UNDERSTANDING GAMMA DELTA T CELL MULTIFUNCTIONALITY - TOWARDS IMMUNOTHERAPEUTIC APPLICATIONS

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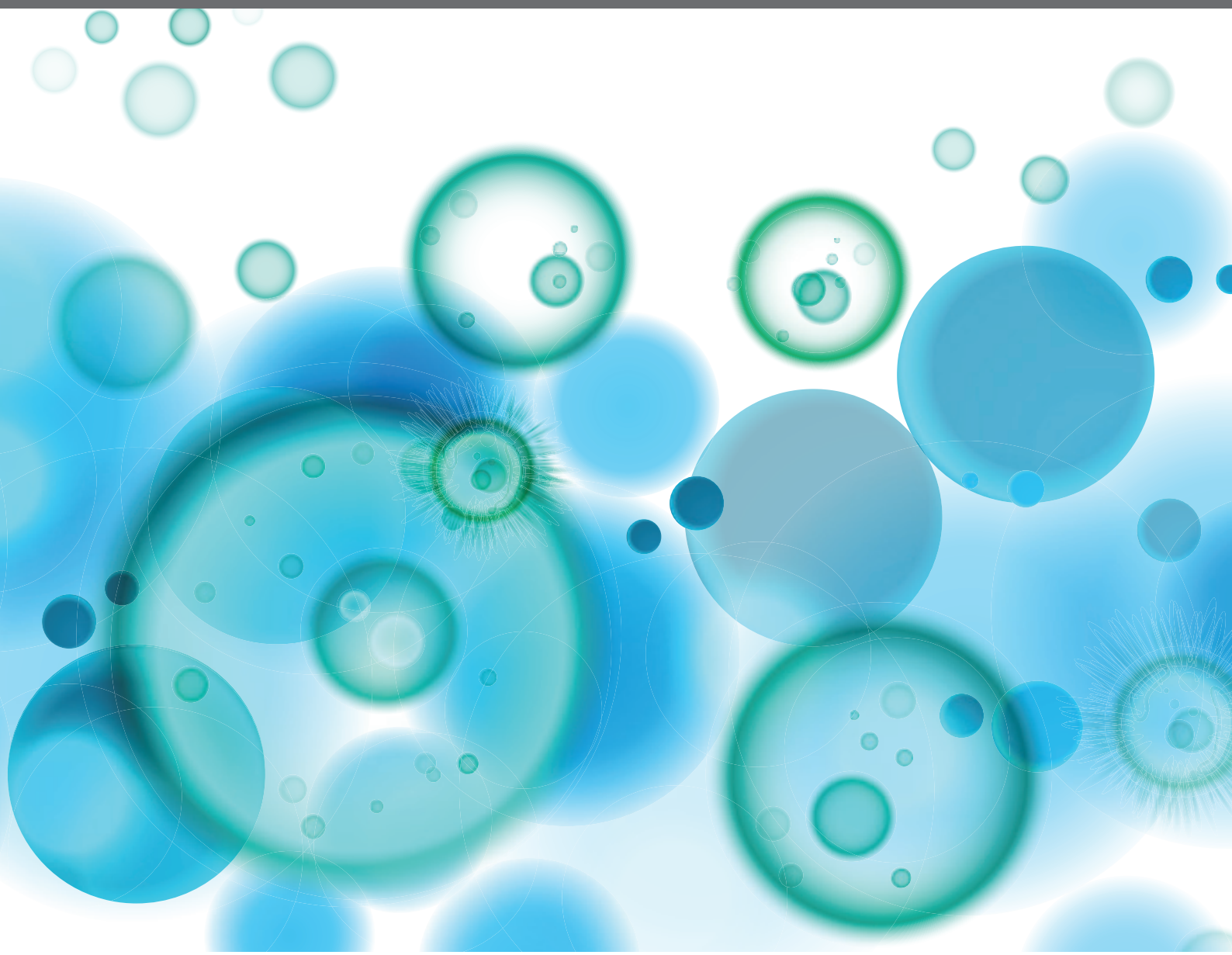

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UNDERSTANDING GAMMA DELTA T CELL MULTIFUNCTIONALITY - TOWARDS IMMUNOTHERAPEUTIC APPLICATIONS

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Editorial: Understanding Gamma Delta T Cell Multifunctionality - Towards Immunotherapeutic Applications

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Editorial on the Research Topic

Understanding Gamma Delta T Cell Multifunctionality - Towards Immunotherapeutic Applications

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INTRODUCTION

γδ T cells have been characterized by the expression of a γδ T cell receptor (TCR). When the γδ TCR and the corresponding αβ TCR were first discovered it was assumed that the corresponding cell types were likely to be functionally very similar. However, some 30 years later, we have realized that they are not. Unlike αβ T cells, γδ T cells (i) sense target antigens independent of MHC molecules; (ii) display NK-cell like innate reactivities, including killing of infected cells as well as microbes; (iii) are able to take up large particulates, including bacteria, and (iv) can act as professional antigen presenting cells.

The "stress sensing" abilities of γδ T cells have led to a great interest in exploring their potential use in novel immunotherapies, not least in cancer. In addition, their capacity to produce various cytokines and to interact with other cells, such as lymphocytes, myeloid cells, and neutrophils, has raised an interest in their potential therapeutic use as immunomodulators. γδ T cells have therefore recently come to stand out as a distinctly unique cell type. At the same time, we have come to realize that γδ T cells are likely to be an ancient cell type with ancestors as far back as in our common ancestors to jawless fish and thereby, potentially predating the development of our adaptive immune system.

It is clear that to make full use of the great potential of γδ T cells in immunotherapeutic interventions, we must significantly improve our understanding of the extensive plasticity and multifunctionality of γδ T cells and of how these cells can be harnessed therapeutically, both safely and effectively.

This Research Topic, contains 26 articles which includes Review, Original Research, and Methods articles in the area of γδ T cell plasticity and multifunctionality, As shortly overviewed in this editorial, these articles shall not represent entire field of γδ T cell biology but primarily focuses on how an increased knowledge in this area can be used and developed further toward improved immunotherapeutic applications in cancer, infectious disease, autoimmunity, and other immunity-related areas.

γδ T CELLS AT BARRIERS

γδ T cells and other "non-conventional" T cells V-gene usage of antigen-receptors often correlates with distinct developmental and functional features. A prominent example is murine dendritic epidermal γδ T cells (DETC). They carry a single "canonical" γδ TCR and depend on the butyrophilin-like Skint-1 molecule for development. Humans lack DETC as Skint-1 is a pseudogene. The mini review of Sutoh et al. discusses the evolution of the Skint-1 family and DETC like cells. They identify two species with a functional Skint-1 gene, namely cattle and the cynomolgous macaque, as species with skin γδ T cells sharing some features with murine DETC. Interestingly, in lamprey, VLRC-expressing cells (one of the three linages of VLR-antigen-receptor expressing lymphocytes) localize to epithelia and exhibit a dendritic morphology in the skin. This led to the hypothesis that stress-sensing lymphocyte at body surfaces provide an evolutionary advantage and might even have existed in the ancestor of jawed- and jawless-vertebrates.

The interplay of DETC and dermal Vγ4 T cells during wound healing is the focus of two papers by the group of Li, Wang et al. and Li, Wu et al.. They review current knowledge on this subject and provide an original research paper which investigates how epidermal invasion of IL-17 producing dermal Vγ4 cells suppresses IGF-1 production by DETC and wound healing via IL-23 and IL-1β producing keratinocytes.

Cruz et al. provides a comprehensive review on immune responses and pathophysiology of the human skin T cells. Although their cellular composition is quite different from that of mice, it is quite intriguing that many molecular players, especially cytokines, may function similarly as in mouse models. A challenge is now to understand the relative contribution of the different T cell subsets, including γδ T cells, to integrity of healthy skin and their function in pathological conditions such as psoriasis, alopecia areata, type II diabetes and melanoma.

The mini review of McCarthy and Eberl covers the two major subsets of human γδ T cells in mucosal immunity and inflammation: stress-responsive, partially resident Vδ1 lymphocytes and microbial phosphoantigens-sensing Vγ9Vδ2 T cells. Both γδ T cell subsets activate other (resident) leukocytes such as neutrophils and myeloid DCs or differentiate to professional APC, as shown for phosphoantigen-activated Vγ9Vδ2 T cells.

The authors discuss how in both situations γδ T cells help to communicate their findings of stressed transformed and infected cells as well as extracellular pathogens downstream to other immune cells. They speculate how this leads to inflammatory responses and T cell activation serving as the boundaries of our insides and the environment and how this knowledge can be used for development of new therapeutic strategies.

Reinhardt and Prinz, discuss the pathogenesis of various forms of spondyloarthritis. Currently, first choice of Spondylitis ankylosans treatment is non-steroidal anti-inflammatory drugs, physiotherapy and anti-TNF but more recently genome wide association studies and promising results with antibodies targeting IL-17 suggest the involvement of IL-17, IL-22, and IL-23. The authors develop a scenario in which enthesis-resident cells sense directly or indirectly mechanical stress and various innate T cells (e.g., MAIT cells or γδ T cells) and/or ILC3 and promote joint inflammation by production of IL-17 and IL-22.

γδ T CELLS IN AUTOIMMUNITY AND INFECTION

γδ T cells are abnormally regulated in some autoimmunities. In an original research article Migalovich Sheikhet et al. show that systemic sclerosis (SSc) γδ T cell subsets are differentially regulated by cardiolipin and zoledronate as compared to healthy donor (HC) γδ T cells. $CD25^+$ γδ T cells were elevated significantly in SSc patient blood compared to HC. The main conclusions and take home messages of this study was that SSc γδ T cell functional responses were abnormal when stimulated by cardiolipin and phosphoantigens and that this may contribute to fibrosis and immunosuppression, both hallmarks of this disease.

Whilst novel forms of cancer immunotherapies is one of the dominating fields of current research and proposed uses of γδ T cells in the clinic, most investigators in the field would agree that γδ T cells, as indeed other parts of the immune system, evolved to combat pathogens. It is in this spirit we have included three articles in this section describing functional involvement of γδ T cells in infectious diseases.

It is well-known that as HIV virus infect $CD4^+$ T-cells, the pool of T cells that are able to produce IL-17 are reduced. γδ T-cells can recognize and kill cells that are infected with virus, including cells infected with HIV virus. As γδ T cells, and in particular Vδ1 γδ T cells, are also able to produce IL-17, they therefore provide an alternative source for IL-17 production in AIDS. However, in an original research paper in this section, Dunne et al. show that such Vδ1 γδ T cells in HIV infected individuals are often exhausted. They show that this exhaustion is likely to be mediated by either PD-1 induction or by downregulation of CD3ε expression.

Malaria is caused by the protozoan parasite *Plasmodium* sp. As malaria causes high mortality and morbidity on several continents and no successful vaccine exists malaria research should be a priority. In a review article in this section Howard et al. summarize what is known about the participation of Vγ9Vδ2 T cells in malaria immune system reactivity. It is well-known that this γδ T cell subset is activated and expanded during a primary *Plasmodium* infection, but not why and what functions this expansion mediates. Following a review of what is known about this subsets innate and adaptive functions, Howard et al. speculate that this subset could serve an important role as an adjuvant in new malaria vaccine formulations.

In an accompanying minireview, Hviid et al. summarize what is known about the participation and possible functions of the Vδ1 γδ T cell subset. They suggest that the more "adaptive" immunological behavior of this subset, as compared to Vγ9Vδ2 T cells, is very likely reflected in their participation in malaria immune reactivity and that this could also be the case for other γ/δ chain combinations other than Vγ9Vδ2.

γδ T CELLS IN TUMOR IMMUNITY

Pioneering studies by Hayday and colleagues in a mouse model of cutaneous carcinogenesis have firmly established an important role of γδ T cells in immune surveillance (1). Subsequently, it has been found that human γδ T lymphocytes display potent HLA-unrestricted cytotoxicity *in vitro* against a variety of tumor cell lines and have demonstrated efficacy *in vivo* when transferred into immunodeficient mice xenografted with different types of tumor cells.

Given their HLA non-restricted method of antigen recognition, the role of γδ T cells in anti-tumor immunity has stimulated great interest to explore their potential for cancer immunotherapy. Interestingly, the production of endogenous PAgs, such as IPP, can be pharmacologically manipulated by aminobisphosphonates (N-BP), such as Zoledronate, which inhibit FPPS, the downstream enzyme of the MVA pathway, leading to accumulation of endogenous IPP and Vγ9Vδ2 T cell activation.

Simões et al. summarize current knowledge on target cell recognition of γδ human T cells. Antigens expressed by target cells are (over) expressed in stressed or transformed cells. Many of them are unknown and knowledge on the molecular basis of their recognition by the γδ TCR is very limited. Vγ9Vδ2 T-cells sense phosphoantigens, isoprenoid synthesis metabolites, which accumulate as after administration of drugs (amino-bisphosphonates), cell-transformation or microbial infections. This sensing is mediated by the Vγ9Vδ2 TCR which "sees" target cells after binding of the phosphoantigens to target cell-expressed butyrophilin (BTN)3A1 molecule. BTN and butyrophilin-like (BTNL) molecules mainly present on epithelial and tumor cells regulate the homing and maturation of certain γδ clonotypes. BTN3A1 is required for Vγ9Vδ2 T cell recognition of PAgs and BTN3A1 modulation *in vitro* and *in vivo* impacts on anti-tumor efficacy of γδ T cells (Blazquez et al.), thus providing the potential for novel Vγ9Vδ2 T cell-based cancer immunotherapy.

Activating NK cell receptors with NKG2D as most prominent example, act either alone or by providing a co-stimulatory signal to the γδ TCR. They serve also as stress-sensors since expression of many NKG2D ligands results from cell-stress (2). Similar to NK cells, γδ T-cells are endowed with anti-leukemia and anti-infection potential and do not mediate graft-vs.-host disease. These features are particularly useful in the setting of HLA haploidentical HSCT depleted of $\alpha\beta^+$ T and B lymphocytes to cure high-risk acute leukemias (Pistoia et al.). In this setting, high numbers of both γδ T cells (Vδ1 and Vδ2) are infused together with $CD34^+$ HSC and may contribute to rapid control of infections and leukemia relapse. In addition, Zoledronate potentiates the cytotoxic activity of γδ T cells *in vitro* and its infusion in patients strongly promotes γδ T cell differentiation and cytolytic activity. Hence, treatment with Zoledronate may contribute to further improve the patient clinical outcome after HLA-haploidentical HSCT depleted of $\alpha\beta^+$ T and B lymphocytes.

Intravenous application of Zoledronate together with low-dose IL-2 has been evaluated as a means of *in vivo* activation of γδ T cells in cancer patients. Hoeres et al. provide a comprehensive review of established and newer strategies exploiting γδ T cells in cancer immunotherapy.

Most likely, strategies aiming to activate γδ T cells *in vivo* will have to be combined with other treatment regimens to obtain optimal anti-tumor activity. Bhat et al. discuss the current development of drugs targeting major pathways of epigenetic regulation and their possible impact on γδ T cell multifunctionality and develop concepts of how some of these approaches might help to improve the efficacy of γδ T cell-based cancer immunotherapy.

Additional strategies to improve the anti-tumor activity of γδ T cells are under study.

These include the use of antibodies to trigger Fc receptor-dependent ADCC, or the use of bispecific antibody constructs to cross-link the γδ TCR with tumor cell surface antigens. Varesano et al. have used the therapeutic anti-EGF-R humanized mAb Cetuximab, in addition to Zoledronate in order to enhance Vδ2 T cell-mediated killing of cancer cells. For this purpose, they have employed a 3-D culture system, consisting of colorectal cancer spheroids, and have shown that Cetuximab triggers Zoledronate-activated Vδ2 T cells to perform ADCC of colorectal cancer and destroy spheroids. Thus, this 3-D system may prove reliable to evaluate the whole anti-tumor effect of combinatorial immunotherapies.

γδ T cells can be redirected to the cancer cell using antibodies. This can be achieved, for instance by the use of bispecific antibodies, in which one binding site recognizes a tumor specific cell surface molecule and the other binding site recognizes CD16 or CD3 or the Vγ9Vδ2 TCR; such bispecific antibodies have shown efficacy in preclinical mouse models. Oberg et al. employed the bispecific antibody HER2xCD16 in the form of a "*tribody*" construct to redirect CD16 expressing γδ and NK cells against HER2 positive tumor cell targets. Compared to Trastuzumab, the HER2xCD16 tribody had superior efficacy in promoting CD16-mediated γδ and NK killing of several types of tumor cells expressing HER2 antigen.

Another approach to improve the efficacy of γδ T cell-based cancer immunotherapy consists in the adoptive transfer of genetically-modified T lymphocytes. This includes lentiviral-mediated transduction of T cells with chimeric antigen receptors (CARs) or with an exogenous TCR of known specificity. To date, both approaches have utilized αβ T cells, but γδ T cells may also be an appealing target. Here, Fisher and Anderson provide a comprehensive review of current engineering approaches in human γδ T cells for cancer immunotherapy. Thus, a tumor-specific αβ TCR can be introduced into γδ T cells without the risk of mispairing between the endogenous and exogenous TCRs. It is also possible to transduce peripheral T lymphocytes with a high-affinity Vγ9Vδ2 TCR (Straetemans et al.). These engineered T cells, named TEGs (T cells engineered to express a defined γδT cell receptor), are broadly reactive against several different tumors and have been produced under GMP conditions to enter a Phase I clinical trial in patients with relapsed/refractory acute myeloid leukemia.

One of the basic observations supporting a role of γδ T cells in tumor immune surveillance is their presence amongst tumor-infiltrating lymphocytes (TIL) in most human tumors.

However, the clinical relevance of γδ TILs is unclear because the relative frequencies of tumor-infiltrating γδ T cells correlate with tumor remission, or with tumor progression or even fail to correlate with prognosis. These findings are strongly suggestive of the fact that γδ T cells in the tumor microenvironment may play opposite functions and thus positive or negative correlation with prognosis may depend on the specific γδ T cell subset present at the tumor site (Pauza et al.). It is clear, however, that an increased presence of γδ T cells within a tumor *per se* is not necessarily associated with a beneficial effect. As discussed here by Lo Presti et al., there are multiple interactions of tumor-infiltrating γδ T cells within the local tumor microenvironment that strongly influence the functional outcome. Relevant factors include (but are not restricted to) tumor-derived immunosuppressive cytokines and metabolites, locally expressed inhibitory checkpoints such as PD-1 (Castella et al.), myeloid-derived suppressor cells (MDSCs, Sacchi et al.) and hypoxia (Siegers et al.). Hence, different conditions occurring in the context of the tumor microenvironment may inhibit γδ T cell functions or even convert γδ T cells into suppressive cells, which negatively influence tumor outcome and patient's prognosis.

Therefore, it is a major challenge for future studies to determine how to specifically boost the anti-tumor effects of γδ T cells while simultaneously shunting their suppressive activity.

CONCLUDING REMARKS

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

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Vγ4 T Cells Inhibit the Pro-healing Functions of Dendritic Epidermal T Cells to Delay Skin Wound Closure Through IL-17A

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Dendritic epidermal T cells (DETCs) and dermal Vγ4 T cells engage in wound re-epithelialization and skin inflammation. However, it remains unknown whether a functional link between Vγ4 T cell pro-inflammation and DETC pro-healing exists to affect the outcome of skin wound closure. Here, we revealed that Vγ4 T cell-derived IL-17A inhibited IGF-1 production by DETCs to delay skin wound healing. Epidermal IL-1β and IL-23 were required for Vγ4 T cells to suppress IGF-1 production by DETCs after skin injury. Moreover, we clarified that IL-1β rather than IL-23 played a more important role in inhibiting IGF-1 production by DETCs in an NF-κB-dependent manner. Together, these findings suggested a mechanistic link between Vγ4 T cell-derived IL-17A, epidermal IL-1β/IL-23, DETC-derived IGF-1, and wound-healing responses in the skin.

Keywords: wound healing, Vγ4 T cells, dendritic epidermal T cells, IL-17A, IL-1β, IL-23, IGF-1

INTRODUCTION

γδ T cells are important components of the skin immune system and participate in psoriasis (1), graft rejection (2), carcinoma (3), antimicrobial barrier function (4), and wound repair (5). Several subsets of γδ T cells with distinctive functions exist in skin tissue: dendritic epidermal T cells (DETCs), which uniformly express an invariant Vγ5Vδ1 TCR (according to Heilig and Tonegawa's nomenclature) and exclusively reside in the murine epidermis; Vγ1 and Vγ4 T cells, two dominant subsets of murine peripheral γδ T cells, have also been found to distribute in the dermal layer of murine skin (6). DETCs are rapidly activated at wound sites after injury and provide a major source of epidermal IGF-1, which is required for efficient wound repair (7). Vγ1 and Vγ4 T cells play distinctive roles in the allergic response (8), autoimmune diseases (9, 10), antitumor responses

(11, 12), as well as infectious immunity (13, 14). However, whether and how Vγ1 and/or Vγ4 T cells affect wound healing remains unknown.

IL-17A is an important pro-inflammatory cytokine that plays a critical role in the initiation and amplification of inflammation responses. At the early stages of inflammation, IL-17A is primarily derived from γδ T cells, though it is secreted by Th17 cells at late stages of inflammation (1). IL-17A is required for efficient skin wound healing, as *Il-17a*$^{-/-}$ mice exhibit defects in wound repair (4). In line with this notion, DETCs have been identified to provide a source of epidermal IL-17A after skin injury, which accelerates wound healing by inducing epidermal keratinocytes to express the host-defense molecules β-defensin 3 and RegIIIγ (4). Vγ4 T cells have been identified as a major source of IL-17A that plays a role in skin diseases (1, 15). Moreover, our previous studies have shown that IL-17A production was impaired in skin around wounds, which explains the impaired wound healing in diabetic settings, and IL-17A-positive Vγ4 T cells transferred to the wound bed could improve diabetic wound healing (16). However, Rodero MP et al. reported a contradictory role of IL-17A in skin wound repair and found that application of an IL-17A neutralizing antibody (Ab) onto the wound bed could significantly promote wound healing (17). Recently, using a skin transplantation model, we showed that Vγ4 T cells were recruited to the epidermis from the dermis in a CCL20-CCR6-dependent manner and primarily provided IL-17A at the wound edge at early stages of transplantation (18). The precise roles of IL-17A and Vγ4 T cells in skin wound healing still need to be clarified.

The IL-1β/IL-23-IL-17A axis is critical for the initiation and amplification of inflammatory responses (9, 19–22). IL-1β and IL-23 can strongly promote γδ T cells to produce IL-17A in γδ T cell-mediated skin diseases (2, 9, 21, 22). Our previous study revealed that IL-1β and IL-23 were required for Vγ4 T cells to accelerate skin graft rejection (18) and that DETCs could upregulate IL-17A production in response to IL-1β plus IL-23 upon TCR engagement (4, 22). IL-1β and IL-23 in the epidermis are mainly derived from keratinocytes and Langerhans cells (9, 22). Intriguingly, the expression of epidermal IL-1β and IL-23 could be enhanced by IL-17A derived from Vγ4 T cells in the transplantation area (18). However, whether and how the IL-1β/IL-23-IL-17A axis affects the engagement of γδ T cells in wound healing needs to be investigated.

IGF-1 is exclusively produced from DETCs in the epidermis and strongly promotes keratinocyte proliferation and migration for efficient wound healing (7). Although IL-17A secreted by DETCs has also been shown to improve skin wound closure (4, 7), IGF-1 plays a much more important role for DETCs in wound repair than IL-17A. The underlying molecular mechanisms and how the interaction of IL-17A and IGF-1 affects wound healing are important questions that need to be answered.

In this study, we observed that Vγ4 T cells were a major source of epidermal IL-17A at the early stages of wounding and were responsible for the delayed wound repair. Moreover, we highlighted that Vγ4 T cell-derived IL-17A indirectly inhibited IGF-1 production in DETCs by enhancing epidermal IL-23/IL-1β expression.

RESULTS

Vγ4 T Cells Delay Skin Wound Healing *via* DETCs

To investigate whether Vγ1 and Vγ4 γδ T cells are involved in skin wound repair, we used a murine wound model with or without contraction and analyzed the cutaneous wound-healing kinetics in age- and sex-matched C67BL/6 wild-type (WT) mice with Vγ1 or Vγ4 T cell depletion treatment [Vγ1 T-cell depletion (Vγ1D) or Vγ4 T-cell depletion (Vγ4D)]. The results showed that mice with Vγ4D compared to isotype controls displayed markedly improved wound healing (Vγ4D vs. control, wound model with contraction, **Figure 1A**; wound model without contraction and **Figure 1C**) and re-epithelialization (wound model with contraction, **Figure 1B**; wound model without contraction and **Figure 1D**), while mice with Vγ1D treatment showed similar results to controls (**Figures 1A–D**), indicating that Vγ4, but not Vγ1 T cells, could delay wound healing. However, the addition of freshly isolated Vγ4 T cells onto the wound bed of *Tcrδ*$^{-/-}$ mice failed to affect skin wound repair of *Tcrδ*$^{-/-}$ animals (**Figure 1E**), suggesting that Vγ4 T cells indirectly affected wound healing.

Because DETCs play a crucial role in skin wound closure (7), we assessed the contribution of DETCs in delayed wound healing mediated by Vγ4 T cells. The wounds of *Tcrδ*$^{-/-}$ mice were supplemented with freshly isolated DETCs alone or DETCs with Vγ4 T cells. Supplementing DETCs onto the wound bed markedly improved wound repair in *Tcrδ*$^{-/-}$ mice (*Tcrδ*$^{-/-}$ + DETCs vs *Tcrδ*$^{-/-}$ control, **Figure 1F**), but the improvement was notably attenuated by the addition of Vγ4 T cells (**Figure 1F**). This finding indicated that DETCs are essential for Vγ4 T cell-delayed skin wound closure.

Vγ4 T Cells Inhibit IGF-1 Production in DETCs to Affect Skin Wound Healing

IGF-1 is exclusively secreted by DETCs in the epidermis and is important for the pro-healing function of DETCs (7). Mice with Vγ4D exhibited markedly enhanced IGF-1 production in the epidermis but not the dermis around wounds compared to control animals (WB, **Figure 2A**; IHC, **Figure 2B**). Furthermore, transferring DETCs onto wound beds notably improved IGF-1 production in the epidermis around wounds of *Tcrδ*$^{-/-}$ mice, but the enhancement was significantly attenuated when Vγ4 T cells were added (**Figure 2C**). Therefore, we considered that Vγ4 T cells inhibited IGF-1 expression in DETCs. Wound healing of WT mice was accelerated by rIGF-1, and wound healing of Vγ4D mice was also promoted when rIGF-1 (200 ng/wound) was supplemented onto wound beds (rIGF-1 + Vγ4D vs. control, **Figure 2D**). Anti-IGF-1 neutralizing Ab (20 μg/wound) markedly delayed wound healing not only in WT but also in Vγ4D mice (IGF-1 Ab + Vγ4D vs. control, **Figure 2E**). According to the results in **Figures 2D,E**, no differences between WT and Vγ4D mice were observed when intervening IGF-1 expression in the epidermis, suggesting that Vγ4 T cells may depend on IGF-1 to affect wound repair.

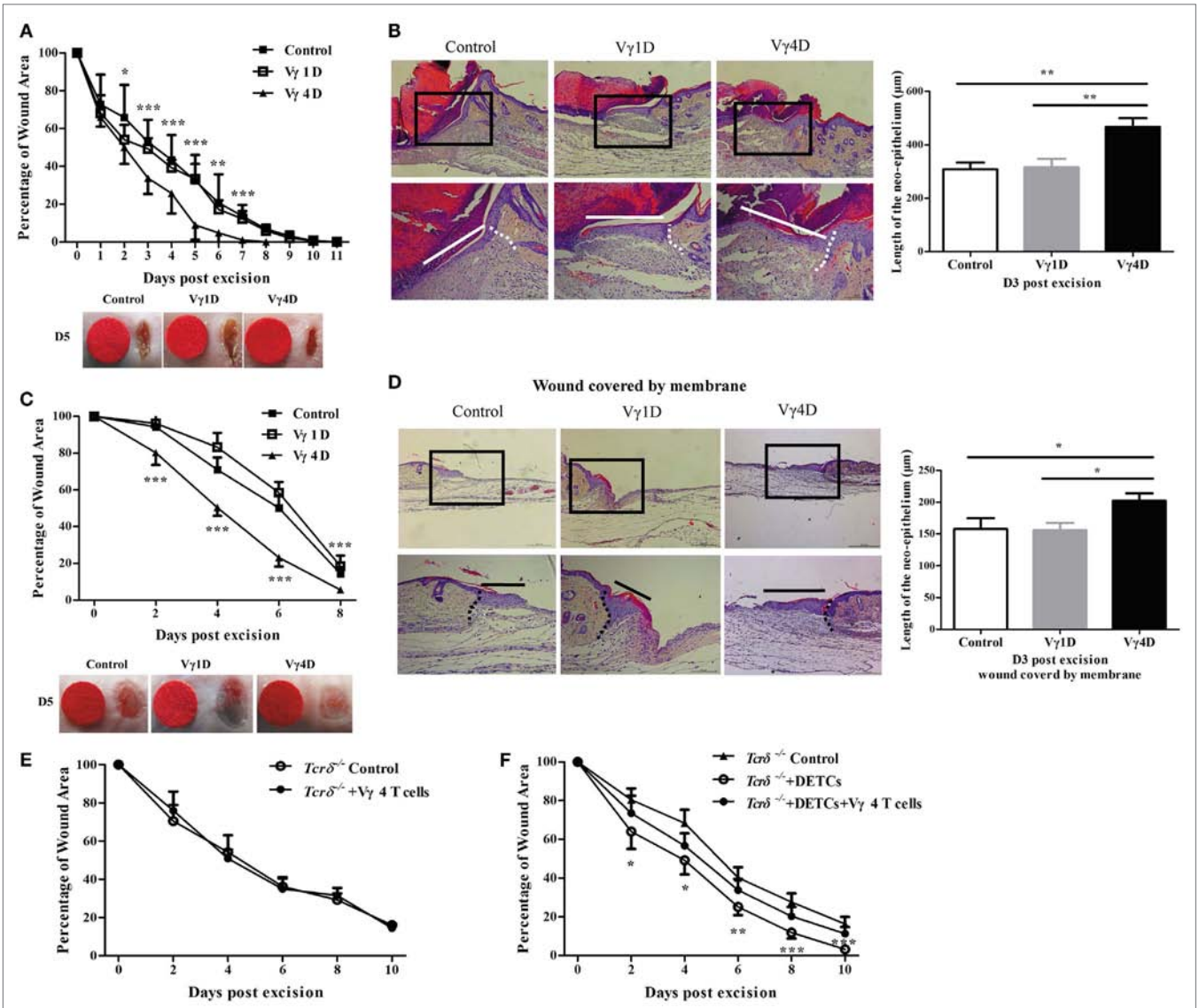

FIGURE 1 | Vγ4 T cells have a negative impact on skin wound healing. **(A–D)** Age- and sex-matched C57BL/6 wild-type mice were intraperitoneally injected with 200 μg/mouse Vγ1 T-cell depletion (Vγ1D) Ab 2.11, Vγ4 T-cell depletion (Vγ4D) Ab UC3, or isotype control Ab (Control) on day 3 before wound excision. Full-thickness wounds were then generated using a sterile 6 mm punch tool on day 0. Wound-closure kinetics was measured over time in wound model with contraction **(A)** or without contraction **(B)**. Stars mark the comparations between Vγ4D and Control in **(A,B)**. On day 3 post-wounding, re-epithelialization in wound model with contraction **(C)** or without contraction **(D)** was analyzed by HE (n = 5–7). **(E)** Fresh isolated Vγ4 T cells (1×10^4 cells/wound) or PBS were added onto wound bed of *Tcrδ*$^{-/-}$ mice immediately after wound excision. **(F)** Fresh isolated dendritic epidermal T cells (DETCs) (5×10^4 cells/wound), DETCs (5×10^4 cells/wound) plus Vγ4 T cells (1×10^4 cells/wound), or PBS was added onto wound bed of *Tcrδ*$^{-/-}$ mice immediately after skin injury. Wound-closure kinetics was measured over time, and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Stars mark the comparations between *Tcrδ*$^{-/-}$ mice + DETCs + Vγ4 T cells and *Tcrδ*$^{-/-}$ control. The values were calculated as the mean ± SD. All data represent at least three independent experiments. P value was calculated by Student's unpaired t-test **(A,C,E,F)** or one-way ANOVA with Bonferroni's multiple comparison test **(B,D)** (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Vγ4 T Cells Suppress IGF-1 Production but Not the Number and Activation of DETCs

Epidermal IGF-1 is primarily derived from DETCs (7), and Vγ4 T cells may decrease epidermal IGF-1 expression by decreasing number, impairing activation, and/or altering IGF-1 production by DETCs. We identified that Vγ4D could not affect the ratios (**Figure 3A**) and numbers (**Figure 3B**) of DETCs located around the wounds after skin injury. Furthermore, the expression of surface activation markers, such as CD25, CD44, CD62L, or CD69, on DETCs around wounds was comparable between Vγ4D and WT controls (**Figure 3C**). Interestingly, Vγ4D could influence the expression of activation receptors on DETCs. With the exception of JAML, TCR δ was slightly increased, and NKG2D was markedly enhanced on DETCs around the wounds (**Figure 3D**). However, IGF-1 production by DETCs at the wound margin was significantly increased

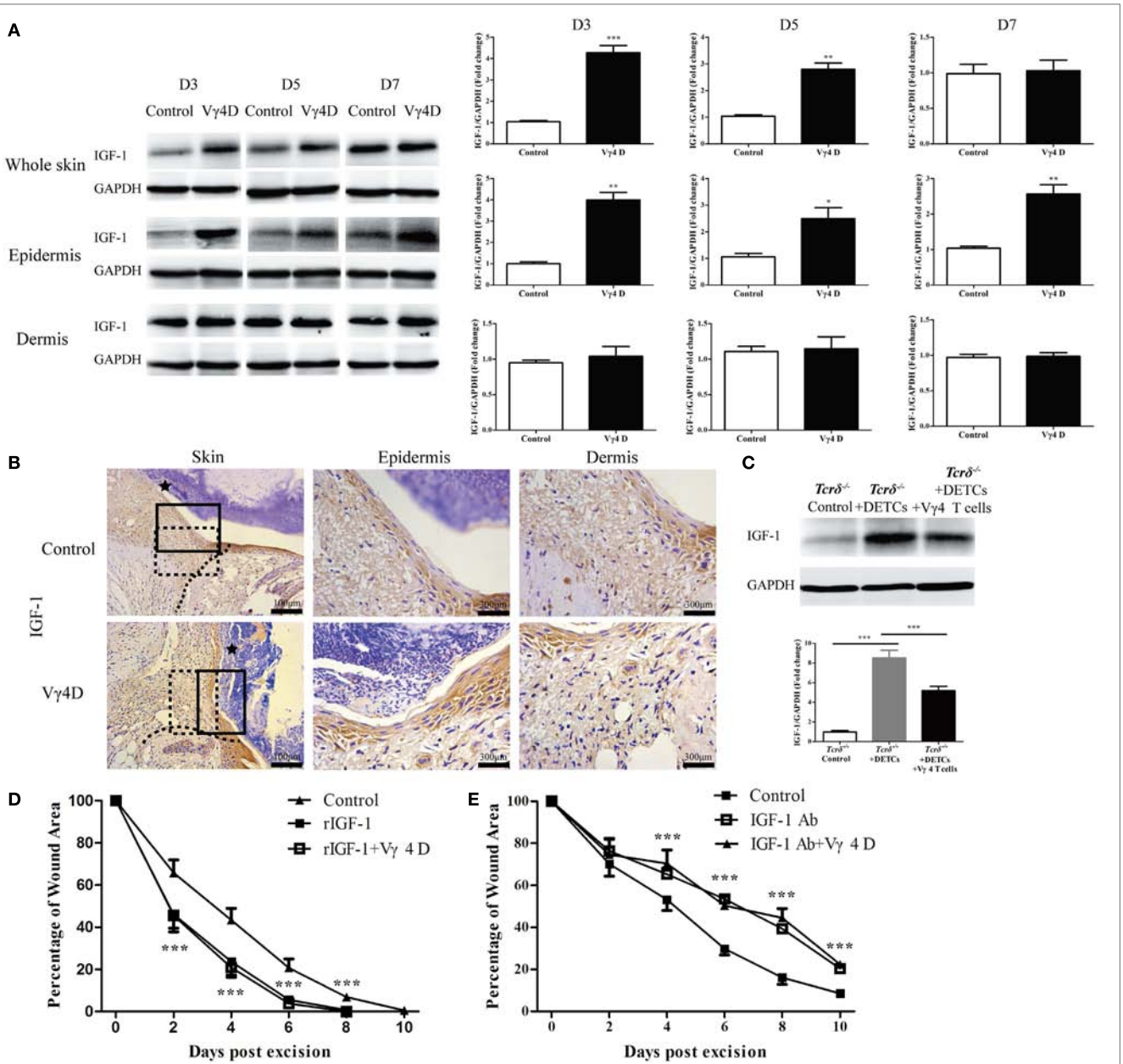

FIGURE 2 | Vγ4 T cells inhibited epidermal IGF-1 production to delay skin wound repair. **(A,B)** Full-thickness wounds were generated in Vγ4 T-cell depletion (Vγ4D) and control mice. On day 3, 5, and 7 post-wounding, each group of epidermis tissues at wound margins was collected from at least three mice for further analysis of IGF-1 production by means of WB **(A)** and IHC **(B)**. The area of wound and neo-epidermis is marked by black star (Skin). Neo-epidermis in solid rectangles and neo-dermis in dashed rectangles were amplified threefolds and labeled as Epidermis and Dermis **(B)**. **(C)** Fresh isolated dendritic epidermal T cells (DETCs) (5×10^4 cells/wound), DETCs (5×10^4 cells/wound) plus Vγ4 T cells (1×10^4 cells/wound), or PBS were added onto wound bed of *Tcrδ*$^{-/-}$ mice ($n = 5$/group) immediately after skin injury, respectively. IGF-1 expression in epidermal wound margin was detected by WB on day 3 post-wounding. **(D,E)** Age- and sex-matched C57BL/6 wild-type mice were intraperitoneally injected with Vγ4D Ab UC3, or isotype control Ab (Control) on day −3. Followed by administration of 200 ng/wound rIGF-1 **(D)** or 20 μg/wound IGF-1 neutralizing Ab **(E)** onto wound margin on days 0, 1, and 2 post excision. Control mice in **(E)** were treated with IGF neutralizing isotype Ab (poly goat IgG). Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds (2 wounds/mouse, 5 mice/group). Star-like markers represent the differences between rIGF-1 + Vγ4D and Control in **(D)**, IGF-1 Ab + Vγ4D and Control in **(E)**. Data are representative of at least three independent experiments with cohorts of the indicated number of mice per group. Bars show mean ± SD. *P* value was calculated by Student's unpaired *t*-test **(A,D,E)** or one-way ANOVA with Bonferroni's multiple comparison test **(C)** (***$P < 0.001$).

in Vγ4D mice compared with WT controls upon PMA plus ionomycin stimulation *ex vivo* (**Figure 3E**). Therefore, the underlying mechanisms of Vγ4 T cells inhibiting epidermal IGF-1 production were likely down-regulating IGF-1 production rather than impacting the number or activation of DETCs *in vivo*.

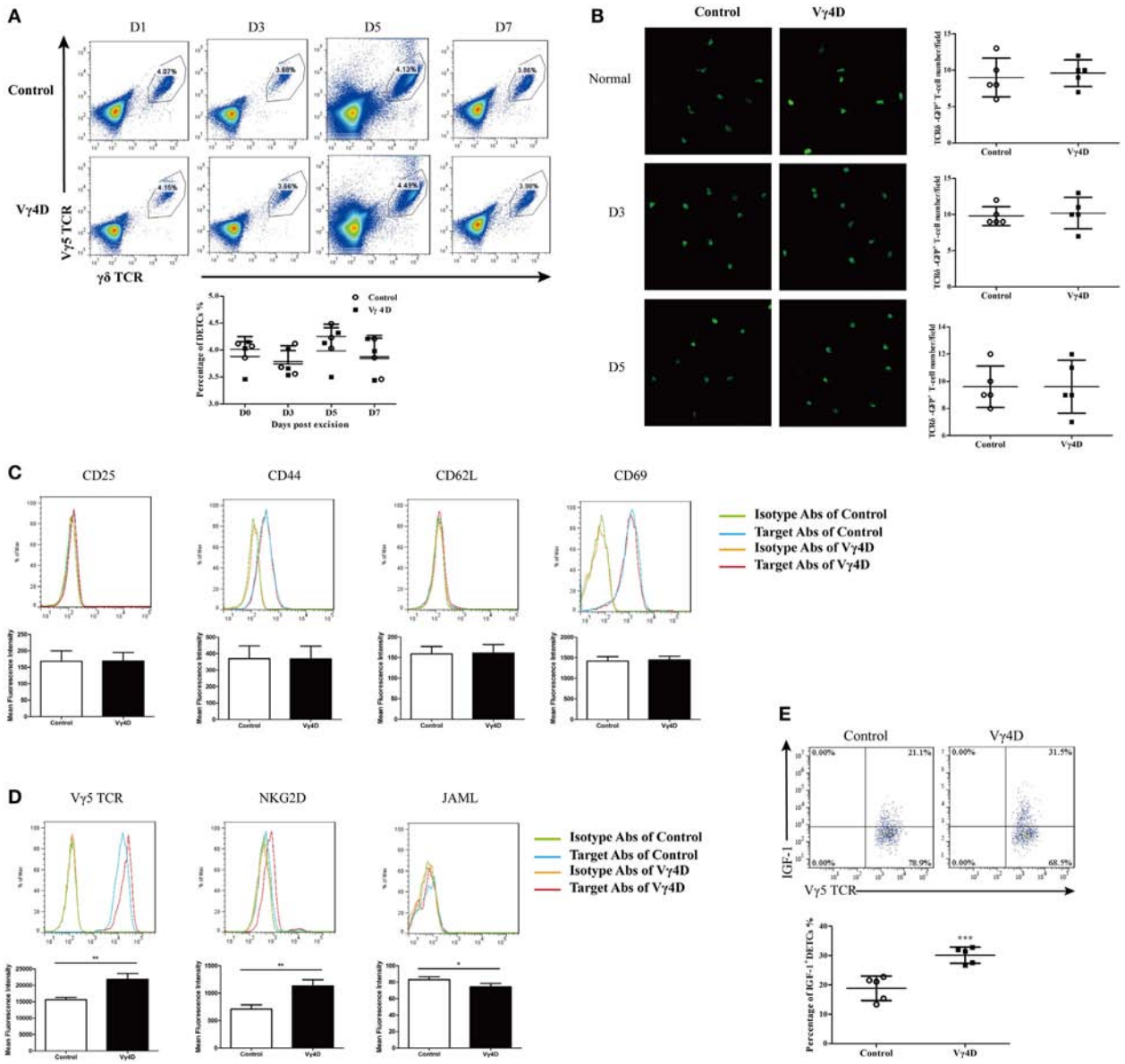

FIGURE 3 | IGF-1 production by dendritic epidermal T cells (DETCs) around wound area was enhanced in Vγ4 T cell depletion mice. **(A)** The percentages of Vγ5 T cells in epidermis at wound margin of Vγ4 T-cell depletion (Vγ4D) and control mice were detected by FACS on days 0, 3, 5, and 7 after wounding. Statistical histogram is showed on the right panel. **(B)** Age- and sex-matched TCRδ-GFP mice were intraperitoneally injected with Vγ4D Ab UC3 or isotype control Ab (Control) on day −3. Epidermal sheets were separated from skin at wound margin of Vγ4D and control TCRδ-GFP mice on days 0, 3, and 5 after wounding. The morphology (left panel) and numbers/field of view (right panel) of DETCs around the wounds in Vγ4D and wild-type (WT) control mice were analyzed by confocal microscopy. **(C–E)** Epidermal cells were isolated from skin at wound margin of Vγ4D and WT control mice on day 3 post wounding. Expressions of T cell activation markers (CD25, CD44, CD62L, and CD69) **(C)** and T cell activation receptors (Vγ5 TCR, NKG2D, and JAML) **(D)** on surface of DETCs were analyzed by FACS. **(E)** The fresh isolated epidermal cells were stimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) for 6 h in the presence of Brefeldin A (100 ng/ml). IGF-1 productions by DETCs were then analyzed by FACS. Error bars represent mean ± SD. Each group of epidermis tissue around wound was gathered from 3 to 6 mice (4 wounds/mouse). All data were representative of three individual experiments. *P* value was calculated using One-way ANOVA with Bonferroni's comparison test **(A)** or Student's unpaired *t*-test in **(B,E)** (***$P < 0.001$).

