ , CD3 γ /CD3 ϵ , and CD3 δ /CD3 ϵ) and a single CD247 homodimer (also termed ζ/ζ). The complex can be abbreviated as $\gamma\delta$ TCR/ $\gamma\delta\epsilon\zeta\zeta$. The TCR γ /TCR δ heterodimer contains variable regions, which allow for antigen recognition, while the other three dimers are invariant and are required for surface TCR expression and for intracellular propagation of the recognition signal (7). Therefore, defects in any chain would expectedly impact $\gamma\delta$ TCR expression and $\gamma\delta$ T cell selection and function.

$\gamma\delta$ T CELLS IN TCRID

$\alpha\beta$ T cells have been extensively studied in TCRID. In contrast, $\gamma\delta$ T cells have been frequently ignored, in part due to their scarcity but also to the lack of markers

other than the TCR to identify them when TCR expression is reduced, as is the case in TCRID. Although their functions are still debated, we believe that their accurate study (relative numbers, Figure 1A, TCR expression, Figure 1B, and main subsets) can help to diagnose TCRID, as reviewed below and summarized in a practical diagnostic flowchart in Figure 1C.

TCR α deficient patients showed combined ID and autoimmune features due to a selective block in $\alpha\beta$ T cell development, as TCR α is part of the TCR α /TCR β ($\alpha\beta$ TCR, Figure 1A inset) antigen-binding heterodimer (9). In contrast, the $\gamma\delta$ TCR was unaffected, as demonstrated by normal surface expression (Figure 1B), which allowed for normal absolute but increased relative numbers of $\gamma\delta$ T cells (Figure 1A). This is unique among TCRID and thus a useful feature in the diagnostic flowchart (Figure 1C). Such $\gamma\delta$ T cells were proposed to be in part protective from infections in the two reported patients. Indeed, $\gamma\delta$ T cells are involved in immune responses against a variety of pathogens including virus, bacteria, and parasites, whereas still other act as antigen-presenting cells (10) or B cell helper cells (11). Their beneficial effects *in vivo* have found recent unexpected recognition in haploidentical allogeneic hematopoietic cell transplantation after depletion of $\alpha\beta$ T and B cells (12),

Abbreviations: DN, double negative; DP, double positive; ID, immunodeficiency; mAb, monoclonal antibody; SCID, severe combined immunodeficiency; SP, single positive; TCR, T cell receptor; TCRID, T cell receptor immunodeficiencies.

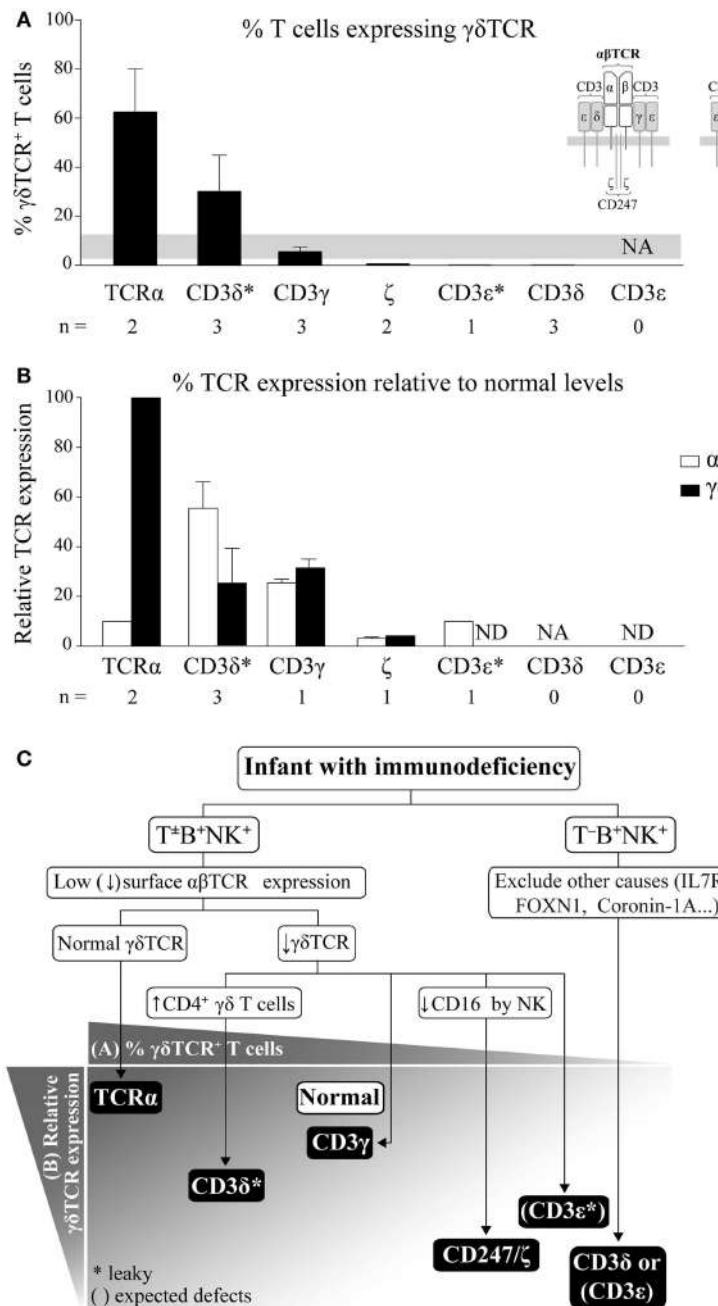


FIGURE 1 | $\gamma\delta$ T cells in TCRID. (A) Proportion of $\gamma\delta$ T cells within the T cell compartment. The percentage of $\gamma\delta$ T cells (mean \pm SEM) was defined as $\gamma\delta$ TCR⁺ using 11F2, IMMU510, or anti-TCR δ monoclonal antibody (mAb) within the T lymphocyte gate (defined as CD3⁺) and ordered from left to right in decreasing values. The gray band indicates the normal range for infants (8). Inset: human TCR isotypes. NA: not analyzable (no T cells); *: leaky mutations (partial defects); n: number of patients for which data was available. **(B)** % TCR surface expression (mean \pm SEM) in $\gamma\delta$ or $\alpha\beta$ T cells relative to healthy donors. TCR surface expression was determined by flow cytometry using different anti-CD3 mAb, $\gamma\delta$ T cells were identified as in **(A)** and $\alpha\beta$ T cells as $\gamma\delta$ TCR⁻CD3⁺ or CD4⁺ cells. ND: not determined. **(C)** Our suggested TCRID diagnostic flowchart using absolute lymphocyte counts for T⁺B⁺NK⁺ or T[±]B⁺NK⁺ phenotype and basic flow cytometry data (top) to point to the most likely culprit TCR chain (bottom). TCR chains are represented by black boxes arranged according to the proportion of $\gamma\delta$ T cells from **(A)** and their surface TCR expression relative to normal controls from **(B)**. The white box indicates normal value. *: as in **(A)**. Brackets represent expected defects, as $\gamma\delta$ T cells values were not available in these TCRID.

which showed that $\gamma\delta$ T cells did not cause graft vs. host disease and may have helped with host immune maintenance and recovery. The fact is that, compared to other complete TCRID, symptoms in both TCR α deficient patients appeared rather late (6 and 15 months of age) and transplantation took place very late (6–7 years of age).