IL-17A Is Required for Vγ4 T Cells to Delay Wound Repair

Vγ4 T cells provide a significant early source of IFN-γ and IL-17A to regulate immune responses (12, 15). It was observed that production of IL-17A was significantly reduced, whereas IFN-γ production was equally distributed in the skin around wounds of Vγ4D mice compared to controls (**Figure 4A**). Relative to the results, IL-17-positive Vγ4 T cells occupied nearly half of the total Vγ4 T cells in the epidermis around wounds, while IFN-γ-positive Vγ4 T cells were less than 5% (**Figure 4B**). Moreover, Vγ4D was

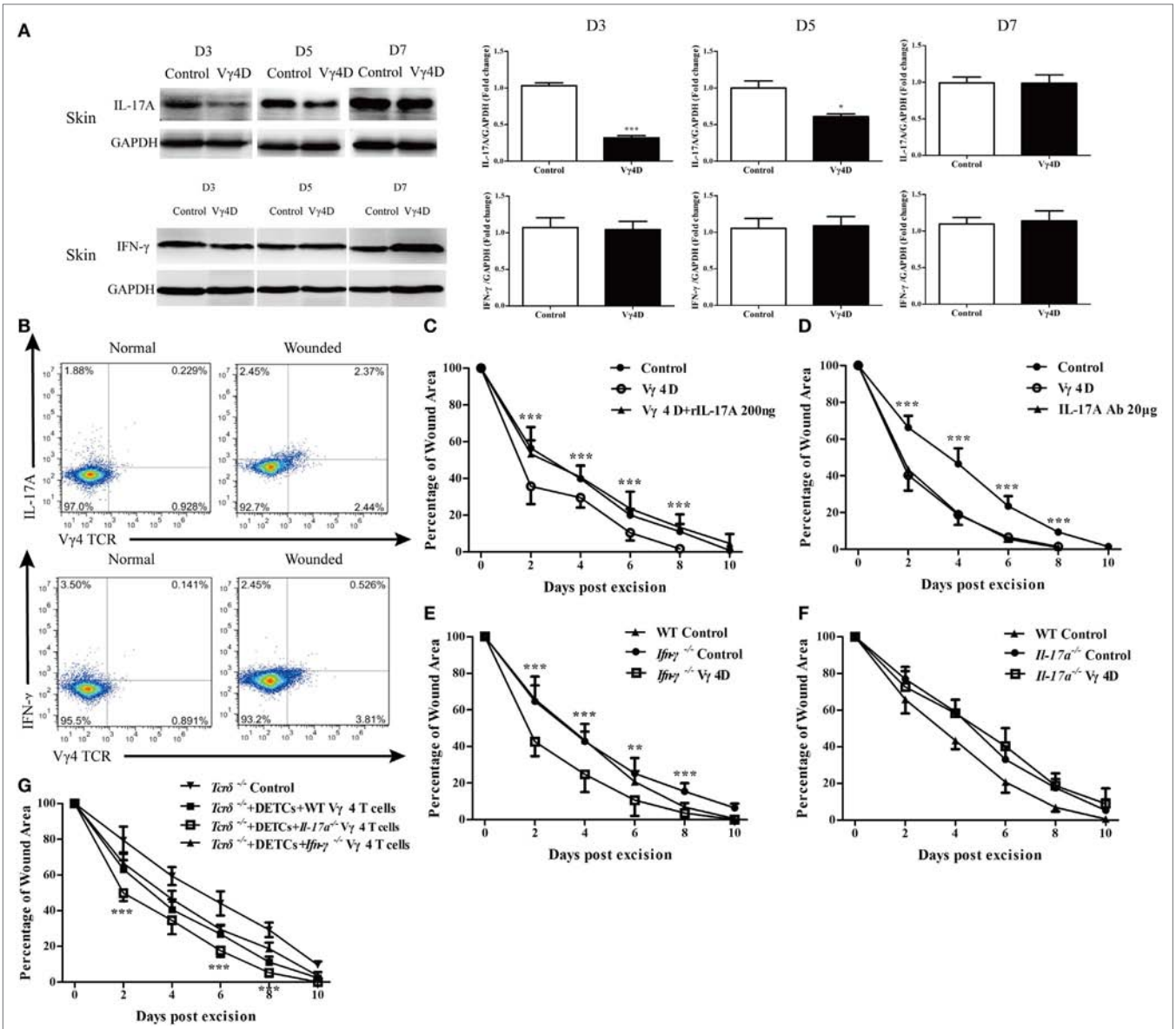

FIGURE 4 | IL-17A was required for Vγ4 T cell-mediated delay of skin wound healing. **(A)** The expressions of IL-17A and IFN-γ in skin around wound of Vγ4 T-cell depletion (Vγ4D) and wild-type (WT) control mice were analyzed by WB on days 3, 5, and 7 after skin injury (n = 3–5/group). **(B)** The IL-17A and IFN-γ production by Vγ4 T cells around wound of Vγ4D and WT control mice were analyzed by FACS on day 3 post excision (4 wounds/mouse, 3–5 mice/group). **(C,D)** Age- and sex-matched C57BL/6 WT mice were intraperitoneally injected with Vγ4D Ab UC3, or isotype control Ab (Control) on day −3. Followed by administration of 200 ng/wound rIL-17A into wound margin of Vγ4D mice **(C)** or 50 μg/wound IL-17A neutralizing Ab into wound margin of WT mice **(D)** on days 0, 1, and 2 post excision. Control mice in **(D)** were treated with IL-17A-neutralizing isotype Ab (mouse IgG1). Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds (2 wounds/mouse, 5 mice/group). Stars represent the comparations between Vγ4D and Vγ4D + rIL-17A in **(C)**, IL-17A Ab and Control in **(D)**. **(E,F)** Age- and sex-matched *Ifn-γ*$^{-/-}$ **(E)** and *Il-17a*$^{-/-}$ **(F)** mice were intraperitoneally injected with Vγ4D Ab UC3, or isotype control Ab on day −3. Full-thickness wounds were then generated on day 0. Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds (2 wounds/mouse, 5 mice/group). WT mice were treated with isotype Ab as WT control. Stars represent the differences between *Ifn-γ*$^{-/-}$ Vγ4D and *Ifn-γ*$^{-/-}$ Control in **(E)**. **(G)** Fresh isolated dendritic epidermal T cells (DETCs) (5 × 10^4 cells/wound) plus WT Vγ4 T cells (1 × 10^4 cells/wound), DETCs (5 × 10^4 cells/wound) plus *Il-17a*$^{-/-}$ Vγ4 T cells (1 × 10^4 cells/wound), DETCs (5 × 10^4 cells/wound) plus *Ifn-γ*$^{-/-}$ Vγ4 T cells (1 × 10^4 cells/wound), or PBS was added onto wound bed of *Tcrδ*$^{-/-}$ mice immediately after skin injury. Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Stars represent the comparations between *Tcrδ*$^{-/-}$ + DETCs + *Il-17a*$^{-/-}$ Vγ4 T cells and *Tcrδ*$^{-/-}$ + DETCs + Vγ4 T cells in **(G)**. Bars represent mean ± SD. All data were representative of three independent experiments. P value was calculated by Student's unpaired t-test (*P < 0.05, **P < 0.01, ***P < 0.001).

shown to accelerate wound repair in **Figure 1**, but the application of rIL-17A (200 ng/wound) on wound beds with Vγ4D nearly eliminated the improvement of wound healing mediated by Vγ4D (Vγ4D + rIL-17A vs Vγ4D, **Figure 4C**). The administration of IL-17A neutralizing Ab (20 μg/wound) on wound beds significantly improved wound healing in WT mice (IL-17A Ab vs. control,

Figure 4D), which was similar to the kinetic curve of Vγ4D animals (**Figure 4D**). In addition, Vγ4D in *Ifn-γ*$^{-/-}$ mice markedly accelerated skin wound closure compared to *Ifn-γ*$^{-/-}$ mice with isotype controls (*Ifn-γ*$^{-/-}$ Vγ4D vs *Ifn-γ*$^{-/-}$ control, **Figure 4E**). However, the wound-healing kinetics was analogous between *Il-17a*$^{-/-}$ mice with Vγ4D and *Il-17a*$^{-/-}$ mice with isotype control (**Figure 4F**). Compared to *Tcrδ*$^{-/-}$ controls, the addition of WT DETCs together with *Il-17a*$^{-/-}$ Vγ4 T cells onto wound beds facilitated wound healing compared to WT Vγ4 T cells (*Tcrδ*$^{-/-}$ + DETCs + *Il-17a*$^{-/-}$ Vγ4 T cells vs. *Tcrδ*$^{-/-}$ + DETCs + WT Vγ4 T cells, **Figure 4G**), while *Ifn-γ*$^{-/-}$ Vγ4 T cells did not contribute to wound repair compared to WT Vγ4 T cells (**Figure 4G**). These findings demonstrated that IL-17A, not IFN-γ, was responsible for Vγ4 T cell-delayed wound closure.

Vγ4 T Cells Provide an Early and Significant Source of Epidermal IL-17A and Infiltrate into the Epidermis Depending on the CCR6-CCL20 Pathway after Skin Injury

As previously mentioned, Vγ4 T cells suppressed wound healing by downregulating IGF-1 production in DETCs. In the skin, Vγ4 T cells and DETCs are physiologically distributed in the dermis and epidermis, respectively. It is reasonable to assume that Vγ4 T cells infiltrated into the epidermis after wounding and thus directly interacted with DETCs. Indeed, the percentage of Vγ4 T cells was significantly increased in the epidermis (**Figure 5A**), decreased in the dermis, and

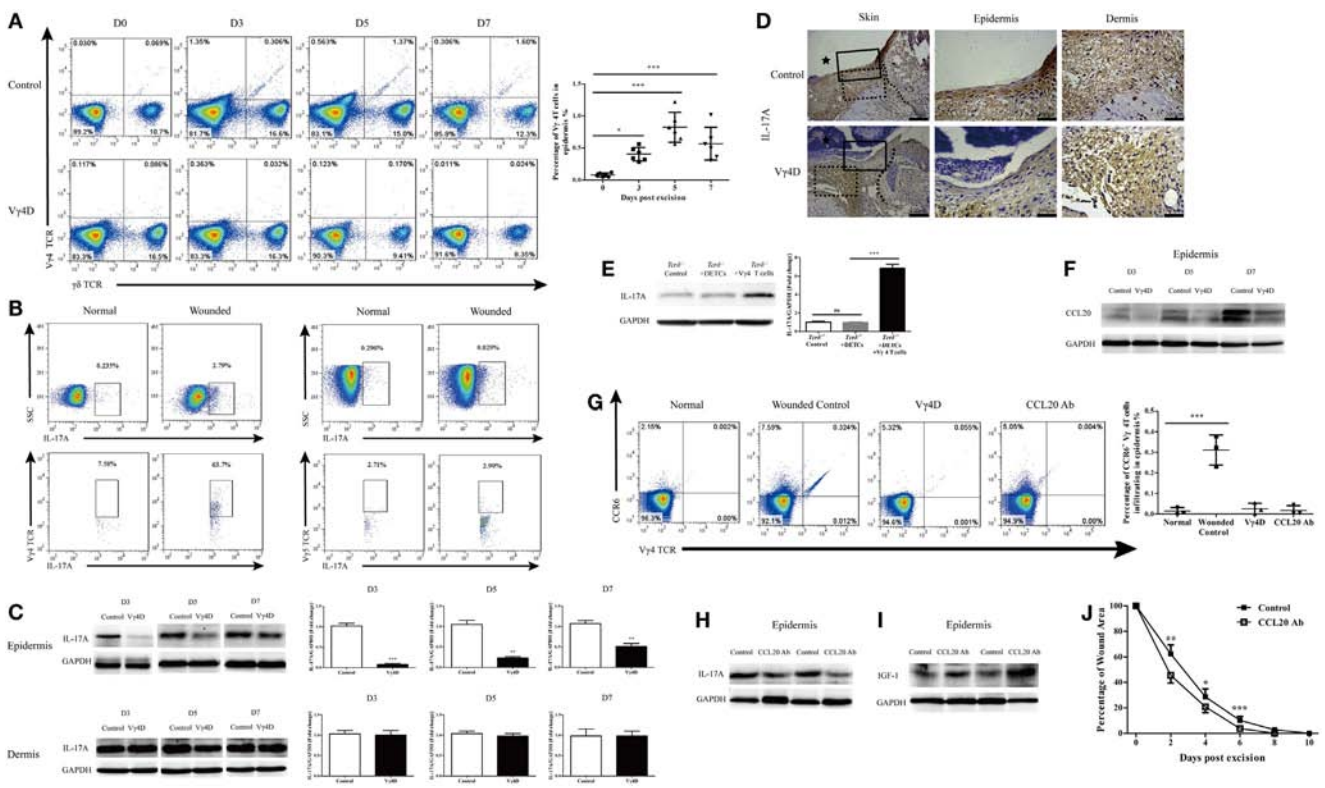

FIGURE 5 | Vγ4 T cells *via* CCR6-CCL20 pathway infiltrated into epidermis and provided major early source of epidermal IL-17A after wounding. **(A)** Vγ4 T cells in epidermis around wound of Vγ4 T-cell depletion (Vγ4D) and wild-type (WT) mice were analyzed by FACS on days 0, 3, 5, and 7 after skin injury (4 wounds/mice, 5–7 mice/group). **(B)** Epidermis infiltrating IL-17A-positive cells around wound of WT mice (4 wounds/mice, 3–5 mice/group) were analyzed by FACS on days 0 and 3 after skin injury (upper panel). Gated on IL-17A-positive cells, the percentage of Vγ4 T cells and dendritic epidermal T cells (DETCs; anti-Vγ5 TCR) are shown (lower panel). **(C)** The expression of IL-17A in epidermis around wound of Vγ4D and WT mice was analyzed by WB on days 3, 5, and 7 after skin injury (n = 3–5/group). **(D)** The expression of IL-17A in epidermis around wound of Vγ4D and WT mice was analyzed by IHC on day 3 post-excision (n = 3–5/group). The area of wound and neo-epidermis is marked by black star (Skin, left panel). Neo-epidermis in solid rectangles and neo-dermis in dashed rectangles are shown on the middle (Epidermis) and right (Dermis) panel. **(E)** Fresh isolated WT DETCs (5×10^4 cells/wound), Vγ4 T cells (1×10^4 cells/wound), or PBS was added onto wound bed of *Tcrδ*$^{-/-}$ mice immediately after skin injury (n = 3/group). On day 3 post-excision, the production of IL-17A in epidermis around wound was determined by WB. **(F)** The expressions of CCL20 in epidermis around wound of Vγ4D and WT control mice were detected by WB on days 3, 5, and 7 after wounding (n = 3/group). **(G)** Addition of CCL20 neutralizing Ab (10 μg/per wound) onto wound bed of Vγ4D and WT control animals at day 0, 1, and 2 after skin injury. On day 3 post-excision, the expressions of CCR6 on surface of Vγ4 T cells in epidermis around wound of animals with Vγ4D, CCL20 neutralizing, or control isotype Abs treatment were detected by means of FACS (4 wounds/group, 3 mice/group). **(H–J)** Addition of CCL20 neutralizing Ab (10 μg/wound) or isotype Ab (rat IgG1) into wound margin on days 0, 1, and 2 after skin injury. The production of IL-17A **(H)** and IGF-1 **(I)** in epidermis around wound was analyzed by WB on day 3 after wounding (n = 3–5/group). **(J)** Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Bars represent mean ± SD. All data were representative of three independent experiments. P value was determined by one-way ANOVA with Bonferroni's comparision test **(A,E,G)** or Student's unpaired t-test **(C,J)** (*P < 0.05, **P < 0.01, ***P < 0.001).

unchanged in draining lymph nodes after wounding (Figure S1 in Supplementary Material). Additionally, more than 50% of the infiltrating IL-17A-positive cells in the epidermis were Vγ4 T cells, and less than 5% of those were DETCs on day 3 after wounding (**Figure 5B**). Moreover, Vγ4D resulted in dramatically reduced IL-17A production in the epidermis rather than the dermis (WB, **Figure 5C**; IHC, **Figure 5D**). Supplementing with freshly isolated Vγ4 T cells rather than DETCs markedly enhanced epidermal IL-17A production around wounds in *Tcrδ*$^{-/-}$ mice (**Figure 5E**). Therefore, we concluded that Vγ4 T cells infiltrated the epidermis around wounds to provide an early, major source of epidermal IL-17A after skin injury.

The CCR6-CCL20 pathway is critical for Th17 to infiltrate into the inflamed area (23). The production of epidermal CCL20 was markedly enhanced after skin injury (**Figure 5F**), and nearly all of the Vγ4 T cells that infiltrated the epidermis were CCR6-positive (**Figure 5G**). Moreover, blocking CCL20 with neutralizing Ab dramatically declined the ratio of infiltrating Vγ4 T cells (**Figure 5G**) and weakened epidermal IL-17A production (**Figure 5H**) but enhanced epidermal IGF-1 production (**Figure 5I**), followed by significantly accelerated skin wound repair (**Figure 5J**). These results demonstrated that Vγ4 cells that infiltrated into the epidermis rely on the CCL20-CCR6 pathway after skin injury.

IL-17A Downregulated Epidermal IGF-1 Production at the Wound Margin

IL-17A has been demonstrated to promote keratinocyte proliferation for efficient skin wound repair (4). Consistent with previous reports, we showed that both IL-17A deficiency (Figure S2 in Supplementary Material) and blocking IL-17A with a high dose of neutralizing Ab (200 μg/wound) in wound margins leads to defective skin wound closure (IL-17A Ab 200 μg vs. control, **Figure 6A**). Interestingly, the addition of a moderate dose of anti-IL-17A neutralizing Ab (20 μg/wound) significantly improved skin wound repair (IL-17A Ab 20 μg vs. control, **Figure 6A**). Moreover, the administration of a high dose of rIL-17A (200 ng/wound) rather than low or medium dose (2 or 20 ng/wound) onto wound beds significantly retarded skin wound closure (rIL-17A 200 ng vs. control, **Figure 6B**). Thus, these findings indicated that the excessive IL-17A has a negative impact on skin wound repair, although IL-17A is essential for efficient wound healing.

Since IGF-1 is also required for efficient skin wound repair, we further investigated whether IL-17A affected IGF-1 production in the epidermis after wounding. As expected, *Il-17a*$^{-/-}$ mice, exhibited a defective skin wound repair and showed a markedly enhanced IGF-1 production in the epidermis around wounds compared to WT controls (**Figure 6C**). The addition of various doses of anti-IL-17A neutralizing Ab onto wound beds notably increased epidermal IGF-1 (**Figure 6D**) and IGF-1 production in DETCs (**Figure 6E**) at wound areas of WT mice in a dose-dependent manner. Moreover, supplementing various doses of rIL-17A onto wound beds markedly decreased IGF-1 production in the epidermis (**Figure 6F**) and IGF-1 production in DETCs (**Figure 6G**) around wounds of WT mice in a dose-dependent manner. These results demonstrated that IL-17A was able to suppress the production of epidermal IGF-1 in DETCs after skin injury.

IL-1β and IL-23 Directly Inhibit IGF-1 Production in DETCs to Delay Wound Healing

To investigate the direct effect of IL-17A on IGF-1 production in DETCs, we isolated, purified, and expanded DETCs *in vitro* (eDETCs) and co-cultured them with rIL-17A. The results showed that rIL-17A failed to inhibit the production of IGF-1 in eDETCs (**Figure 7A**). Although we observed that IL-17A could inhibit the pro-healing function of DETCs *in vivo*, DETCs did not directly respond to IL-17A. However, in the co-culture system of eDETCs and primary keratinocytes isolated from newborn C57 mice, rIL-17A notably suppressed IGF-1 production in eDETCs (**Figure 7B**), suggesting that IL-17A retarded IGF-1 production in DETCs *via* epidermal cells.

The IL-1β/23-IL-17A axis plays a crucial role in inflammation (9, 19–22). It has been shown that the production of IL-17A by DETCs could be markedly enhanced in response to IL-1β and IL-23 upon TCR stimulation (4, 22). We investigated whether IL-1β and IL-23 could directly affect IGF-1 production in DETCs. The results showed that rIL-1β and rIL-23 markedly inhibited IGF-1 production by eDETCs *in vitro* (WB, **Figure 7C**; FACS, **Figure 7D**). Furthermore, application of rIL-1β and rIL-23 onto wound beds of WT mice markedly decreased IGF-1 production in the epidermis (**Figure 7E**) and DETCs (**Figure 7F**) around wounds and subsequently delayed skin wound repair (**Figure 8D**). The supplement of IL-1β/IL-23 neutralizing antibodies onto wound beds of WT mice notably enhanced IGF-1 production in the epidermis (**Figure 7G**) and DETCs (**Figure 7H**) around wounds, followed by improved skin wound closure (**Figure 8G**). These results suggest that IL-1β and IL-23 could directly affect IGF-1 production in DETCs.

IL-1β and IL-23 Are Intermediaries between IL-17A and IGF-1 in the Epidermis around Wounds

In the co-culture system of eDETCs and primary keratinocytes, rIL-1β and rIL-23 reduced IGF-1 expression by eDETCs *in vitro* (**Figure 8A**). rIL-17 also inhibited the production of IGF-1 by eDETCS in the co-culture system (**Figure 8A**), while blocking IL-17A together with the application of rIL-1β and rIL-23 failed to rescue the reduction of IGF-1 in eDETCs *in vitro* (**Figure 8A**). However, blocking IL-1β and IL-23 with neutralizing antibodies together with the addition of rIL-17A almost eliminated the reduction of IGF-1 in eDETCs mediated by IL-17A *in vitro* (**Figure 8A**). *In vivo*, the production of IGF-1 in eDETCs was enhanced by blocking IL-17A (20 μg/wound) on wound beds of WT mice, while the expression of IGF-1 was decreased when adding rIL-1β plus rIL-23. Blocking IL-17A could not rescue the declined production of IGF-1 (**Figure 8B**). The epidermal expression of IGF-1 around

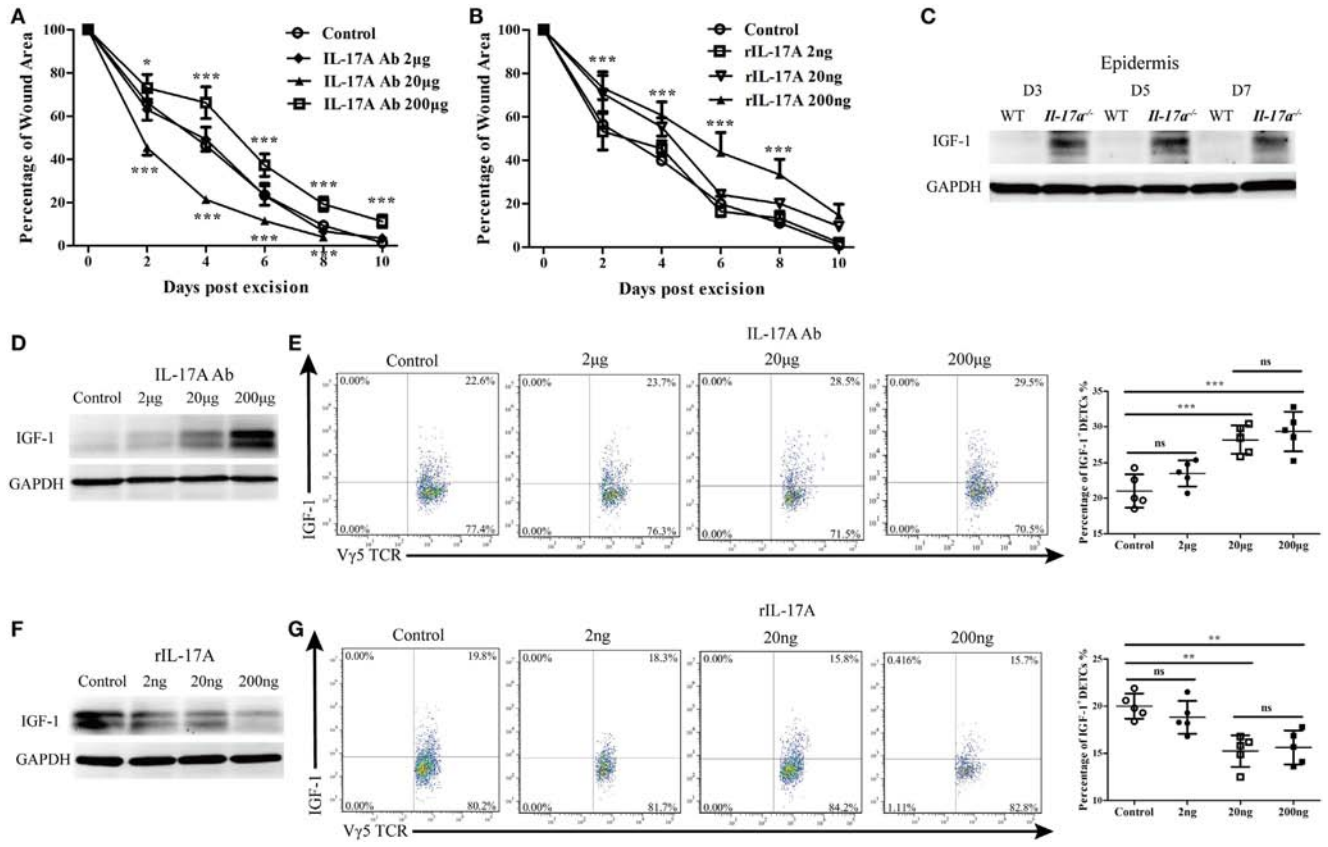

FIGURE 6 | IL-17A inhibited the production of IGF-1 in epidermis around wound after skin injury. **(A)** Addition of various doses of IL-17A neutralizing Ab (0, 2, 20, and 200 μg/wound) into wound margin of WT mice on days 0, 1, and 2 after skin injury. Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Upper stars stand for the comparations between IL-17A Ab 200 μg and Control, and lower stars represent the comparations between IL-17A Ab 20 μg and Control. **(B)** Wound closure kinetics in WT mice treated with various doses of rIL-17A (0, 2, 20, and 200 ng/wound) on days 0, 1, and 2 after skin injury (n = 10 wounds/group). Stars stand for the comparations between rIL-17A 200 ng and Control. **(C)** The production of IGF-1 in epidermis around wound of WT and *IL-17a*$^{-/-}$ mice on days 3, 5, and 7 post-excision was examined by WB. **(D,E)** Addition of various doses of IL-17A neutralizing Ab (0, 2, 20 and 200 μg/wound) into wound margin of WT mice on days 0, 1, and 2 after skin injury. Epidermis around wound was collected on day 3 post-excision. IGF-1 expression was determined by WB **(D)** and FACS **(E)**. **(F,G)** Addition of various doses of rIL-17A (0, 2, 20, and 200 ng/wound) into wound margin of WT mice on days 0, 1, and 2 after skin injury. Epidermis around wound was collected on day 3 post-excision. The production of IGF-1 was detected by WB **(F)** and FACS **(G)**. Epidermal sheets around wounds were collected from 3–7 mice with 4 wounds/mice in **(C–G)**. Data are representative of three individual experiments. Bars are shown mean ± SD. P value was determined by one-way ANOVA with Student's unpaired t-test **(A,B)** or Bonferroni's comparision test **(E,G)** (*P < 0.05, **P < 0.01, ***P < 0.001).

wounds was assessed by western blot and showed a similar result to FACS (**Figure 8C**). Wound repair was significantly promoted by blocking IL-17A alone compared to the application of rIL-1β and rIL-23 together with blocking of IL-17A (IL-17A Ab vs. IL-17A Ab + rIL-1β/23, **Figure 8D**). Supplying rIL-17A to wound margins could weaken IGF-1 production in eDETCs (**Figure 8E**) and the epidermis around wounds (**Figure 8F**), whereas the addition of rIL-17 with IL-1β, and IL-23 neutralization failed to display this effect (**Figures 8E,F**). Compared to the addition of rIL-17 alone, rIL-17 supplied with IL-1β combined with IL-23 neutralization completely reversed wound healing kinetics to control levels (rIL-17A vs. rIL-17A + IL-1β/IL-23 Abs, **Figure 8G**). These findings indicated that IL-1β and IL-23 acted as intermediaries between IL-17A and IGF-1 to impact wound closure.

IL-17A and IL-1β/IL-23 Form a Positive Feedback in the Epidermis around Wounds

After showing that IL-1β and IL-23 were required for IL-17A to inhibit IGF-1 production in DETCs, we wanted to determine the precise role of IL-17A in epidermal IL-1β/IL-23 production *in vivo* and *in vitro*. The *in vivo* production of both IL-1β and IL-23 in the epidermis around wounds was markedly reduced in *Il-17a*$^{-/-}$ mice compared to WT controls (**Figure 9A**). IL-17A neutralization attenuated IL-1β and IL-23 production in the epidermis, as increasing doses of IL-17 neutralization led to the reduced expression of IL-1β and IL-23 (**Figure 9B**). Moreover, the administration of rIL-17A enhanced the production of epidermal IL-1β and IL-23 in a dose-dependent manner (**Figure 9C**). rIL-17A notably enhanced the *in vitro* production of IL-1β and IL-23

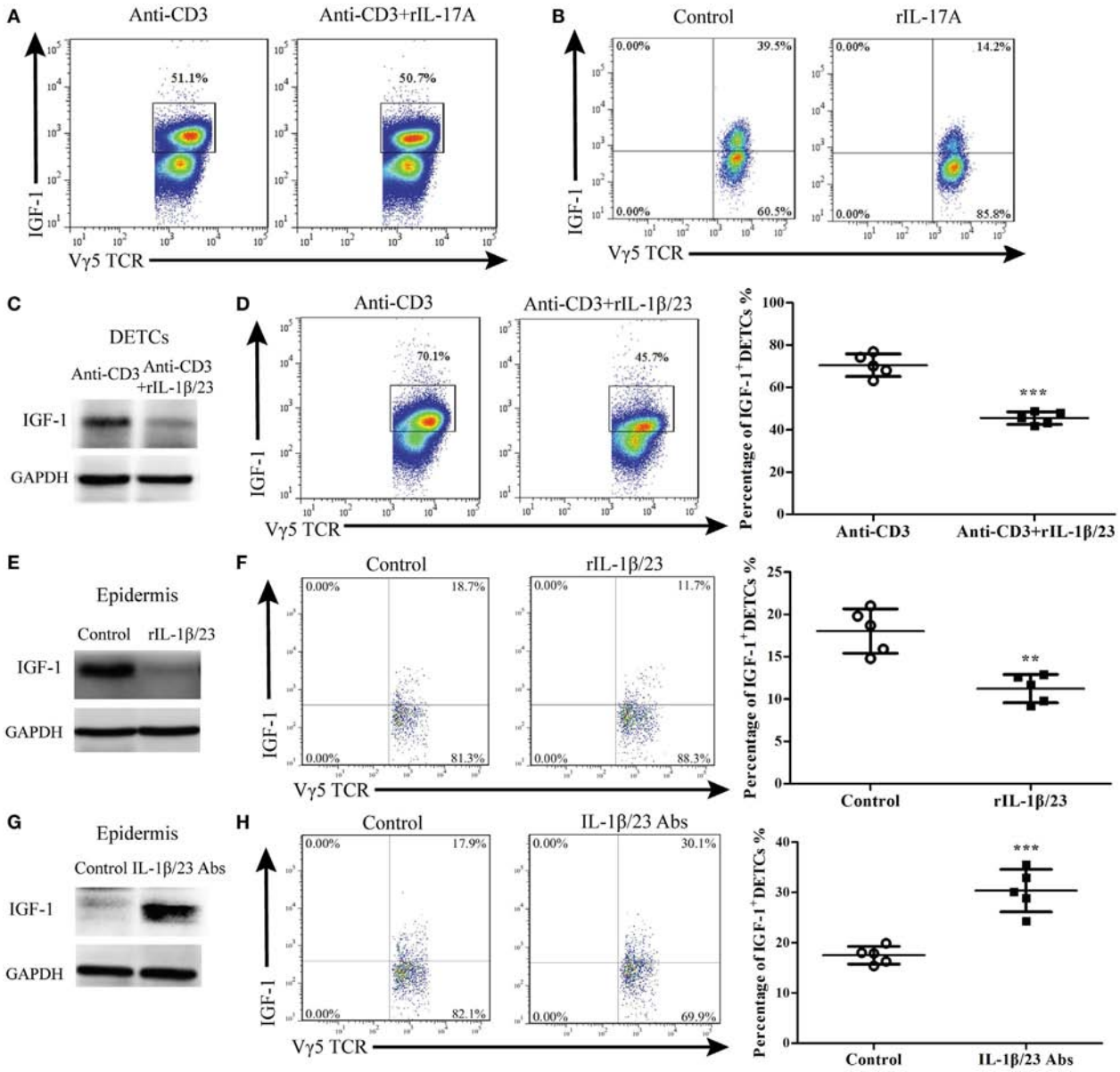

FIGURE 7 | IL-1β and IL-23 directly inhibited IGF production by dendritic epidermal T cells (DETCs). **(A)** DETCs were isolated from wild-type (WT) mice and expanded with Con A stimulation for 4 weeks. The expanded DETCs (eDETCs) (purity > 95%) were rested without Con A for 2 weeks before further analysis. eDETCs were stimulated for 6 h with anti-CD3ε (5 μg/ml) either alone or combined with rIL-17 (100 ng/ml) in the presence of brefeldin A (BFA) (100 ng/ml). IGF-1 productions by eDETCs were analyzed by FACS. **(B)** eDETCs were co-cultured with keratinocytes (1:1, 1×10^6/ml) and stimulated by rIL-17 in presence of anti-CD3ε for 6 h. IGF-1 expression in eDETCs was detected by FACS. **(C,D)** eDETCs were stimulated with anti-CD3ε either alone (Anti-CD3) or combined with rIL-1β (100 ng/ml) plus rIL-23 (100 ng/ml) (anti-CD3 + rIL-1β/23) in the presence of BFA for 6 h. The expression of IGF-1 in eDETCs was detected by WB **(C)** and FACS **(D)**. **(E,F)** Age- and sex-matched WT mice were treated with rIL-1β (20 ng/wound) plus rIL-23 (20 ng/wound) on days 0, 1, and 2 after wounding. Epidermis around wound was collected from these animals on day 3 post-excision. The productions of IGF-1 in epidermis around wound were detected by WB **(E)** and FACS **(F)**. **(G,H)** Age- and sex-matched WT mice were treated with IL-1β neutralizing Ab (20 μg/wound) plus IL-23 neutralizing Ab (20 μg/wound) on days 0, 1, and 2 after wounding. Animals with IL-1β isotype Ab (armenian hamster IgG) plus IL-23 isotype Ab (rat IgG2a) treatment were used as control. Epidermis around wound was collected from these animals on day 3 post-excision. IGF expression was determined by WB **(G)** and FACS **(H)**. Epidermal sheets around wounds were gained from 3 to 5 mice with 4 wounds/mice in **(E–H)**. Data represent three individual experiments. Bars are shown mean ± SD. *P* value was assessed by Student's unpaired *t*-test **(D,F,H)** (**$p < 0.01$, ***$p < 0.001$).

by primary keratinocytes at both the mRNA (**Figure 9D**) and protein (**Figure 9E**) levels, suggesting that IL-17A is an important factor to enhance epidermal IL1/23 production in the epidermis around wounds after skin injury.

Furthermore, we investigated the effect of Vγ4 T cells on epidermal IL-1β and IL-23 production. Vγ4D markedly reduced IL-1β and IL-23 production in the epidermis rather than the dermis around wounds in WT mice (WB, **Figure 9F**; IHC, **Figure 9G**).

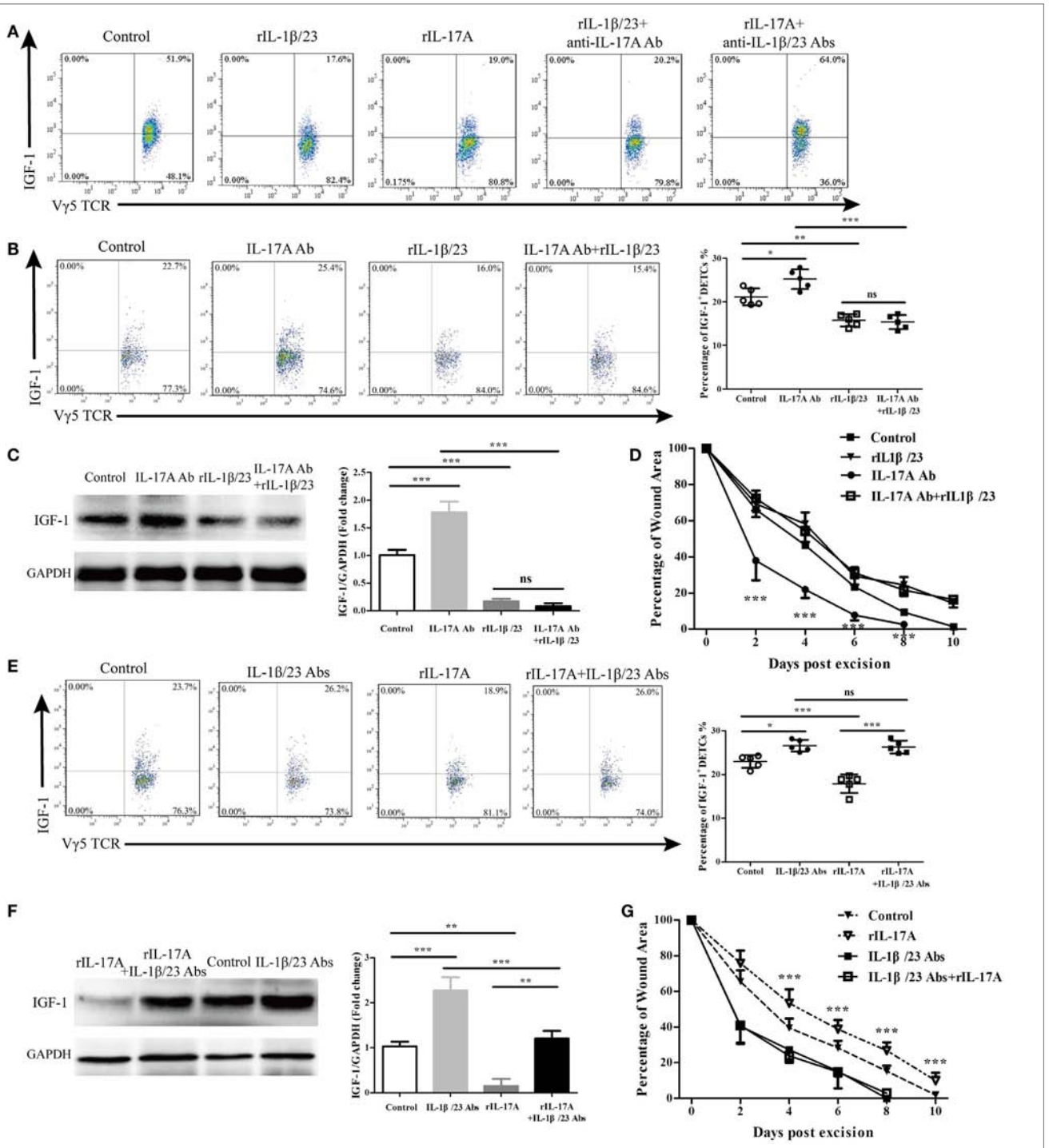

FIGURE 8 | IL-17A *via* IL-1β and IL-23 reduced IGF-1 production by dendritic epidermal T cells (DETCs). **(A)** eDETCs co-cultured with keratinocytes (1:1, 1 × 10^6/ml) in presence of rIL-1β/rIL-23 (100 ng/ml), rIL-17A (100 ng/ml), rIL-1β/rIL-23 plus anti-IL-17A Ab (10 μg/ml), or rIL-17A plus anti-IL-1β/anti-IL-23 Abs (10 μg/ml) for 6 h. IGF-1 production by eDETCs was detected by FACS. **(B–D)** Age- and sex-matched wild-type (WT) mice were treated with IL-17A neutralizing Ab (20 μg/wound), rIL-1β/rIL-23 (20 ng/wound), or IL-17A neutralizing Ab plus rIL-1β/rIL-23 (20 ng/wound) for continuously 3 days after wounding. IGF-1 expression in DETCs **(B)** and epidermal sheet **(C)** around wound was detected by FACS and WB, respectively, on day 3 post-excision (4 wounds/mice, 3 mice/group). **(D)** Wound closure kinetics over time was shown (n = 10 wounds/group). Stars mark the comparations between IL-17A Ab and IL-17A Ab + rIL-1β/rIL-23. **(E–G)** Age- and sex-matched WT mice were treated with IL-1β/IL-23 neutralizing Abs (20 μg/wound), rIL-17A (20 ng/wound), rIL-17A plus IL-1β/IL-23 neutralizing Abs for continuously 3 days after wounding. IGF-1 expression in DETCs **(E)** and epidermis **(F)** around wound was analyzed by FACS and WB on day 3 post-excision (4 wounds/mice, 3 mice/group). **(G)** Wound closure kinetics over time was shown (n = 10 wounds/group). Stars represent the differences between rIL-17A and rIL-17A + IL-1β/IL-23 Abs. Data represent three individual experiments. Bars are shown mean ± SD. P value was assessed by One-way ANOVA with Bonferroni's comparison test (*P < 0.05, **P < 0.01, ***P < 0.001).