Similar to TCR α deficient patients, patients with partial CD3 δ deficiency (CD3 δ^* in **Figure 1**) due to a leaky splicing mutation showed strongly reduced $\alpha\beta$ T cell numbers and normal absolute but high relative numbers of $\gamma\delta$ T cells (**Figure 1A**), although with low surface TCR expression [(13) and **Figure 1B**]. In contrast to TCR α deficiency, partial CD3 δ deficiency showed early severe combined ID (SCID) features and required very early transplantation (before 2 years of age), thus their $\gamma\delta$ T cells were not protective, perhaps as a consequence of their impaired TCR expression and function (13). Unexpectedly, partial CD3 δ deficiency caused a stronger impact in $\gamma\delta$ (**Figure 1B**) than in $\alpha\beta$ TCR surface expression (25 vs. 55% relative to controls (13)). A detailed study of their $\gamma\delta$ T cells showed an enrichment in a subset of otherwise rare CD4⁺ $\gamma\delta$ T cells, which exhibited an activated phenotype and were refractory to further TCR stimulation (14). This CD4-expressing $\gamma\delta$ T cell subset seems to be pathognomonic for partial CD3 δ deficiency, since: (i) it has been ascertained in three of three tested patients with this condition and (ii) it was not found in other TCRID (14, 15). Its developmental origin deserves further comment. $\alpha\beta$ and $\gamma\delta$ T cells differentiate within the thymus from a late DN common progenitor (16). In humans, development of most $\gamma\delta$ T cells seems to mimic that of $\alpha\beta$ T cells: from DN progenitors through a CD4⁺CD8⁺ double positive (DP) pathway (17, 18), to DN and either CD4⁺ or CD8⁺ SP populations. DN and CD8⁺ SP $\gamma\delta$ T cells are minor intrathymic subsets but become the major $\gamma\delta$ T cell subsets in the periphery, while CD4⁺ SP are the main intrathymic subset, followed by DP (19). Notably, the last two subsets can be found in peripheral blood in pathological conditions, and most bone marrow and peripheral blood $\gamma\delta$ T cells from patients with $\gamma\delta$ T cell acute lymphoblastic leukemia are either CD4⁺ SP or DP (20). Thus, we believe that the 10-fold enrichment of CD4⁺ SP $\gamma\delta$ T cells observed

in patients with partial CD38 deficiency is due to low TCR-dependence for positive selection of CD4⁻ $\gamma\delta$ T cells and disrupted negative selection of CD4⁺ $\gamma\delta$ T cells (14).

CD3 γ deficient patients, most of which showed mild ID (21), had normal numbers of polyclonal peripheral blood $\gamma\delta$ T cells [absolute and relative, (22) and **Figure 1A**] with low surface TCR [around 30% of control levels (22) and **Figure 1B**], similarly to their $\alpha\beta$ T cell counterparts (23), likely with an abnormal $\gamma\delta$ TCR/ $\delta\epsilon\delta\epsilon\zeta\zeta$ stoichiometry. Despite their high homology, the invariant CD3 γ and δ chains show different roles in human vs. mouse $\gamma\delta$ T cell development. Indeed, CD3 γ -deficient mice exhibited a severe $\gamma\delta$ T cell developmental block (24).

$\gamma\delta$ T cells were studied in only two of three reported CD247 deficient patients (21). The patients showed SCID features and reduced absolute and relative $\gamma\delta$ T cells numbers (**Figure 1A**). Surface $\gamma\delta$ TCR expression was also reduced (4% vs. healthy controls, **Figure 1B**). The number of $\alpha\beta$ T cells was only slightly reduced despite their similarly reduced surface TCR, with all reported cases showing reduced numbers of CD4⁺ T cells but normal or high numbers of CD8⁺ T cells (21, 25–27).

A single patient with partial CD3 ϵ deficiency (28–30) showed very low surface $\alpha\beta$ TCR expression (10% of normal levels, **Figure 1B**, CD3 ϵ^*), mild ID, normal CD8⁺, and reduced CD4⁺ ($\alpha\beta$) T cells, but no $\gamma\delta$ T cells as determined with the anti-TCR δ -1 monoclonal antibody (mAb) (**Figure 1A**). We have however considered for **Figure 1C** that surface $\gamma\delta$ TCR expression might have been similar to $\alpha\beta$ TCR expression.

Three studied CD38 deficient patients [out of 16 reported, all with severe T cell lymphopenia and SCID (31, 32)], showed a few circulating CD3 $^{+}$ T cells, which were DN but $\gamma\delta$ TCR⁻ (33). $\gamma\delta$ TCR⁺ cells were indeed undetectable by flow cytometry in peripheral blood or by immunohistochemistry in the thymus, lymph nodes, spleen, or gut. However, gene microarray analysis and protein expression of patient thymocytes showed increased levels of TCR γ and TCR δ transcripts and proteins (33), which could be interpreted as presence and thus significant selection of $\gamma\delta$ T cells unable however to leave the thymus, perhaps due to insufficient surface TCR compared to partial CD38 deficiency.

Finally, $\gamma\delta$ T cells have not been studied in SCID patients with complete CD3 ϵ deficiency (31). Nevertheless, given their severe T cell lymphopenia, we can safely presume for **Figure 1C** that they were absent.

In summary, the proportion of $\gamma\delta$ T cells within total T lymphocytes (**Figure 1A**) and the level of surface $\gamma\delta$ vs. $\alpha\beta$ TCR expression (**Figure 1B**), as well as the severity of lymphopenia (T⁻B⁺NK⁺ or T[±]B⁺NK⁺ phenotype), can be used to generate a practical TCRID diagnostic flowchart (**Figure 1C**). For instance, if an infant has SCID and no T cells but normal B and NK cell numbers (T⁻B⁺NK⁺ phenotype) and other causes have been ruled out, CD38 or CD3 ϵ deficiency should be considered (**Figure 1C**). In contrast, if some T cells are present (T[±]B⁺NK⁺ phenotype) and $\gamma\delta$ TCR expression is low, TCR α deficiency can be ruled out. If CD16 expression by NK cells is normal, CD247 deficiency can be excluded, and the presence or absence of high absolute numbers of CD4⁺ $\gamma\delta$ T cells will rule out CD3 γ or partial CD38 deficiency, respectively.

LAB TRICKS TO IDENTIFY $\alpha\beta$ AND $\gamma\delta$ T CELLS IN TCRID

When surface TCR expression is low, $\alpha\beta$ T cells can be identified by the expression of CD4 or CD8 $\alpha\beta$ (i.e., CD8^{bright}) within the lymphoid subset (23), whereas $\gamma\delta$ T cells are identified only by expression of the $\gamma\delta$ TCR. We have reported that most CD3 $^{+}$ cells within normal DN lymphocytes are $\gamma\delta$ T cells (34), and this may also help in certain TCRID.

Despite their reduced numbers and surface TCR expression, an appropriate multicolor flow cytometry approach can help to identify $\gamma\delta$ T cells in TCRID. To avoid underestimation due to low TCR surface expression, we recommend: (i) the use of bright fluorochromes such as PE, PE-Cy5.5, PE-Cy7, or APC, rather than FITC, (ii) an appropriate choice of CD3 mAb such as UCHT-1, F101.01, or S4.1 due to their high signal-to-noise ratio in TCRID, (iii) two-color stainings with CD3 and $\gamma\delta$ TCR mAb, which can also help to single out $\gamma\delta$ T cells as a DP subset, and (iv) to avoid mixing $\alpha\beta$ TCR and CD3 mAb, as they sometimes compete (UCHT-1, for instance).

CD4 and CD8 expression by $\gamma\delta$ T cells should also be tested to rule out partial CD38 deficiency (see above). CD4, CD8,

$\gamma\delta$ TCR (IMMU510 or 11F2), and CD3 (UCHT-1 or S4.1) is a useful combination, to this end. Lastly, intracellular stainings for invariant TCR chains has been shown to be useful to identify T cells expressing very low surface TCR, such as those with CD247 (21) or partial CD38 deficiency (14).

CONCLUSION AND PERSPECTIVES

Human $\gamma\delta$ T lymphocytes are still puzzling in terms of development, function, and TCR stoichiometry in ways that mouse models do not wholly recapitulate. Human TCRID share defects in T cell development and function and in TCR expression. While their $\alpha\beta$ T cells have been studied in detail, $\gamma\delta$ T cells have been frequently ignored, in part due to their scarcity and to the lack of appropriate markers to identify them when TCR expression is reduced. Here, pooling published studies, we proposed some technical tricks to identify $\gamma\delta$ T cells in TCRID patients and made the point that their careful analysis can help to inform a rapid differential diagnosis using a flowchart, with clinical benefit.

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