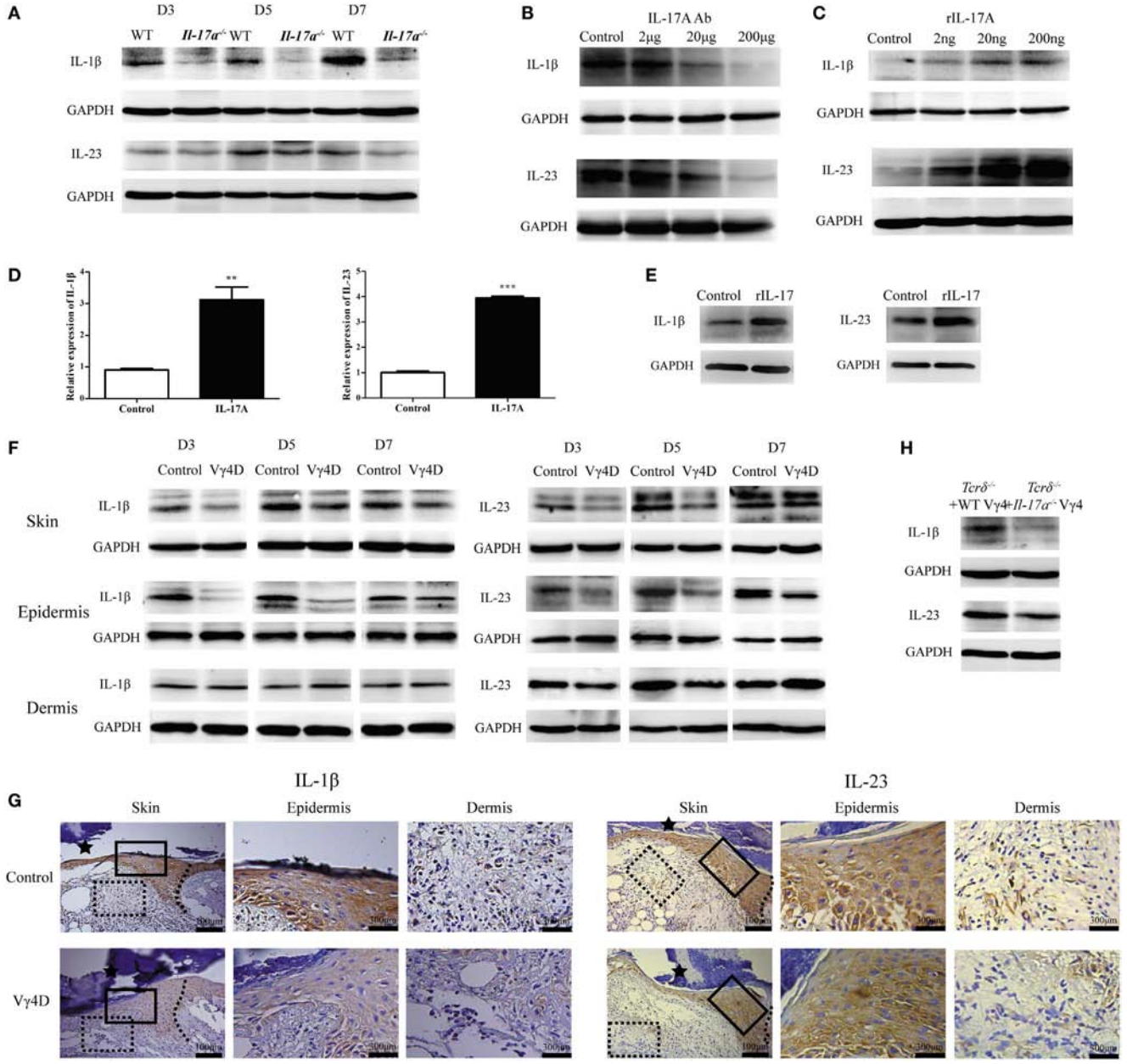

FIGURE 9 | Vγ4 T cell-derived IL-17A enhanced the production of IL-1β and IL-23 in epidermis around wound. **(A)** The expression of epidermal IL-1β and IL-23 around wound of wild-type (WT) and *Il-17a*$^{-/-}$ mice was analyzed by WB on days 3, 5, and 7 post-excision (n = 3/group). **(B)** Age- and sex-matched WT mice received various doses of IL-17A neutralizing Ab treatment (doses of 0, 2, 20, and 200 μg/wound) in the wound margin on days 0, 1, and 2 post excision. WB analysis of IL-1β and IL-23 expression in epidermal wound margin on day 3 post excision (n = 3/group). **(C)** WT mice were injected with various doses of rIL-17A treatment (doses of 0, 2, 20, and 200 ng/wound) in the wound margin for continuous 3 days following excision. IL-1β and IL-23 expression in epidermal wound margin was analyzed by WB on day 3 post excision (n = 3/group). **(D,E)** Fresh isolated primary keratinocytes cultured with IL-17A (100 ng/ml) for 24 h. The expression of IL-1β and IL-23 was measured by qPT-PCR **(D)** and WB **(E)**. **(F,G)** The expression of IL-1β and IL-23 in whole skin, epidermis, and dermis around wound of Vγ4D and WT control mice was assessed by WB on days 3, 5, and 7 post-excision **(F)** and IHC **(G)** on day 3 post-excision. **(H)** WT or *Il-17a*$^{-/-}$ Vγ4 T cells were added onto wound bed of *Tcrδ*$^{-/-}$ mice upon wounding (n = 4/group). The production of IL-1β and IL-23 in epidermis at wound edge was analyzed by WB on day 3 post-excision. Data represent three independent experiments using the indicated number of mice in brackets for each group or mouse strain. P value was assessed by Student's unpaired t-test (**P < 0.01, ***P < 0.001).

Compared to *Il-17a*$^{-/-}$ Vγ4 T cells, supplementing WT Vγ4 T cells onto wound beds of *Tcrδ*$^{-/-}$ mice markedly improved epidermal IL-1β and IL-23 production around wounds (**Figure 9H**). These results indicated that IL-17A was not only stimulated by IL-1β and IL-23 but also promoted IL-1β/IL-23 production in the epidermis to form a positive feedback loop after skin injury.

IL-1β Rather Than IL-23 Plays a Crucial Role in the Regulation of IGF-1 Production by DETCs in an NF-κB-Dependent Manner

We further investigated the precise roles of IL-1β and IL-23 in inhibiting IGF-1 production by DETCs *in vivo* and *in vitro*. The application of rIL-1β, rIL-23, or rIL-1β plus rIL-23 onto wound beds markedly decreased IGF-1 production by DETCs around wounds after skin injury (**Figure 10A**). Interestingly, the IGF-1 production by DETCs was significantly reduced in mice with rIL-1β plus rIL-23 treatment compared to those with rIL-1β or rIL-23 treatment alone (**Figure 10A**). Moreover, the administration of IL-1β neutralizing Ab alone or IL-1β plus IL-23 neutralizing Abs together onto wound beds notably increased IGF-1 production by DETCs around wounds after skin injury. However, IL-23-neutralizing Ab alone did not exhibit a similar effect (**Figure 10B**). Similar to the *in vivo* results, the addition of rIL-1β alone or rIL-1β plus rIL-23 rather than rIL-23 alone could remarkably inhibit IGF-1 production by eDETCs *in vitro* (WB, **Figure 10C**; FACS, **Figure 10D**). These findings suggested that IL-1β suppressed IGF-1 production in DETCs, and IL-23 could only enhance the inhibition mediated by IL-1β.

We next examined the IL-1β/IL-23 downstream pathways such as phosphorylated NF-κb and STAT3 (24–27). As expected, IL-1β and IL-23 markedly enhanced the phosphorylation (**Figure 10E**) and the translocation of NF-κb and STAT3 from the cytoplasm to the nucleus (**Figure 10F**) in eDETCs upon anti-CD3e stimulation *in vitro*. Moreover, blocking NF-κb signaling rather than STAT3 remarkably increased the expression of IGF-1 by eDETCs upon anti-CD3e stimulation *in vitro* (WB, **Figure 10G**; FACS, **Figure 10H**). Thus, we concluded that NF-κb signaling may play a more critical role in the IL-1β/IL-23 regulation of IGF-1 production in DETCs.

DISCUSSION

Wound healing is a complex process divided into four phases of hemostasis, inflammation, proliferation, and remodeling (28). Murine γδ T cells are the major T lymphocyte subsets that are distributed in skin tissue and engage in inflammation and re-epithelialization in wound repair (7, 28, 29). Skin γδ T cells consist of several subsets with distinctive functions. DETCs are the major source of epidermal IGF-1 that promote re-epithelialization (7), and Vγ4 T cells provide IL-17A in the early stages of skin inflammation (1, 15). It has been demonstrated that both impaired and excessive inflammation suppresses wound healing (4, 17). However, whether inflammation affects wound healing and how skin γδ T cell subsets are involved in this process remain unknown. In this study, we demonstrated that Vγ4 T cells provided IL-17A in the epidermis around wounds at early stages after skin injury, and IL-17A inhibited IGF-1 production by DETCs around wounds to delay wound healing. It is noteworthy that we used anti-Vγ4 TCR Ab (UC3-10A6) purchased from BioXcell to deplete Vγ4 T cells according to Hartwig et al. and Suryawanshi et al. (30, 31). However, Koenecke et al. identified that *in vivo* treatment with both GL3 and UC7-13D5 antibodies against TCR did not deplete γδ T cells but instead caused TCR internalization (32). Therefore, we analyzed the percentages of Vγ1 T cells and Vγ4 T cells in the spleen or lymph nodes 5 weeks after Vγ1 or Vγ4 T cell depletion and confirmed that the efficacy of Vγ1 or Vγ4 T cell depletion was excellent and could last for at least 5 weeks (data not shown). Furthermore, we have examined Vγ4 T cell percentages in the dermis from 3 days to 28 weeks after Vγ4 T cell depletion and confirmed that the efficacy of Vγ4 T cell depletion was excellent and could last for at least 3 months (data not shown). These results at least partially confirm that InVivoMAb treatment can deplete Vγ4 T cells *in vivo* because the downregulation of Vγ4 TCR complexes is impossible to recover after some time. But, we used the same clone Ab (UC3-10A6) to deplete and detect Vγ4 T cells, which cannot distinguish cells that have had their TCR downregulated from depletion. Therefore, we could still not exclude the possibility that Ab treatment does not deplete γδ T cells but rather reduces their TCR complexes on the cell surface.

Dendritic epidermal T cell-derived IGF-1 is necessary for epithelial homeostasis and protects keratinocytes in injured areas from apoptosis to aid re-epithelialization (7). DETCs around wounds provide IL-17A to assist in wound healing by inducing epidermal keratinocytes to express antimicrobial peptides and proteins such as β-defensin 3, S100A8, and RegIIIγ. Therefore, DETC-derived IL-17A is regarded as essential to reestablish the antimicrobial skin barrier after skin injury (4). *Tcrδ*$^{-/-}$ mice exhibit wound healing defects, which are rescued by the reconstitution of DETCs or addition of recombinant IGF-1 (7, 28). Similarly, wound-healing defects in *Il-17a*$^{-/-}$ animals could be restored by the addition of rIL-17A, IL-17A-producing DETCs (4), or IL-17A-producing Vγ4 T cells (data not shown). In addition, our previous studies demonstrated that both IGF-1 and IL-17A production in diabetic wounds were impaired, and the application of DETCs or IL-17A-positive Vγ4 T cells could improve the defects of diabetic wound healing (16, 33). Although IL-17A is required for efficient skin wound healing, excessive IL-17A displayed a harmful impact on skin wound closure (34). In this study, we revealed that epidermal IGF-1 production, which is required for efficient wound healing, could be downregulated by IL-17A after skin injury. Deficiency or blockage of IL-17A enhanced IGF-1 production in the epidermis and DETCs around wounds *in vivo*, whereas the addition of rIL-17A attenuated this effect. In the co-culture system of keratinocytes and eDETCs, blocking IL-17A with neutralizing Ab increased IGF-1 production by eDETCs *in vitro*, whereas the addition of rIL-17A resulted in the opposite outcome. Furthermore, we determined that Vγ4 T cell-derived IL-17A inhibited IGF-1 production by DETCs to delay wound repair. Vγ4 T cell depletion enhanced epidermal IGF-1 production and promoted wound healing. Addition of WT rather than *Il-17a*$^{-/-}$ Vγ4 T cells onto wound beds of *Tcrδ*$^{-/-}$ mice significantly attenuated epidermal IGF-1 production and wound healing, which was facilitated by supplementing WT DETCs. Thus, these findings suggested a previously unrecognized role of IL-17A to downregulate epidermal IGF-1 production, which was critical for Vγ4 T cells to impair wound healing.

Vγ4 T cells provide a major source of IL-17A to participate in skin autoimmune diseases (1, 19). In this study, we further

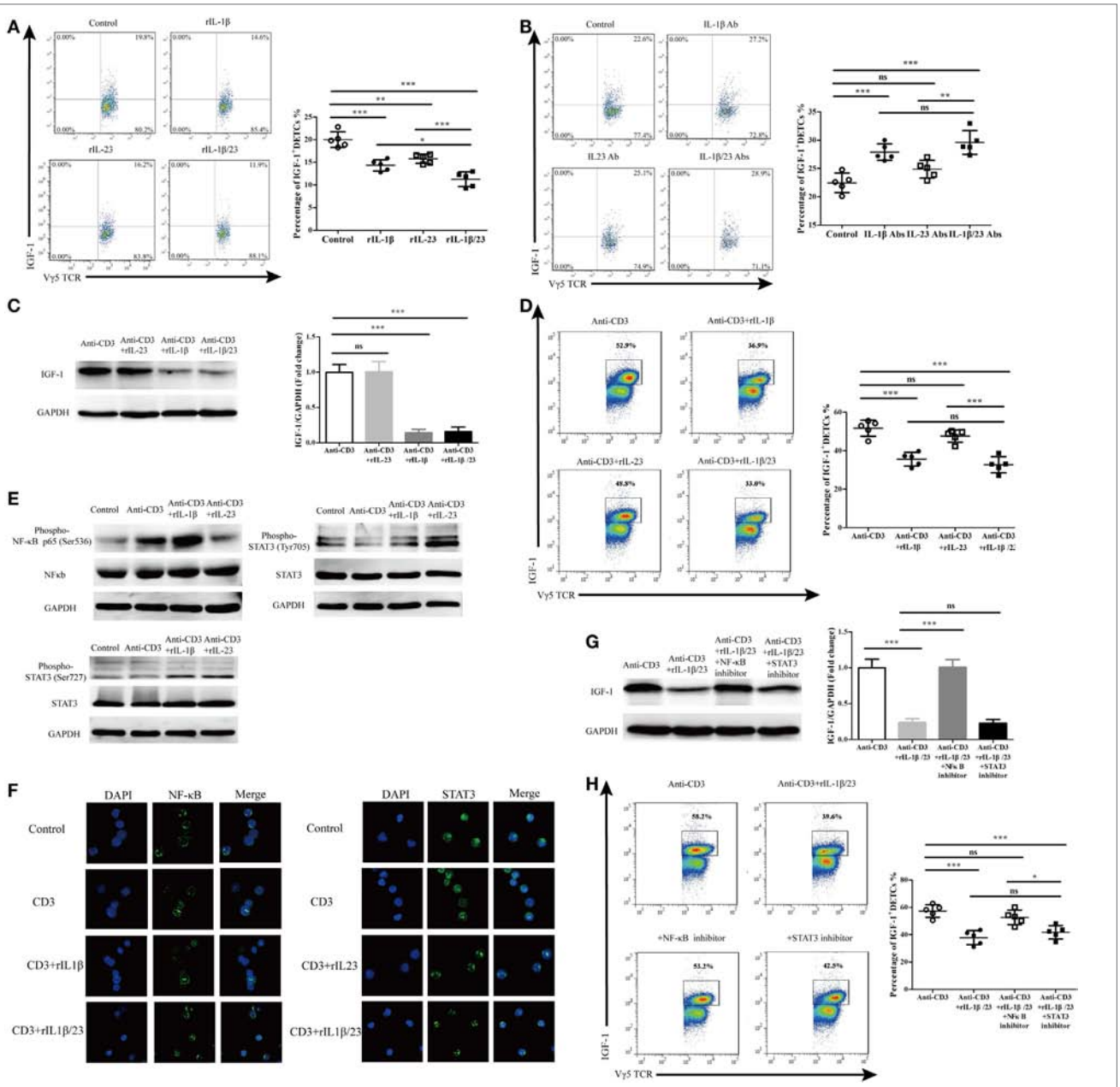

FIGURE 10 | IL-1β rather than IL-23 significantly decreased IGF-1 production by dendritic epidermal T cells (DETCs) in NF-κB-dependent manner. **(A)** FACS analysis of IGF-1 production in DETCs around wound of wild-type (WT) mice with rIL-1β, rIL-23, or rIL-1β plus rIL-23 treatment in the wound margin for continuous 3 days following excision (4 wounds/mice, 3 mice/group). **(B)** FACS analysis of IGF-1 production by DETCs around wound of WT mice with IL-1β-neutralizing Ab, IL-23-neutralizing Ab, or IL-1β-neutralizing Ab plus IL-23-neutralizing Ab treatment in the wound margin for continuous 3 days following excision (4 wounds/mice, 3 mice/group). **(C,D)** eDETCs were rested for 2 weeks without ConA and then co-cultured with rIL-1β, rIL-23, or rIL-1β plus rIL-23 upon anti-CD3ε stimulation for 6 h. The production of IGF-1 in eDETCs was measured by WB **(C)** and FACS **(D)**. **(E,F)** eDETCs co-cultured with rIL-1β or rIL-23 upon anti-CD3ε stimulation for 30 min. **(E)** Expression of phospho-NF-κB p65 (Ser536), total NF-κB p65, phosphor-STAT3 (Ser727), phosphor-STAT3 (Ser705), and total STAT3 was measured by WB. **(F)** Confocal immunofluorescence was used to check nucleus translocation of NF-κB p65 and STAT3. **(G,H)** eDETCs co-cultured with rIL-1β plus rIL-23, rIL-1β/rIL-23 plus NF-κB inhibitor (PDTC), or rIL-1β/rIL-23 plus STAT3 inhibitor (S3I-201) upon anti-CD3ε stimulation for 6 h. WB **(G)** and FACS **(H)** were performed to analyze IGF-1 expression by eDETCs. Data are representative of three individual experiments. Bars are shown mean ± SD. Data are representative of at least three independent experiments. Error bars represent mean ± SD. One-way ANOVA with Bonferroni comparison test was used to calculated *P* value (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

confirmed that Vγ4 T cells provided major source of IL-17A in the epidermis at early stages of wounding. Approximately half of the epidermal IL-17A-positive cells are Vγ4 T cells on day 3 after wounding. Depletion of Vγ4 T cells dramatically decreased epidermal IL-17A production in WT mice, and the addition of freshly isolated Vγ4 T cells onto wound beds significantly

enhanced epidermal IL-17A production in *Tcrδ*$^{-/-}$ animals. It is worth mentioning that DETCs have also been reported as a source of epidermal IL-17A for efficient wound healing (4, 7). Indeed, more than 15% of eDETCs, which expanded *in vitro* with ConA for 2–4 weeks, expressed IL-17A upon anti-CD3 stimulation (Figure S3 in Supplementary Material). However, few IL-17A-positive DETCs were detected in the epidermis around wounds after skin injury, and the addition of freshly isolated DETCs onto wound beds failed to significantly enhance epidermal IL-17A production in *Tcrδ*$^{-/-}$ animals. Therefore, Vγ4 T cells rather than DETCs provided a major source of IL-17A in the epidermis after wounding.

The IL-1β/IL-23-IL-17A axis is critical for the initiation and amplification of inflammatory responses (9, 19–22). Interestingly, our previous results suggested that IL-17A might also act upstream to enhance epidermal IL-1β/IL-23 production in a skin graft transplantation model (18). Here, we defined that IL-17A and IL-1β/IL-23 form a positive feedback loop in the epidermis around wounds to amplify local inflammation after skin injury. The epidermal IL-1β and IL-23 production at wound edges was weakened by a deficiency or blockage of IL-17A but enhanced by the addition of rIL-17A. Moreover, we revealed that Vγ4 T cell-derived IL-17A impacted epidermal IL-1β/IL-23 production after wounding. Depletion of Vγ4 T cells resulted in decreased epidermal IL-1β/IL-23 production, and supplementing WT rather than *Il-17a*$^{-/-}$ Vγ4 T cells onto wound beds promoted epidermal IL-1β/IL-23 production in *Tcrδ*$^{-/-}$ mice. In addition, IL-17A-producing γδ T cells express high levels of CCR6 on their surface and are recruited by CCL20 to migrate toward inflammatory sites (35). We observed that CCL20 neutralization dramatically decreased the infiltration of Vγ4 T cells into the epidermis around wounds and reduced epidermal IL-17A production. Together, IL-1β/IL-23 and CCL20 are thought to amplify epidermal IL-17A production by Vγ4 T cells and thereby exacerbate local inflammation.

Both Vγ4 T cells and DETCs have been shown to produce IL-17A in the presence of IL-1β and IL-23 (4, 19, 22). Here, we identified that IL-1β/IL-23 rather than IL-17A directly inhibited IGF-1 in DETCs. Furthermore, we revealed that IL-1β and IL-23 were crucial for IL-17A to inhibit IGF-1 production by DETCs. In the co-culture system of keratinocytes and eDETCs, blocking IL-1β/IL-23 totally reversed the reduction of IGF-1 in eDETCs caused by rIL-17A, whereas blocking IL-17A failed to rescue the reduction of IGF-1 by eDETCs mediated by rIL-1β/rIL-23. Blocking IL-1β/IL-23 consistently eliminated the inhibition of epidermal IGF-1 and the delay of wound healing, which were caused by rIL-17A *in vivo*, whereas blocking IL-17A failed to rescue the reduction of epidermal IGF-1 and the delay of wound healing, which were mediated by rIL-1β/rIL-23 *in vivo*.

Activated DETCs not only secrete IGF-1 but also produce IL-17A in the presence of IL-1β and IL-23 (4, 7). Here, we found a negative correlation between IGF-1 and IL-17A production in DETCs, which was closely regulated by IL-1β rather than IL-23. The addition of rIL-1β together with rIL-23 markedly reduced IGF-1 but enhanced IL-17A production by eDETCs *in vitro* (Figure S3 in Supplementary Material). Moreover, IL-1β rather than IL-23 has been shown to regulate IGF-1 and IL-17A production by DETCs in an NF-κb-dependent manner. Applying rIL-1β rather than rIL-23 had similar effects on eDETCs to rIL-1β together with rIL-23 (Figure S3 in Supplementary Material). Blocking NF-κb rather than STAT3 signaling with drugs completely eliminated the reduction of IGF-1 and enhancement of IL-17A by eDETCs mediated by rIL-1β/rIL-23 *in vitro* (Figure S3 in Supplementary Material). In addition, blocking IL-1β rather than IL-23 notably enhanced IGF-1 production by DETCs around wounds *in vivo*. However, rIL-1β and rIL-23 have exhibited a limited ability to enhance IL-17A production by DETCs that were freshly isolated from intact or wounded skin (Figure S4 in Supplementary Material). This suggests that, with long-term strong stimulation, DETCs may change to pro-inflammation cells to provide IL-17A in refractory wounds. This issue needs to be deeply investigated in the future.

TCR signaling is required for the survival and activation of T lymphocytes. DETCs rely on the recognition of TCR Vγ5 to unidentified ligands on epidermal cells under steady and stressed states (36). Interestingly, part of Vγ4 T cells, isolated from spleen/lymph nodes and expanded with anti-CD3 or anti-Vγ4 Ab *in vitro*, produces IL-17A upon PMA plus ionomycin or anti-CD3 re-stimulation (data not shown). Whether epidermal-infiltrating Vγ4 T cells that produce IL-17A are associated with some ligands expressed on stressed epidermal cells is still unclear. With the exception of TCR signaling, NKG2D has been demonstrated to activate γδ T cells (37). Rae1 and H60, ligands of NKG2D, are expressed on the surface of keratinocytes and could be markedly enhanced by inflammatory mediators (38, 39). Our data showed that Vγ4D significantly improved the expression of NKG2D on DETCs around wounds. Whether and how NKG2D regulates IGF-1 production by DETCs need to be investigated. Moreover, BTLA has been reported as a negative regulatory signal for γδ T cell activation until now (40). Whether and how BTLA affects the pro-inflammatory function of Vγ4 T cells and pro-healing function of DETCs need to be clarified in the future.

Our data established a mechanistic link between Vγ4 T cell-derived IL-17A, epidermal IL-1β/IL-23, DETC-derived IGF-1, and wound repair in the skin. The functional balance between Vγ4 T cells and DETCs resulted in efficient skin wound closure. Since that microbiological status at the wound area is very important for the outcome of skin wound healing, further investigation is needed into how Vγ4 T cell-mediated inflammation affects the defense from pathogen invasion after wound after skin injury.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 WT and *Il-17a*$^{-/-}$ mice were obtained from the Animal Center of the Third Military Medical University (Chongqing, CN, USA). TCRδ-GFP, *Tcrδ*$^{-/-}$ and *Ifn-γ*$^{-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Sex-matched mice aged 6–8 weeks were used for all experiments. All experiments were performed under specific pathogen-free conditions in conformity with ethical guidelines

and approved by the Animal Ethics Committee of the Third Military Medical University.

Preparation of Full-Thickness Excision Wound

Excision wounds were cut down to the musculus panniculus carnosus with a diameter of 6-mm sterile punch. For wound model without contraction, glue the biological membrane (negative pressure wound therapy kit, China) with the adhesive dressings immediately onto the surface of the wound before contraction and single-house the mice. Wound area was recorded daily by macroscopic digital photographs and estimated at each time point relative to day 0 until completely closure.

Mouse Models of Vγ1/Vγ4 T Cell Depletion, IL-17A/IFN-γ/IL-1β/IL-23/CCL20 Neutralization and rIGF-1/rIL-17A/rIL-1β/rIL-23 Addition

Mouse model of Vγ1D was implemented by intraperitoneal injection of 200 μg anti-Vγ1 Ab (clone 2.11; BioXcell, USA) 3 days before wound excision. Vγ4D was generated by anti-Vγ4 Ab (clone UC3-10A6; BioXcell, USA). Control mice were intraperitoneally administrated isotype control (armenian hamster IgG) (BioXcell, USA). Neutralization mouse models were conducted by injecting subcutaneously into wound margin 0, 1, and 2 days following excision with 20 μg/wound of anti-IGF-1 Ab (R&D Systems, Minneapolis, MN, USA), anti-IL-17A Ab (clone 17F3; BioXcell, USA), anti-IFN-γ Ab (clone XMG1.2; BioXcell, USA), anti-IL-1β Ab (clone B122; BioXcell, USA), anti-IL-23 Ab (clone BE0051; BioXcell, USA), anti-CCL20 Ab (clone 114906; R&D Systems, USA), respectively. IL-17A neutralization of low and high doses was performed by 2 and 200 μg/wound of anti-IL-17A Ab, respectively. Isotype control mice received equivalent doses of isotype Abs subcutaneously. Isotype control Abs of IGF-1, IL-17A, IL-1β, IL-23, CCL20 are poly goat IgG, mouse IgG1, armenian hamster IgG, rat IgG2, and rat IgG1 (BioXcell), respectively. Mouse models of recombinant cytokine addition were generated by subcutaneously injecting rIGF-1/rIL-17A/rIL-1β/rIL-23 (R&D Systems, Minneapolis, MN, USA) with the doses of 2, 20, 200 ng/wound, respectively, in the wound margin. Control mice were administrated with sterile PBS.

Preparation of WT, *Il-17a*$^{-/-}$, and *Ifn-γ*$^{-/-}$ Vγ4 T Cells

Vγ4 T cells were isolated from lymph node cells and splenocytes of C57BL/6 WT, *Il-17a*$^{-/-}$, and *Ifn-γ*$^{-/-}$ mice by the EasySep positive cell isolation systems (PE labeled anti-Vγ4 Ab; BD, USA; EasySep mouse PE positive selection kit; StemCell Technologies, Canada). The purity of isolated Vγ4 T cells was identified by FACS analysis. 1×10^4 cells/wound of WT, *Il-17a*$^{-/-}$, and *Ifn-γ*$^{-/-}$ Vγ4 T cells were placed onto the wound bed immediately following excision.

Isolation of Epidermal Sheets and Single-Cell Suspensions

Skin that is less than 5 mm away from wound edge was regarded as wound margin. Each group of epidermis tissue around wound was collected from 3–7 mice (4 wounds/mouse) for further analysis. Subcutaneous tissue of skin was wiped off and then skin was cut into small pieces of 0.5×0.5 cm^2. Epidermal sheets were torn off carefully from wound margins after incubated with 5 mg/ml dispase II at 37°C for 2 h. Epidermal single-cell suspensions were isolated by dissociating epidermal sheets with 0.3% Trypsin/GNK solution for 30 min at 37°C, then washed by PBS and filtered through a 70-μm strainer. Epidermal cells were cultured in the concentration of 5×10^5–1×10^6/ml with 10% FBS 1640 medium. Primary keratinocytes were isolated from skin of newborn mice in the same method as above. According to the manufacturer's instructions (Miltenyi, GER), CD11c$^+$ cells were removed from primary keratinocytes. The purity of isolated keratinocyte population was >95%.

Preparation of DETCs

After epidermal single-cell suspension was prepared, lymphocyte-M (Cedarlane Laboratories, Canada) was used to enrich DETCs (purity = 15–20%). For reconstitution assays, DETCs were further purified by the EasySep positive cell isolation systems (PE labeled anti-TCRδ Ab; BD, USA; EasySep mouse PE positive selection kit; StemCell Technologies, Canada). The purity of isolated DETCs was identified by FACS analysis (purity > 90%). Then, DETCs were transferred to wound bed immediately following excision (5×10^4 cells/wound). For *in vitro* assays, DETC population was expanded in 48-well plates ($1–2 \times 10^6$/ml) with RPMI containing 10% FBS, 1 μg/ml Con A (Sigma-Aldrich, Germany), 10 ng/ml mouse rIL-2, 1 mM Na Pyruvate (Sigma-Aldrich, Germany), 2 mM glutamine, 25 mM HEPES, 50 μM 2-ME (Biosharp, CN, USA), 100 M non-essential amino acids (Gibco, USA), 100 μg streptomycin and 100 U penicillin for 2–4 weeks. The purity of expanded DETCs (eDETCs) was > 95% by means of FACS analysis. eDETCs were rested without ConA for 2 weeks before used for further experiments.

Flow Cytometry Analysis and Intracellular Cytokine Staining

The following fluorochrome-labeled murine monoclonal antibodies (mAbs) were purchased from BD, Biolegend, eBioscience, and Sungene Biotech: CD16/32(2.3G2), γδTCR (GL3), Vγ1 (2.11), Vγ4 (UC3-10A6), Vγ5 (536), CD25 (3C7), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), NKG2D (CX5), JAML (4E10), CCR6 (29-2L217), IL-17A (TC11-18H10.1), IFN-γ (XMG1.2). IGF-1 (H70, Santa Cruz Biotechnology, USA) was stained followed by fluorescent-labeled secondary reagents FITC goat-anti-rabbit mAb (Boster, CN), PE donkey-anti-rabbit mAb (Biolegend, USA). For surface staining, cells were first blocked with anti-CD16/32 mAb for 15 min and then incubated with different cellular surface mAbs for 30 min at 4°C. For further intracellular staining, cells were fixed and permeabilized according to the manufacturer's recommendations (BD Biosciences, USA). For *ex vivo* detection of intracellular cytokines, epidermal

cells were stimulated with 50 ng/ml PMA, 750 ng/ml ionomycin (BD Biosciences), and 100 ng/ml Brefeldin A (Beyotime, CN, USA) at 37°C, 5% CO_2 for 6 h. For *in vitro* stimulation, eDETCs or eDETCs plus primary keratinocytes cultured with murine rIL-1β/rIL-23/rIL-17A (100 ng/ml, R&D system, USA), IL-1β/IL-23/IL-17A neutralizing Ab (10 μg/ml, BioXcell, USA), or signal inhibitors NFκB inhibitor (PDTC, 5 μM), STAT3 inhibitor (S3I-201, 100 μM) (Selleck Chemicals, USA) for 6–24 h. Cells were stained as above. Stained cells were detected on Attune Acoustic Focusing Cytometer (Life Technologies, USA) and analyzed with FlowJo software (Tree Star Incorporation, USA).

Western Blot Analysis

Tissue protein extracts of whole skin, dermis, and epidermis were prepared by grinding the tissue into powder in liquid nitrogen. Then, the powder was lysed in lysis buffer (KeyGEN BioTECH, CN) with 1 μg/ml protease inhibitor, 5 μg/ml PSMF, and 10 μg/ml phosphatase inhibitor, followed by low-speed rotation at 4°C for 20 min and centrifugation at 14,000 × *g* for 15 min. Concentration of protein extraction was determined by BCA protein assay kit (Thermo Scientific, Rockford, USA). 25 μg/sample was run on 10% or 12% SDS-PAGE, then transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon, USA). PVDF membranes were incubated with 3% bull serum albumin (BSA) (Biosharp, CN, USA) at room temperature for 2 h, then with following Abs at 4°C overnight: IL-17A, IGF-1 (1:200, Santa Cruz Biotechnology, USA), CCL20 (1:200, R&D system, USA), IL1β/IL23 Ab (1:1,000, Abcam, UK), NF-κB p65 (D14E12), phospho-NF-κB p65 (Ser536, 93H1), STAT3 (D3Z2G)/phospho-STAT3 (Tyr705, D3A7), phospho-STAT3 (Ser727, D4X3C) (1:1,000, Cell signaling technology, USA). Anti-GAPDH Ab (1:2,000, SunGene Biotech, CN, USA) was as loading control. The membranes were visualized with goat-anti-rabbit/mouse/rat-HRP IgGs (1:5,000, SunGene Biotech, CN, USA) at room temperature for 1 h and chemiluminescence liquid for seconds (Solarbio, CN). ChemiDoc™ XRS detection system (Bio-Rad, USA) was used to analyze blots.

HE Staining and Immunohistochemistry

Specimens of wound margin were embedded in paraffin by routine methods. The sections were stained with H&E and new epithelial length was measured under the microscope in a blinded manner. For immunohistochemistry, skin sections were deparaffinized and hydrated in xylene and graded alcohol series. Antigen retrieval was conducted by incubating sections in 10 mM citric acid (pH 6) at 95°C for 15 min. Sections were blocked and stained with goat-anti-rabbit serum or 3% BSA with 0.3% Triton X-100 (Sigma-Aldrich, Germany) at room temperature for 60 min and followed by primary antibodies: anti-IL-1β, anti-IL-23, anti-IGF-1, and anti-IL-17A (1:100–200, Abcam, UK) overnight at 4°C. Samples were washed and incubated with secondary antibodies at room temperature for 60 min. Slides were incubated with drops of diaminobenzidine solution (Boster, CN, USA) and counterstained with hematoxylin (Beyotime, CN, USA). Stained sections were examined under Olympus BX51 microscope (Tokyo, Japan).

Immunofluorescence of DETCs and Epidermis Sheets

eDETCs were stimulated with anti-CD3ε Ab (5 μg/ml), rIL-1β, or/and IL-23 (100 ng/ml) for 30 min, then were washed with PBS. eDETCs were re-suspended by PBS to concentration of 1×10^6/ml. 20 μl cell suspension was dropped to slide and went dry at room temperature. Then cells were fixation with acetone at 4°C for 30 min and washed by PBS for 5 min × 3 times. Cells were permeabilized with 0.5% Triton X-100 at 4°C for 15 min and washed three times with PBS. Cells were then incubated in 5% BSA at room temperature for 1 h. Cells were washed with PBS and incubated with anti-NF-kB p65 or anti-STAT3 Ab (1:500, Cell signaling technology, USA) in PBS at 4°C overnight. Cells were stained with DAPI (4′,6-diamidino-2-phenylindole) for 30 s and washed in PBS for 1 min. Cells were covered by mounting solution (Boster, CN, USA) and preserved at 4°C. Epidermis sheets isolated from TCRδ-GFP mice were lay on slice and directly covered by mounting solution. Slices were photographed using Zeiss Axioplan fluorescent microscope.

RNA Extraction and Real-Time Quantitative PCR (qRT-PCR)

RNAs were extracted by a Qiagen RNeasy kit or TRIzol reagents (Invitrogen, USA). cDNA was reverse transcripted by 0.5 mg total RNA with a TaqMan reverse transcription kit (Life Technologies, USA). qRT-PCR was detected on Bio-Rad RT-PCR system using SYBR Green Supermix and gene-specific primer pairs: IL-1β: 5′—3′(forward) ACCTTCCAGGATGAGGACATGA, 5′—3′ (reverse) CTAATGGGAACGTCACACACCA; IL-23p19: 35′—3′ (forward) CTGAGCCACCCAGGAAAGTA, 5′—3′(reverse) TG AGAAAACCCAGAGCATCA; GAPDH: 5′—3′(forward) CGTG CCGCCTGGAGAAAC, 5′—3′(reverse) AGTGGGAGTTGCTG TTGAAGTC. Gene expression level was normalized to GAPDH and represented as fold differences by the method where $F = 2^{-\Delta\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_{\text{induced}} - \Delta Ct_{\text{reference}}$.

Statistical Analysis

All data were shown as mean ± SD. Statistical differences were calculated by two-tailed unpaired Student's *t*-test or one-way ANOVE with Bonferroni comparison test on SPSS19.0 software (IBM, USA), and graphs were manufactured by GraphPad Prism software (GraphPad Software, Inc., USA). $P < 0.05$ was considered significant.

ETHICS STATEMENT

All experiments were performed under conventional animal raising conditions in conformity with ethical guidelines and approved by the Animal Ethics Committee of the Third Military Medical University.

AUTHOR CONTRIBUTIONS

Conceptualization, WH; methodology, YL, YW, ML, LZ, and XH; validation, RY, JH, XZ, XH, and YH; formal analysis, YL, LZ, and

YW; investigation, YL and YW; resources, GL, YJ, RW, and ZY; writing-original draft, WH, YL, and YW. Writing-reviews and editing, WH, GL, and JW; funding acquisition, WH, GL, and JW; supervision, WH, GL, and JW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00240/full#supplementary-material.

FIGURE S1 | The number of Vγ4 T cells was decreased in dermis rather than draining lymph nodes. Wild-type and Vγ4 T-cell depletion mice were sacrificed on days 0, 3, 5, and 7 after excision. **(A)** Dermal sheets around wounds were collected from 3 mice with 4 wounds/mice. Percentage of dermal Vγ4 T cells was analyzed by FACS. **(B)** Percentage of Vγ4 T cells in draining lymph nodes was analyzed by FACS. Representative data of three individual experiments were shown. Dots represent individual animals. All error bars represent mean ± SD. *P* value was assessed by one-way ANOVA with Bonferroni's comparison test (***P* < 0.01, ****P* < 0.001).

FIGURE S2 | Wound healing was defected in IL-17A knock out mice. Age- and sex-matched C57BL/6 wild-type mice and *Il-17a*$^{-/-}$ mice were anesthetized and full-thickness wounds were then generated using a sterile 6 mm punch tool. Wound-closure kinetics was measured over time and dots in wound closure kinetics represent 10 wounds per group (2 wounds/mouse, 5 mice/group). Bars represent mean ± SD. All data were representative of three independent experiments. *P* value was calculated by Student's unpaired *t*-test (***P* < 0.01, ****P* < 0.001).

FIGURE S3 | IL-17A production on dendritic epidermal T cells (DETCs) was significantly increased by IL-1β and IL-23. eDETCs were stimulated for 6 h with anti-CD3ε, anti-CD3ε plus rIL-1β, anti-CD3ε plus rIL-23, anti-CD3ε plus rIL-1β and rIL-23, anti-CD3ε plus rIL-1β and rIL-23 with NF-κB inhibitor (PDTC), or anti-CD3ε plus rIL-1β and rIL-23 with STAT3 inhibitor (S3I-201) in the presence of brefeldin A. IL-17A expression on eDETCs was detected using FACS. Data are representative of three individual experiments. All error bars represent mean ± SD.

FIGURE S4 | IL-1β and IL-23 accelerated IL-17A production in epidermis. Cell suspensions were isolated from epidermis of normal skin **(A)** or wound margin **(B)** on day 3 post excision (4 wounds/mice, 3–5 mice/group). Cells were stimulated with anti-CD3ε Ab, rIL-1β, and IL-23, or anti-CD3ε Ab + rIL-1β + IL-23 for 6 h *in vitro*. Rectangles in the upper lane represent for Vγ5T cells and rectangles in the down lane represent for IL-17A positive population of Vγ5T cells gated in the upper rectangles. Statistical figures are on the right side. Data are representative of three individual experiments. All error bars represent mean ± SD. *P* value was assessed by one-way ANOVA with Bonferroni's comparison test (*P < 0.05, **P < 0.01, ***P < 0.001).

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OPINION
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Immunotherapy With Human Gamma Delta T Cells—Synergistic Potential of Epigenetic Drugs?

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INTRODUCTION

Epigenetics has emerged as one of the fastest growing concepts, adding more than 45 new publications every day, spreading through various fields (1). Conrad Waddington coined the term "epigenetics" in 1942; however, a multitude of definitions has been endorsed by different researchers. In essence, Waddington's definition of "epigenetics" and its redefinition by Holiday is at the heart of cellular function. Hence, it is obvious that epigenetic regulation plays a central role also in the specification, differentiation, and functional plasticity of T lymphocytes (2). T-cell fate decision in progenitor cells, functional CD4 T-cell plasticity, CD8 T-cell differentiation, but also T-cell memory, are all substantially governed by epigenetic mechanisms (3–7). Here, we focus on the current development of drugs targeting major pathways of epigenetic regulation and their possible impact on γδ T-cell multifunctionality. We aim to develop concepts of how some of these approaches might help to improve the efficacy of γδ T-cell-based immunotherapies.

The dynamic construction of chromatin organization exists in two principal states, i.e., transcriptionally repressive "heterochromatin" and active "euchromatin." The heterochromatin formation (**Figure 1A**) is mediated by SET domain, the chromodomain, and plant homeodomain finger, found in the heterochromatin protein 1 (HP1)/chromobox, and the chromodomain helicase-DNA-binding subfamilies, recognizing histone methylation (e.g., H3K9 di- and tri- methylation) (8). Histone deacetylase (HDAC) associates with HP1, then recognize histone methyltransferases and methylated DNA *via* methyl-binding proteins such as MeCP2. HDACs also interact with DNA methyltransferases (DNMTs; the enzymes catalyze DNA methylation), thus forming the regulatory axis of a multiprotein complex responsible for transcriptional repression (9–11). DNA demethylation can be achieved "actively" by the hydroxylation of 5-methylcytosine to 5-hydroxymethyl cytosine mediated by the ten-eleven translocation (TET) enzymes (12, 13). In contrast, the "euchromatin" formation is a complex, multistep process involving post-translational modifications (PTM) of histones and also chromatin-remodeling complex (**Figure 1B**). In addition to other PTM, the acetylation of histone leading to the "euchromatin" formation has already been reported during the 1990s (14). This process of histone lysine acetylation is mediated by HAT and is recognized by the bromodomain (Brd) proteins, additionally recruiting proteins. The Brd proteins are thus categorized as components of HAT complexes, components of chromatin-remodeling complexes, and Brd and ExtraTerminal domain (BET) proteins. BET proteins, particularly Brd2 and Brd3, play a multifaceted role by maintaining euchromatin status and simultaneously "reading" both acetylated histones and transcription factors. By recruiting and coupling the transcriptional machinery to the target gene promoter and/or enhancer sites,

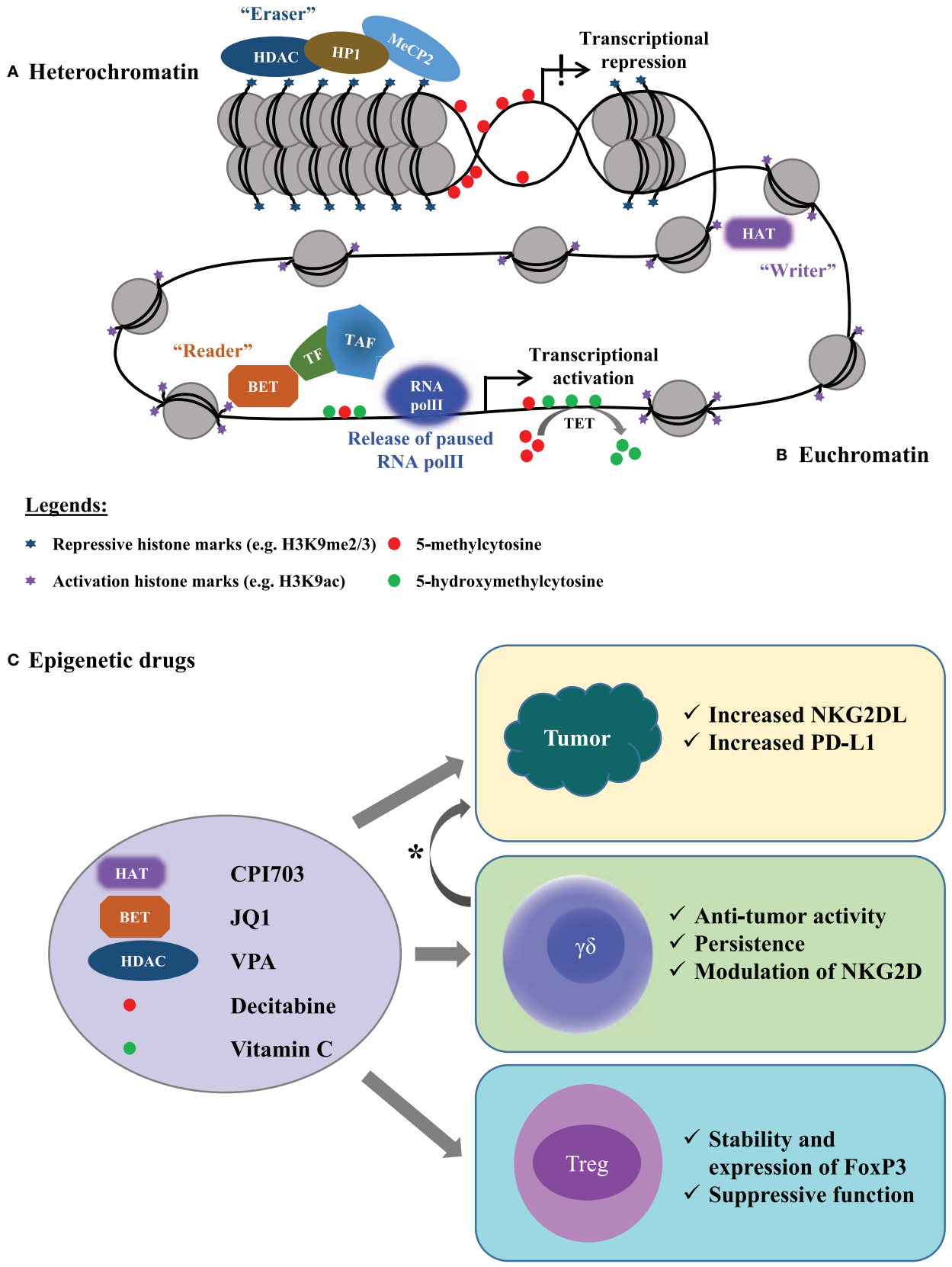

FIGURE 1 | Continued

FIGURE 1 | An overview of epigenetic mechanisms governing cellular processes and the drugs targeting respective epigenetic processes. There are two possible states of chromatin organization: **(A)** the "closed" chromatin associated with heterochromatin formation and transcriptional repression drives gene silencing. **(B)** The mechanistic organization of euchromatin maintains the "open" chromatin confirmation and allows active gene expression. **(C)** Examples of how epigenetic drugs modulate the γδ T-cell/Treg/tumor interaction. In the circle (left part), the epigenetic drugs (on the right-hand side) that are either in pre-clinical development or clinically approved are listed along with the respective target proteins (on the left-hand side). These are the key proteins for diverse epigenetic processes. The effect of the listed epigenetic drugs on immune cells (γδ T cells and Treg) and tumor cells are shown in the rectangles (right part). As marked by asterisk (*), the epigenetic drugs are proposed to synergize, leading to increased efficacy of γδ T cell-based immunotherapy. HDAC, histone deacetylase; HP1, heterochromatin protein 1; MeCP2, methyl-CpG binding protein 2; HAT, histone acetyltransferase; BET, Bromodomain and ExtraTerminal; TF, transcription factor; TAF, transcription-associated factors; RNA polII, RNA polymerase II; TET, ten-eleven translocation; VPA, valproic acid; NKG2D, natural-killer group 2, member D receptor protein; NKG2DL, ligands for NKG2D receptor protein; PD-L1, programmed death ligand 1; FoxP3, forkhead Box P3; Treg, regulatory T cells.

BET proteins further release paused RNA polymerase II for the respective gene activation (15–19). Additionally, the proteins involved in the principal states of chromatin organization have multiple functions including enzymatic activity. Such an important complexity in protein/enzyme function provides a leverage for the epigenetic drugs.

Nonetheless, it is important to realize (but currently not yet a major focus of epigenetic research) that any enzymatic activity (and thus epigenetic regulation) depends on the appropriate cellular metabolism. While the central role of the cellular metabolism for the maintenance of stem cell pluripotency (which is drastically influenced by epigenetics) is well known, the respective roles of metabolic pathways and nutrients availability versus epigenetics for the differentiation and plasticity of immune cells have only recently been appreciated (20, 21).

EPIGENETIC DRUGS

In view of the central role of epigenetic regulation for developmental biology and cellular activation, proliferation, and differentiation, it comes as no surprise that many drugs targeting specific steps of epigenetic regulation have been developed (**Figure 1C**). If suitable for clinical application, such drugs might have broad applications for the treatment of (certain types of) cancer but also autoimmune and chronic inflammatory diseases.

Currently, two hypomethylating agents targeting epigenetic "erasers," decitabine (5-aza-2′-deoxycitidine) and azacitidine (5-azacitidine) are approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndromes, but are also used in other clinical conditions (22). The major effect of such agents is to induce hypomethylation of CpG islands thereby allowing re-expression of suppressed genes including tumor suppressor genes. Not unexpectedly, hypomethylating drugs have major effects on immune cells including the stabilization of FoxP3 expression and Treg activity (23). In addition, numerous studies have investigated effects of hypomethylating agents on NK cells, dendritic cells, and T cells [see Ref. (22)]. It is difficult to draw general conclusions as the reported effects may be linked to specific experimental conditions or treatment regimens, but immunomodulatory effects are quite obvious (22). Immunogenicity of tumors might increase due to re-expression of tumor-associated antigens. However, hypomethylating agents might also promote tumor resistance through upregulation of inhibitory molecules like PD-1 and/or PD-L1 (24, 25). Obviously, the complexity of the effects of epigenetic drugs needs to be carefully evaluated. A major breakthrough in cancer immunotherapy has been the introduction of checkpoint inhibitors into clinical practice. Currently, several trials have been initiated where azacitidine is combined with PD-1/PD-L1 or CTLA-4 checkpoint inhibitors in hematological malignancies and colorectal cancer (26). Another regulator of DNA methylation is Vitamin C (VC). In addition to its antioxidant activity, VC also activates TET enzyme activity and thereby promotes 5-hydroxymethylation of DNA (27, 28).

Like hypomethylating agents, HDAC inhibitors (HDACi) have multiple effects on tumor cells but also on immune cells. In fact, their therapeutic efficacy against cancer is likely to depend on the simultaneous modulation of the immune system (29). Several structural classes of HDCAi have been developed. While some HDACi inhibit all HDACs, others are specific for class I and class IIa HDACs (e.g., valproic acid, VPA) or only class I HDAC (e.g., entinostat). Some HDACi including VPA upregulate the expression of NKG2D ligands on tumor cells and thereby augment the susceptibility to recognition and lysis by NK cells and γδ T cells (30, 31). As of today, several HDACi have been approved by the FDA either as monotherapy or in combination with other drugs, such as with PD-1 or CTLA-4 checkpoint inhibitors (26), for the treatment of hematological malignancies and some solid tumors [see Ref. (26)].

Epigenetic drugs which target epigenetic "readers" are BET inhibitors. The inhibition of BET proteins has a broad impact on gene regulation and may have a therapeutic effect in cancer (18). JQ-1, a pan-BET inhibitor blocks Th17 differentiation and thereby suppresses Th17-related inflammatory diseases in mouse models (19, 32). Importantly, recent studies point to a selective effect of JQ-1 on PD-L1 expression. PD-L1 is a direct target gene of the BET family member Brd4, and BET inhibition by JQ-1 has been found to enhance anti-tumor immunity by suppressing the PD-L1 expression on tumor cells and antigen-presenting cells but also through upregulation of NKG2D ligand MICA on tumor cells (33–35). BET inhibition also affects T-cell differentiation. A recent study reported superior *in vivo* persistence and anti-tumor activity of tumor antigen-specific murine T cells upon adoptive transfer (36). Moreover, BET proteins appear to be interesting targets for synergistic anti-tumor effects in combination with other inhibitors targeting, e.g., PI3-kinase (37), Bcl-2 (38), PARP (39), or HDAC (40). Last but not least, BET inhibitors like JQ-1 might also synergize with checkpoint inhibitors to facilitate efficient anti-tumor immune responses (41). Based on promising pre-clinical results, BET inhibitors have entered clinical trials. However, many details of how BET inhibitors work at

the molecular level and which cells and tissues are differentially affected, are not yet precisely known; therefore, the adverse side effect profile of various BET inhibitors needs to be studied in detail (42).

PLASTICITY OF γδ T CELLS

γδ T cells are considered to link innate and adaptive immunity because they can be rapidly activated *via* their T-cell receptor (TCR) in an MHC-independent manner (e.g., recognition of pyrophosphates in the case of human Vγ9Vδ2 T cells) but also express functional innate receptors such as toll-like receptors (43). Importantly, human Vγ9Vδ2 T cells cannot only differentiate into different cytokine-producing subsets, but also may acquire regulatory activity and "professional" antigen-presenting capacity (44). Moreover, γδ T cells are usually potent cytotoxic effector cells which kill various tumor target cells independent of HLA restriction. Human Vγ9Vδ2 T cells recognize pyrophosphates accumulating in tumor cells exhibiting a dysregulated mevalonate metabolic pathway in a butyrophilin 3A-dependent manner (45). However, most γδ T cells also express the activating NKG2D receptor, which endows them with a TCR-independent second activation pathway *via* recognition of NKG2D ligands (e.g., MICA/B) on tumor cells. Based on their HLA-independent mode of target cell recognition, γδ T cells have recently attracted substantial interest as potential effector cells in cell-based cancer immunotherapy (46). This includes the perspective of using allogeneic γδ T cells from healthy donors since γδ T cells from the blood of tumor patients are sometimes difficult to expand *in vitro*. The experience of one of us (Zhinan Yin) with over 140 adoptive γδ T-cell transfers in more than 45 patients with different malignancies indicates that such γδ T-cell transfers are safe and are well tolerated.

In the murine system, genome-wide histone (H3) acetylation and methylation profiling have identified distinct molecular programs in interferon-γ versus IL-17 producing γδ T cells (47). It is also well established that epigenetic mechanisms regulate the chromatin accessibility of the TCR γ locus during intrathymic T cell development (48, 49). Currently, however, there is only limited information available as to how epigenetics contributes to the multifunctionality of human γδ T cells. We have performed a comprehensive analysis of peripheral blood αβ T cell subsets ($CD4^+$, Treg, $CD8^+$) and γδ T cells. In this ongoing work, we expect to obtain information on how γδ T cells differ from (subsets of) αβ T cells at the transcriptome and epigenetic level (Bhat et al., unpublished). Moreover, we have investigated the effects of the HDACi VPA on the Vγ9Vδ2 subset of human γδ T cells upon *in vitro* culture. VPA differentially modulated the expression of certain surface markers (notably CD86, CD54, and NKG2D) on γδ T cells compared with αβ T cells (50). For instance, NKG2D receptors on γδ T cells and their respective ligands on tumor cells were even more affected after VPA treatment (Bhat et al., under revision). We also observed that VPA induced the expression of a non-secreted isoform of IL-4 (IL-4δ13) which is known to have regulatory properties (51). Ongoing studies in our laboratories analyze the effects of VC on the *in vitro* differentiation of human γδ T cells. VC increases and stabilizes the expression of FoxP3 in transforming growth factor-β (TGF-β)-treated Vγ9Vδ2 T cells and augments the proliferative capacity of Vγ9Vδ2 T cells upon pyrophosphate-induced growth arrest (Kouakanou et al., to be published). RNA-seq and reduced representation bisulfite sequencing analyses of VC-treated human γδ T cells will provide insights how VC globally affects human γδ T-cell plasticity at the transcriptional and DNA methylation level. Though our study has been focused on the Vγ9Vδ2 subset, the effect of epigenetic drugs needs to be addressed in the context of distinct subsets of γδ T cells. Hence, the implication of epigenetic modulation needs to be investigated using different settings. Interestingly, we also found that TGF-β, usually considered as an immunosuppressive cytokine (52), can actually increase the cytotoxic activity of purified γδ T cells activated by pyrophosphate antigens in the presence of TGF-β (Peters et al., submitted). Thus, a variety of strategies are available to modulate the plasticity of human γδ T cells.

HOW TO MODULATE THE ANTI-TUMOR POTENTIAL OF MULTIFUNCTIONAL γδ T CELLS?

Based on the outlined principles, we can envisage a multitude of approaches to enhance the cytotoxic anti-tumor activity of human γδ T cells, or to modulate their subset phenotype (Bhat et al., under revision), or to revert their detrimental activity (e.g., regulatory activity and/or high-PD-L1 expression of tumor-infiltrating γδ T cells) (53). DNMT inhibitors and HDACi already in clinical use modulate antigens relevant for γδ T-cell activation including NKG2D receptor and ligands (22, 26) and thus may increase the efficacy of adoptive γδ T-cell immunotherapy. Of special interest, however, are established and emerging new BET inhibitors. It will be important to find out whether BET inhibitors like JQ-1 can increase the functionality of *in vitro* expanded γδ T cells and eventually their persistence and anti-tumor activity similar to what has been described for tumor-reactive $CD8^+$ T cells (36). BET inhibitors might also augment γδ T-cell immunotherapy *via* increasing the expression of NKG2D ligands on tumor cells (35). Furthermore, the recently reported BET inhibitor-mediated inhibition of PD-L1 expression on tumor cells [associated with improved anti-tumor immunity; Ref. (33)] might also extend to the inhibition of PD-L1 expression on tumor-infiltrating γδ T cells, which has been shown to restrain effective αβ T-cell responses in pancreatic oncogenesis (54). Last but not least, novel inhibitors have been developed selectively inhibiting the Brd interaction of CBP/EP300, which plays a crucial role in Treg biology (55). By dampening Treg activity, such small molecule inhibitors might also increase the efficacy of γδ T-cell immunotherapy in cancer patients. Overall, we have a plethora of strategies at hand to potentially increase the efficacy of γδ T-cell immunotherapy. The challenge is to design the best possible (pre-clinical and clinical) studies to identify efficacious synergistic strategies with acceptable adverse risk profile.

AUTHOR CONTRIBUTIONS

DK and JB wrote the manuscript. LK, CP, ZY, JB, and DK contributed to the discussion of the draft and made final corrections.

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Dysregulated CD25 and Cytokine Expression by γδ T Cells of Systemic Sclerosis Patients Stimulated With Cardiolipin and Zoledronate

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Objectives: γδ T cells, a non-conventional innate lymphocyte subset containing cells that can be activated by lipids and phosphoantigens, are abnormally regulated in systemic sclerosis (SSc). To further evaluate the significance of this dysregulation, we compared how exposure to an autoantigenic lipid, cardiolipin (CL), during co-stimulation with an amino-bisphosphonate (zoledronate, zol), affects the activation and cytokine production of SSc and healthy control (HC) γδ T cells.

Methods: Expression of CD25 on Vγ9$^+$, Vδ1$^+$, and total CD3$^+$ T cells in cultured peripheral blood mononuclear cells (PBMCs), their binding of CD1d tetramers, and the effect of monoclonal antibody (mAb) blockade of CD1d were monitored by flow cytometry after 4 days of *in vitro* culture. Intracellular production of IFNγ and IL-4 was assessed after overnight culture.

Results: Percentages of CD25$^+$ among CD3$^+$ and Vδ1$^+$ T cells were elevated significantly in short-term cultured SSc PBMC compared to HC. In SSc but not HC, CL and zol, respectively, suppressed %CD25$^+$ Vγ9$^+$ and Vδ1$^+$ T cells but, when combined, CL + zol significantly activated both subsets in HC and partially reversed inhibition by the individual reagents in SSc. Importantly, Vδ1$^+$ T cells in both SSc and HC were highly reactive with lipid presenting CD1d tetramers, and a CD1d-blocking mAb decreased CL-induced enhancement of %SSc CD25$^+$ Vδ1$^+$ T cells in the presence of zol. %IFNγ$^+$ cells among Vγ9$^+$ T cells of SSc was lower than HC cultured in medium, CL, zol, or CL + zol, whereas %IFNγ$^+$ Vδ1$^+$ T cells was lower only in the presence of CL or CL + zol. %IL-4$^+$ T cells were similar in SSc and HC in all conditions, with the exception of being increased in SSc Vγ9$^+$ T cells in the presence of CL.

Conclusion: Abnormal functional responses of γδ T cell subsets to stimulation by CL and phosphoantigens in SSc may contribute to fibrosis and immunosuppression, characteristics of this disease.

Keywords: cardiolipin, systemic sclerosis, fibrosis, γδ T cells, zoledronate, phosphoantigens, interferon γ, interleukin-2

KEY MESSAGES

Cardiolipin activates human Vδ1$^+$ γδ T cells co-stimulated with zoledronate.
In systemic sclerosis, cardiolipin and zoledronate suppress the activation of Vγ9$^+$ and Vδ1$^+$ γδ T cells, respectively.
The CD1d molecule is involved in cardiolipin activation of Vδ1$^+$ T cells in systemic sclerosis.

INTRODUCTION

T cells are thought to be involved in the immune pathogenesis of systemic sclerosis (SSc) by their recognition of foreign or self-antigens that activate effector programmers leading to pathological fibrosis (1–3). While abnormal responses to conventional peptidic autoantigens have been identified, a few studies suggest that abnormal responses to lipid antigens and phosphoantigens may also play a role in the pathogenesis of SSc (4, 5). In this regard, γδ T cells expressing the Vδ1 gene segment in their T cell receptor (TCR) (Vδ1$^+$ T cells) were found to be dysregulated in early and late SSc (6, 7). Thus, these cells were highly activated in the peripheral blood (PB) of patients, infiltrated the skin in early disease, and oligoclonally expanded in damaged internal organs (6, 7). Recent evidence indicates that a fraction of Vδ1$^+$ TCR recognize lipid antigens presented by CD1d molecules expressed by antigen-presenting cells (APCs), suggesting a possible role for lipid antigens in the pathogenesis of SSc *via* effects on Vδ1$^+$ T cells (8–10). In support of this, 10–20% of SSc patients have antibodies to cardiolipin (CL), a mitochondrial autolipid that is also present in microorganisms (11). Moreover, the T cell response to CL in a murine model of autoimmunity was independent of classical lipid responsive αβ TCR$^+$ invariant natural killer T (iNKT) cells, suggesting that lipid reactive γδ T cells, rather than iNKT cells, may play a more critical role in disease-related autoimmune responses to CL (12). However, there is no available evidence to indicate that human γδ T cells in SSc recognize and respond to CL.

The second class of γδ T cells, characterized by expression of the Vγ9 gene in the γδ TCR (Vγ9$^+$ γδ T cells), is also abnormally regulated in SSc. Thus, amino-bisphosphonate (ABP) compounds inhibit farnesyl pyrophosphatase, leading to increased levels of intracellular phosphoantigens [mainly isopentenyl pyrophosphate (IPP)] in APC that bind to and induce a conformational change in butyrophilin 3A1 (CD277) cell surface molecules on APC (13). This alteration is recognized by Vγ9$^+$ TCR leading to Vγ9$^+$ T cell activation (14, 15). In some previous publications, Vγ9$^+$ T cells were shown to maintain functionality as cytotoxic effectors and cytokine producers in SSc and respond, albeit in a suppressed manner, to phosphoantigens, relative to healthy controls (HC) (5, 16). Other researchers, on the other hand, detected no significant difference between productivity of TNFα and IFNγ by γδ T cells in SSc patients and HC (17). Furthermore, intravenous treatment with zoledronate (zol), a potent ABP, adversely affected the clinical course in a SSc patient, suggesting that this reagent may have activated disease relevant pathogenic γδ T cells (18).

Indeed, the results presented in this article indicate for the first time, to our knowledge, that the functional programmes and activation of human Vδ1$^+$ γδ T cells *in vitro* can be modulated by CL. Furthermore, activation is dependent on the CD1d lipid-presenting molecule and co-stimulation with zol. Importantly, the responses of γδ T cells to these stimuli differ between SSc and HC in a manner that could adversely affect immune responses and the fibrotic process characteristic of this devastating disease.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board (Helsinki Committee) of the Sheba Medical Center, Ramat Gan, and Rambam Health Care Campus, Haifa, Israel. All patients and controls signed informed consent forms. Patients, described in **Table 1**, were treated in the Rheumatology Clinic at Sheba Medical Center in Ramat Gan, Israel, and at the B. Shine Rheumatology Unit at Rambam Health Care Campus in Haifa, Israel. All patients recruited for the study fulfilled criteria of the American College of Rheumatology for SSc (19). Controls included healthy donors from the hospital staff.

Reagents

OCH, an α-galactosylceramide analog with a truncated side chain that stimulates Th2-biased cytokine production in NKT, was obtained from the National Institutes of Health (NIH), USA, and stored at 0.2 mg/ml in an aqueous solution containing 0.5% Tween 20, 56 mg sucrose, and 7.5 mg histidine (20). CL (Sigma-Aldrich, Israel) was dissolved according to manufacturers' instructions (stock solution 5 mg/ml) in methanol and stored at −2°C. The monoclonal antibodies (mAbs) for blocking experiments were anti-CD1d (Biolegend, USA) or IgG2b isotype control (Miltenyi Biotec, Germany). The mAbs for staining cell surface antigens included anti-CD3 APC (Tonbo Bioscience), anti-CD25 PE (BD Pharmingen, USA), anti-Vδ1-FITC (Endogen, Pierce, USA), and anti-Vγ9-FITC (Immunotech, USA). zol (Novartis, USA) and interleukin-2 (IL-2; Boeheringer–Mannheim, Germany) were used at the indicated concentrations.

Isolation of PB Mononuclear Cells and Cell Culture

Peripheral blood mononuclear cells (PBMCs) were separated from 10 to 20 ml of heparinized blood on Lymphoprep gradients (AXIS-SHIEL, Oslo, Norway) by density centrifugation at 1,500 rpm as previously described (21). The mononuclear fraction was removed from the interface washed in phosphate-buffered saline (PBS) solution PH 7.4 and frozen in liquid nitrogen. Since our goal was to evaluate the effects of activation *in vitro*, and blood samples were relatively small, it was not always feasible to perform detailed cytometric analysis of freshly isolated cells. Thus, on the day of initiation of the experiments, freshly isolated PBMC of patients 1–12 in **Table 1** and HC, per well placed at 2×10^5 cells/well in 200 µl of final growth medium (FM) consisting of RPMI-1640 (Taassiot Biologiot, Beit Haemek Israell) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and penicillin–streptomycin solution (100 µg/ml) in 96-well round bottom culture plates. CL (2.5–10 µg/ml) or zol (2 µM) or IL-2

TABLE 1 | Clinical characteristics of systemic sclerosis patients.

Patient	Age	Sex	ANA	SCI70	ACA	Organ involvement	Disease duration (years)	Subset	Treatment
1	58	F	+	–	+	GI, muscle	5	Limited	MTX, iloprost, IVIG
2	55	F	NA	NA	NA	Joints	0.5	Limited	Iloprost, HCQ
3	41	F	+	+	NA	Joints	4	Diffuse	MTX, iloprost, MabThera
4	67	F	NA	+		Kidney, lung, APS, cardiac	9	Diffuse	Iloprost, bosentan, prednisone
5	48	F	NA	NA	NA	Lung, GI, joints	15	Limited	Iloprost, bosentan, HCQ
6	83	F	+	–	NA	Lung	6	Limited	MTX, prednisone
7	44	M	+	+		Lung, pulmonary HTN	16	Limited	CYC
8	61	F	+	NA	NA		23	Limited	Iloprost, tracleer, plaquenil
9	58	M	+	–		Pericardium, joints, lungs	1	Limited	NA
10	42	M	+	NA	NA		20	Linear	HCQ, prednisone
11	60	M	+	NA	NA	Lung, pulmonary HTN, GI, cardiac	8	Diffuse	CYX, MTX, prednisone, bosentan, CCB
12	64	M	+	+		Lung, kidney, heart, joints	3	Diffuse	Rituximab, leflunomide, SSZ
13	48	F	+	+		Lung, digital ulcers, GI, joints	4	Limited	Prednisone, omeprazole, CYC, iloprost, MTX, bosentan
14	57	F	+	+		GI, digital ulcers	15	Limited	Prednisone, HCQ, iloprost, bosentan
15	47	F	+	–	+	GI, trigeminal neuralgia	6	Limited	Omeprazole
16	68	F	+	–	+	Digital ulcers	25	Limited	CCB
17	44	F	+	+		Digital ulcers	7	Diffuse	MMF, bosentan
18	58	F	+	+		Lungs, joints, GI	11	Diffuse	MMF, bosentan, prednisone, HCQ, omeprazole, iloprost
19	54	F	+	+		Lungs, joints, GI, digital ulcers, acroosteolysis	30	Diffuse	AZA, bosentan, prednisone, HCQ, omeprazole, iloprost

ANA, antinuclear antibodies; APS, antiphospholipid syndrome; AZA, azathioprine; CCB, calcium channel blockers; CYC, cyclophosphamide; f, female; GI, gastrointestinal; HCQ, hydroxychloroquine; HTN, hypertension; IVIG, intravenous immunoglobulin; M, male; MTX, methotrexate; scl70, scleroderma 70 antibodies; SSZ, salazopyrin.

(100 IU/ml) or the mAb indicated above was added at the initiation of the cultures as indicated in individual experiments. After 4–5 days, cells were collected from the cultures, washed twice in PBS, and prepared for staining with mAbs for flow cytometry. In experiments involving analysis of cytokines, we used PBMC from patients 13–19 and corresponding HC samples that had been previously been frozen in liquid nitrogen.

Flow Cytometry

Prior to analysis, cells were removed from culture, stained with trypan blue, and determined to be >95% viable by trypan blue exclusion. Cell staining procedures were as previously described (21). Lymphocytes were gated according to typical forward and side scatter plots using a BD FACS Calibur flow cytometer (Becton Dickinson, Mountain View CA, USA), and analysis of gated cells was performed using FlowJo software. Cells within designated gates (e.g., $CD3^+$) were further analyzed. Percentage of cells expressing a marker (e.g., CD25) within the specific subset (e.g., $CD3^+$, or $V\gamma9^+$ or $V\delta1^+$) was calculated as follows: % dual positive cells/(% dual positive + % marker negative within subset); e.g., % $CD25^+V\gamma9^+$ within subset = $\%CD25^+V\gamma9^+/(\%CD25^+V\gamma9^+ + \%CD25^-V\gamma9^+)$.

Cytokines

For intracellular cytokine staining, freshly isolated PBMC were cultured overnight alone, with CL (2.5 μg/ml), zol (2 μM), or both or with 50 ng/ml PMA (Sigma-Aldrich) and 1 mM ionomycin (Sigma-Aldrich), in the presence of brefeldin A (Sigma-Aldrich). After stimulation, cells were stained with APC-labeled mAb to Vδ1 (Miltenyi) or PC5-labeled mAb to Vγ9 (Beckmann Immunotech) or APC-labeled anti-CD3 (Tongo). The cells were fixed and permeabilized using a Cytofix/Cytoperm kit (eBioscience) and then stained using a mixture of FITC-labeled anti-IFNγ (eBioscience) and PE-labeled anti-IL-4 mAb (eBioscience). Intracellular cytokine staining was analyzed by flow cytometry.

CD1 Tetramers Staining

CD1d tetramers bound to APC obtained from the NIH included: PBS-47 = a CD1d tetramer linked to an analog of α-galactosylceramide; $CD1d^-$ OCH = a CD1d tetramer linked to an α-galactosylceramide analog with a truncated side chain; CD1d unbound = tetramers not linked to an exogenous antigen.

Statistical Analysis

Results are expressed as mean ± 1 SEM. Statistical comparisons were drawn using a two-tailed Student's *t*-test except where indicated using Excel Microsoft, Redmond WA software. A p value <0.05 was considered as statistically significant.

RESULTS

Activation Status of γδ T Cell Subsets in Non-Stimulated Short-Term Cultures

γδT cells in SSc patients are highly activated *in vivo* to express HLA-DR (6, 22). We evaluated whether cell surface membrane expression of CD25, the IL-2 receptor α-chain, which is induced by TCR-mediated T cell activation, is likewise upregulated in SSc γδ T cells (23). Thus, we recorded, by flow cytometry, $\%V\delta1^+$ and $V\gamma9^+$ γδ T cells among the $CD3^+$ lymphocytes in PBMC derived from SSc patients and HC and the percentage of $CD25^+$ T cells within each subset, after brief *in vitro* culture in medium

containing a low dose of IL-2 (100 IU/ml, FMIL-2). There were non-significant increases of %Vδ1$^+$ and Vγ9$^+$ T cells within the CD3$^+$ population in cultures of PBMC derived from SSc patients ($n = 12$) relative to HC ($n = 8$) (**Figure 1A**). However, there was a significant increase of %CD25$^+$ Vδ1$^+$ and Vγ9$^+$ T cells relative to the respective complementary Vδ1$^-$ or Vγ9$^-$ CD3$^+$ populations in both SSc and HC cultures (**Figure 1B**). Moreover, % CD25$^+$ within total CD3$^+$ and also within the Vδ1$^+$, but not Vγ9$^+$ T cell populations, were significantly more highly represented in SSc than HC PBMC cultures (**Figure 1B**). The gating procedure

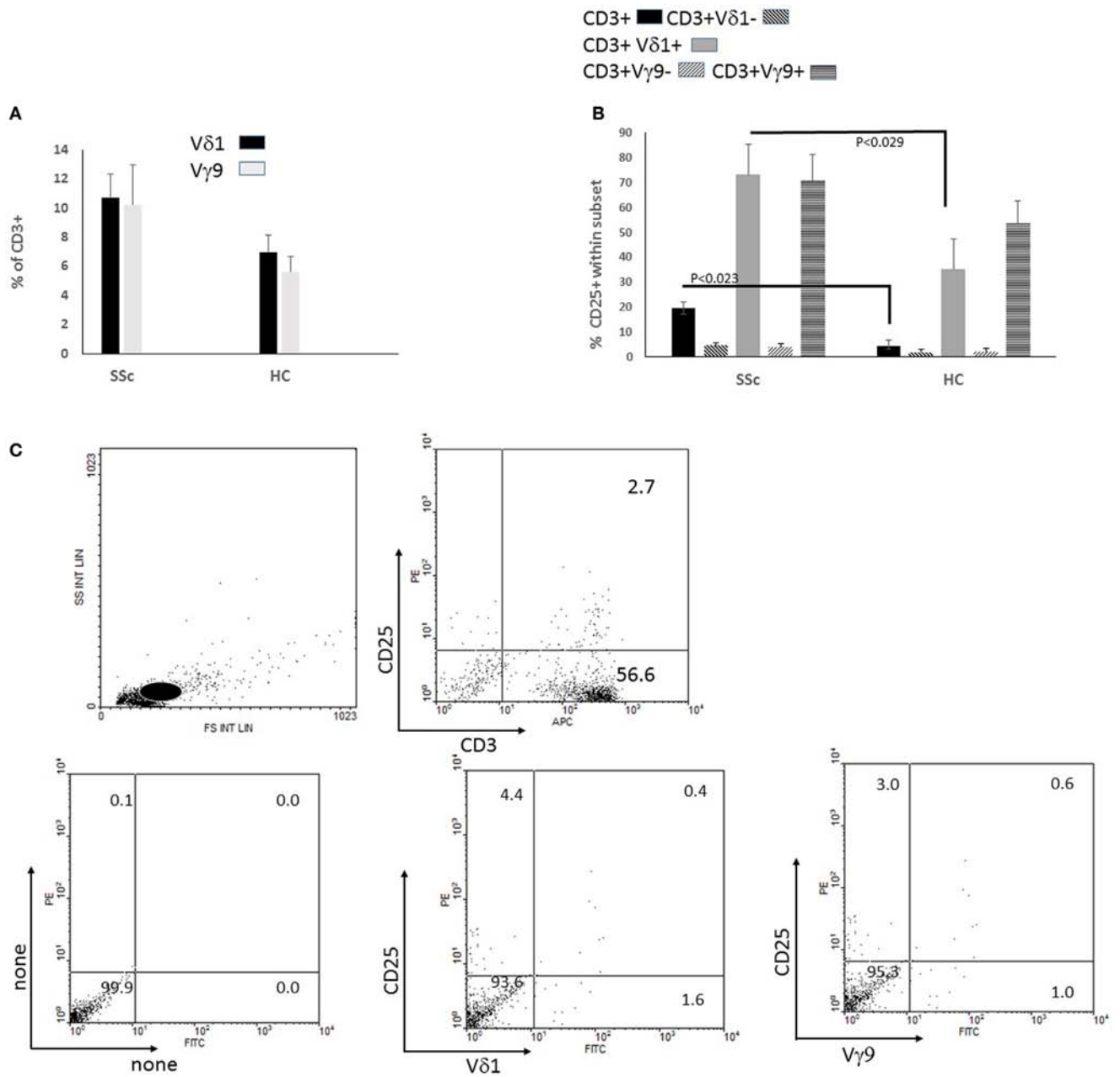

FIGURE 1 | Percent of CD25$^+$ cells in cultures of peripheral blood mononuclear cells (PBMCs) from patients with systemic sclerosis (SSc) vs healthy controls (HCs). PBMCs of 12 SSc patients (patients 1–12 in **Table 1**) and 8 HCs were incubated in medium alone for 4 days and then stained for flow cytometric analysis with fluorochrome-labeled monoclonal antibodies (mAbs) anti-CD25-PE, anti-Vδ1-FITC, anti-Vγ9-FITC, and anti-CD3-APC. **(A)** Data for the anti-Vδ1-FITC, anti-Vγ9-FITC staining of T cells in the cultures obtained by gating on CD3$^+$ cells as shown in **(C)**. **(B)** Results for percent of cells within each of indicated subsets that were stained with anti-CD25-PE in **(B)** calculated as indicated in Section "Materials and Methods." Bars represent the mean and SEMs. Significant differences between means by two-tailed Student *t*-test are indicated. **(C)** Dot plots represent on the upper left forward and side scattergram. The Lymphocyte gate is indicated with a white circle. On the upper right, dot plots of cells within the lymphocyte gate were stained with mAb to CD3 and CD25. The lower panel shows dot plots of cells within the gated CD3$^+$ population unstained on the left, or with mAb to Vδ1 or Vγ9 bound to FITC, and CD25-PE as indicated.

for these analyses and an example of such analysis are shown in **Figure 1C**. These results suggested that T cells, consisting chiefly of αβ T cells, and the Vδ1$^+$ T cell subset in particular maintain an abnormally highly activated status as reflected by CD25 expression after brief *in vitro* culture of SSc PBMC in the presence of IL-2.

Effect of CL and zol on CD25 Expression on γδ T Cells Subsets in HC and SSc

Next, we evaluated how CL, which is a potential ligand for γδ and NKT cells, and zol, which activates Vγ9$^+$ γδ T cells, influence %CD25$^+$ T cells in the cultures (24–26). PBMCs were cultured in FMIL-2 or in FMIL-2 supplemented with zol (FMIL-2 zol), with or without CL, and expression of CD25 on subsets of T cells was evaluated by flow cytometry as indicated in an example using PBMC of a HC (**Figure 2A**). Neither CL (2.5 μg/ml) nor zol (2 μM) altered mean %CD25$^+$CD3$^+$ T cells in PBMC cultures of SSc patients ($n = 10$), whereas zol, but not CL, induced an increase of this subset in cultures of HC ($n = 8$) from 40.1 ± 12.1% to 71.6 ± 7.8% ($p < 0.043$; **Figure 2B**). In contrast, zol decreased the mean frequency of CD25$^+$ cells in Vδ1$^+$ T cells, and CL reduced it, in Vγ9$^+$ T cells of SSc but not HC PBMC cultures. Furthermore, culture with combined CL + zol increased both mean %CD25$^+$ Vδ1$^+$ and Vγ9$^+$ T cells in HC cultures significantly (**Figure 2B**). In cultures of SSc PBMC, however, CL + zol only partially abrogated the significant decrease induced by each reagent separately (**Figure 2B**).

We next examined the dose response of %CD25$^+$ γδ T cell subsets in the cultures to increasing concentrations of CL (**Figure 3**). In HC PBMC cultured in FMIL-2, CL decreased mean %CD25$^+$ Vδ1$^+$ and Vγ9$^+$ T cells non-significantly in a dose-dependent manner. In contrast, when added to FMIL-2 zol, it increased mean %CD25$^+$ Vδ1$^+$ T cells significantly and Vγ9$^+$ T cells non-significantly. In SSc PBMC cultured in FMIL-2, % CD25$^+$Vδ1$^+$ TC increased slightly in the presence of 2.5 μg/ml of CL followed by a decrease at higher concentrations (**Figure 3**), along with a dose-dependent significant decrease of %CD25$^+$Vγ9$^+$ T cells. In FMIL-2 zol, %CD25$^+$Vδ1$^+$ and Vγ9$^+$ T cells of SSc patients, respectively, increased or decreased in a CL dose-dependent manner, but in contrast to HC, the increase in the %CD25$^+$Vδ1$^+$ subset was not statistically significant. Taken together, these results suggest that, in the presence of zol and IL-2, CL induces the expression of CD25 on healthy human Vδ1$^+$ T cells, whereas the response in SSc is blunted.

CD1d Tetramer Binding to SSc and HC Vδ1$^+$ T Cells

We first determined whether Vδ1$^+$ T cells of SSc patients can bind the tetramers of CD1d described in the Section "Materials and Methods." Briefly cultured PBMC of SSc patients and HC were co-stained with mAb to Vδ1 and with fluorescently labeled native CD1d tetramers (CD1d unbound), CD1d tetramer complexed with a natural ligand, alpha galactosylceramide (PBS-47), or with an OCH derivative of this compound (CD1d-OCH) (27). By gating on lymphocytes, in a preliminary experiment as shown in the upper panel of **Figure 4A**, we ascertained the ability of the native CD1d tetramer to clearly detect cells within either Vδ1$^+$ or Vδ1$^-$ as well as on CD8$^+$ and CD8$^-$ cells in these brief cultures. In a further representative experiment, shown in the middle and lower panels of **Figure 4A**, 8–12% of the cells in HC cultures and 3–4% in an SSc patient-derived culture expressed Vδ1. Unbound, PBS-47 and CD1d-OCH stained both Vδ1$^+$ T cells as well as Vδ1$^-$ T cells in the cultures with the frequencies (relative to total cells in the cultures) indicated in each dot plot. Importantly, binding of all types of the tetramers was observed, even those not chemically attached to known lipids (CD1d unbound). Since CL binds to CD1d and is presented to human and murine iNKT cells and to murine γδ T cells (24, 25), it is possible that unbound CD1d tetramer, which is presumed to be loaded with lipid antigens endogenously expressed by the cells in which it is produced, includes a small proportion of CL-loaded CD1d tetramers.

Similar experiments of short-term PBMC cultures revealed that a mean of 21.2, 37.3, and 45.6% ($n = 5$, SSc) vs 55.5, 58.1, and 54.2% ($n = 2$, HC) of the Vδ1$^+$ T cells, respectively, were co-stained by PBS-47, CD1d unbound, or CD1d-OCH tetramers (**Figure 4B**). Importantly, and as expected, due to known binding of CD1d to CD4$^+$ and other NKT cells, we also detected a significant level of expression of CD1d tetramer-positive cells in the Vδ1$^-$ populations of lymphocytes in the cultures, which included CD4$^+$ and CD8$^+$ T cells (**Figure 4A** and data not shown for CD4). In additional experiments, only inconsistent binding to Vγ9$^+$ T cells was noted (not shown). However, an enhanced potential of briefly cultured Vδ1$^+$ T cells of both SSc and HC to bind CD1d tetramers relative to Vδ1$^-$ cells in the cultures was evident ($p < 0.043$, $p < 0.042$, and $p < 0.082$ for the respective tetramers for SSc patient; **Figure 4B**). These results suggested that the TCR of Vδ1$^+$ T cells in SSc, similar to that of HC, bear the potential to recognize lipid antigens and possibly autoantigens presented by CD1d.

Role of CD1d in Activation of SSc Vδ1$^+$ T Cells in the Presence of CL and zol

We then asked whether the observed decrease of %CD25$^+$ Vδ1$^+$ T cells in SSc-derived PBMC cultures in the presence of zol could be related to an effect of zol that leads to decreased binding of CD1d lipid antigen-presenting elements to the Vδ1$^+$ T cells in SSc cultures. PBMCs from two SSc patients were cultured in FMIL-2 or FMIL-2 zol and after five days stained with CD1d tetramers. We detected decreased binding of the different CD1d tetramers to Vδ1$^+$, but not to Vδ1$^-$ T cells that had been cultured in FMIL-2 zol relative to those cultured in FMIL-2 alone (**Figure 5A**). Since CD25 expression is induced on T cells as a response to TCR binding of antigen-presenting molecules, these results suggest that zol-induced reduction of CD25 may be due to zol-mediated downregulation of CD1d binding to the Vδ1$^+$ γδ TCR.

To confirm that CD1d-mediated interactions plays a role in activation of SSc Vδ1$^+$, we tested the ability of mAb to CD1d to reverse the low level activation of %CD25$^+$ Vδ1$^+$ T cells induced by CL in the presence of zol (**Figure 3**). PBMC of SSc patients ($n = 5$) were cultured in medium containing zol with or without added 2.5 μg/ml CL, in the presence of either an irrelevant

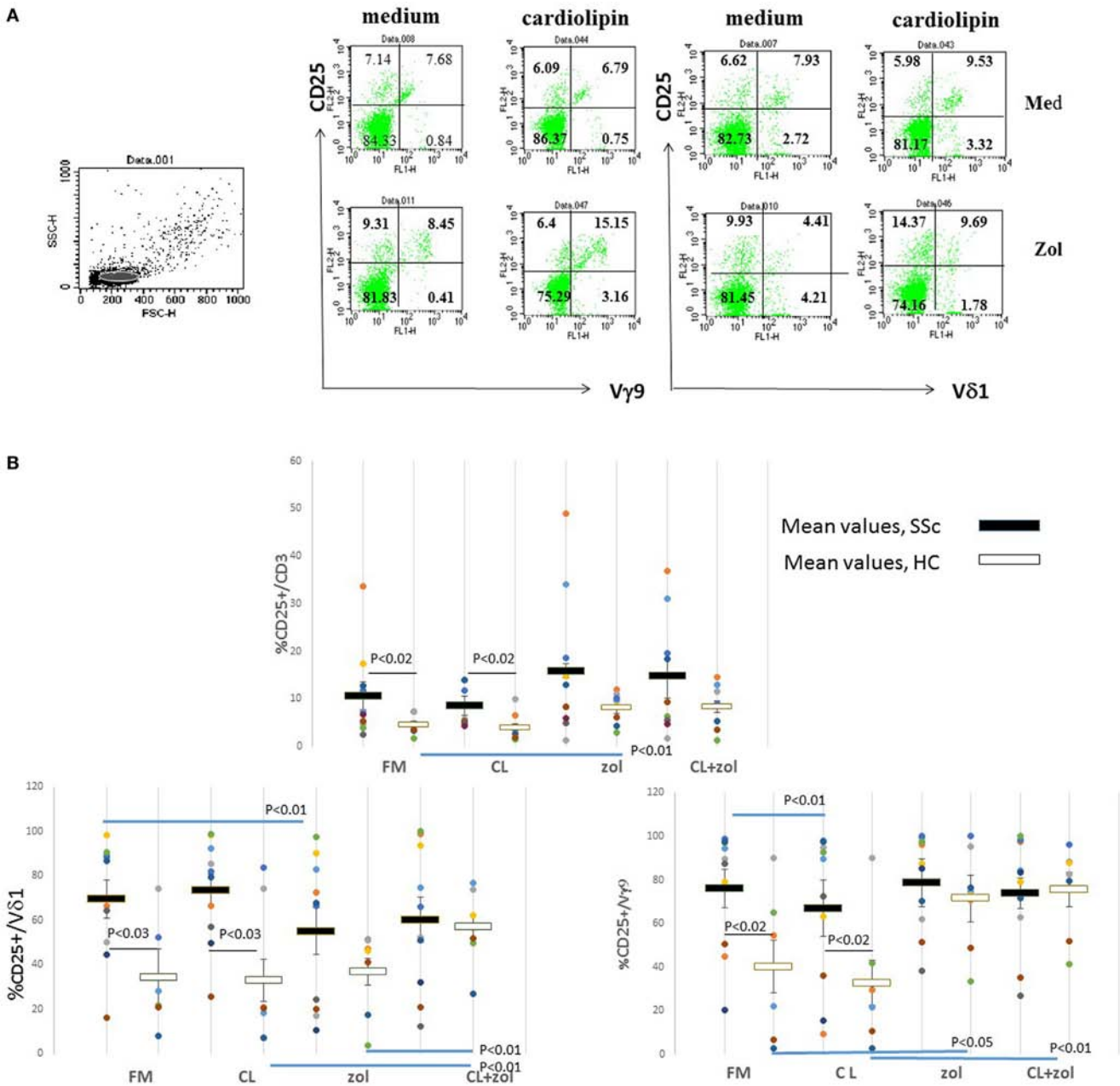

FIGURE 2 | CD25 expression on γδ T cells in response to cardiolipin (CL) and zoledronate (zol). **(A)** Peripheral blood mononuclear cells (PBMCs) from a healthy control (HC) were incubated in medium with interleukin-2 (100 IU) alone or with added CL (2.5 μg/ml), either with or without zol (2 μM) for 4 days and analyzed by flow cytometry after staining with fluorochrome-labeled monoclonal antibodies (mAbs) anti-CD25-PE, anti-Vδ1-FITC, anti-Vγ9-FITC, and anti-CD3-APC. Forward and side scatter of the sampled cells are shown on the left; the lymphocyte population is highlighted in gray. On the right are shown dot plots of cells within total $CD3^+$ population, representing both the $CD3^+V\delta1^-$ (containing most $V\gamma9^+$ T cells) and $CD3^+V\delta1^+$ populations after co-staining with mAb to CD25 and either Vδ1 or Vγ9. Percent of cells within indicated quadrants (set on the basis of non-stained cells) are shown. **(B)** PBMC from 10 systemic sclerosis (SSc; patients 1–10 **Table 1**) and 8 HC were cultured as mentioned in **(A)** and stained with mAbs to CD25, CD3, Vδ1, and Vγ9. Dots represent values for each culture of an individual. The mean percent and 1 SEM of $CD3^+$ T cells, or of the indicated subsets, expressing CD25 in each of the culture conditions are indicated (medium with IL-2 = FM; medium with CL 2.5 μg/ml with IL-2 = CL; medium with IL-2 and zol 2 μM = z; zol with CL 2.5 μg/ml = CL + zol. Black horizontal bars represent mean for SSc and white for HC. *P* values calculated from two-tailed Student's *t*-test comparing groups connected by horizontal lines are indicated.

isotype control mAb or a blocking mAb to CD1d. As illustrated in **Figure 5B**, in the presence of the isotype control mAb, $\%CD25^+V\delta1^+$ T cells increased 25% in CL-containing cultures compared to cultures without CL. In contrast, in the presence of mAb to CD1d, $\%CD25^+V\delta1^+$ T cells decreased 9% relative to cultures without CL ($p < 0.0502$). These results suggest that

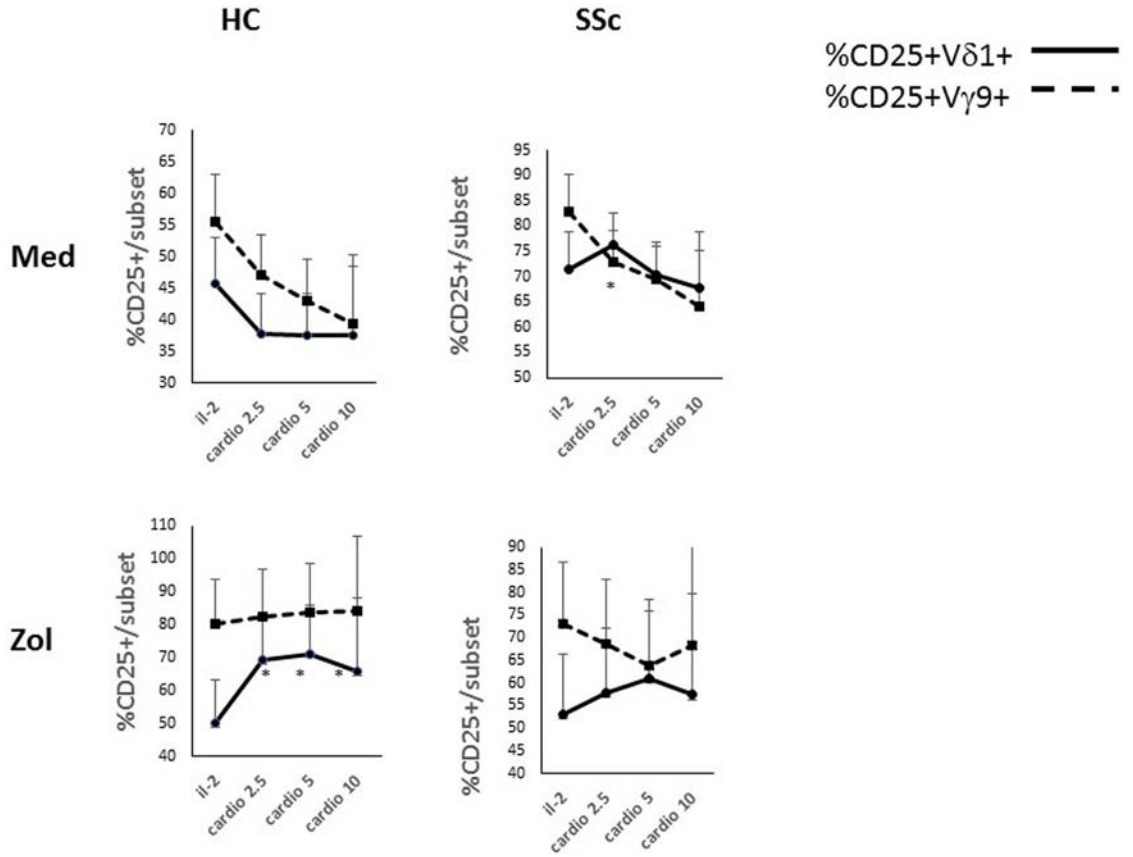

FIGURE 3 | Dose effects of cardiolipin (CL) on cultured T cells in the presence and absence of zoledronate (zol). Dose response of % CD25$^+$ Vδ1$^+$ or Vγ9$^+$ T cells as indicated (%CD25$^+$ within a subset/total% of subset) after *in vitro* culture for 4 days in medium containing interleukin-2 (IL-2) 100 IU or, in addition, increasing concentration of CL (in microgram per milliliter) as indicated on the *x*-axis are shown. In parallel cultures, zol (2 μM) was added. Results represent mean and 1 SEM for cultures derived from three HCs and from five systemic sclerosis patients. Asterisks indicate a significant difference ($p < 0.05$) for comparison of the mean at the relevant concentration of CL to IL-2 alone.

CL is recognized by CD1d, which may lead to enhancement of %CD25$^+$Vδ1$^+$ T cells.

IFNγ and IL-4 Production by SSc and HC Vδ1$^+$, Vγ9$^+$, and Total T Cells

IFNγ and IL-4, respectively, oppose or support fibrosis, a central pathological feature of SSc. We previously reported decreased expression of IFNγ by SSc relative to HC Vγ9$^+$ γδ T cells in response to zol, and also secretion of IL-4 by Vδ1$^+$ T cells that was induced by zol in an SSc patient (18, 20). Here, we analyzed by flow cytometry (see Materials and Methods) how CL and zol affect intracellular cytokine production by the T cells from SSc patients and HC, after brief *in vitro* activation. The mean % IL-4$^+$ cells among CD3$^+$, Vγ9$^+$, and Vδ1$^+$ T cells in PBMC of SSc ($n = 7$, patients 13–19 in **Table 1**) and HC ($n = 5$) in the presence of non-TCR-dependent stimulation (PMA and ionomycin) were virtually identical (respectively, 28.2 ± 22.2 vs 35.0 ± 8.22, $p < 0.75$, 34.0 ± 9.18 vs 32.1 ± 9.18, $p < 0.49$, 15.6 ± 4.7 vs 16.5 ± 8.7, $p < 0.91$). In contrast, the frequency of IFNγ-secreting cells trended to be uniformly yet non-significantly lower in SSc (28.4 ± 6.97 vs 48.96 ± 9.05, $p < 0.09$, 47.8 ± 11.37 vs 75.9 ± 2.34, $p < 0.083$, 40.4 ± 9.62 vs 71.9 ± 9.68, $p < 0.058$). Contrasting with the non-significant differences in the presence of PMA + ionomycin, % IFNγ$^+$ CD3$^+$ T cells were significantly lower in SSc PBMC in medium alone or in the presence of both CL and zol (**Figure 6**). %IFNγ$^+$ Vγ9$^+$ T cells were uniformly and significantly reduced in SSc PBMC in all culture conditions, including medium alone and medium with CL, zol, or both reagents. In contrast, %IFNγ$^+$ Vδ1$^+$ T cells in SSc PBMC was lower in medium and zol than in HC but significantly only in the presence of CL or CL + zol. On the other hand, while there was no significant difference in % IL-4-secreting cells among CD3$^+$ and Vδ1$^+$ T cells of SSc and HC in any of the culture conditions, % IL-4 $^+$Vγ9$^+$ T cells in SSc cultures were significantly increased relative to HC but only in the presence of CL.

Taken together, these results indicate that T cells in SSc retain a reduced potential to secrete the antifibrotic IFNγ. Importantly, the Vγ9$^+$ T cell subset appeared to be most highly significantly suppressed in its ability to secrete IFNγ relative to HC, whereas the lower production of this cytokine in the Vδ1$^+$ T cell subset of SSc patients strictly required CL. Moreover, CL increased

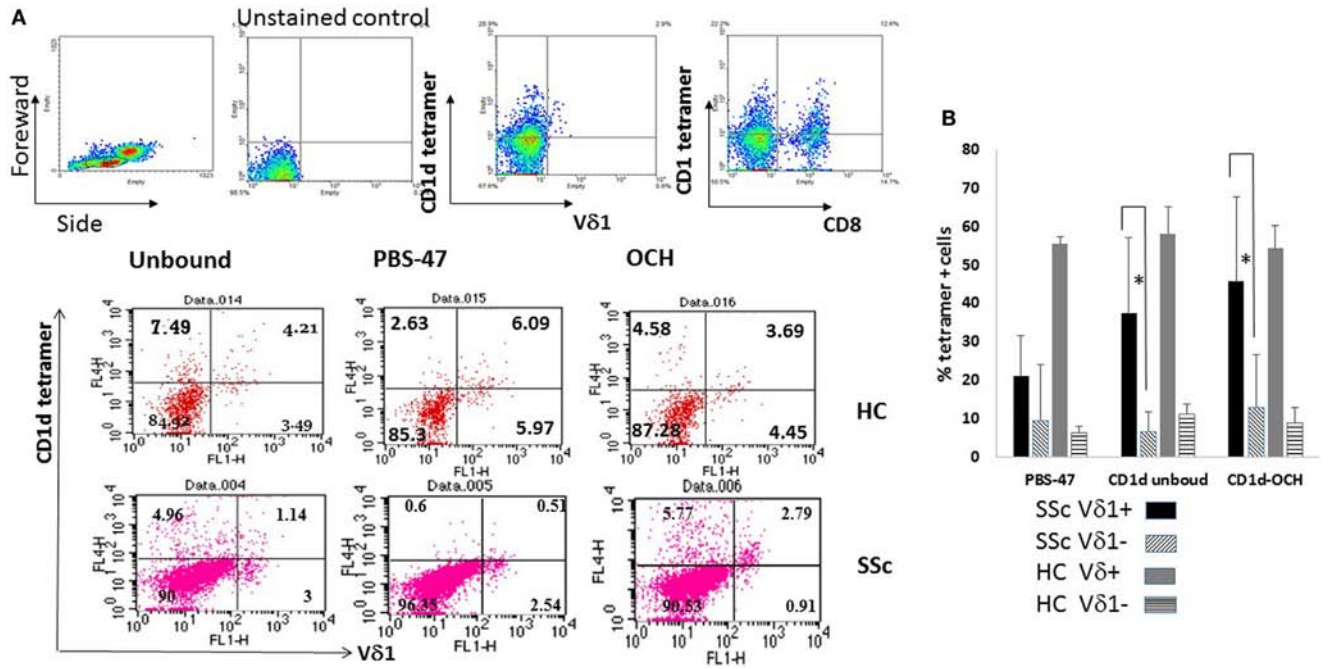

FIGURE 4 | CD1d tetramer binding to cultured Vδ1$^+$ T cells in systemic sclerosis (SSc) patients and healthy controls (HCs). **(A)** The upper panel shows gating strategy for analysis of CD1d tetramer binding using HC peripheral blood mononuclear cells (PBMCs) cultured in FM containing interleukin-2 (IL-2) 100 IU/ml for 6 days. Cells selected from the lymphocyte gate as indicated in the Forward/Side scattergram on the left were analyzed untouched or after staining with the CD1d unbound tetramer and monoclonal antibody (mAb) to either Vδ1 or CD8. The second and third panels represent flow cytometric dot plots of PBMC of a HC and SSc patient (patient 1; **Table 1**) cultured in FM containing IL-2 100 IU/ml for 6 days. The cells were stained with APC labeled CD1d tetramers (unbound, PBS-47 and bound to OCH) as indicated above the dot plots and Vδ1-FITC. Percent cells in quadrants set according to a non-stained control are shown. **(B)** Bars represent mean and 1 SEM of %Vδ1$^+$ and Vδ1$^-$ cultured cells binding the indicated tetramers in cultures as described in **Figure 4A** for five SSc patients and two HCs. * denotes $p < 0.05$ for adjacent indicated means.

secretion of the profibrotic IL-4 cytokine by the Vγ9$^+$ T cell subset in SSc relative to HC. Together these results suggest that exposure to CL may induce a profibrotic phenotype within the γδ T cell subset in SSc.

DISCUSSION

Dysregulated T cell functions are thought to contribute to the profibrotic phenotype in SSc (28). Here, we focused on the role of a self-lipid antigen, CL, a putative autoantigen, and of an inducer of phospho-antigens (zol), in the regulation of activation and cytokine secretion by γδ T cells in SSc. Despite the small sample number of patients studied and large individual variability, our results revealed a statistically significant *a priori* heightened state of activation of Vδ1$^+$ γδ T cells in SSc manifested by increased expression of CD25 in short-term cultures absent of any additional external antigenic stimulation. Next, we found that CL exerted several statistically significant effects, including:

a. Decreased CD25 expression of the Vγ9$^+$ T cells of SSc patients but not of HC.
b. In conjunction with co-stimulation by a Vγ9$^+$ T cell activator, zol, significant enhancement of CD25 expression in HC and, to a lesser degree, in SSc Vδ1$^+$ T cells.
c. Decreased secretion of IFNγ by SSc Vδ1$^+$ T cells relative to HC while inducing an increase, relative to HC, in the secretion of IL-4 by patient Vγ9$^+$ T cells.

Furthermore, although we have not directly shown binding of a CL-bound CD1d tetramer to SSc Vδ1$^+$ T cells, the involvement of CD1d lipid-presenting molecules in CL-induced SSc-specific perturbations of γδ T cell subset activation was suggested by the finding that CL + zol activation of SSc Vδ1$^+$ T cells was inhibited by a mAb to CD1d. Together, these *in vitro* findings suggest that CL could contribute to the immune dysregulation in SSc in a direction that could enhance fibrosis by cytokine dysregulation. In addition, cross-interactions between CL activated SSc Vδ1$^+$ T cells and phosphoantigen activated Vγ9$^+$ T cells may cross-modulate activation of the respective subsets.

CD25 is a marker of the state activation of T cell subsets induced by interactions of the TCR with its ligands (23). An increased CD25 expression on Vδ1$^+$ T cells in short-term cultures of SSc (relative to HC) in the absence of any additional triggers other than low-dose IL-2, suggests that enhanced activation of these cells *in vivo* as previously demonstrated by Giacomelli et al., which is presumably triggered by autoantigens, is maintained *in vitro* (**Figure 1B**) (6). Since a subset of Vδ1$^+$ T cells can recognize lipids, these results suggest that an abnormal response to autoantigenic or foreign lipids could be

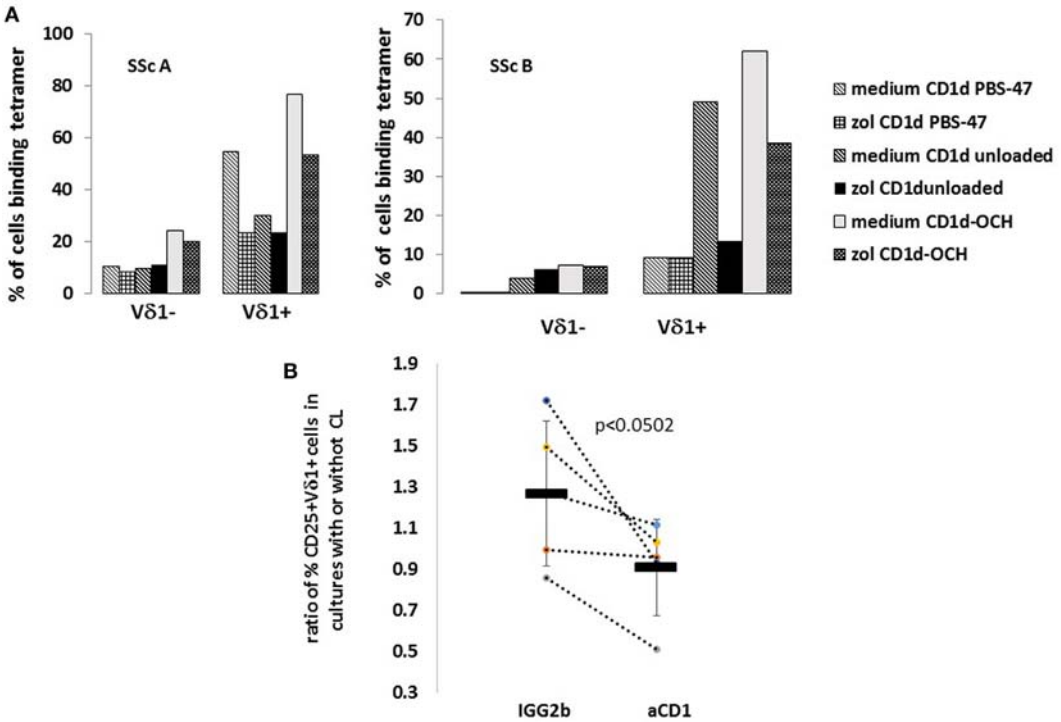

FIGURE 5 | CD1d involvement in activation of Vδ1⁺ T cells by zoledronate (zol) and cardiolipin (CL) in systemic sclerosis (SSc) patients and healthy controls (HCs). **(A)** Peripheral blood mononuclear cells (PBMCs) from two SSc patients (SScA and SScB, patients 11 and 12; **Table 1**) were cultured in FMIL-2 alone (medium), or with 2 μM zol for 3 days, then co-stained with indicated antigen-presenting cell-labeled tetramers (CD1d unbound, CD1d PBS-47, and CD1d-OCH) and monoclonal antibody (mAb) to Vδ1-FITC, as described in figure. Bars show percent of cells binding the specific tetramers indicated in the legend within Vδ1⁻ and Vδ1⁺ cells in the cultures. **(B)** PBMC of five SSc patients (patients 8–12; **Table 1**) were cultured for 4 days with zol (2 μM) with or without CL (2.5 μg/ml), in the presence of a control non-binding mAb IgG2b or a mAb to CD1d. Subsequently, the expression of CD25 on the Vδ1⁺ T cells was assayed by flow cytometry and the %CD25⁺Vδ1⁺ calculated. The y axis represents the ratio of %CD25/Vδ1⁺ T cells in cultures with and without CL. Each dot represents an individual patient; results in the presence of the different mAb are connected by dotted lines. Means of values in the presence of control mAb and anti-CD1d are represented by horizontal bars; 1 SEM is indicated by vertical bars. *p* Value comparing means (Student's one-tailed *t*-test) is indicated.

involved in the increased level of activation found in SSc Vδ1⁺ T cells. The autoantigen we chose to study in this context, CL, is known to elicit a humoral immune response during infections, including hepatitis, HIV, *Treponema pallidum*, and *Coxiella burnetii* as well as in a variety of autoimmune diseases (29–31). More specifically, in localized SSc, antibodies to CL are highly prevalent, suggesting a link to fibrotic processes in this disease even in the absence of other systemic features (11). Abnormal levels and structure of CL play a role in idiopathic pulmonary fibrosis, as well as in bleomycin-induced fibrosis, which is often used as a murine model for SSc (32). Thus, lysocardiolipin acyltransferase, a CL-remodeling enzyme, protects against lung fibrosis induced by bleomycin, suggesting a critical role for CL in the fibrotic process. This is thought to be related to correction of bleomycin-induced decrease of the CL level and of the unsaturated to saturated fatty acid ratio and unsaturation index in the CL. These findings thus suggest a pivotal pathogenic role for CL in SSc (32).

Moreover, CL reactive γδ T cells have been described in the T cell repertoire of rodents, suggesting that such cells might exist in the human γδ compartment as well (26). Although it is known that CL binds to human CD1d tetramers that are recognized by the TCR of human Vδ1⁺ T cells and NKT cells, the data presented here are the first to provide evidence that this autoantigen indeed plays a role in human Vδ1⁺ T cell activation (24). Moreover, our data suggest, for the first time, that CL elicits different responses in HC and in SSc γδ T cells cultured *in vitro*.

Interestingly, we found that the enhancement of the percentage of HC Vδ1⁺ T cells expressing CD25 induced by CL was dependent on co-stimulation of PBMC cultures with zol. These results suggest that CL activation of Vδ1⁺ T cells *in vitro* requires simultaneous signals that can be supplied by phosphoantigen-triggered Vγ9⁺ T cells (**Figures 2**, **3** and **7**). Zolendronate is taken up by monocytes, increasing production of intracellular phosphoantigens, mainly IPP, in the mevalonate pathway by inhibition of farnesyl pyrophosphate synthase (33). IPP binds intracellularly to butyrophilin 3A1 (CD277) expressed on APCs, which triggers a cognitive TCR-mediated activation of the Vγ9⁺ γδ T cells to secrete cytokines, express activation markers, and also, remarkably, to differentiate into APCs (14, 33–35). A recent study demonstrated that this activation may also result in the

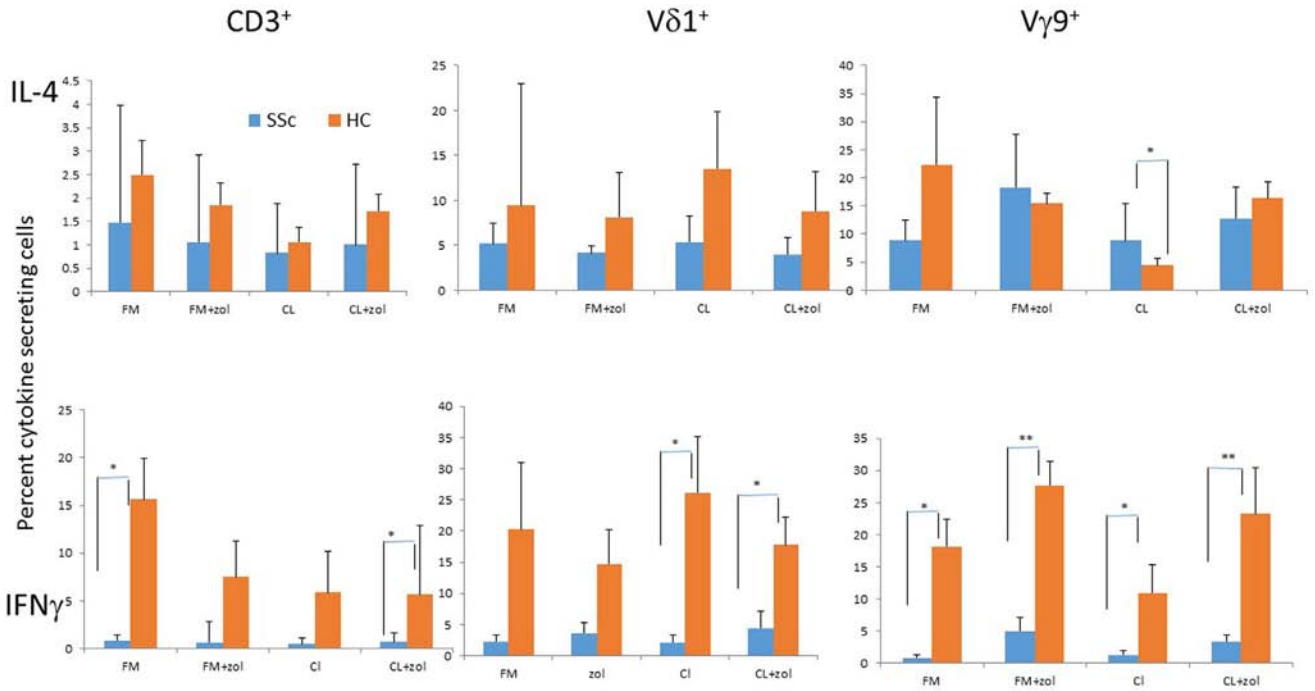

FIGURE 6 | Cytokine production by systemic sclerosis (SSc) patients and healthy controls (HCs) T cells. Peripheral blood mononuclear cells from seven SSc patients (patients 13–19; **Table 1**) and five HC were cultured overnight in medium alone (FM), cardiolipin (CL), zoledronate (zol), or both (FM + zl or CL + zol). Subsequently, cell surface membranes were stained with monoclonal antibody (mAb) to CD3, Vγ9, or Vδ1, and cells were stained intracellularly with mAb to IL-4 (upper panel) or IFNγ. Bars indicate the mean ± 1 SEM of %cytokine+ cells for the subsets as indicated. *$p < 0.05$ and **$p < 0.005$ for comparison of adjacent SSc and HC mean values, respectively.

expression of CD1d on the cell surface membrane of the Vγ9+ T cells *via* trogocytosis of CD1d-expressing B cell and activated macrophage membranes (36). Thus, the "helper" role of zol in terms of activation of the Vδ1+ T cell subset of HC in the presence of CL could be related to its effects on cytokines, induction of co-stimulatory molecules on APC expressing CD1d, or *de novo* CD1d-dependent presentation of CL by the Vγ9+ T cells themselves. To resolve the critical question of cross-regulation of the γδ T cell subsets and how APC and other CL-responsive cells may be involved, future experiments using isolated populations and studies to investigate the role of direct cell contact as opposed to that of soluble mediators are required. Nevertheless, irrespective of the mechanisms involved, we postulate that the *a priori* elevated expression of CD25 on SSc Vδ1+ T cells *ex vivo* (**Figure 1**) may have been elicited *in vivo* by exposure to CL in the presence of activated Vγ9+ T cells (**Figure 7**).

Plausible scenarios leading to activated SSc Vδ1+ T cells *in vivo* include viral infections, intracellular bacterial infections, and malignant transformation, all of which are associated with overproduction of IPP (37). Vγ9+ T cells activated by IPP induce apoptosis of the affected cells, exposing CL on the surface membrane, which may be taken up by Vγ9+ T cells and dendritic cells for CD1d-mediated presentation to responding iNKT and Vδ1+ T cells (5, 36, 38). Since CL enhanced CD25 expression by Vδ1+ T cells in SSc during zol co-stimulation was inhibited by a blocking mAb to CD1d, we suggest that activation of SSc Vδ1+ T cells in the presence of co-stimulation with zol is dependent on Vδ1+ TCR interactions with CD1d on the APC.

It is noteworthy that %CD25+ Vδ1+ T cells of SSc patients decreased in the presence of co-stimulation with zol in the absence of CL, suggesting that, in the absence of the autoantigen CL, SSc IPP-activated Vγ9+ T cells subset may suppress Vδ1+ T cell activation. Interestingly in this regard, whereas the direct IPP Vγ9+ T cell stimulator can quite efficiently activate SSc Vγ9+ T cells, zol is quite inefficient in this regard, consistent with our previous report (4, 5) Our data suggest that zol-induced decrease of CD25 expression might involve decreased binding of CD1d (presumably *via* their TCR) to the Vδ1+ T cells, by an unknown underlying mechanism (**Figure 5**). Conversely, Vγ9+ T cell activation manifested by CD25 expression was impeded by CL in the absence of zol, in SSc specifically. CL inhibition of Vγ9+ T cells in SSc could be mediated by Vδ1+ CD1d-CL complex reactive cells, since their partial inhibition in the presence of both CL and zol was reversed by anti-CD1d (data not shown). One possibility is that decreased CD25 expression entails alterations of cytokine secretion since CL, even in the absence of zol, decreased IFNγ in Vδ1+ T cells and enhanced IL-4 in Vγ9+ T cells relative to what is found in HC (**Figure 6**). Further studies of how interactions between activated γδ T cell subsets in HC and SSc are regulated by antigens and autoantigens could shed new light on dysregulated immune responses in SSc. It is important to note, however, that our patient population was not

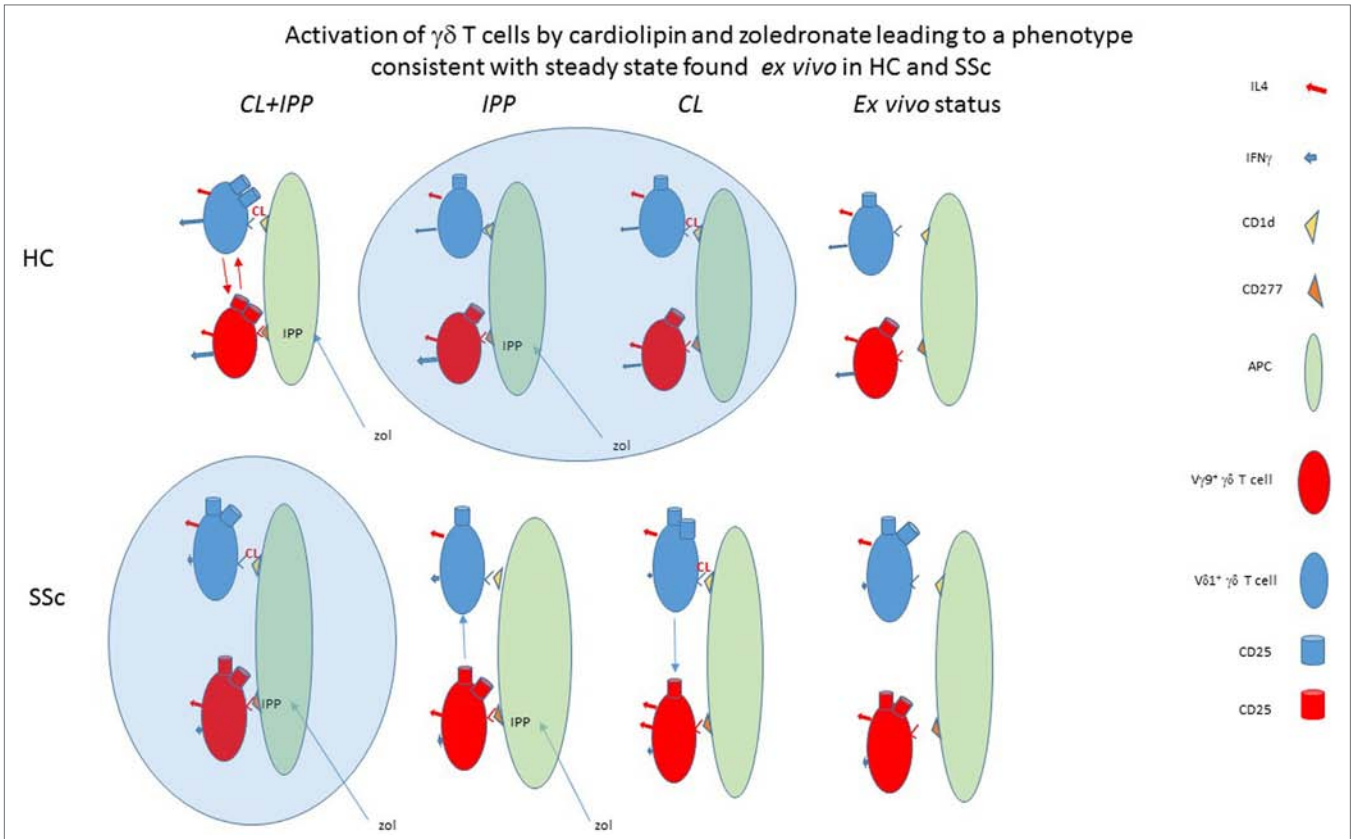

FIGURE 7 | Model of differential cardiolipin (CL) and zoledronate (zol) effects in systemic sclerosis (SSc) patients and healthy controls (HC). The cartoon shows Vδ1⁺ or Vγ9⁺ T cells interacting with an APC bearing CD1d and CD277, in the presence or absence of CL and/or isopentenyl pyrophosphate (IPP) induced by zol, respectively, presented by CD1d or CD277. Response of the antigen-triggered γδ T cells to antigen stimulation is reflected by expression of CD25 and release of cytokines. Magnitude of response is represented by the number of CD25 symbols or size of the cytokine arrow, using HC as the standard. The phenotype of *ex vivo* cultured cells without any stimulation is depicted on the right as indicated. As seen, the *ex vivo* non-stimulated phenotype is consistent with concurrent dual stimulation (CL and zol) of the Vδ1⁺ and Vγ9⁺ T cells in SSc and with each separately in HC.

homogeneous, and most were treated with immunosuppressive drugs, which might have affected the outcome of these experiments (**Table 1**). Interestingly in this regard, our previous study failed to reveal any consistent effect of a potent immunosuppressive agent, cyclophosphamide, on percentages of Vγ9⁺ or Vδ1⁺ T cells among CD3⁺ T cells in a small number of SSc patients (4, 5). Furthermore, our data presented in **Figure 1** show that both CD3⁺ and Vδ1⁺ and, to a lesser extent, Vγ9⁺ T cells showed an increased, rather than decreased, proportion of CD25⁺ T cells in the absence of antigenic stimulation. Thus, it is unlikely that the immunosuppressant reagents used in the patients might have significantly affected the results of antigen stimulation *in vitro*. Nevertheless, further experiments of untreated patients will be necessary to rigorously exclude the effects of medications used in SSc on the γδ T cell response to zol and CL.

A recent report indicated that CD161⁺ Vδ1⁺ T cells secreting diminished amounts of the antifibrotic cytokine IFNγ are expanded in SSc patients (39). Our results suggest that exposure to a lipid autoantigen such as CL plays an active role in suppression of IFNγ production in SSc Vδ1⁺ T cells (**Figure 6**). In addition, in the experimental conditions used in this article, Vγ9⁺ T cells of SSc patients also produced less IFNγ than HC, consistent with our previous independent experiments, and this relative decrease was maintained in the presence of CL (16). On the other hand, although IL-4 was not significantly different in SSc and HC T cells and was not suppressed by CL or zol in SSc relative to HC, % IL-4 secreting Vγ9⁺ T cells surprisingly increased (relative to HC) in the presence of CL. The mechanism underlying this shift is not clear, but could hypothetically also be mediated by CL reactive Vδ1⁺ T cells as postulated in the cartoon, as a consequence of the reduction of IFNγ induced in these cells by CL (**Figure 7**). In all, the abnormal responses of the γδ T cell subset in the SSc population to CL appear to shift the balance of cytokines in SSc, to a more profibrotic profile *via* IL-4 increase and IFNγ decrease, and to a more immune suppressive one via decreased activation of Vγ9⁺ T cell responses to phosphoantigens. Thus, we speculate that, in the setting of infection or other IPP-inducing cellular stresses such as cancer, CL-induced activation of IL-4-producing γδ T cells in SSc, together with a decrease of IFNγ-producing γδ T cells, may contribute to organ fibrosis. This idea is based on the known antifibrotic effect of IFNγ, vs the profibrotic effects of IL-4 (40). Interestingly, these changes may also have impacted

the vascular aspects of SSc, as occurred in a previously described patient whose disease dramatically deteriorated after intravenous zol therapy (18, 41). Because the increase of IL-4 detected in these *in vitro* experiments was relatively small, further work to determine the role of IL-4 vs IFNγ-producing γδ T cells *in vivo* in biopsies of afflicted tissues from SSc patients will be required. Furthermore, it remains to be determined whether the effect of CL is able to overcome the anti-fibrotic effect (mediated by cytotoxicity against fibroblasts) of IPP-activated $V\gamma9^+$ T cells (4, 5).

In summary, effects induced by CL and possibly by other autoantigenic lipids or foreign lipid antigens, may have important implications for the dysregulation of γδ T cell subsets and, perhaps, for the pathogenesis of the fibrotic phenotype in SSc. Further experiments, including isolation of different mononuclear cells subsets, including those other than the γδ T cell subset, and studies of the roles of cell contact and soluble mediators are warranted to fully understand the role of autoantigenic lipids and phosphoantigens in SSc.

ETHICS STATEMENT

The study was approved by the Institutional Review Board (Helsinki Committee) of the Sheba Medical Center, Ramat Gan, and Rambam Health Care Campus, Haifa, Israel.

AUTHOR CONTRIBUTIONS

HS: substantial contributions to the conception or design of the work; the acquisition, analysis, and interpretation of data for the work; drafting the work; and final approval of the version to be published. JH: substantial contributions to the conception or design of the work; analysis and interpretation of data for the work; and final approval of the version to be published. PF: substantial contributions to the conception or design of the work; analysis and interpretation of data for the work; and final approval of the version to be published. AB-G: substantial contributions to the conception or design of the work; the acquisition, analysis, and interpretation of data for the work; patients' care and sampling; drafting the work and revising it critically for important intellectual content; and final approval of the version to be published. YB-M: interpretation of data for the work; patients' care and sampling; drafting the work and revising it critically for important intellectual content; and final approval of the version to be published. IB: substantial contributions to the conception and design of the work; the acquisition, analysis, and interpretation of data for the work; patients' care and sampling; drafting the work and revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tribody [(HER2)$_2$xCD16] Is More Effective Than Trastuzumab in Enhancing γδ T Cell and Natural Killer Cell Cytotoxicity Against HER2-Expressing Cancer Cells

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An enhanced expression of human epidermal growth factor receptor 2 (HER2, ErbB2) often occurs in an advanced stage of breast, ovarian, gastric or esophageal cancer, and pancreatic ductal adenocarcinoma (PDAC). Commonly, HER2 expression is associated with poor clinical outcome or chemoresistance in ovarian and breast cancer patients. Treatment with humanized anti-HER2 monoclonal antibodies, such as trastuzumab or pertuzumab, has improved the outcome of patients with HER2-positive metastatic gastric or breast cancer, but not all patients benefit. In this study, the bispecific antibody [(HER2)$_2$xCD16] in the tribody format was employed to re-direct CD16-expressing γδ T lymphocytes as well as natural killer (NK) cells to the tumor-associated cell surface antigen HER2 to enhance their cytotoxic anti-tumor activity. Tribody [(HER2)$_2$xCD16] comprises two HER2-specific single chain fragment variable fused to a fragment antigen binding directed to the CD16 (FcγRIII) antigen expressed on γδ T cells and NK cells. Our results revealed the superiority of tribody [(HER2)$_2$xCD16] compared to trastuzumab in triggering γδ T cell and NK cell-mediated lysis of HER2-expressing tumor cells, such as PDAC, breast cancer, and autologous primary ovarian tumors. The increased efficacy of [(HER2)$_2$xCD16] can be explained by an enhanced degranulation of immune cells. Although CD16 expression was decreased on γδ T cells in several PDAC patients and the number of tumor-infiltrating NK cells and γδ T cells was impaired in ovarian cancer patients, [(HER2)$_2$xCD16] selectively enhanced cytotoxicity of cells from these patients. Here, unique anti-tumor properties of tribody [(HER2)$_2$xCD16] are identified which beyond addressing HER2 overexpressing solid tumors may allow to treat with similar immunoconstructs combined with the adoptive transfer of γδ T cells and NK cells refractory hematological

malignancies. A major advantage of γδ T cells and NK cells in the transplant situation of refractory hematological malignancies is given by their HLA-independent killing and a reduced graft-*versus*-host disease.

Keywords: human γδ T cells, human natural killer cells, human epidermal growth factor receptor 2, trastuzumab, bispecific antibody, tribody, cancer, CD16

INTRODUCTION

The human epidermal growth factor receptor 2 [(HER2), ErbB2] stimulates tumor cell proliferation *via* the Ras-MAP-kinase pathway and its expression is often associated with an aggressive tumor phenotype, advanced stage diseases, and poor clinical outcome (1, 2). Since anti-HER2 therapies are successful for the treatment of HER2-expressing tumors, HER2 is often selected as a tumor target antigen (3). HER2 expression in cardiomyocytes does not exclude an anti-HER2 therapy when the cardiac function in patients receiving anti-HER2 therapy is closely monitored. A dysfunction of cardiomyocytes, which is induced in 4% of the cancer patients receiving anti-HER2 therapy, is reversible (4). However, several HER2-positive tumors are resistant against anti-HER2 therapy or develop a resistance often accompanied by loss of anti-HER2-directed Th1 immunity (5). In an attempt to optimize anti-HER2 therapies, the initial monotherapy with humanized anti-HER2 mAb trastuzumab (Herceptin®, Genentech, South San Francisco, CA, USA) against metastatic gastric or breast cancer was gradually replaced by combination therapies with cytostatic agents (e.g., docetaxel, capecitabine, paclitaxel) and/or other anti-HER2 mAb (e.g., pertuzumab), and/or tyrosine kinase inhibitors (e.g., lapatinib) (2, 3, 6–12). Alternatively, the antibody-drug conjugate (ADC) trastuzumab emtansine (T-DM-1) consisting of the anti-HER2 mAb trastuzumab linked to the cytotoxic agent emtansine (DM-1), which enters and destroys the HER2-overexpressing cells by binding to tubulin, was successful in patients with advanced breast cancer (13, 14). Trastuzumab and pertuzumab induce antibody-dependent cell-mediated cytotoxicity (ADCC) and/or cell death of tumor cells by inhibition of HER2 signaling (15–17). ADCC is mediated by activating Fcγ-receptor (FcγR) bearing myeloid cells as well as by natural killer (NK) cells or γδ T lymphocytes (10, 18–20). Regarding γδ T cells, Capietto and colleagues recently reported that adoptive transfer of human Vγ9Vδ2-expressing γδ T lymphocytes from healthy donors (HDs) together with trastuzumab reduced growth of HER2-expressing breast cancer tumors grafted into immunocompromised mice. In their study, γδ T cells bound to mAb-labeled breast cancer tumors *via* FcγRIII (CD16) and thereby exerted ADCC (21).

Differential clinical responses toward therapeutic antibodies such as trastuzumab or rituximab related to polymorphisms in *FCGRIIA* and *FCGRIIIA* genes have promoted the development of Fc engineered antibodies, which improve cellular cytotoxicity against tumors (16, 17, 22, 23). Besides, enhanced cytotoxicity was also obtained with bispecific antibodies (bsAb), which allow redirecting of distinct effector cell populations including T lymphocytes to the tumor-site (24). The development of bsAb recruiting T cells has been successfully introduced into clinical application for blinatumomab and catumaxomab for treatment of relapsed or refractory B-cell precursor acute lymphoblastic leukemia and malignant ascites, respectively (25–27). Targeting solid tumors with bsAb is more complex and is under investigation (28, 29). bsAb also offer the ability to selectively trigger a distinct activating FcγR with high affinity and allow recruitment of FcγR expressing effector cells. For example, bispecific [HER2xCD16] antibodies retargeting NK cells to tumors have proven efficacy (30–32).

γδ T lymphocytes are attractive effector cells for bsAb based on their HLA-independent recognition of antigens, their capacity to present antigens to αβ T cells and not to induce a severe graft-*versus*-host disease (33–36). Previously, we have shown the efficacy of bsAb, such as [HER2xCD3] and [(HER2)$_2$xVγ9], which selectively target CD3- and/or Vγ9-expressing T cells to HER2-expressing pancreatic ductal adenocarcinoma (PDAC) cells. HER2 is overexpressed on tumor cells of patients with PDAC (37). In particular, [(HER2)$_2$xVγ9] selectively enhanced the cytotoxicity of Vγ9-expressing T cells from PDAC patients *in vitro* by inducing the release of perforin and granzyme B as well as *in vivo* in xenograft models of PDAC upon transfer of human γδ T cells (38). These data are of great interest because PDAC is an extremely aggressive disease with poor prognosis and limited therapeutic options due to the resistance of PDAC to chemotherapy (39). Similar to PDAC, ovarian cancer (OC) is also regarded as an aggressive malignancy with a characteristic immunosuppressive tumor microenvironment (40). Ultimate recurrence of (serous) OC after surgery is often associated with poor prognosis and resistance against chemotherapy (41). In this study, we focused on a bsAb in the tribody format, [(HER2)$_2$xCD16], to redirect CD16-expressing γδ T cells in addition to NK cells to lyse HER2-expressing tumor cells. For this purpose, γδ T cells and NK cells were used for cytotoxity assays, and applied either as peripheral blood lymphocytes (PBL) or tumor-infiltrating lymphocytes (TIL), as purified cells isolated out of PBL or TIL (freshly isolated) or as short-term *in vitro* expanded cells (suitable for adoptive cell transfer). The potential of tribody [(HER2)$_2$xCD16] to enhance the cytotoxicity of these cells against allogeneic cancer cell lines and autologous primary OC cells was compared to the cytotoxic activity mediated by trastuzumab.

Abbreviations: BrHPP, bromohydrin-pyrophosphate; bsAb, bispecific antibodies; HD, healthy donors; HER2, human epidermal growth factor receptor 2; mAb, monoclonal antibodies; n-BP, nitrogen-containing bisphosphonates; OC, ovarian cancer; PAg, phosphorylated antigen; PBL, peripheral blood lymphocytes; pCR, pathological complete response; PDAC, pancreatic ductal adenocarcinoma; RTCA, real-time cell analyzer; TCR, T cell receptor; TIL, tumor-infiltrating lymphocytes.

MATERIALS AND METHODS

Patient Cohorts

Leukocyte concentrates from healthy adult blood donors were obtained from the Department of Transfusion Medicine of

the University Hospital Schleswig-Holstein (UKSH) in Kiel, Germany. Heparinized blood was drawn from healthy donors (HDs) of the Institute of Immunology [UKSH, Christian-Albrechts University (CAU)], whereas blood from patients was obtained from the Department of General and Thoracic Surgery or the Clinic of Gynecology and Obstetrics, both of the UKSH in Kiel. In accordance with the Declaration of Helsinki, written informed consent was obtained from all donors, and the research was approved by the relevant institutional review boards (ethic committee of the Medical Faculty of the CAU to Kiel, code number: D405/10, D403/14, D404/14). Twenty-four patients with histologically verified PDAC (10 females, age 67.2 ± 12.1 years; 14 males, age 66.6 ± 6.9 years, stage pT1-4, pN0-1, L0-1, V0-1Pn 0-1, R0-1) were enrolled. None of the patients had been treated with chemo- or radio-therapy prior to this investigation. Moreover, 20 patients (7 females, age 72.3 ± 6.5 years; 13 males, age 67.2 ± 7.5 years) with other (cancer) diseases were included as follows: 3x papillary carcinoma, pancreaticobiliary type, 3x (multi-cystic) serous cystadenoma of the pancreas, 3x neuroendocrine papilla carcinoma, 2x cholangiocarcinoma, 2x bile duct carcinoma, 2x gastrointestinal carcinoma, 1x tubular adenocarcinoma with high-grade epithelial dysplasia, 1x lymph nodes in the pancreatic head, 1x jejunum metastasis, 1x hepatocellular carcinoma, 1x B cell Non-Hodgkin lymphoma. For functional assays, one patient with advanced stage of breast cancer (age 55 years, stage IIIB, high grading) and one patient with OC (OC1, age 60, high-grade serous, stage IIIC) at first diagnosis shortly after surgery and before chemotherapy were additionally enrolled (code number: AZ A157/11, AZ B3277/10). From another OC patient (OC11, age 52, high-grade serous, stage IIIC) tumor material was obtained after exploratory laparotomy before chemotherapy and blood before and after neoadjuvant chemotherapy with carboplatin/paclitaxel (4x, q3w) plus bevacizumab administered at the second cycle. The adjuvant therapy was followed by three further cycles with carboplatin/paclitaxel (3x, q3w). For flow cytometric analysis, PBL and TIL of 15 patients with advanced OC [age 56 ± 13 years, FIGO-stage IB-IVB (serous or mucinous carcinomas)] were enrolled (AZ B3277/10).

Isolation and *In Vitro* Culture of Lymphocyte Populations

Peripheral blood lymphocytes were isolated from the leukocyte concentrates, from heparinized blood, or from EDTA blood by Ficoll–Hypaque (Biochrom, Berlin) density gradient centrifugation. To separate freshly isolated γδ T cells or NK cells, negative selection kits [T cell receptor (TCR) γδ$^+$ T Cell Isolation Kit or NK cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach], according to the manufacturer's instructions were used.

To establish short-term γδ T cell lines, PBL were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM Hepes, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FCS (complete medium), and stimulated with their selective antigens 300 nM phosphorylated antigen (PAg) Bromohydrin-pyrophoshate [(BrHPP); kindly provided by Innate Pharma, Marseille, France] or 5 µM of nitrogen-containing bisphosphonates (n-BP) zoledronic acid (Novartis, Basel, Switzerland). 50 U/mL rIL-2 (Novartis) was added every 2 days over a culture period of 14–21 days. After 2–3 weeks, most γδ T cell lines had a purity of >95% Vδ2 γδ T cells. γδ T cells with a purity of <98% were labeled with anti-TCRαβ mAb clone IP26 (Biolegend, San Diego, CA, USA) and subjected to magnetic separation in order to deplete remaining αβ T cells. Magnetically depleted γδ T cells as well as γδ T cells of donors of whom we initially received only 1 mL blood with a low percentage of γδ T cells were re-stimulated to ensure large-scale expansion of pure γδ T cell lines as previously described (42, 43).

Separation of Tumor-Infiltrating Lymphocytes and Primary Tumor Cells

Tumors from patients with advanced OC removed during surgery were dissected in tumor tissues by the pathologist of the UKSH. Tumor tissue was washed in 10 cm dishes with PBS to remove blood debris. Subsequently, the tumor tissue was minced into approximately 1 mm^3 pieces and digested in 5–10 mL PBS with enzymes A, K, and R of the Tumor Dissociation Kit (Miltenyi Biotec) for 1 h at 37°C. Digested cell suspension was then passed through a 100 µm cell strainer (Falcon, BD Bioscience, Heidelberg), visually controlled by light microscopy and centrifuged at 481 *g* for 5 min. Cell pellet was resuspended in complete medium and cultured in 75 cm^2 culture flasks to establish primary tumor cell lines. TILs were isolated by Ficoll–Hypaque density gradient centrifugation. Polyploidy of primary tumor cells was analyzed by using the tricolor probe TERC (3q26)/MYC (8q24)/SE 7 TC (Kreatech/Leica, #KBI-10704) for fluorescence *in situ* hybridization (FISH) as previously described (44). Polyploidy was detected in 60–100% of cells in each investigated primary ovarian tumor sample which classified them together with a high expression of HER2 and epithelial cell adhesion molecule as cancer cells.

Tumor Cell Lines

Pancreatic ductal adenocarcinoma cell lines, such as PancTu-I, Panc1, and Panc89 were kindly provided by Dr. Christian Röder, Institute for Experimental Cancer Research UKSH/CAU, Kiel. PDAC cell lines, Burkitt lymphoma Raji cells (DSMZ, Braunschweig) as well as OC cell line SK-OV-3 or IGROV-1 and esophageal cell line OE33 (ATCC, Manassas, VA, USA) were cultured in complete medium. The genotype of tumor cell lines was recently confirmed by short tandem repeats analysis and mycoplasma negativity routinely once per month by RT-PCR. For removing adherent tumor cells from flasks, cells were treated with 0.05% trypsin/0.02% EDTA.

Generation of bsAb

The tribodies [(HER2)$_2$xCD16] (45), [(CD20)$_2$xCD16] (45), and [(HER2)$_2$xCD89] (unpublished) were expressed as published previously (45). Briefly, Lenti-X™ 293T-cells were co-transfected with corresponding expression vectors coding for light or heavy chain derivatives of the tribodies using the calcium phosphate technique including 5 mM chloroquine. Heterodimeric tribody molecules composed of a light chain and a heavy chain tagged with a C-terminal hexahistidine motif were purified from

supernatant by two successive steps of affinity chromatography using CaptureSelect Fab kappa affinity matrix (BAC B.V., Naarden, The Netherlands) and nickel-nitrilotriacetic acid agarose beads (Qiagen, Hilden, Germany) as described earlier (38, 45). Purity and integrity of bsAb were verified by capillary electrophoresis using an Experion™ Automated Electrophoresis System (Bio-Rad, München).

Flow Cytometry

Fluorochome-labeled mAb were used for six-color surface staining as follows: anti-CD3 clone SK7, anti-TCRγδ clone 11F2 (both from BD Biosciences), anti-TCRαβ clone IP26 (Biolegend), anti-TCRVδ2 clone Immu389 (Beckman Coulter, Krefeld), anti-TCRVδ1 clone TS8.2 (Thermo Fisher Scientific, Schwerte), and anti-CD56 clone NCAM16.2 (BD Biosciences), and corresponding isotype controls (BD Biosciences or Biolegend). Fluorochome-labeled mAb were used for three-color surface staining as follows: anti-TCRγδ clone 11F2 (BD Biosciences), anti-TCRVδ2 clone Immu389 (Beckman Coulter), and anti-CD16 clone B73.1 (BD Biosciences) or anti-CD3 clone SK7 (BD Biosciences) plus corresponding isotype controls (BD Biosciences or Biolegend). To analyze HER2 expression, we used 20 µg/mL trastuzumab (Roche Pharma AG, Grenzach Wyhlen), and the corresponding humanized IgG control (20 µg/mL) followed by a second step staining with goat-anti-human $F(ab)_2$ (Medac, Hamburg). The binding properties of tribody [(HER2)$_2$xCD16] and tribody [(HER2)$_2$xCD89] as a control were analyzed by using HER2-positive tumor cells, CD16-expressing immune cells as well as antigen-negative cells as a control. Briefly, 3–5 × 10^5 cells were incubated with or without purified bispecific antibody derivatives in wash buffer (1% BSA, 0.1% NaN_3 in PBS) for 30 min at 4°C. Cells were washed twice with washing buffer. After incubation with 10 µg/mL anti-Penta-His™ Alexa Fluor® 488 labeled IgG at 4°C for 30 min, cells were washed and analyzed by a flow cytometer.

For intracellular staining of granzyme B, 5 × 10^5 cells were washed with staining buffer, fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences). Thereafter, cells were washed twice with Perm/Wash by centrifugation and stained with PE-conjugated anti-granzyme B mAb clone GB11 (BD Biosciences) for 30 min, washed and measured.

All samples were analyzed on a FACS-Calibur or LSR-Fortessa flow cytometer (both from BD Biosciences) using CellQuestPro, Diva, or FlowJo software.

^{51}Cr-Release Assay

Cytotoxicity against tumor cells was analyzed in a standard 4 h ^{51}Cr-release assay with titrated numbers of γδ T effector cells supplemented with 12.5 U/mL IL-2 as described elsewhere (38). To investigate possible increase of cytotoxic activity, cells were treated with 300 nM BrHPP, the indicated concentrations of trastuzumab, tribody [(HER2)$_2$xCD16], or tribody [(CD20)$_2$xCD16] as a control construct. Supernatants were measured in a Micro-Beta Trilux β-counter (Perkin Elmer). Specific lysis was calculated as $[(cpm_{test} - cpm_{spontaneous})/(cpm_{max} - cpm_{spontaneous})] \times 100$, where spontaneous release was determined in medium only and maximal release in Triton-X-100-lysed target cells. Spontaneous release did not exceed 15% of the maximal release.

Real-Time Cell Analyzer (RTCA)

As an alternative assay to measure cytotoxicity against adherent cells a real-time cell analyzer (RTCA, xCELLigence, ACEA, San Diego, CA, USA) was used as described elsewhere (35, 43, 46). Briefly, 5,000–15,000 adherent tumor cells/well were added to 96-well micro-E-plate in complete medium to monitor the impedance of the cells *via* electronic sensors every 5 min for up to 24 h. The impedance of the cells is expressed as an arbitrary unit called cell index (CI) which reflects changes in cellular parameters such as morphological changes (e.g., spreading, adherence), cell proliferation, and cell death. Since the initial adherence in different wells can differ slightly, the CI was normalized to 1 after having reached the linear growth phase and before the addition of constructs, substances, or suspended cells. Then, trastuzumab or tribody [(HER2)$_2$xCD16] and corresponding control constructs were added in the previously titrated optimal concentrations. Thereafter, PBL, freshly isolated γδ T cells or NK cells, or short-term activated γδ T cells together with the indicated 12.5–50 U/mL IL-2 were added to the RTCA-single plate (SP) assay (xCELLigence). Where indicated, short-term activated Vδ2 γδ T cells were stimulated with 300 nM BrHPP. When effector cells induced lysis of the tumor cells, the loss of impedance of tumor cells shown as decrease of the normalized CI was analyzed. As positive control for killing, tumor cells were treated with a final concentration of 1% Triton X-100. For the precise analysis of cytotoxicity, the cells were monitored every minute for the indicated time points.

CD107a-Degranulation Assay and ELISA

Twenty thousand PDAC cells in 96-well microtiter plates (Nunc, Wiesbaden) were cultured overnight. Medium or 1 µg/mL [(HER2)$_2$xCD16] or control constructs with or without 300 nM BrHPP together with short-term activated γδ T cells (E/T ratio: 12.5:1) supplemented with 12.5 U/mL rIL-2 were added after 24 h. For CD107a-assay, 10 µL FITC-labeled anti-human CD107a mAb clone H4A3 (50 µg/mL, Biolegend) was added directly, whereas 3 µM monensin was added 1 h after co-culturing the cells. After additional 3 h, γδ T cells were stained with PE-labeled anti-TCRγδ mAb, and analyzed by flow cytometry.

For assessment of granzyme B with ELISA, cell culture supernatants of duplicates were collected after 4 h and stored at −20°C until use. Human granzyme B was measured by a sensitive sandwich ELISA following the procedures outlined by the manufacturer (R&D System, Wiesbaden, Germany).

Statistical Analysis

The statistical analysis was assessed by Wilcoxon rank sum test or ANOVA using Graph Pad Prism (Graph Pad Software, Inc., La Jolla, CA, USA). Data from at least three independent biological replicates were used to test for normal distribution with the Shapiro–Wilk test (Graph pad Prism) followed by a parametrically *t* test using Microsoft Excel. All statistical tests were two-sided and the level of significance was set at 5%.

RESULTS

Differential Trastuzumab-Mediated Lysis of Cancer Cells

In this study, we asked whether the treatment of HER2-expressing PDAC cells or OC cells with trastuzumab in the absence or presence of patients' PBL can induce tumor cell lysis comparable to that of breast cancer cells. In order to analyze trastuzumab-mediated lysis of HER2-expressing cancer cells by inhibition of HER2 signaling after its homodimerization and ADCC-mediated lysis by FcγRIII (CD16)-expressing cells, such as NK cells or γδ T cells, we applied a RTCA-assay. The RTCA system allows monitoring of cytotoxic activity of a low number of innate and innate-like cells such as NK cells and γδ T cells within PBL, respectively, in real time without the incorporation of labels over time periods of several days. As shown in **Figure 1A**, the addition of trastuzumab alone induced a strong, but incomplete cell death of breast cancer cell line MCF-7 (**Figure 1A**), whereas there was almost no lysis of PDAC cell line PancTu-I and primary OC cells OC1 by trastuzumab. The co-culture of tumor cells with IL-2 stimulated allogeneic breast cancer, PDAC, or OC patients' PBL, respectively, in the absence of trastuzumab induced a delayed lysis of MCF-7 cells, a low lysis rate of PancTu-I cells and an obvious lysis of OC1 cells (**Figure 1A**) in comparison to the trastuzumab-mediated lysis suggesting an IL-2-mediated activation of PBL effector cells. PBL without IL-2 did not lyse these tumor cells (data not shown). The combination of trastuzumab and PBL enhanced lysis of all applied tumor cells (**Figure 1A**) compared to trastuzumab alone. This effect was more pronounced in MCF-7- and OC1-cells than in PancTu-I cells and other PDAC cells such as Panc1 co-cultured with allogeneic PBL from HDs (Figure S1A in Supplementary Material, upper figure). In contrast, the OC cell line SK-OV-3 or primary OC cells OC11, which highly expressed HER2 (Figure S1B in Supplementary Material), but not IGROV-1 cells (data not shown), were lysed very efficiently after co-culture with allogeneic PBL of HDs in the presence of IL-2 and trastuzumab suggesting a higher heterogeneity within OC cells (**Figure 1A**; Figure S1A in Supplementary Material). The differential intensity of lysis induced by trastuzumab alone was not due to a heterogenous HER2 expression as shown by similar staining patterns of the tumor cells with trastzumab (**Figure 1B**; Figure S1B in Supplementary Material). Taken together, in the presence of patients' PBL trastuzumab induced lysis of HER2-expressing tumor cells but was unable to eradicate all target cells. In further experiments, we asked for possible reasons for this and strategies to further enhance cytotoxicity by cells of the innate and innate-like immunity.

Characterization of Immune Cells of Tumor Patients Which Can Be Engaged by Trastuzumab

Besides varying direct effects of trastuzumab on the different tumor cells, one possible additional explanation for the weak lysis of PDAC cell lines by PBL from cancer patients is our observation of a significantly decreased CD16 expression on Vδ2-positive γδ T cells within the PBL of PDAC patients (**Figure 2A**, right panel), in contrast to TCR γδ-negative NK cells (**Figure 2A**, left panel), which was not present in age-matched HDs or patients with other different (cancer) diseases (shortly before surgery, listed in Section "Materials and Methods") (**Figure 2A**). Of note expression of other activating Fcγ receptors, i.e., Fcγ RI (CD64) and Fcγ RIIA (CD32A) on γδ T cells were not observed (data not shown). Independent of our *in vitro* assay studies with PBL and tumor cells, a low number of CD16-expressing immune cells within the tumors can be responsible for a reduced response toward trastuzumab in tumor patients. Thus, we exemplarily analyzed fresh tumor material of advanced OC patients, where the majority of the analyzed tumors expressed HER2 and compared the distribution of NK cells and γδ T cells within the tumors and the blood of these patients (**Figure 2B**). Interestingly, a significantly reduced number of NK- and γδ-T cells within TIL compared to PBL was observed (**Figure 2B**).

Bispecific Antibody as an Alternative to Trastuzumab

As trastuzumab did not optimally inhibit tumor cell proliferation, we employed a bispecific antibody in the tribody format (**Figure 3A**). [(HER2)$_2$xCD16] consists of a CD16 Fab fragment and two single chain fragments variable (scFv), which were derived from trastuzumab variable regions and which were genetically fused with a flexible linker to the CH1- or CL-domains of the CD16 Fab. [(HER2)$_2$xCD16] was transiently expressed in Lenti-X 293 T cells, purified, and analyzed by capillary electrophoresis (**Figure 3B**). Tribody [(HER2)$_2$xCD16] specifically bound HER2-positive tumor cells (**Figure 3C**) and CD16-expressing NK cells as well as γδ T cells (**Figure 3D**), but not antigen-negative cells such as Raji or αβ T cells (**Figures 3C,D**). In addition, tribody [(HER2)$_2$xCD89] was used as a control, which did not bind to CD89-negative NK cells, γδ-, or αβ-T cells (**Figure 3D**). The cytotoxic activity triggered by the tribody [(HER2)$_2$xCD16] was initially analyzed at varying concentrations and varying effector to target cell ratios (E/T ratio) (data not shown and following Figures). We measured a saturating concentration of tribody [(HER2)$_2$xCD16] between 0.1 and 1 μg/mL. In contrast, the saturating concentration of trastuzumab was 10 μg/mL.

Enhanced PBL-Mediated Lysis of HER2-Expressing Tumor Cells by Tribody [(HER2)$_2$xCD16] in Comparison to Trastuzumab

To compare the cytotoxic potential of [(HER2)$_2$xCD16] tribody with that of trastuzumab, we initially analyzed freshly isolated PBL from different PDAC patients ($n = 9$) and HDs ($n = 6$) that had been co-cultured with HER2-expressing PDAC cells (PancTu-I- or Panc89 cells) or breast cancer cells (MCF-7 cells) in the RTCA system (**Figure 4A**). Previously titrated saturating concentrations of 10 μg/mL trastuzumab and 1 μg/mL of tribody [(HER2)$_2$xCD16] together with IL-2 were applied (**Figure 4A**). Although the concentration of tribody [(HER2)$_2$xCD16] was 10-fold lower than that of trastuzumab, the tribody significantly and more potently enhanced the PBL-mediated lysis of HER2-expressing tumor cells (**Figure 4A**).

Moreover, PBL or TIL of patient OC11 were co-cultured with autologous primary ovarian tumor cells OC11 in the presence or absence of 1 μg/mL tribody [(HER2)$_2$xCD16] together with IL-2 (**Figure 4B**). Interestingly, lysis of HER2-expressing primary ovarian tumor cells by autologous PBL or TIL was also significantly increased after the addition of tribody [(HER2)$_2$xCD16] (**Figure 4B**). The TIL-mediated lysis of autologous primary ovarian tumor cells was not complete, but very impressive due to the very

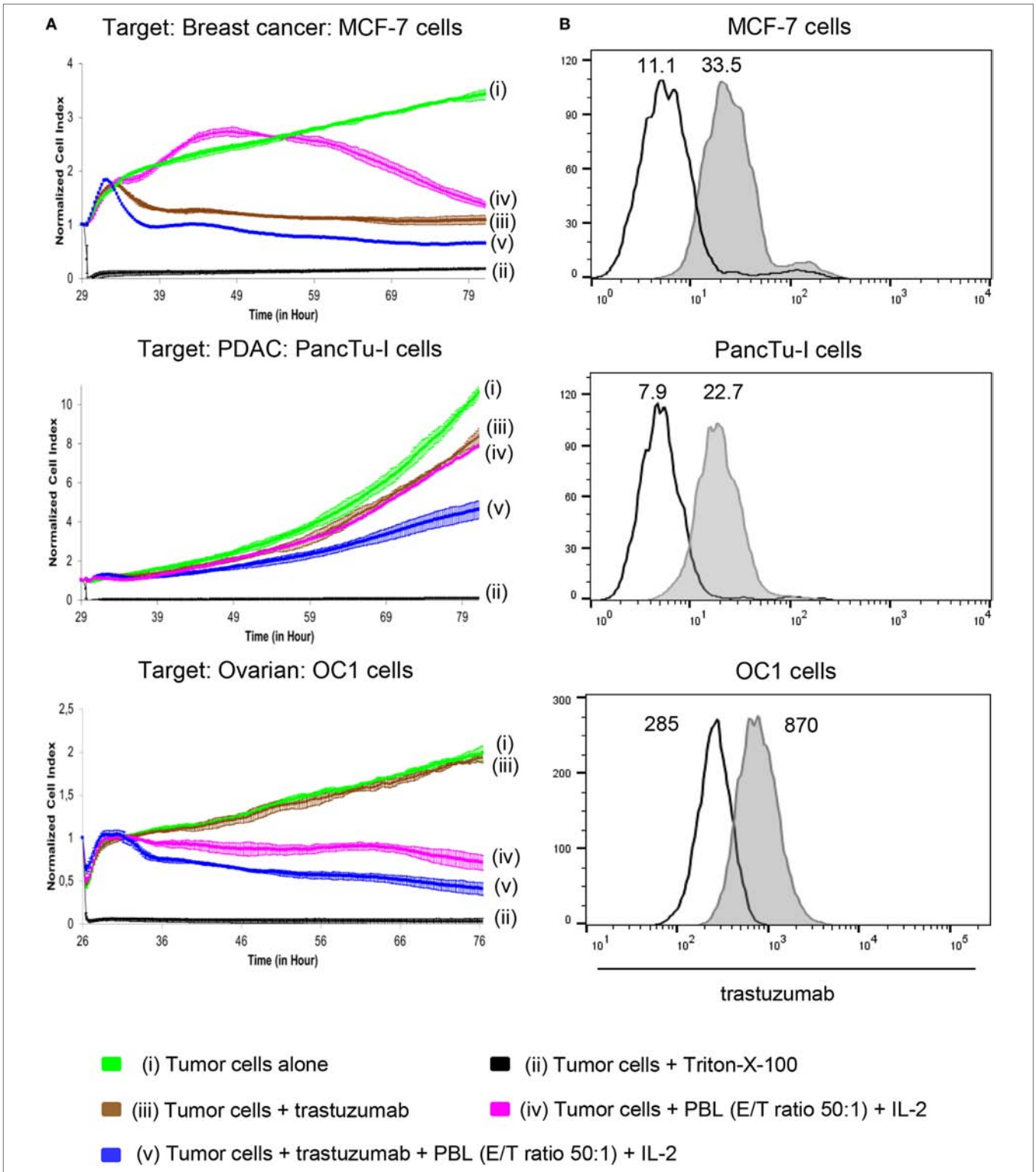

FIGURE 1 | Continued

FIGURE 1 | Effect of trastuzumab on breast cancer, pancreatic ductal adenocarcinoma (PDAC), and ovarian cancer (OC) cells with different human epidermal growth factor receptor 2 (HER2) expression. **(A)** 10 × 10^3 MCF-7 cells, 5 × 10^3 PancTu-I cells, and 5 × 10^3 primary OC cells, OC1 [(i), green lines] were cultured in complete medium for 26–29 h on an E-plate covered at the bottom with electronic sensors that measured the impedance of the adherent tumor cells expressed as an arbitrary unit called cell index (CI) every 5 min. The CI was normalized to 1 shortly before the addition of substances as follows: (ii) Triton-X-100 to induce maximal lysis (black line), (iii) 10 µg/mL trastuzumab (brown line), (iv) peripheral blood lymphocytes (PBL) of allogeneic breast cancer, PDAC, or OC patients (E/T ratio 50:1), respectively, together with 50 IU/mL rIL-2 (purple line), or (v) a combination of trastuzumab with PBL (blue line). CI was then measured every minute for additional 40 h. The loss of tumor cell impedance and thus a decrease of CI correlated with lysis of tumor cells. The average of triplicates and SD were calculated; one representative experiment out of four is shown. **(B)** HER2 expression of MCF-7 cells, PancTu-I cells, and primary OC cells OC1 cells was analyzed by staining the cells with 10 µg/mL trastuzumab (gray histograms) and appropriate isotype controls (open black lines) as indicated, followed by appropriate second step Ab and measuring by flow cytometry. Numbers indicate the median fluorescence intensity of the isotype control and staining with trastuzumab, respectively.

low percentage of <0.5% NK cells and 0.9% γδ T cells compared to 5% NK cells and 1.5% γδ T cells within the PBL (**Figure 4B**; **Figure 2B** filled circle). Further, tribody [(HER2)$_2$xCD16] alone or control constructs such as tribody [(HER2)$_2$xCD89] which has no specificity for NK cells or γδ T cells or tribody [(CD20)$_2$xCD16] targeting CD20 being not expressed on the applied tumor cells, did not trigger target cell lysis (Figure S2 in Supplementary Material).

NK Cells as Well as γδ T Cells Are Activated by Tribody [(HER2)$_2$xCD16]

To analyze whether the cytotoxicity of both NK cells and γδ T cells can be enhanced by the tribody [(HER2)$_2$xCD16], we magnetically isolated both cell populations from PBL of HDs. HER2-expressing PDAC cells such as Panc89 cells were used as targets to analyze the effect of the tribody [(HER2)$_2$xCD16] on freshly isolated NK cell- or γδ T cell cytotoxicity in several RTCA-assays. We observed that the tribody [(HER2)$_2$xCD16] enhanced cytotoxic activity of freshly, negatively isolated NK cells which was dependent on the number of NK cells as expected (**Figures 5A–C**). At a higher E/T ratio all tumor cells were lysed. At lower E/T ratios tribody [(HER2)$_2$xCD16] triggered lysis was more pronounced in the presence of IL-2 (**Figures 5B,C**). Regarding a reduced percentage of innate immune cells at the tumor site as described for NK cells (**Figure 2B**), a parallel activation of additional innate-like immune cells can be beneficial. For instance, freshly, negatively isolated γδ T cells can be activated *via* the TCR through their selective antigens [e.g., phosphorylated antigens (PAg)] in the presence of exogenous IL-2 to kill HER2-expressing Panc89 cells (**Figure 5D**, light blue and orange line indicating different E/T ratios), which can be further increased by the addition of tribody [(HER2)$_2$xCD16] (**Figure 5D**, dark blue and red line indicating different E/T ratios). In contrast to NK cells, the addition of exogenous IL-2 as well as the TCR engagement is absolutely essential for inducing optimal cytotoxic activity of freshly isolated γδ T cells (**Figure 5D**, upper panel, pink and purple line indicating different E/T ratios). Tribody [(HER2)$_2$xCD16] alone (central **Figure 5D**, brown line) and control constructs such as tribody [(HER2)$_2$xCD89] or tribody [(CD20)$_2$xCD16] did not trigger target lysis (Figure S2 in Supplementary Material).

Tribody [(HER2)$_2$xCD16] Increased Cytotoxic Activity of γδ T Cells Suitable for Adoptive Cell Transfer

In general bsAb can be applied in cancer immunotherapy in various clinical settings. Besides application as single agent, they can be combined in regimens including adoptive cell transfer. Patient-derived γδ T cells expanded with their selective antigens and IL-2 *in vitro* might be combined with bsAb to enhance cytotoxic activity of transferred γδ T cells. The latter approach requires an examination of the cytotoxic activity of short-term expanded γδ T cells in comparison to freshly isolated ones. As shown in ^{51}chromium-release experiments, we observed a limited cytotoxic capacity of short-term expanded γδ T cells from HDs ($n = 5$) as well as from PDAC patients ($n = 5$) against the PDAC cell line PancTu-I (**Figure 6B**, medium, left two panels). The limited cytotoxicity of short-term expanded γδ T cells was somewhat enhanced by the addition of their selective antigens such as PAg (**Figure 6B**, phosphoantigen, right two panels), whereas the addition of trastuzumab only weakly increased the cytotoxicity of these γδ T cells (**Figure 6C**, medium). Lysis induced by trastuzumab (**Figure 6C**, medium) correlated with the CD16 expression on the surface of the γδ T cells (**Figure 6A**). Thus, short-term expanded γδ T cells of HD donor 5 (**Figure 6A**, quadrat) and PDAC donor 3 (**Figure 6A**, circle), which both had a high percentage of CD16-positive cells, exerted an enhanced cytotoxic activity (**Figure 6C**, medium). In contrast, short-term expanded γδ T cells of other donors, which expressed lower numbers of CD16 (**Figure 6A**), were only slightly affected (**Figure 6C**, medium). Similar to the γδ T cells within the PBL, we screened the CD16 expression on all established short-term expanded γδ T cells of HD ($n = 43$) as well as of PDAC patients ($n = 20$) which were used in different experiments in this studies (Figure S3 in Supplementary Material). In general, we observed a high variability in CD16 expression between 1 and 70% which drastically influenced the effectiveness of trastuzumab (Figure S3 in Supplementary Material; **Figure 6C**). Although we observed that the combination of trastuzumab and PAg BrHPP enhanced γδ T cell-mediated killing of PancTu-I cells (**Figure 6C**, phosphoantigen), the application of tribody [(HER2)$_2$xCD16] was more potent in enhancing γδ T cell-mediated killing (**Figure 6D**, medium) than trastuzumab (**Figure 6C**, medium) presumably due to the higher CD16 binding affinity. Interestingly, the combination of tribody [(HER2)$_2$xCD16] and BrHPP enhanced the cytotoxic activity of γδ T cells from PDAC patients more prominent than γδ T cells from HDs (**Figure 6D**, phosphoantigen). This can be explained by the lower CD16 expression on γδ T cell lines of PDAC patients (**Figure 6A**). Importantly, it demonstrates the effectiveness of tribody [(HER2)$_2$xCD16] on low CD16-expressing cells.

In another assay with additional short-term expanded γδ T cell lines of three different donors as effector cells and HER2-expressing PancTu-I cells, we analyzed in parallel HER2-negative, CD20-positive Raji tumor cell line as target cells to prove specificity

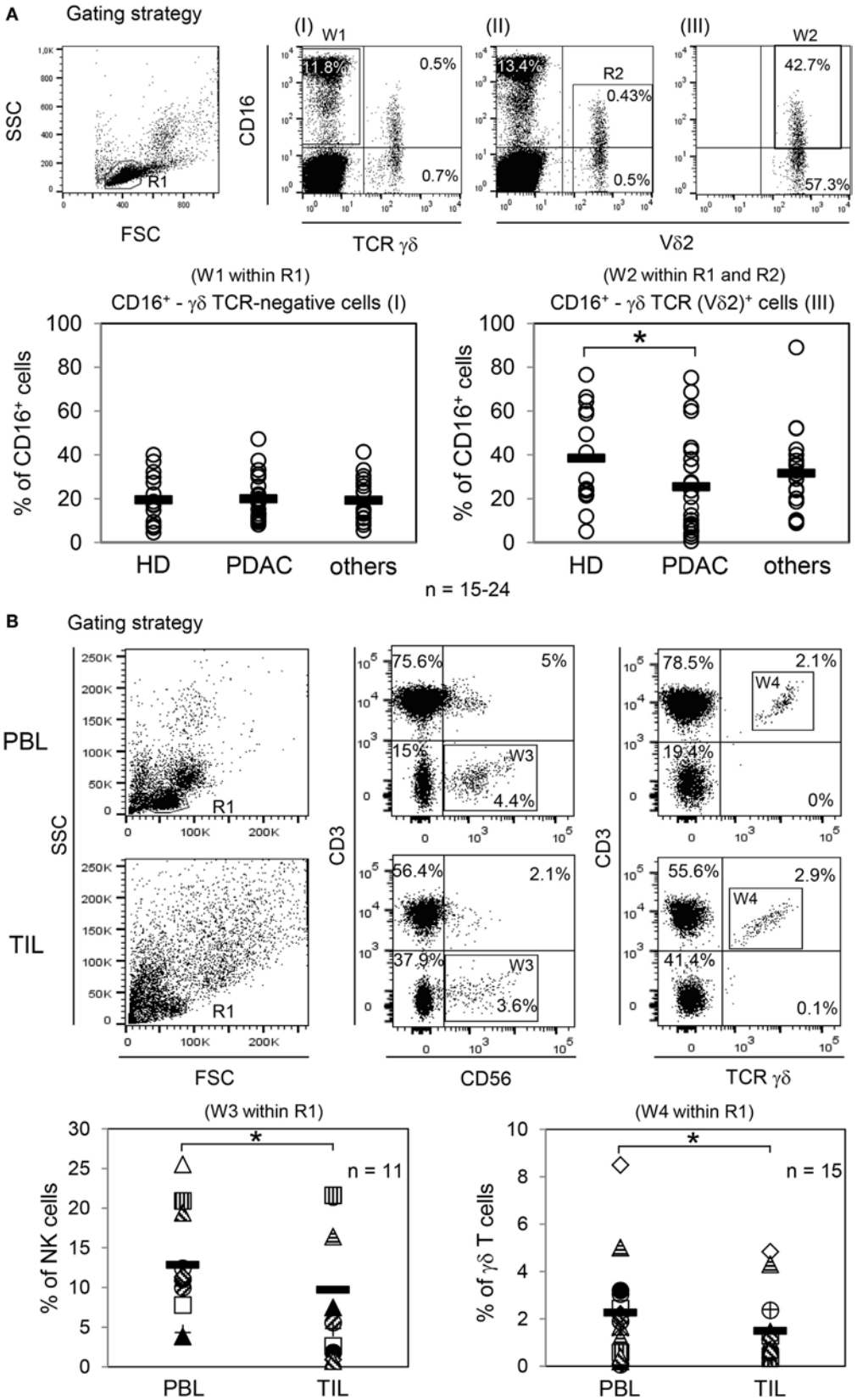

FIGURE 2 | Continued

FIGURE 2 | Analysis of CD16 expression within peripheral blood lymphocytes (PBL) and distribution of natural killer (NK) cells and γδ T cells within PBL and tumor-infiltrating lymphocytes (TIL) of ovarian cancer (OC) patients. **(A)** Gating strategy with one healthy donor (HD) for determination of CD16$^+$ γδ T cell-negative or -positive cells. CD16$^+$ γδ TCR-negative cells were calculated within lymphocytes (R1; FSC/SSC) by pan T cell receptor (TCR) γδ-negative and CD16-positive expression (W1; I). CD16$^+$ TCR γδ (Vδ2)-positive T cells were determined within lymphocytes (R1; FSC/SSC) by CD16-positive as well as by pan TCR γδ-positive (I) and Vδ2-positive (II) cells. For precise CD16 expression on Vδ2-positive T cells, an additional gate (R2; II) was set and the expression was calculated in the logical gate of "R1 AND R2" (W2; III). In parallel, CD3 expression of all analyzed γδ T cells was determined (data not shown). The relative percentage of CD16-expressing γδ T cell-negative cells [NK cells, (W1 within R1)] and TCR Vδ2 γδ-positive T cells (W2 within R1 and R2) were analyzed from PBL of age-matched HDs (HD, $n = 24$, 12 females, 12 males; age 65 ± 10 years), from PDAC patients ($n = 24$, 12 females, 12 males; age 64 ± 11 years), and from patients with other cancer diseases ($n = 15$, age 64 ± 11 years). Each symbol represents the data of one donor, and the thick bars represent the mean value of different experiments. Significances were performed by the Wilcoxon rank sum test and are shown as P value; $^*P < 0.05$. **(B)** Gating strategy of NK cells and γδ T cells within PBL or TIL of one OC patient. NK cells were defined by CD3-negative and CD56$^+$ cells (W3) within R1 (FSC/SSC) and γδ T cells by CD3$^+$ pan TCR γδ$^+$ cells (W4) within R1. The relative percentage of NK cells ($n = 11$; age 57 ± 10) is shown in "W3 within R1" and for γδ T cells ($n = 15$; age 56 ± 13) in "W4 within R1." Each symbol presents the data of one donor. Mean values of different experiments are indicated (thick bars). Significances were assessed by the Wilcoxon rank sum test and are shown as P value, $^*P < 0.05$.

of the tribody [(HER2)$_2$xCD16]. Tribody [(HER2)$_2$xCD16] triggered lysis of HER2-expressing tumor cells, but not of HER2-negative Raji cells, which, however, were efficiently killed in the presence of PAg BrHPP or tribody [(CD20)$_2$xCD16] (Figure S4 in Supplementary Material).

In addition, the results of the ^{51}Cr-release assay for PancTu-I cells were confirmed by the RTCA-assay (**Figure 7**). Again, the tribody [(HER2)$_2$xCD16] enhanced the γδ T cell-mediated cytotoxicity against PancTu-I cells at different E/T ratios more extensively than trastuzumab independent of whether short-term expanded γδ T cells from PDAC patients ($n = 7$) or from HDs ($n = 4$) were used (**Figure 7A**). Similar to the results with PancTu-I cells, we observed that γδ T cell cytotoxicity against other HER2-expressing tumor cells such as established breast cancer cell line MCF-7 (**Figure 7B**) or autologous primary OC cells OC11 (**Figure 7C**) was increased. The lysis of the latter one was enhanced after addition of autologous short-term expanded γδ T cells isolated from TIL together with tribody [(HER2)$_2$xCD16]. As expected, the control constructs had no cytolytic effect (data not shown). We observed no significant enhancement of γδ T cell cytotoxicity by the tribody [(HER2)$_2$xCD16] compared to trastuzumab when we used a high E/T ratio and Panc89 cells as target cells, which were lysed more efficiently than other PDAC cells (e.g., PancTu-I- or Panc1 cells; Figure S5 in Supplementary Material). Interestingly, a reduced lysis of Panc89 cells by short-term expanded γδ T cells, which was due to a lower E/T ratio (5:1) was more effective with tribody [(HER2)$_2$xCD16] than with trastuzumab, although γδ T cells from some donors expressed CD16 very weakly at the cell surface (Figures S3 and S5 in Supplementary Material). Enhanced γδ T cell-mediated lysis with tribody [(HER2)$_2$xCD16] was also observed at a lower E/T ratio when other HER2-expressing tumor cells such as the esophageal cancer cell line OE33 were used (Figure S5 in Supplementary Material).

Enhanced γδ T Cell Cytotoxicity Against HER2-Expressing Tumor Cells by Tribody [(HER2)$_2$xCD16] Is Mediated by Cytolytic Granules

Besides the aspect that several PDAC cells are almost resistant to the CD95- or TRAILR-induced cell death, γδ T cells mainly mediate their cytotoxic activity through the release of cytolytic granules (47). As shown in **Figure 8**, the CD107a-degranulation assay revealed a significantly enhanced exocytosis after application of tribody [(HER2)$_2$xCD16] to short-term expanded γδ T cells from HDs or PDAC patients co-cultured with PancTu-I cells which was accompanied by an increase in granzyme B release (**Figures 8A,B**). Degranulation was *per se* higher after PAg-stimulation, but was further significantly enhanced after additional application tribody [(HER2)$_2$xCD16] (**Figure 8A**). Interestingly, the intracellular content of granzyme B was not influenced by the treatment with tribody [(HER2)$_2$xCD16] suggesting a permanent intracellular production of granzyme B (**Figure 8C**).

DISCUSSION

In this study, the tribody [(HER2)$_2$xCD16] efficiently enhanced the cytotoxic activity of NK cells and γδ T cells of healthy persons as well as of cancer patients against HER2-expressing tumor cells including breast-, pancreatic-, ovarian-, and esophageal cancer cells. While NK cells can be activated directly by tribody [(HER2)$_2$xCD16], the addition of exogenous IL-2 as well as the TCR engagement is essential for inducing cytotoxicity in resting γδ T cells pointing to a pre-activation of γδ T cells by their selective antigens (e.g., PAg or n-BP) *in vivo* or an adoptive transfer of short-term activated γδ T cells together with the tribody as potential therapeutic strategies.

In contrast to trastuzumab, tribody [(HER2)$_2$xCD16] enhanced the cytotoxic activity even of CD16 low-expressing γδ T cells of PDAC patients together with their selective antigens (e.g., PAg or nBP) and IL-2 against HER2-low-expressing PDAC cell lines. Interestingly, we observed a reduced number of NK cells within TIL of OC patients before surgery and chemotherapy which may explain the superiority of PBL compared to TIL (both from OC patients) against autologous tumor cells. An activation of γδ TIL of OC patient OC11 after neoadjuvant chemotherapy stimulated *via* PAg together with Th1 cytokine IL-2 and tribody [(HER2)$_2$xCD16] efficiently increased lysis of autologous HER2-expressing primary OC cells (OC11) suggesting a compensation of the low number of NK cells within TIL by γδ T cell activation. In this context, our own additional data reveal that the absolute γδ T cell number in a cohort of 26 breast cancer patients receiving chemotherapy did not differ from the number before chemotherapy, in contrast to decreased NK cells and αβ T cell numbers (48).

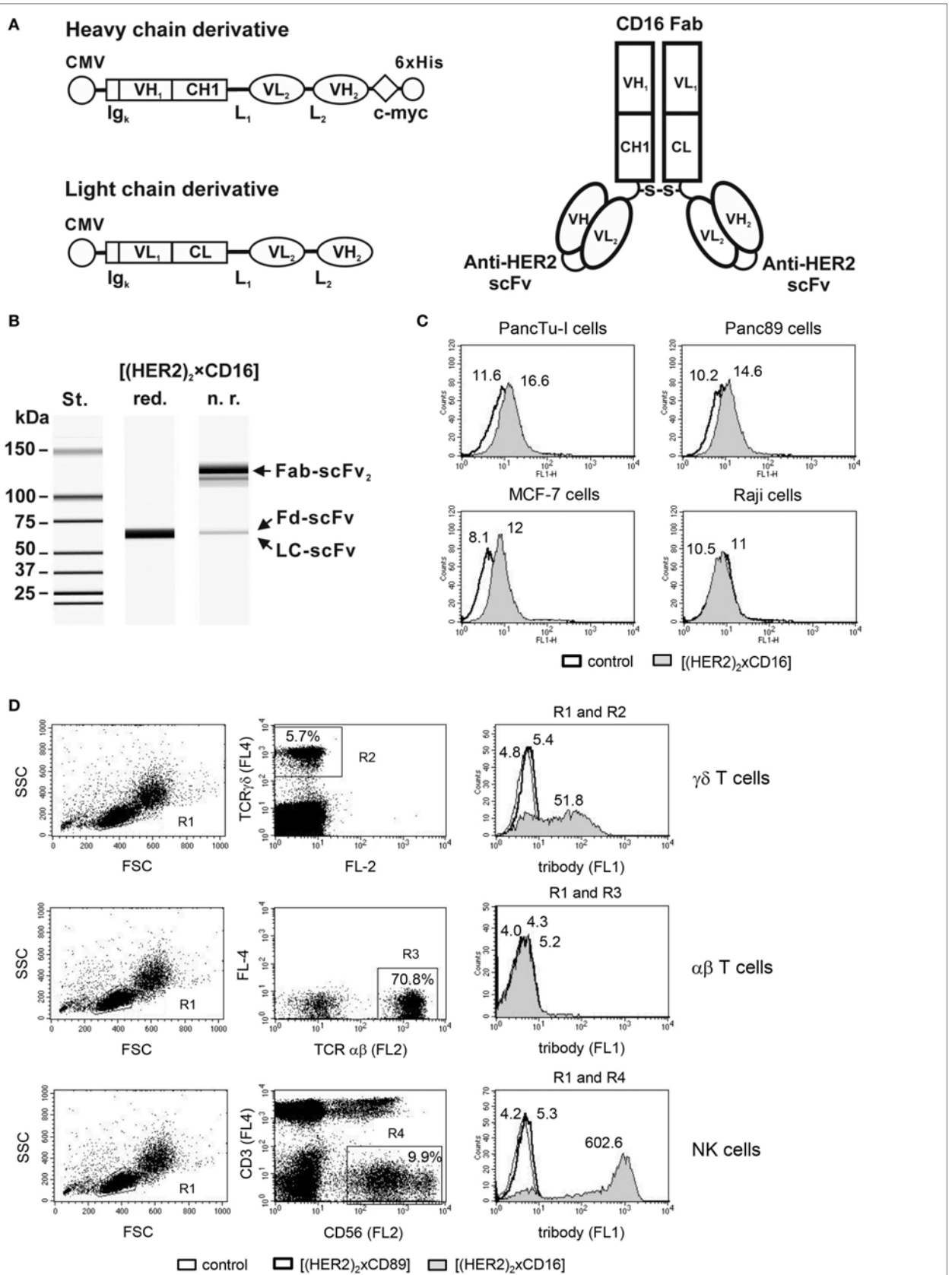

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FIGURE 3 | Design and purification of [(HER2)$_2$xCD16]. **(A)** Scheme of the expression cassettes for (left) the tribody protein and (right) the assembled tribody molecule. CMV, cytomegalovirus immediate early promoter; Ig$_\kappa$, murine Ig kappa secretion leader; VH$_1$, VL$_1$, and CH1, CL, sequences coding for the human immunglobulin (Ig) variable (V) or constant (C) heavy and light chain regions from the CD16 mAb, respectively; VH$_2$, VL$_2$, sequences coding for the variable heavy and light chain regions building a scFv with specificity for HER2; L$_1$, L$_2$, sequence coding for a 15 amino acid flexible linker $(G_4S)_3$ and a 20 amino acid flexible linker $(G_4S)_4$, respectively; c-myc and 6xHis, sequence coding for the c-myc epitope and a hexahistidine tag, respectively. S-S, disulfide bond. **(B)** Evaluation of the purity of the [(HER2)$_2$xCD16] tribody by capillary electrophoresis; Fd, fragment difficult; LC, light chain. Specific binding of **(C)** tumor cells (e.g., PancTu-I, Panc89, MCF-7, and Raji) and **(D)** γδ T and NK cells within PBL and lack of binding to αβ T cells within PBL was analyzed by flow cytometry. Representative experiments out of three analyzed are presented. [**(D)**, left and middle panel] The percentage of γδ T cells were defined by pan TCR γδ$^+$ cells (R2), αβ T cells by pan TCR αβ$^+$ cells (R3), and NK cells by CD3-negative and CD56$^+$ cells (R4), respectively, all within the lymphocyte gate R1 (FSC/SSC). [**(D)**, right panel] The median fluorescence intensity of isotype or tribody staining was presented by histogram blots for γδ T cells (R1 and R2), αβ T cells (R1 and R3), or for NK cells (R1and R4). Numbers indicate the median fluorescence intensity of the isotype control [bold line in **(C)** and thin line in **(D)** as indicated] and staining with [(HER2)$_2$xCD89] [bold line in **(D)**] or [(HER2)$_2$xCD16] [gray filled in **(C,D)**] respectively.

Human epidermal growth factor receptor 2 can be overexpressed in advanced breast, gastric, colorectal, pancreatic, and OC cells (6). In general, HER2-expressing tumors are regarded as biologically aggressive neoplasms frequently associated with chemo-resistance and poor clinical outcome. Therapy with humanized HER2 mAb targeting different members of the epidermal growth factor receptor (EGFR) family combined with cytostatic agents e.g., docetaxel and potentially tyrosine kinase inhibitors such as, e.g., lapatinib or neratanib/afatinib has clearly improved the outcome of patients with metastatic breast or gastric cancer (6, 10–12). Trastuzumab as well as pertuzumab induce cell death *in vitro* by inhibiting the Ras-MAP-kinase- or PI3K/Akt/mTOR pathway in tumors or ADCC (2, 49). In neoadjuvant settings, trastuzumab and pertuzumab in combination with chemotherapy have been shown to improve pathological complete response (pCR) in early or locally advanced breast cancer. In adjuvant settings, trastuzumab with taxane-based chemotherapy is considered standard of care (2, 11, 50–53).

Combining trastuzumab with chemotherapy has been also described in a few clinical trials to significantly improve overall survival compared with chemotherapy alone in HER2-positive advanced gastric and in one clinical trial in metastatic pancreatic cancer (37, 54, 55). In addition, several case reports described an effect of trastuzumab on HER2-expressing uterine serous and high-graded endometrioid tumors varying from complete response to stable disease for 11 months (56–58). However, single drug trastuzumab in HER2-overexpressing OC show only moderate activity (59). Anti-HER2 monotherapy is currently not a standard therapy for OC patients. Several previously unpublished or ongoing phase I or II trial studies (https://clinicaltrials.gov/.) treated OC patients with either trastuzumab combined with chemotherapy (NCT00433407) or HER2 cytotoxic T-cell (CTL) peptide-based vaccine (NCT00194714) or anti-CD3xanti-HER2/neu (HER2Bi) armed anti-CD3 activated T cells (aATC) plus low-dose aldesleukin and sargramostim (NCT02470559). The application of our tribody [(HER2)$_2$xCD16] to OC patients could be an option to enhance avidity to HER2-expressing OC cells due to the format with the two HER2-binding sites as well as the possibility to target innate cells (NK cells) as well as cells linking innate and adaptive immunity (γδ T cells) instead of a polyclonal activation of all T cells after stimulation with anti-CD3.

In our studies, we observed a direct effect of trastuzumab on breast cancer cells MCF-7 *in vitro* and enhanced trastuzumab-mediated lysis in the presence of PBL which mediate ADCC. In addition, we demonstrated an enhanced lysis of HER2-expressing tumor cells by the combination of trastuzumab and PBL in PDAC cell lines established from primary cells (stage G1–G3, e.g., PancTu-I and Panc1) as well as in OC cells such as cisplatin-resistant SK-OV-3 cells and primary tumor cells OC1 and OC11.

Besides an initial existing resistance against anti-HER2 treatment, a further not completely understood problem seems to be an evolving secondary HER2-resistance. Several reasons are suggested as follows (6, 10, 49): (i) diminished HER2 expression, (ii) accumulation of an intracellular truncated active kinase p95-HER2 which lacks an extracellular domain (60, 61), (iii) overexpression of other EGFR family members, (iv) hindrance of trastuzumab binding, (v) hyper-activation of the downstream PI3K-Akt-mTOR pathway based on genetic alterations of the tumors (62), (vi) competing circulating immunoglobulins for FcγR (e.g., CD16) binding, (vii) cleavage of CD16 from the cell surface of NK cells by matrix metalloproteinases (63), (viii) undesired binding of inhibitory FcγR instead of activating ones, or (ix) loss of Th1 CD4$^+$ T cells in the tumor microenvironment which supports cytotoxic T cells, such as CD8$^+$ αβ TCR$^+$ or γδ TCR$^+$ cells (5, 64, 65).

Novel strategies are in development aiming to achieve more effective and durable responses. An attempt to combat trastuzumab resistance was the development of the ADC trastuzumab emtansine (T-DM-1). The cytotoxic agent emtansine (DM-1), which destroys tumor cells by binding to tubulin, has been conjugated to trastuzumab which inhibits the PI3K signaling (13, 49). An improvement of progression-free and overall survival in heavily pretreated patients with advanced HER2$^+$ breast cancer and an acceptable toxicity profile has been demonstrated in phase III clinical trials (66, 67). Ongoing trials evaluate the role of T-DM-1 in neoadjuvant treatment of HER2$^+$ breast cancer (68). In parallel, the development of further ADC such as SYD985 which are tested in ongoing phase I clinical trials, is enforced (49).

Another possibility can be a Th1 cytokine-induced senescence in tumor cells (5, 64, 65). The treatment of HER2-expressing breast cancer cells with Th1 cytokines, IFN-γ and TNF-α can cause oncogene inactivation of HER2 followed by a cell-cycle arrest of the tumor cells (5, 64). Since IFN-γ/TNF-α treatment of patients is not advisable, HER2$^+$ breast cancer patients were treated with HER2-pulsed dendritic cells (DC) to restore depressed Th1 response which seems to be due to a functional and reversible cytokine shift (69). Datta et al. reported that a preserved

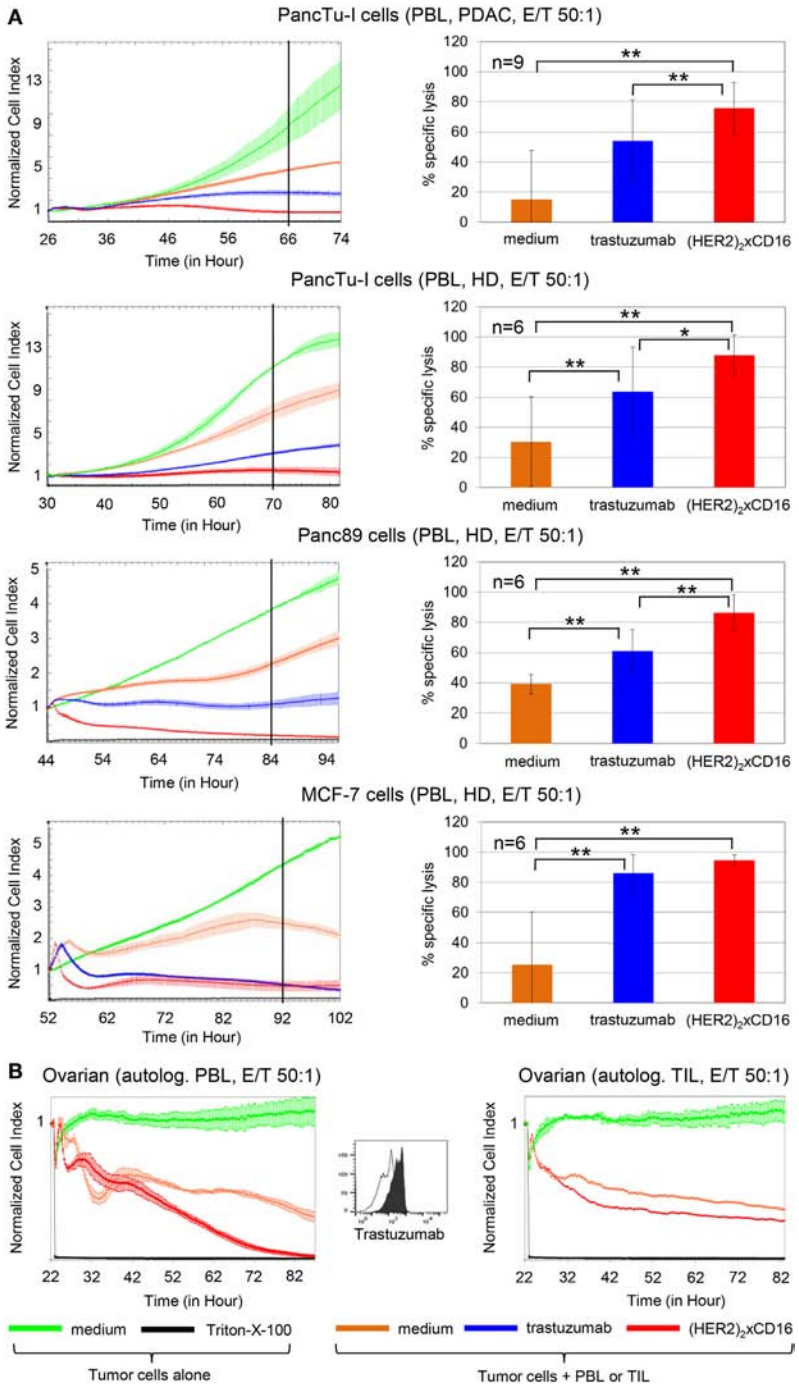

FIGURE 4 | Tribody [(HER2)$_2$xCD16] increased cytotoxicity compared to trastuzumab. **(A)** After culturing 5 × 10^3 pancreatic ductal adenocarcinoma (PDAC) cells (PancTu-I- and Panc89 cells), 10 × 10^3 breast cancer cells (MCF-7 cells) overnight, cells were cultured without or with allogeneic peripheral blood lymphocytes (PBL) at an E/T ratio of 50:1 in the presence of 50 IU/mL rIL-2 with medium (orange line), 10 µg/mL trastuzumab (blue line), or 1 µg/mL tribody [(HER2)$_2$xCD16] (red line). As control cells were left untreated (green line) or treated with Triton-X-100 (black line). **(B)** 5 × 10^3 primary ovarian cancer (OC) cells OC11 had been cultured overnight in medium before the addition of medium control (green line) or Triton-X-100 (black line) in the absence of effector cells. Autologous PBL or TIL of human epidermal growth factor receptor 2 (HER2)-expressing OC patient OC11 cultivated with 50 IU/mL rIL-2 were added at an E/T ratio of 50:1 alone (orange line) or with 1 µg/mL tribody [(HER2)$_2$xCD16] (red line). **(A,B)** The cell index was analyzed in 5 min steps over ~24–52 h and after normalization to 1 (after addition of PBL ± substances) in 1 min steps for >50 h as indicated. The average of three replicates with SD is presented for each tumor cell line with PBL **(A,B)** or TIL [**(B)**, right figure for OC] of one representative healthy donor or cancer patients (PDAC, OC, left panel) in independent experiments. For validation, experiments were replicated several times under equal conditions using different PBL of different donors in independent experiments (right panel). The cytotoxic capacity of PBL against the indicated tumor cells in the presence of medium (orange bars), 10 µg/mL trastuzumab (blue bars), or 1 µg/mL tribody [(HER2)$_2$xCD16] (red bars) was calculated 40 h (black line, left panel) after addition of effector cells (PBL) as % of specific lysis compared to control sample (green line) and maximal lysis (black line). Statistical analysis was performed by *t* test. Significances are shown as *P* value; $^*P < 0.05$ and $^{**}P < 0.01$.

anti-HER2 Th1 response was associated with pCR to neoadjuvant trastzumab application combined with chemotherapy (70). In addition, the anti-HER2 DC vaccination was combined with anti-estrogen therapy which improved regional nodal immune response and pCR rate in patients with estrogen receptor$^+$/HER2$^+$ early breast cancer (71).

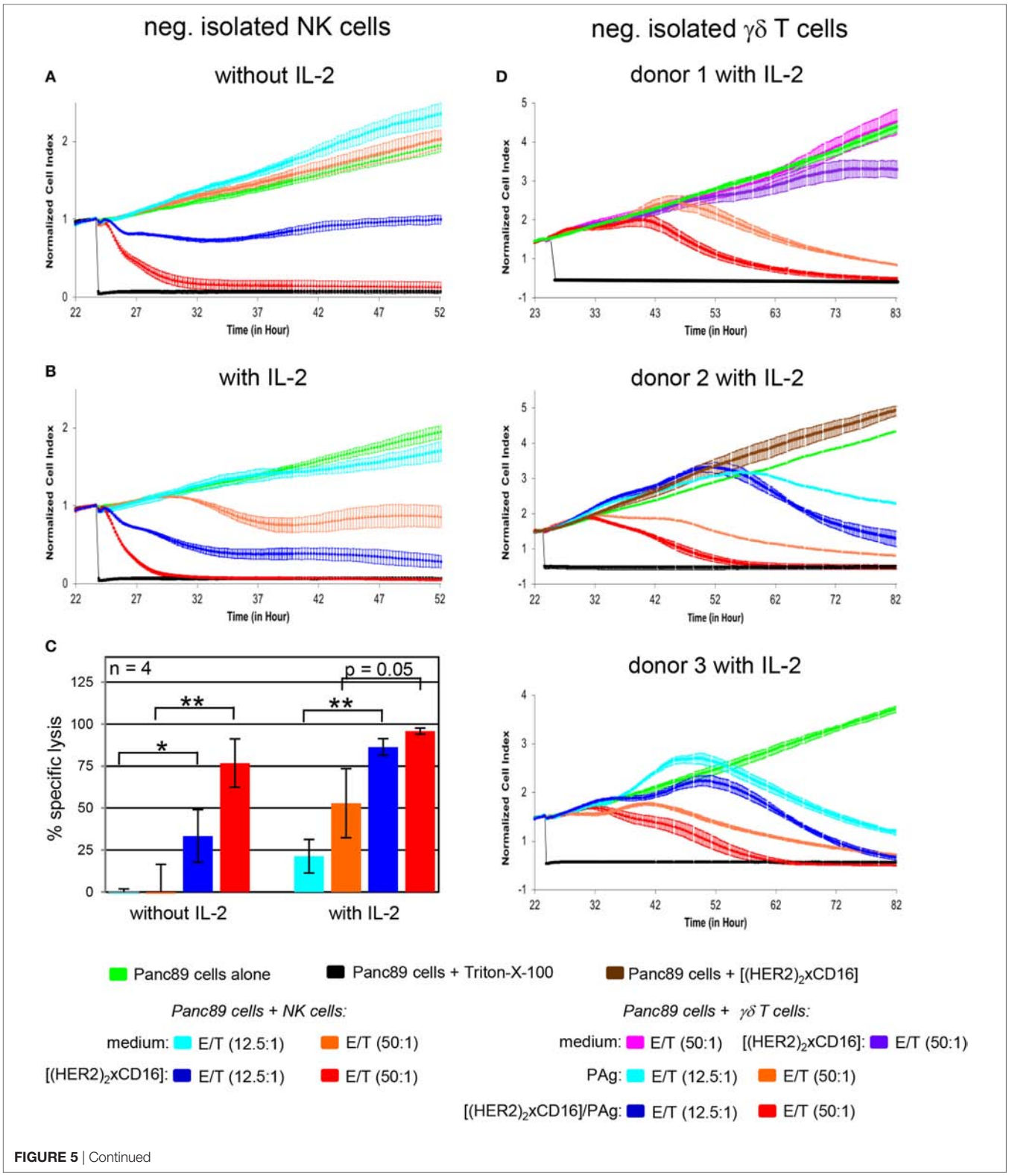

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FIGURE 5 | Enhancement of freshly isolated natural killer (NK) cell- and γδ T cell-mediated lysis of Panc89 cells by tribody [(HER2)$_2$xCD16]. 5 × 10^3 Panc89 cells were seeded overnight in complete medium. Cell index was analyzed in 5 min steps over ~22 h. After overnight adherence, Panc89 cells were cultured with additional complete medium (green line), 1 µg/mL tribody [(HER2)$_2$xCD16] (brown line), or positive control Triton-X-100 (black line). Additionally, Panc89 cells were co-cultured with **(A–C)** allogeneic negatively, freshly isolated NK cells or **(D)** γδ T cells at the indicated E/T ratio with or without 50 IU/mL rIL-2 (as indicated) in the presence of medium [light blue line (E/T ratio 12.5:1); orange line (E/T ratio 50:1)] or 1 µg/mL tribody [(HER2)$_2$xCD16] [dark blue line (E/T ratio 12.5:1); red line (E/T ratio 50:1)] and for γδ T cells with 300 nM phosphorylated antigen bromohydrin-pyrophosphate (BrHPP) or without BrHPP in medium (pink line) or 1 µg/mL tribody [(HER2)$_2$xCD16] at an E/T ratio 50:1. Lysis of tumor cells was measured after normalization to 1 in 1 min steps for >30 h as indicated. **(A,B,D)** The average of three replicates with SD is represented for each tumor cell line with effector cells of one representative healthy donor (HD) in independent experiments. **(C)** Several replications of the experiments under equal conditions using different NK cells of different donors in independent experiments were performed. The NK cell cytotoxicity against Panc89 cells was calculated 30 h after addition of NK cells without or with IL-2. The % of specific lysis was calculated by comparing measured samples to control sample (green line) and maximal lysis (black line). Statistical analysis was performed by *t* test. Significances are shown as *P* value; **P* < 0.05 and ***P* < 0.01.

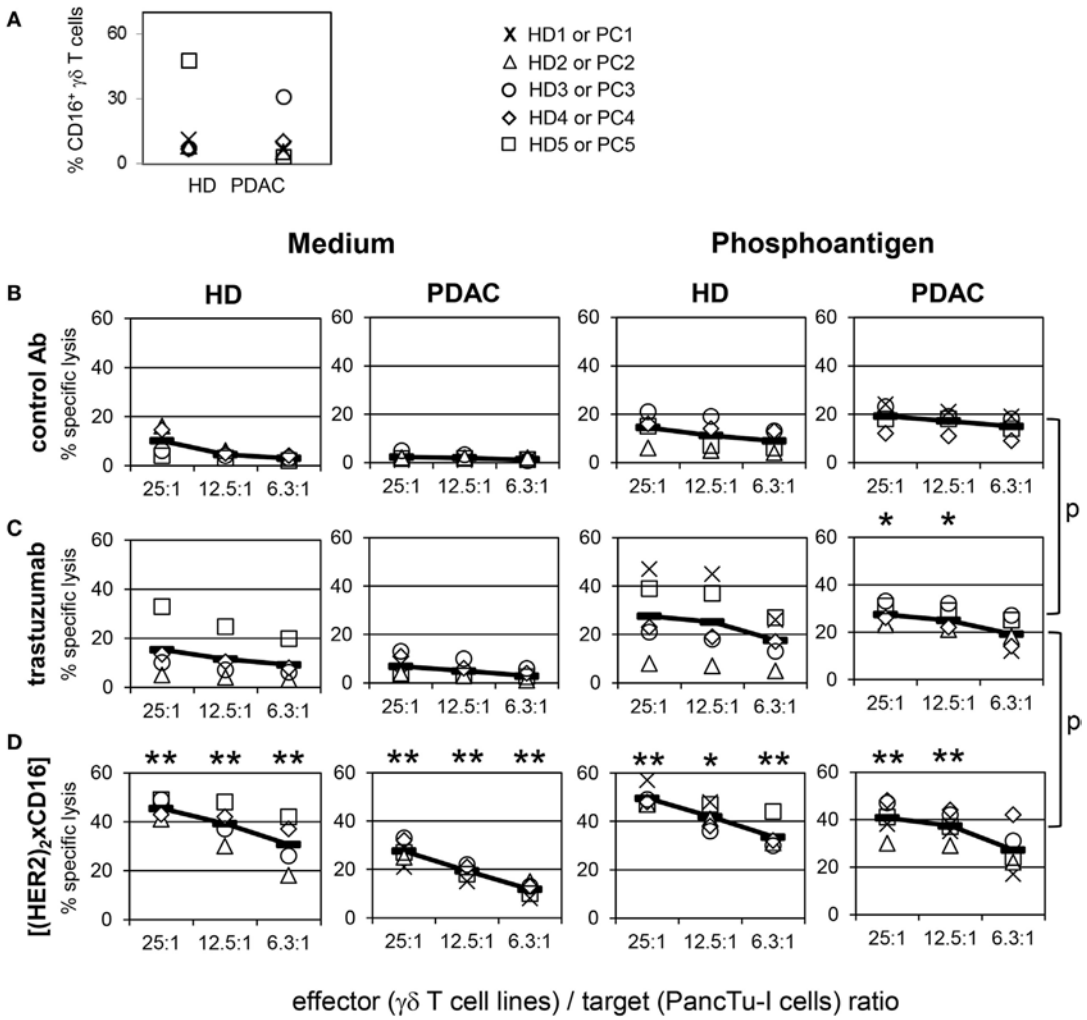

FIGURE 6 | Enhancement of γδ T cell-mediated pancreatic ductal adenocarcinoma (PDAC) lysis by tribody [(HER2)$_2$xCD16]. **(A)** Vγ9Vδ2 γδ T cell lines of healthy donors (HDs) or PDAC patients were stained with anti-CD16 monoclonal antibodies (% CD16$^+$ γδ T cells) or cultured with **(B)** control Ab, **(C)** 10 µg/mL trastuzumab, or **(D)** 1 µg/mL tribody [(HER2)$_2$xCD16] in complete medium (left panel) or with 300 nM phosphoantigen bromohydrin-pyrophosphate (right panel) as indicated. **(B–D)** γδ T cell lines at the indicated E/T ratios were added to ^{51}Cr-labeled PancTu-I cells for 4 h. Each symbol represents the mean value of triplicate assays of one donor. Representative results from five different HDs and five different PDAC patients are shown. Statistical analysis was performed by *t* test. Significances are shown as *P* value; **P* < 0.05 and ***P* < 0.01.

A very promising strategy to overcome certain limitations of mAb and above-mentioned HER2-resistance mechanisms may be the use of bsAb with an enhanced cytotoxic activity against tumor cells (24). Sahied et al. designed bispecific minibodies targeting HER2 and CD16 which possess the advantages of a native IgG with respect to flexibility but in a different format.

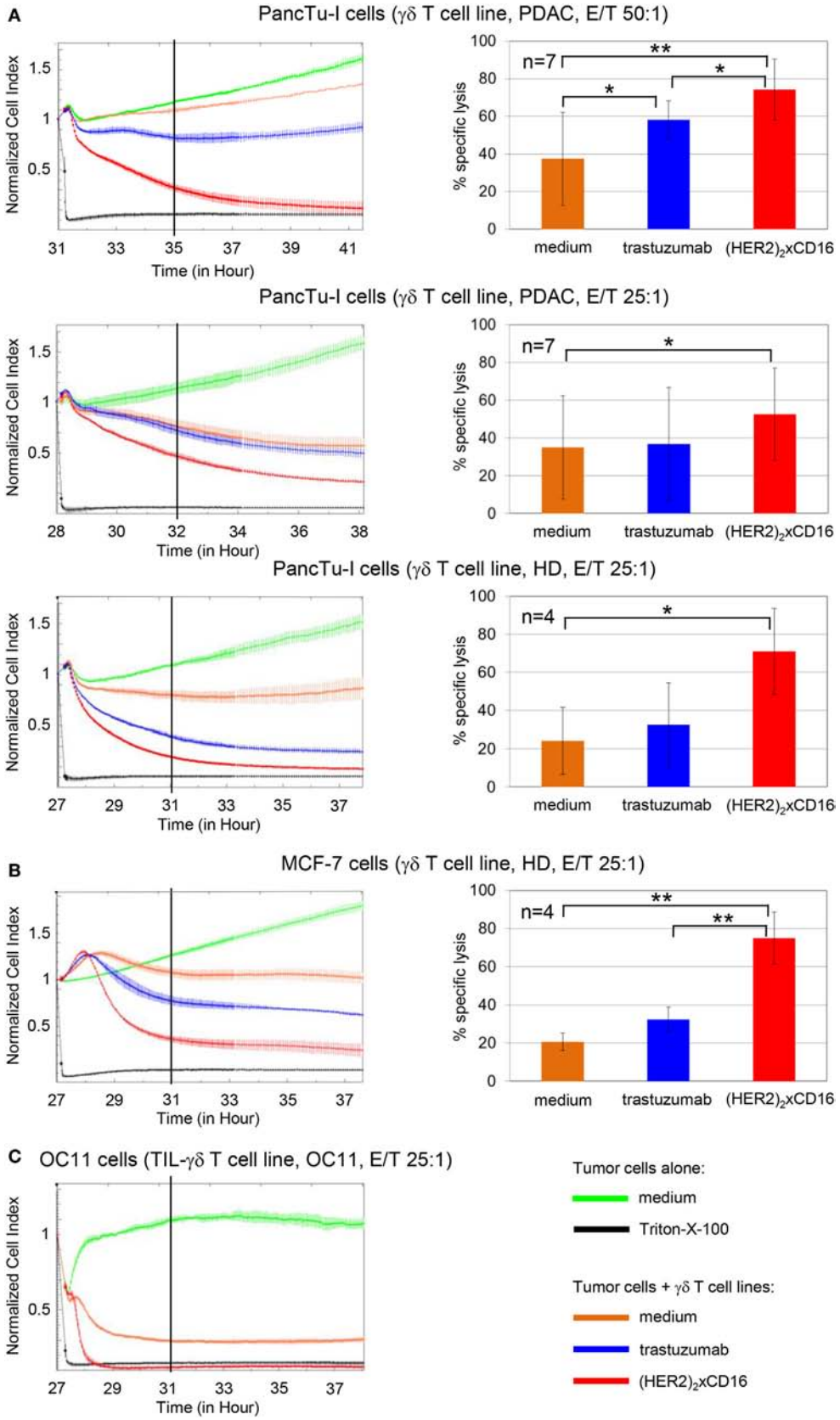

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FIGURE 7 | Enhanced cytotoxicity of tribody [(HER2)$_2$xCD16] compared to trastuzumab. 5 × 10^3 PDAC cells (PancTu-I and Panc89 cells), 10 × 10^3 breast cancer cells (MCF-7 cells), or 5 × 10^3 ovarain cancer (OC) OC11 cells were seeded overnight. Cell index was analyzed in 5 min steps over ~27–31 h. After reaching the linear growth phase, tumor cells were co-cultured with medium (green line) or with **(A,B)** allogeneic or **(C)** autologous Vγ9Vδ2 T cell lines at the indicated E/T ratio with 12.5 IU/mL rIL-2 in the presence of medium (orange line), 10 µg/mL trastuzumab (blue line), or 1 µg/mL [(HER2)$_2$xCD16] tribody (red line). Lysis of tumor cells was measured after normalization to 1 in 1 min steps for >10 h as indicated and compared to maximal lysis (black line). The average of three replicates with SD is represented for each tumor cell line with allogeneic Vγ9Vδ2 T cell lines of one representative healthy donor or cancer patients (PDAC) or autologous Vγ9Vδ2 T cell line established out of the TIL of patient OC11 (left panel) in independent experiments. Several replications of the experiments using different Vγ9Vδ2 T cell lines of different donors in independent experiments were performed (right panel). The cytotoxicity of Vγ9Vδ2 T cell lines against the indicated tumor cells in the presence of medium (orange bars), 10 µg/mL trastuzumab (blue bars), or 1 µg/mL tribody [(HER2)$_2$xCD16] (red bars) was calculated 4 h after addition of Vγ9Vδ2 T cell lines. The % of specific lysis was calculated by comparing measured samples to control sample (green line) and maximal lysis (black line). Statistical analysis was performed by *t* test. Significances are shown as *P* value; $^*P < 0.05$ and $^{**}P < 0.01$.

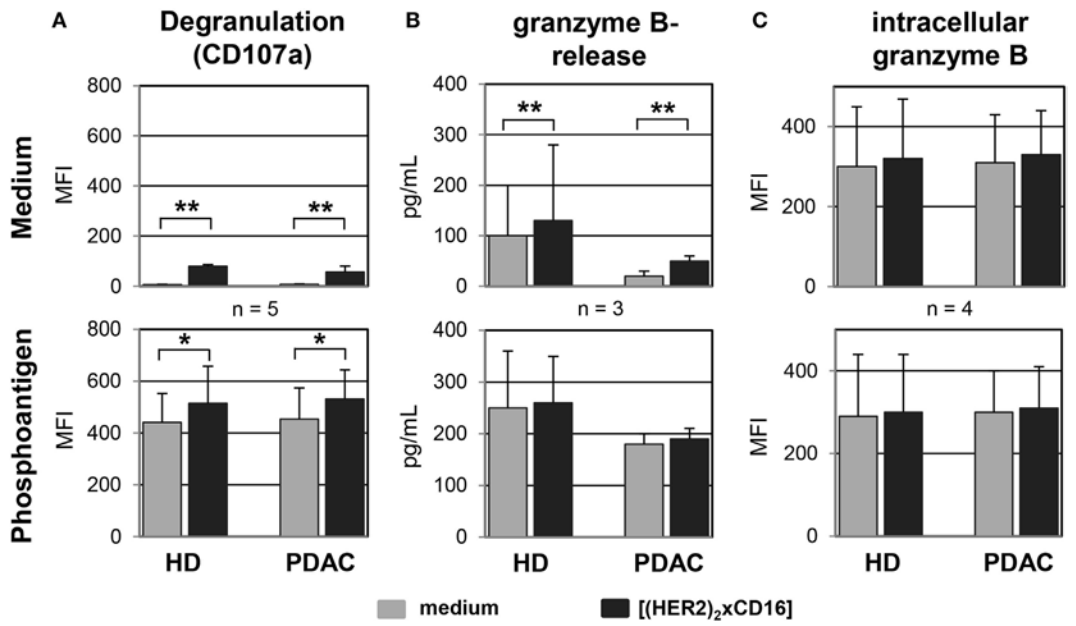

FIGURE 8 | Tribody [(HER2)$_2$xCD16] induces an enhanced degranulation and release of granzyme B. Short-term expanded γδ T cells were co-cultured with PancTu-I cells (E/T ratio of 12.5:1) in medium (upper panel) or 300 nM phosphoantigen bromohydrin-pyrophosphate (lower panel) in the presence of medium or 1 µg/mL of the tribody [(HER2)$_2$xCD16] and **(A)** degranulation was analyzed by staining γδ T cells with anti-Vδ2 TCR and anti-CD107a monoclonal antibodies and then determining the median fluorescence intensity (MFI) of CD107a on Vδ2 TCR-expressing γδ T cells using flow cytometry or by **(B)** measuring the release of granzyme B in the supernatants using specific ELISAs or **(C)** the intracellular MFI of granzyme B using a flow cytometer after 4 h of co-culturing. Result ± SD of three donors is shown. Statistical analysis was performed by *t* test. Significance is presented as $^*P < 0.05$ and $^{**}P < 0.01$.

These minibodies have the potential to target HER2-expressing tumor cells and promote their lysis by NK cells and mononuclear phagocytes. Here, we employed the tribody [(HER2)$_2$xCD16] to examine whether γδ T cells within PBL or TIL of PDAC and OC patients can be activated additionally to NK cells. Besides their HLA-independent recognition of antigens and their reduced induction of a graft-*versus*-host disease, an additional advantage of simultaneously activated γδ T cells is their capacity to present antigens, their possibility to induce maturation of DC, and their production of Th1 cytokines such as IFN-γ and TNF-α, which could compensate for a decreased number of CD4$^+$ T cells (producing Th1 cytokines) at the tumor-site of PDAC patients (33–35, 72). Regarding the role of γδ T cells in the uptake of antigens *via* Ab-opsonization and CD16 and their possibility to cross-present antigens to αβ T cells after their activation (33, 73), we can speculate that tumor antigens could be taken up by γδ T cells and presented to αβ T cells after activation based on our observation that PAg and tribody [(HER2)$_2$xCD16] stimulation induce a γδ T-APC phenotype. In contrast to the activation of NK cells by [(HER2)$_2$xCD16], our results revealed that γδ T cells have to be concomitantly activated by their selective antigens and IL-2 in addition to the tribody to exert enhanced cytotoxic activity. Regarding a pre-activation of γδ T cells, an initial application of clinically licensed n-BP or PAg together with IL-2 is necessary for γδ T cells expansion to guarantee their enhanced cytotoxic activity by tribody [(HER2)$_2$xCD16].

Our results clearly demonstrate that γδ T cells within PBL or TIL can be activated *via* a combined stimulation by their selective antigens plus IL-2 plus tribody [(HER2)$_2$xCD16] to effectively lyse allogeneic as well as autologous tumor cells *via* the release of granzyme B. In general, cytotoxic γδ T cells often release

granzymes out of their cytolytic granules after their activation by bsAb, which could be an advantage regarding the aspect that several tumor cells are almost resistant to the CD95- or TRAILR-induced cell death (38, 47). In addition, an adoptive transfer of γδ T cells with [(HER2)$_2$xCD16] or [(HER2)$_2$xVγ9] could enhance their cytotoxicity and support the anti-tumor response of innate cells activated *via* [(HER2)$_2$xCD16]. Regarding the reported anti-tumor response of macrophages and neutrophils, an additional activation of γδ T cells at the tumor-site can be promising with respect to the differential infiltration of innate cells and γδ T cells in different tumor entities (74). If a bsAb targeting two HER2 molecules should be replaced by a bsAb targeting HER2/HER3 or HER2/HER4 or other families of the EGFR family has to be evaluated but the cognate activation of innate and innate-like cells (e.g., γδ T cells) *via* CD16 or other common ligands such as NKG2D-specific ligands for NK cells and γδ T cells seems to be promising (75).

ETHICS STATEMENT

Patient cohorts leukocyte concentrates from healthy adult blood donors were obtained from the Department of Transfusion Medicine of the University Hospital Schleswig-Holstein (UKSH) in Kiel, Germany. Heparinized blood was drawn from HDs of the Institute of Immunology [UKSH, Christian-Albrechts University (CAU)], whereas blood from patients was obtained from the Department of General and Thoracic Surgery or the Clinic of Gynecology and Obstetrics, both of the UKSH in Kiel. In accordance with the Declaration of Helsinki, written informed consent was obtained from all donors, and the research was approved by the relevant institutional review boards (ethic committee of the Medical Faculty of the CAU to Kiel, code number: D405/10, D403/14, D404/14, AZ A157/11, AZ B3277/10).

AUTHOR CONTRIBUTIONS

HO, CK, MP, and DW performed the conception and the experimental design. The experiments, the analysis, and interpretation of the data and the preparation of the figures were done by HO, CK, MP, DG, and DW. SS and DB organized and provided the blood and tissue from PDAC and ovarian cancer patients, respectively. DK and MG provided the infrastrcuture for experimental set up and were helpful with the interpretation of the results. DW and HO wrote the manuscript. CK, MP, DK, SS, DB, DG, and MG revised the manuscript critically. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00814/full#supplementary-material.

FIGURE S1 | Impact of trastuzumab on cancer cells with different human epidermal growth factor receptor 2 (HER2) expression. **(A)** 5 × 10^3 Panc1 cells, 5 × 10^3 SK-OV-3 cells and 5 × 10^3 primary ovarian cancer OC11 [(i), green lines] had been cultured in 10 % FCS medium for 26 h in plates which measured the impedance of the adherent tumor cells with electronic sensors. Impedance is expressed as an arbitrary unit called cell index (CI) every 5 min, which was normalized to 1 shortly before the addition of substances as follows: 10 µg/mL trastuzumab [(iii), brown line], PBL of healthy donors (E/T ratio 50:1) together with 50 IU/mL rIL-2 [(iv), purple line], or a combination of (iii) and (iv) as indicated [(v), blue line]. Triton-X-100 was added as positive control to induce maximal lysis [(ii), black line, positive control]. CI was then measured every minute for analysis of precise cytotoxicity time point for additional 40 h. The trastuzumab and/ or PBL-mediated lysis of HER2-expressing tumor cells correlated with the loss of tumor cell impedance and thus a decrease of the normalized CI. The average of triplicates and SD are presented; one representative experiment out of three is shown. **(B)** HER2 expression of Panc1 cells, SK-OV-3 cells as well as primary ovarian cancer cells OC11 was analyzed by staining the cells with 10 µg/mL trastuzumab (grey histograms) and appropriate isotype controls (open black lines) as indicated, following by appropriate second step Ab and measuring by flow cytometry. Numbers indicate the median fluorescence intensity of the appropriate staining with trastuzumab.

FIGURE S2 | Control constructs and tribody [(HER2)$_2$xCD16] did not modulate impedance of tumor cells. 5 × 10^3 pancreatic ductal adenocarcinoma cells (Panc89 and PancTu-I) were cultured with medium (green line), control constructs such as 1 µg/mL tribody [(HER2)$_2$xCD89] (purple line) or [(CD20)$_2$xCD16] (light blue line), respectively or 1 µg/mL [(HER2)$_2$xCD16] (red line) or with Triton-X-100 (black line) for the indicated time points. The cell index (CI) was analyzed in 5 min steps over ~24 h and in 1 min steps after 24 h. The average of three replicates with SD is presented for each tumor cell line in independent experiments.

FIGURE S3 | Analysis of CD16 expression on short-term activated γδ T cells. For flow cytometric analysis, a gate was set on expanded Vδ2 γδ T cells with a purity of >95% (based on forward and side scatter properties to exclude dead cells) and on pan T cell receptor γδ-positive cells to determine the relative percentage of CD16-expressing Vδ2 γδ-positive T cells from healthy donors (n = 11) and from pancreatic ductal adenocarcinoma patients (n = 11). Each symbol presents the data of one donor, and the thick bars represent the mean value of different experiments. Statistical analysis was performed by t test. Significances are shown as P value; $^*P < 0.05$.

FIGURE S4 | γδ T cell cytotoxicity against human epidermal growth factor receptor 2 (HER2)-positive PancTu-I cells and HER2-negative Raji cells with

tribody [(HER2)$_2$xCD16]. Vγ9Vδ2 γδ T cell lines of three healthy donors were co-cultured with complete medium (no bispecific antibodies), 1 µg/mL control tribody [(CD20)$_2$xCD16] or 1 µg/mL tribody [(HER2)$_2$xCD16] in the presence or absence of 300 nM phosphoantigen bromohydrin-pyrophosphate as indicated. γδ T cell lines at the indicated E/T ratios were added to ^{51}Cr-labeled HER2-positive PancTu-I cells or HER2-negative Raji cells for 4 h. Each bar represents the mean value of three independent donors in triplicate assays. Statistical analysis was performed by ANOVA. Significances are shown as *P* value; $^{*}P < 0.05$ and $^{**}P < 0.01$.

FIGURE S5 | Tribody [(HER2)$_2$xCD16] enhanced lysis of human epidermal growth factor receptor 2 (HER2)-expressing cancer cells in comparison to trastuzumab. 5×10^3 Panc89 cells or esophageal cell line OE33 were cultured overnight in complete medium. After reaching the linear growth phase, Vγ9Vδ2 T cell lines at the indicated E/T ratio and substances were added as follows: without effector cells: complete medium (green line) or Triton-X-100 (black line); with effector cells and 12.5 IU/mL rIL-2 in medium (orange line), with 10 µg/mL trastuzumab (blue line), or with 1 µg/mL tribody [(HER2)$_2$xCD16] (red line). The cell index was analyzed in 5 min steps over ~28–53 h and after normalization to 1 (after addition of PBL ± substances) in 1 min steps for >10 h. The average of three replicates with SD is represented for each tumor cell line with effector cells of one representative healthy donor (HD) in independent experiments (left panel). For validation, experiments were replicated several times under equal conditions using different Vγ9Vδ2 T cell lines of different donors in independent experiments (right panel). The cytotoxic capacity of Vγ9Vδ2 T cell lines against the indicated tumor cells calculated 4 h after addition of effector cells as % of specific lysis compared to control sample (green line) and maximal lysis (black line). Statistical analysis was performed by t test. Significances are shown as *P* value; $^{**}P < 0.01$.

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REVIEW
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Improving the Efficiency of Vγ9Vδ2 T-Cell Immunotherapy in Cancer

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Increasing immunological knowledge and advances in techniques lay the ground for more efficient and broader application of immunotherapies. gamma delta (γδ) T-cells possess multiple favorable anti-tumor characteristics, making them promising candidates to be used in cellular and combination therapies of cancer. They recognize malignant cells, infiltrate tumors, and depict strong cytotoxic and pro-inflammatory activity. Here, we focus on human Vγ9Vδ2 T-cells, the most abundant γδ T-cell subpopulation in the blood, which are able to inhibit cancer progression in various models *in vitro* and *in vivo*. For therapeutic use they can be cultured and manipulated *ex vivo* and in the following adoptively transferred to patients, as well as directly stimulated to propagate *in vivo*. In clinical studies, Vγ9Vδ2 T-cells repeatedly demonstrated a low toxicity profile but hitherto only the modest therapeutic efficacy. This review provides a comprehensive summary of established and newer strategies for the enhancement of Vγ9Vδ2 T-cell anti-tumor functions. We discuss data of studies exploring methods for the sensitization of malignant cells, the improvement of recognition mechanisms and cytotoxic activity of Vγ9Vδ2 T-cells. Main aspects are the tumor cell metabolism, antibody-dependent cell-mediated cytotoxicity, antibody constructs, as well as activating and inhibitory receptors like NKG2D and immune checkpoint molecules. Several concepts show promising results *in vitro*, now awaiting translation to *in vivo* models and clinical studies. Given the array of research and encouraging findings in this area, this review aims at optimizing future investigations, specifically targeting the unanswered questions.

Keywords: gamma delta T-cell, cancer immunotherapy, tumor metabolism, ADCC, NKG2D, immune checkpoints, programmed cell death protein 1, vascular endothelial growth factor

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INTRODUCTION

Following the discovery in the 1980s, gamma delta (γδ) T-cells have become increasingly recognized as important players in natural host defense against infections and malignancies. Early evidence of an anti-tumor functionality of γδ T-cells came from the experiments in mice (1) and it is now well established (2). In humans, γδ T-cells can be found in various cancer tissue samples [e.g., melanoma (3, 4) and epithelial tumors (5–11)]. More recently, analysis of microarray data also described patterns of γδ T-cells in a large collection of malignancies (12) and a prior extensive gene expression study demonstrated that γδ T-cell infiltration into tumors represents a positive prognostic marker in many types of cancer (13). Offering some hints for a functional role in tumor rejection, γδ T-cell infiltration in melanoma, colorectal cancer, and lung tumors were found to be associated with lower stage and lack in metastasis. Additionally, γδ T-cells extracted from such cancer tissues were able to kill malignant cells *in vitro* (4, 14, 15). In cancer patients, γδ T-cells were also repeatedly found reduced or defective and depicted a diminished proliferative capacity (16–18)

and exhaustion (19–23). Patients with higher γδ T-cell count following allogenic stem cell transplantation for acute leukemia had a significant survival advantage (24). In connection with their suspected function in natural tumor defense, the utilization of γδ T-cells has become a promising concept in the field of cancer immunotherapy.

Definition

γδ T-cells express variables Vγ and Vδ chains (25, 26) as part of a T-cell receptor (TCR) complex that is structurally and functionally distinctive from the major histocompatibility complex (MHC) binding TCR of αβ T-cells (27). In humans, it is feasible to further divide γδ T-cells into "Vδ2" and "non-Vδ2 cells," the latter consisting of mostly Vδ1- and rarely Vδ3- or Vδ5-chain expressing cells. Despite unrestricted and the theoretically high combinatory diversity (28), the Vδ2 chain is found preferentially paired with the Vγ9 chain (29). These Vγ9Vδ2 T-cells account for approximately 5% of peripheral blood T-cells, representing the dominant γδ T-cell subpopulation in this compartment in healthy human adults (30). Interestingly, the preferential appearance of Vγ9- and Vδ2-chains develops in the fetus (31), but the overall clonal repertoire of blood γδ T-cells is further contracting after birth (32). The latter is probably a response to a uniform stimulus, like a ubiquitous pathogen or conserved stress molecule (33).

Functional Aspects

Genetic and functional studies indicate that γδ T-cells have developed and act as an intermediate between the innate and the adaptive immune system. Features representative of an innate phenotype is their ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis and to rapidly react toward pathogen-specific antigens without prior differentiation or expansion (28). Notably, the gene expression signature of Vγ9Vδ2 T-cells was characterized as a hybrid of αβ and NK-cells (34). Typical characteristics of the adaptive immune system, found in γδ T-cells, are their capabilities for somatic recombination of receptor genes, memory formation (35), and professional antigen presentation (36). Unlike αβ T-cells, γδ T-cells respond directly to proteins and non-peptide antigens (37) and are therefore not MHC restricted (38). At least some γδ T-cell specific antigens display evolutionary conserved molecular patterns, found in microbial pathogens and "induced self-antigens," which become upregulated by cellular stress, infections, and transformation (28). Following the observation on stimulatory effects of certain non-peptide mycobacterial components on Vγ9Vδ2 T-cells (39, 40), the responsible substances could be isolated and characterized and are commonly termed as phosphoantigens (PAgs) (41). We consider PAgs the primary trigger of Vγ9Vδ2 γδ T-cell activation and discuss them in greater detail in the following. However, Vγ9Vδ2 γδ T-cells may also respond to other antigens and ligands *via* TCR and (co-)receptors (42).

Vγ9Vδ2 T-Cells in Cancer Immunotherapy

Subsets of Vγ9Vδ2 T-cells can be defined analyzing the expression of surface markers (e.g., CD27, CD45RA, CCR7, and CD16) or regarding their dominant cytokine production and correlate with functional differences like proliferative capacity or cytotoxic potential (43, 44). It has been extensively demonstrated *in vitro* (45–55) and using *in vivo* models (22, 56–68) that γδ T-cells are able to recognize various tumor cells and exert strong anti-tumor effects. Tumor growth is inhibited *via* different mechanisms including the release of pro-inflammatory cytokines, granzymes and perforin, and the engagement of apoptosis inducing receptors (69).

Several drugs and treatment concepts might improve the activity of Vγ9Vδ2 T-cells against cancer. Most candidates are still at a pre-clinical stage, some were tested in animal models, and very few went into clinical tests so far. Although Vδ1+ cells shown promising results pre clinically (70), all previous clinical trials focused on the usage of Vγ9Vδ2 T-cells. Reasons for the earlier therapeutic employment of Vγ9Vδ2 T-cells include their relatively high abundance in the peripheral blood and the possibility to efficiently culture them *ex vivo* or to stimulate and expand them *in vivo* using amino-bisphosphonates (N-BP) or synthetic PAgs (45), as discussed later.

Here, we divide the existing clinical studies according to the used strategy into two main groups: (1) *in vivo* activation (17, 18, 23, 71–74) and (2) adoptive cell transfer strategies (75–84). In the latter case, the adoptively transferred cells originally were extracted, activated, and cultured autologous blood cells. Varieties include the transfer of processed haploidentical cell preparations with subsequent *in vivo* stimulation (82), as well as local administration of cultured cells into the tumor or the peritoneal cavity (85, 86). Well organized and comprehensive analyses of the performed clinical studies involving γδ T-cells have recently been published by others (45, 87, 88) and an overview is given in **Table 1**.

Outline

Much has been learned by studying γδ T-cells from animals, especially those from mice. However, there are major distributional, structural, and functional differences between the species, especially the lack of Vγ9Vδ2 T-cells or functional homologs in mice (91, 92). In this review, we focus on human γδ T-cells, their anti-tumor capabilities, and strategies for improving the effectiveness of Vγ9Vδ2 T-cells in cancer immunotherapy. Current publications contain additional information on the topics not covered here, especially the biology of non-Vδ2 cells (93) and their role in cancer and cancer therapy (2). We also refer to more detailed literature regarding the differences of rodent and human γδ T-cells (28), γδ T-cells acting as professional antigen-presenting cells (36), concerning B-cell help (94) and potential use as a vaccine (95), cell ontogeny (33), phylogenetic aspects (28, 42), genetically modified γδ T-cells (e.g., CARs) (96, 97), as well as molecular details of receptor signaling (98, 99). We discuss approaches especially that aim to sensitize target cells and the local interaction of tumor and effector cells in connection with the underlying mechanisms.

TARGETING THE CELLULAR METABOLISM

Survival and growth of cancer cells are connected to specific metabolic alterations which have been considered a distinctive

TABLE 1 | Clinical studies.

Reference	Year	Disease	*N*	Reported outcome	Systemic therapy/comments
***In vivo* stimulation**					
Wilhelm et al. (18)	2003	MM, indolent, lymphomas	19	16% PR, 16% SD	+PAM +IL-2/response correlates with *in vitro* expansion
Dieli et al. (23)	2007	HRPC	18	16% PR, 27% SD	+ZOL +IL-2
Bennouna et al. (73)	2010	RCC, GYN-, GI-cancers	28	42% SD	+BrHPP +IL-2
Laurent et al. (89) abstract only	2010	Follicular lymhoma	45	26% CR, 18% PR	+BrHPP +IL-2 +RTX
Meraviglia et al. (71)	2010	Breast cancer	10	10% PR, 20% SD	+ZOL +IL-2/response correlates with *in vivo* expansion
Lang et al. (74)	2011	RCC	12	16% SD	+ZOL +IL-2
Kunzmann et al. (72)	2012	RCC, melanoma, AML	21	16–42% SD AML: 25% PR	+ZOL +IL-2
Pressey et al. (17)	2016	Neuroblastoma	4	25% SD, 75% PD	+ZOL +IL-2
Adoptive transfer					
Kobayashi et al. (78)	2007	RCC	7	Delayed tumor doubling times in 4/7 patients	–
Bennouna et al. (75)	2008	RCC	10	60% SD	–
Abe et al. (80)	2009	MM	6	66% SD	–
Nakajima et al. (81)	2010	Lung cancer	10	30% SD	–
Kobayashi (79)	2011	RCC	11	9% CR, 45% SD	+ZOL +IL-2
Nicol et al. (84)	2011	Solid tumors	18	16% SD, 16% PR and CR	+ZOL +other tumor-specific treatments
Noguchi et al. (77)	2011	Solid tumors	25	12% SD, 12% PR	+other tumor-specific treatments
Sakamoto et al. (76)	2011	Lung cancer	15	40% SD	–
Cui et al. (86)	2014	HCC	62	Longer PFS and OS	–/in addition to radiofrequency ablation
Wilhelm et al. (82)	2014	Hematological malignancies	4	75% CR	+ZOL +IL-2 +Chemo/*in vivo* stimulation following transfer of haploidentical cells
Wada et al. (85)	2014	Gastric cancer	7	Reduction in ascites in 2/7 patients	–/intraperitoneal administration of γδ T-cells
Aoki et al. (90)	2017	Pancreatic cancer—adjuvant	28	Higher recurrence free survival in patients with sustained higher γδ T-cell numbers	+Chemo

AML, acute myeloid leukemia; BrHPP, bromohydrin pyrophosphate; Chemo, chemotherapy; CR, complete remission, GI, gastrointestinal; GYN, gynecological; HCC, hepatocellular carcinoma; HRPC, hormone refractory prostate cancer; MM, multiple myeloma; N, number of patients; OS, overall survival; PAM, pamidronic acid; PD, progressive disease; PFS, progression free survival; PR, partial remission; RCC, renal cell carcinoma; RTX, rituximab; SD, stable disease; ZOL, zoledronic acid.

"hallmark of cancer" (100). Most prominent example of such adaptation is the "Warburg effect," the preferential utilization of aerobic glycolysis by various tumor cells, described by Warburg in 1924 (101). Obvious elements of this phenotype are the inhibition of oxidative phosphorylation despite sufficient oxygenation, an elevated glucose consumption, and an increased production of lactic acid (LA). Changes in the tumor metabolism can be complex and beside glucose metabolism also affect lipid and amino acid pathways (102). Correspondingly, our idea of Vγ9Vδ2 T-cell natural anti-tumor functions is based on their ability to distinguish normal and transformed cells due to their metabolic phenotype. In particular, they might recognize an intrinsic overproduction of PAgs arising from isoprenoid biosynthesis in tumor cells.

Many PAgs are naturally occurring prenyl-pyrophosphates (41) originating from isopentenyl pyrophosphate (IPP) of the eukaryotic mevalonate pathway as well as those generated in the microbial non-mevalonate (also termed as MEP or DOX-P) pathway (103). A dysregulated mevalonate pathway, conjoined with a higher abundance of mevalonate pathway products was described in certain malignant cell types (104, 105) and may indeed be important to support the survival of malignant cells (106). PAg accumulation has been explained by increased buildup, especially of IPP due to upregulation of the gate-keeping enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (107) and other mevalonate pathway enzymes (104). We currently lack sufficient information to decide if a dysregulated mevalonate pathway associated with increased PAgs is indeed a "general hallmark of tumorigenesis" rather than an outlier. In any case, several therapeutic concepts focus on Vγ9Vδ2 T-cells' metabolic sensor and potent effector mechanisms.

N-BPs and PAgs

Activation of Vγ9Vδ2 T-cells with PAgs and N-BPs is the most commonly used strategy for *in vitro* research and both *in vivo* stimulation as well as application of adoptive cell therapy. The potency of the individual PAg molecule to elicit response from Vγ9Vδ2 T-cells differs (108) and is especially high for microbial (E)-4-hydroxy-3-methyl-butenyl pyrophosphate (HMBPP), certain synthetic compounds like bromohydrin pyrophosphate (BrHPP) (109) or nucleotides derived from HMBPP (110). However, so far only BrHPP and N-BPs have been used clinically. N-BPs were found to trigger activation and expansion of Vγ9Vδ2 T-cells as well as their interferon-γ (IFN-γ) release (46, 111) and were later recognized as indirect acting PAgs (112). This class of substances is structurally related to direct PAgs, but acts by inhibition of farnesyl diphosphate synthase and the accumulation of upstream metabolites like the direct PAg IPP (113). In immunotherapy N-BPs serve a double purpose. First, they sensitize target cells, rendering many primarily resistant tumor

cells vulnerable to γδ T-cell mediated attack (114). Second, they induce expansion of γδ T-cells *in vivo* and *in vitro*. The degree of inhibition of farnesyl diphosphate synthase thereby correlates well with important anti-tumor functions of Vγ9Vδ2 T-cells over various tumor cell lines (115). Apart from sensitization of tumor cells, N-BPs exert additional direct anti-neoplastic effects, like an increased production of toxic mevalonate pathway products and a decrease of essential downstream metabolites (113, 116).

Ex Vivo Culture and *In Vivo* Models

Potent natural and synthetic PAgs, like the patented drug BrHPP (termed as IPH1101 or Phosphostim®) can be used for effective *in vitro* (117) and *in vivo* (22, 75) expansion of Vγ9Vδ2 T-cells.

Protocols for *ex vivo* culture of human Vγ9Vδ2 T-cells vary regarding the culturing conditions, timing and dosage of used N-BPs or PAgs, and added co-stimulators like IL-2 (88, 118) and may result in different phenotypes and effector cell characteristics. Zoledronic acid (ZOL) is a potent N-BP and commonly used about 1 μM *in vitro*, a concentration also in the range of the peak plasma level following a single standard dose of 4 mg intravenously (88). Repetitive administration of exogenous IL-2 is commonly used as it drives proliferation of PAg stimulated Vγ9Vδ2 T-cells resulting in an increased yield (63, 67). Results of *in vitro* expansion are highly donor dependent and may also predict the respective *in vivo* expansion efficacy, which can be additionally restricted in cancer patients (18). Currently, an optimal dose of ZOL as well IL-2 has not been determined *in vivo* (88) and a recent study indicated that the efficacy of ZOL stimulation depends on drug concentration and duration of exposure with an individual optimum (67).

The ability to recognize the PAgs is linked to germline-encoded regions of the γδ TCR (119) and so far functionally only described in primates (120). Even though homologs sequences of human Vγ9 and Vδ2 genes were recently described in other species, such as alpaca and armadillo (121, 122). As wild type mice lack PAg-responding γδ T-cells the *in vivo* expansion of human Vγ9Vδ2 T-cells has been studied using xenograft mice (57, 123) or cynomolgus monkeys (59). Results from such models show that sensitizing tumor cells with N-BPs, combined with adoptive transfer of *ex vivo* expanded human Vγ9Vδ2 T-cells with or without exogenous IL-2 administration is feasible and induces moderate anti-tumor responses (58, 65, 66, 68, 124). The role for additional systemic application of N-BPs in context with adoptive cell transfer strategies remains uncertain. On one side it has been reported to promote engraftment of *ex vivo* stimulated and adoptively transferred human cells in mice (124), on the other side there are indications that repetitive application of these drugs *in vivo* induces Vγ9Vδ2 T-cells exhaustion (23, 71, 74).

Clinical Experience

One may speculate that the observed anti-tumor effects of N-BPs or high-dose IL-2 monotherapy as well as allogenic stem cell transplantation are influenced by γδ T-cells without being recognized as such (125–127). Implementation of clinical Vγ9Vδ2 T-cell studies benefited from the fact that side effects and pharmacological profiles of N-BPs and IL-2 monotherapy were already known. IL-2 is established as an effective treatment for several types of cancer for about 30 years (128) and N-BPs are widely used for osteoporosis, hypercalcemia, and the treatment of bone metastasis (125). The first prospective trial focusing on the *in vivo* stimulation of anti-tumor functions by γδ T-cells used the N-BP pamidronic acid (18), later studies the more potent ZOL (17, 23, 71, 72, 74) in combination with IL-2. These N-BPs have also been used to stimulate Vγ9Vδ2 T-cells *ex vivo* for adoptive cell therapy (76, 77, 80, 81, 83). Additionally, a few studies applying adoptive cell transfer included the systemic administration of ZOL with (79, 82) or without additional IL-2 (84). Taken together the clinical studies involving the use of N-BPs to increase the anti-tumor effects of Vγ9Vδ2 T-cells in different types of malignancies depicted a tolerable toxicity but revealed inconsistent responses and overall only a modest efficacy (compare **Table 1**).

Similarly, BrHPP was tested in early clinical studies with small success, for both *ex vivo* stimulation and consecutive adoptive transfer of cells in combination with IL-2 in metastatic renal cell carcinoma (75) and for *in vivo* stimulation targeting solid tumors (73). A strategy combining BrHPP stimulation and the tumor targeting antibodies rituximab (RTX) (89) is discussed separately.

Current Obstacles

Several reasons might explain the limited therapeutic effectiveness of both N-BPs and synthetic PAgs *in vivo*. Maybe most importantly N-BPs and synthetic PAgs lack cancer specificity regarding uptake or molecular targeting and also affect other cells. Also, N-BPs and BrHPP both have short plasma half-life periods (22, 67). BrHPP is quickly degraded by plasma phosphatases and common N-BPs cannot passively cross the plasma membrane, and is preferentially rooted to the bone due to their calcium binding characteristics (112). Cancer cells in other compartments are those that lack adequate active transport mechanisms might therefore not be affected. It is established that monocytes/macrophage type cells take up N-BPs *via* fluid endocytosis and induce activation of Vγ9Vδ2 T-cells (129, 130). Unfortunately ZOL also induces killing of human macrophages (131) and, additionally, uptake of N-BP by neutrophils impairs γδ T-cell proliferation *via* production of reactive oxygen species (132). Indeed treatment with N-BP can decrease circulating γδ T-cell count (133) and repetitive stimulation with BrHPP lead to progressive exhaustion of γδ T-cell activation and expansion *in vivo* (22). A new strategy to stimulate Vγ9Vδ2 T-cells and avoid exhaustion might be the application of an attenuated, live vaccine with genetically engineered metabolic profile that overproduces HMBPP. Adapting traits of a bacterial infection with *Salmonella enterica* indeed elicited a prolonged Vγ9Vδ2 T-cell immunity in monkeys (134). A different concept to increase N-BP concentration in the tumor tissue is to administer drugs (and *ex vivo* stimulated cells) locally (135). Nevertheless, this is not a working concept for systemic diseases. It also has to be taken into account that although commonly well tolerated, N-BPs and exogenous IL-2 have considerable and dose limiting toxicities, including inflammatory and cytokine reactions, osteonecrosis of the bone, and hypocalcemia (128, 136).

Modified PAgs and N-BPs

The development of new direct and indirect PAgs may overcome pharmacodynamic restrictions and improve clinical efficacy (112). Newly designed PAgs (137) and bisphosphonate prodrugs (138, 139) have chemically masked phosphate groups, allowing these compounds to enter cells without the need for active transmembrane transport (140) and should not accumulate in the bone. Following intracellular uptake they are converted to their active forms, which are potent stimulators of Vγ9Vδ2 T-cells and sensitize different tumor cell lines toward γδ T-cell anti-tumor effects *in vitro* (138–140). Bisphosphonate prodrugs already depicted some effect in combination with adoptive cell transfer in an animal model of bladder carcinoma and human fibrosarcoma (138, 139).

Nano-technology based carriers for N-BP delivery (141) as well as lipophilic bisphosphonate (60, 142, 143) and synthetic nucleotide pyrophosphates (110) are additional pharmacotherapeutic strategies that may improve Vγ9Vδ2 T-cell immunotherapy in the future.

Butyrophilin 3A (BTN3A)

More recently, Butyrophilin 3A (BTN3A, CD277) was described as essential for γδ T-cell activation by direct PAgs (144, 145). BTN3A belongs to the important B7 family of co-stimulatory molecules (146) and consists of three isoforms: BTN3A1, BTN3A2, and BTN3A3. BTN3A2 differs as it lacks an intracellular B30.2 domain that is needed for PAg recognition. However, when using the mouse anti-human-CD277 antibody clone 20.1 directed against an extracellular domain, all BTN3A isoforms support Vγ9Vδ2 T-cell activation (144). The molecular details of signal transduction are a current research topic and matter of debate, especially regarding two different models: originally, the "antigen presenting model" by Vavassori et al. (145) assuming that CD277 and the TCR interact directly following PAg binding to an extracellular CD277 domain. Recent experimental evidence rather supports a second, so called "allosteric model" by Harly et al., postulating that PAgs interact with the intracellular B30.2 domain of CD277 (147) either directly (148) or indirectly (149, 150) and induce a conformational change that is transferred to the extracellular parts of the CD277 molecule (147, 151). PAg sensing may additionally involve molecules like Rho-GTPase (152) or Periplakin and is modulated by mechanisms enabling transmembranous PAg transport or *via* hydrolyzation of PAgs by ecto-ATPase CD39 (106, 153).

Development of mouse anti-human-CD277 antibodies has been very useful in deciphering the activation processes of Vγ9Vδ2 T-cells (144, 154) and also holds therapeutic potential. The mode of action of these antibodies was proven to be downstream and independent of IPP (144, 149). Furthermore, activating anti-CD277 clone 20.1 has similar but not identical stimulatory capabilities compared with PAg stimulation (155) and might be restricted to certain Vγ9Vδ2 T-cells with specific complementarity-determining region sequences of the TCR (156). Still, anti-CD277 antibodies might outperform N-BPs or other metabolic sensitizers in target cells that fail to internalize drugs or which have decreased mevalonate pathway activity. It was shown that anti-CD277 antibodies enhance anti-tumor functions of Vγ9Vδ2 T-cells *in vitro* (144) and in a xenotransplant mouse model of human acute myeloid leukemia (AML) (157). We also observed that primary chronic lymphatic leukemia (CLL) cells are hardly affected by ZOL sensitization become lysed by Vγ9Vδ2 T-cells following their incubation with activating anti-CD277 antibody (158). Unfortunately, antibodies with a murine background seem inappropriate for clinical use and development of a humanized version or a human homolog of the clone 20.1 antibody has not been reported. A further drawback is the widespread expression of the CD277 molecule in human tissues (146, 159), which is why additional strategies for enhancement of selectivity might be required. One solution could be the development of antibody constructs combining both CD277 activating and tumor-antigen specificity.

Other Agents

Therapeutic specificity might also be achieved by targeting tumor cell specific metabolic alterations. Therefore, we tested whether the pyruvate dehydrogenase activator dichloroacetate (DCA) might improve Vγ9Vδ2 T-cell anti-tumor functions *in vitro*. DCA inhibits aerobic glycolysis, malignant cell proliferation and indirectly facilitates mitochondrial oxidative decarboxylation of pyruvate to acetyl-coenzyme A (160). Indeed, we found that DCA + ZOL treated leukemia cell lines induce higher IFN-γ production by Vγ9Vδ2 T-cells compared with ZOL treatment alone. We also suspected that DCA increases the supply of metabolites upstream of IPP and therefore increases PAg accumulation when combined with ZOL (161). Still, alternative explanations are possible as DCA decreases tumor cells' LA production (160) and LA can directly inhibit several immune functions. Tumor LA efflux is, therefore, an attractive target and could be targeted by inhibition of lactate transporters and nonsteroidal anti-inflammatory drugs (NSAID) (101). Concerning potential anti-tumor effects of NSAIDs, the use of indomethacin as well as specific cyclooxygenase-2 inhibitors resulted in an increase of Vγ9Vδ2 T-cell dependent tumor cell lysis. If this observation is connected to LA release has not been investigated but was attributed to the inhibition of prostaglandin effects (162). Finally, the enzymes CD39 and CD73 that regulate ATP/adenosine balance and thereby the function of immune cells might represent interesting targets for immunotherapy (163). Here, CD39 might be of special interest in the context of Vγ9Vδ2 T-cell therapy as it was shown to be capable of PAg hydrolyzation (164).

Summary

Adoptive transfer of *ex vivo* cultured cells and various combinations of N-BPs, BrHPP, and IL-2 have demonstrated clinical effects but are rather disappointing compared to the promising pre-clinical results. The discrepancy suggests that the *in vivo* characteristics of stimulated Vγ9Vδ2 T-cells are still insufficiently understood. To overcome the current limitations, we need to learn more about differentiation and functionality of PAg activated γδ T-cells, its subpopulations and migration patterns. PAgs and N-BPs with improved pharmacokinetics and potency are very promising new developments, but their toxicity profile and clinical effectiveness have yet to be established. A breakthrough

would be the development of PAg or N-BP analogs with strong molecular tumor cell specificity.

Beside these innovations, we should search for additional tumor-specific transport mechanisms and metabolic peculiarities. A good example for the exploitation of a "metabolic weak spot" in cancer is the use of asparaginase in acute lymphatic leukemia (165). We need to identify such targets in the context of γδ T-cell sensing and will hopefully be able to design specific and effective compounds at least for certain types of cancer. Finally, we should consider the metabolic needs of immune cells as well. They may also rely on mevalonate pathway products or upregulate aerobic glycolysis following activation (166) and therefore become negatively affected by certain therapeutic interventions.

TARGETING ACTIVATING AND INHIBITORY RECEPTORS

NKG2D and Its Ligands

In innate immune responses mediated by NK-cells, NKG2D serves as a primary activating receptor and ligand binding triggers cytotoxicity and cytokine production (167–169). In humans, one NKG2D homodimer assembles with four DAP10 adaptor proteins that become phosphorylated upon ligand binding and activation (170). Ligands from distinctive families, the MHC class I polypeptide related sequence A (MICA) and B (MICB) and the cytomegalovirus UL16-binding protein (ULBP) family bind NKG2D even though they share little sequence similarity (171). The expression of NKG2D ligands (NKG2DL) is induced or upregulated primarily in tissues of epithelial origin, as a result of cellular stresses such as viral infection, malignant transformation, or classical heat shock (172, 173). All NKG2DLs are not functionally equivalent and can enable immune cells to recognize of a broad range of different types of infections and indicate malignant transformation in different tissues (170, 171, 174).

NKG2D is also expressed by γδ T-cells and provides important (co-)stimulatory signals in T-cell-mediated immune responses by amplifying T-cell cytokine production, proliferation, and cytotoxicity *in vitro* (52, 98, 169, 175). The NKG2D pathway is also relevant in the context of N-BP treatment and the expression of ULBP1 was found correlated with the sensitivity of AML blasts toward TCR-mediated killing by Vγ9Vδ2 T-cells (114). Additionally, the results of Wrobel et al. indicated that the NKG2D pathway is involved in anti-tumor effects of γδ T-cells against melanoma and various epithelial cancers (55).

MICA-Polymorphism and Soluble MIC (sMIC)

The general concept is that cell stress and transformation increase the expression of MICA antigens and activate immune cells *via* NKG2D. However, MICA is a highly polymorphic human stress antigen and Shafi et al. showed that MICA coding sequence polymorphisms substantially affected RNA and protein expression (176). Some examined individuals showed better response to higher, others to lower MICA expression, and challenging the concept of an invariable direct correlation between stress molecule abundance and immune cell activation (176, 177).

Tumors also adopt evasion strategies, like shedding of free or the exosome form of MICA/MICB. These released molecules can inhibit immune effector cells due to interaction with NKG2D (178). Märten et al. found elevated levels of sMIC levels in sera of patients with pancreatic carcinoma correlated with tumor stage. The cytotoxic response of immune toward tumor cells was found impaired with in the presence of high sMIC levels but restored by neutralization of sMIC (179).

Temozolomide (TMZ) and Other Chemotherapeutics

Glioblastoma multiforme (GBM) is an extremely aggressive brain tumor, which is not very sensitive to either classical chemotherapy or immunotherapeutic approaches. Lamb et al. showed that *ex vivo* expanded γδ T-cells recognize malignant glioma *via* NKG2DL and lyse glioma cell lines and primary GBM specimens. Additionally TMZ, a DNA methylating chemotherapeutic agent licensed for GBM therapy, increased NKG2DL also on TMZ-resistant glioma cells. They also demonstrated that immune effector cells can be genetically modified to resist the toxicity of TMZ without changing their phenotype or their cytotoxicity against GBM target cells (180). Similarly, Chitadze et al. investigated the NKG2DL system in different GBM cell lines and confirmed that TMZ increased the cell surface expression of NKG2DL and sensitizes GBM cells to γδ T-cell mediated lysis. TMZ might therefore enhance the potential of adoptive transfer of *ex vivo* expanded γδ T-cells for glioblastoma treatment (181, 182).

Dacarbazine is a cytotoxic drug used for treatment of Hodgkin's lymphoma and melanoma. Although dacarbazine does not directly affect immune cells, it triggers the upregulation of NKG2DL on tumor cells, leading to NK-cell activation and IFN-γ secretion in mice and humans (183). Apart from TMZ and dacarbazine, studies suggest that other chemotherapeutics, like fluorouracil, doxorubicin, or vincristine sensitize tumor cell lines toward a NKG2D-dependent cytotoxic activity of Vγ9Vδ2 T-cell (184, 185). This could be a target cell or drug specific phenomenon as we were unable to boost γδ T-cell induced lysis of several leukemia cell lines with other cytostatic drugs (186).

Bortezomib and Epigenetic Drugs

Niu et al. reported that multiple myeloma (MM) cells can be sensitized toward killing by γδ T-cells and NK-cells using low-dose bortezomib. Additionally, bortezomib increases the expression of NKG2D and induces apoptosis of MM-cells, but not γδ T-cells and NK-cells (187). Treatment with 5-azacytidine, its derivate decitabine or histone deacetylase inhibitors may also increase the expression of NKG2DL in different types of malignancies prompting Bhat et al. to consider those epigenetic drugs a promising approach in γδ T-cell immunotherapy (188). Suzuki et al. evaluated possible additive effects of valproic acid (VPA), a histone deacetylase inhibitor, on γδ T-cell mediated cytotoxicity against bladder cancer cell lines TCCSUP and 253J (189). VPA did increase expression of NKG2DL and sensitivity toward cytolysis by γδ T-cells for both cancer cell types, whereas ZOL pre-treatment was only effective against TCCSUP. 253J cells were preferentially engaged *via* NKG2D-NKG2DL interaction, while TCCSUP cells were mainly recognized through the γδ TCR (189). Chávez-Blanco et al. showed that hydralazine in combination

with VPA increase the expression of MICA and MICB ligands by target cells, as well as NK-cell cytotoxicity *via* NKG2D. Additionally it reduces the shedding of MIC molecules to the supernatant (190). Satwani et al. incubated acute lymphoblastic leukemia and non-Hodgkin lymphoma cell lines for 24 h with 10 ng/mL of romidepsin (191). They demonstrated an approximately 50- to 1,300-fold increase in the number of cells positive for the surface expression of MICA/B in these cell lines. They further demonstrated a significant increase in NK-cell-mediated *in vitro* cytotoxicity (191).

Inhibitory Receptors

The development of immune checkpoint inhibitors targeting the cytotoxic T-lymphocyte-associated Protein 4 (CTLA4) or programmed cell death protein 1 (PD-1) and its ligand (PD-L1) has substantially extended the possibilities of immunotherapy. These substances are able to induce enduring remissions in a considerable subset of patients with treatment refractory types of cancer, for example melanoma, non-small cell lung cancer, and Hodgkin's lymphoma (192). Considering their clinical significance, relatively little is known about the role of γδ T-cells in immune checkpoint therapy and also regarding the role of inhibitory axes for γδ T-cell biology.

Programmed Cell Death Protein 1

Programmed cell death protein 1 is a key inhibitory receptor in inflammation, responsible for induction of tolerance, and immunosuppression in cancer (193). Following interaction with its ligands programmed death-ligand 1/2, the PD-1 receptor inhibits TCR and PI3K/AKT signaling and decreases proliferation and IL-2 release (194). It is interesting that both the PD-Ls and the CTLA4 ligands (CD80 and CD86) are members of the B7 family of proteins and therefore interrelated to BTN3A/CD277. Several types of malignancies have a relevant susceptibility to therapeutic PD-1/PD-L1 blockade, but it is barely predictable which individual patient will respond. The initially assumed direct relationship between tumor cell expression of PD-Ls and response rate following therapeutic PD-1 blockade might not be universally valid and the strength of PD-1 dependent immunosuppression is influenced by the topographic organization of the tumor microenvironment (195).

An early *in vitro* study addressed the expression profile and functionality of PD-1/PD-L1 in γδ T-cells following stimulation with HMBPP and suggested that the PD-1/PD-L1 axis is important for regulation of anti-tumor mechanisms of γδ T-cells (196). Later it was found that PD-1 expression is more frequent on Vδ1, compared with Vδ2 T-cells (197) and equably distributed over several functionally distinctive subsets of Vγ9Vδ2 T-cells (44). A report that *ex vivo* cultivated Vδ2 T-cells depict stable, low cell surface expression of PD-1 following adoptive transfer (198) might fit the observations that PD-1 is only temporarily upregulated following *in vitro* stimulation as it has been reported both for HMBPP and ZOL (196, 198). Vδ2 T-cells derived from neonates may behave differently as they depict prolonged PD-1 expression following activation and function as a regulator of tumor necrosis factor-α (TNF-α) production and cell degranulation, both being part of fetal inflammatory response (199).

Programmed cell death protein 1 expression might contribute to insufficient expansion of Vγ9Vδ2 T-cells in cancer patients, as a diminished response to PAg stimulation was demonstrated in bone marrow derived Vγ9Vδ2 T-cells from patients with MM. Such cells depicted a significantly increased PD-1 expression and were located in proximity to PD-L1+ MM-cells and myeloid-derived suppressor cells (200). Additional treatment with PD-1 antibody resulted in a twofold increase in proliferative response and an increased mobilization of CD107a following ZOL stimulation *in vitro* (200). Beside the bone marrow of MM patients, PD-1 positive γδ T-cells were also found in neuroblastoma infiltrated bone marrow (201).

Other Inhibitory Receptors

Alongside PD-1 several other inhibitory molecules are currently investigated regarding their function in limiting anti-tumor responses and potential therapeutic prospects (202). This is of special interest as there are indications for compensatory upregulation of alternative inhibitory receptors during anti-PD-1 therapy (203). Examples are the B- and T-lymphocyte attenuator (BTLA), CTLA4, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte activation gene-3 (LAG-3) and their respective ligands.

B- and T-lymphocyte attenuator was suggested to inhibit late phases of immune reactions and has structural and functional similarities to PD-1 and CTLA4 (204). It is expressed on Vγ9Vδ2 T-cells and engagement by its ligand, the herpesvirus entry mediator, reduced activation, proliferation, and anti-lymphoma response (205). Differing from PD-1 expression kinetics (196, 198), BTLA is initially downregulated following stimulation with PAgs but upregulated upon IL-7 treatment (205).

Compared with PD-1 and BTLA, even less is known concerning the functional implications of CTLA4, LAG-3, and TIM-3 on γδ T-cells. Melanoma patients with a higher ratio of Vδ1 to total γδ T-cells had poorer overall survival and *vice versa* higher frequencies of Vδ2 cells were associated with longer survival in a study using CTLA4 inhibitory antibody ipilimumab (16). Expression of LAG-3 indicates inhibition of PD-1 + T-cells in the tumor tissue and poorer prognosis in follicular lymphoma (206). From studies examining distinctive T-cell populations, we know that CTLA4 can inhibit T-cell activity *via* signaling mechanisms distinctive from PD-1 (207), but we still lack mechanistic studies conclusively demonstrating CTLA4 expression and function for Vγ9Vδ2 T-cells. In women with pre-eclampsia γδ T-cells with low TIM-3 expression depict a higher IFN-γ production (208) and in the context of malaria infection a high TIM-3 level was found correlated with reduced pro-inflammatory cytokine production (209). Similar to anti-PD-L1 antibodies, the inhibition of the TIM-3 ligand galactine-9 that is expressed by γδ T-cells, increases tissue infiltration by αβ T-cells in a pancreatic tumor model (5).

Summary

The referred data provide interesting prospects to enhance immunotherapy by means of modulating the expression of NKG2DL. Even though several of the referred effects were shown for NK-cells, these strategies might also apply for sensitizing tumor

cells toward γδ T-cell dependent cytotoxicity. Negative aspects like possible adverse effects on immune cell functionality or tumor escape mechanisms like sMIC and MICA-polymorphism need to be considered in future studies.

The physiological relevance of the currently known inhibitory receptors for γδ T-cells biology remains vague and additional observational and experimental studies are required. Based on the current evidence we assume that PD-1 is important for regulation of Vγ9Vδ2 T-cell functionality under specific conditions only, for example in an immunosuppressive tumor microenvironment. In inflammation and in the tumor microenvironment, γδ T-cells can become inhibited *via* PD-1 and also inhibit other PD-1 + immune cells *via* PD-L1 expression (5, 210). However, inhibitory effects of PD-1 may be overruled upon strong (co-) stimulation, for example *via* the TCR or with IL-2. Beside the local tissue distribution of receptors and ligands, expression kinetics are important to understand the function of the inhibitory receptors for immune homeostasis. Unfortunately, many studies do not distinguish whether tissue infiltrating T-cells are αβ or γδ T-cells in the first place. Combination therapy of adoptive transfer or *in vivo* stimulation of γδ T-cells with PD-1, PD-L1, CTLA4, or BTLA antibodies therefore seems feasible but the pre-clinical rational is currently not well established.

ADCC AND ANTIBODY CONSTRUCTS

Cytotoxicity of γδ T-cells against target cells can be significantly enhanced using specific monoclonal antibodies (mAbs) that induce ADCC. ADCC of γδ T-cells is thought to depend on Fc-γ receptor III (CD16) as it has been demonstrated that anti-CD19 antibody triggered CD107a, IFN-γ, and TNF-α expression is correlated to the amount of CD16+ γδ T-cells in an *in vitro* cytotoxicity assay (211). Furthermore, γδ T-cell mediated ADCC increases with higher numbers of CD16+ γδ T-cells (212) and was found inhibited with CD16 blocking antibodies (213). CD16 expression is usually low in unstimulated γδ T-cells, but increases following activation, for example with PAgs (213, 214).

B-Cell Malignancies

Rituximab

Several lymphoma and B-cell lineage leukemia subtypes were studied using stimulated γδ T-cells in combination with monoclonal anti-CD20 antibodies (212–216). Tokuyama et al. found RTX to increase the killing of several lymphoma cell lines and to improve ADCC of γδ T-cells against CLL and autologous follicular lymphoma cells (213). Furthermore, BrHPP stimulated γδ T-cells demonstrated stronger CD107a expression and increased ADCC toward individual B-cell lymphoma cell lines and patient CLL cells in combination with anti-CD20 antibodies (214). One single clinical phase I/IIa study used RTX plus BrHPP and IL-2 for *in vivo* stimulation of γδ T-cells in patients with relapsed follicular lymphoma (89). Altogether, 45 patients were treated according to protocol and the treatment was generally well tolerated, with low grade pyrexia being the most common side effect (89). Despite the 45% overall response rate (26% complete response) (89), it seems like development of BrHPP containing therapies is no longer pursued by the company in charge.

Second Generation Anti-CD20 Antibodies and Anti-CD52

The newer anti-CD20 antibodies ofatumumab and obinutuzumab were also tested regarding the efficacy inducing ADCC in connection with γδ T-cells (215). Obinutuzumab is an Fc engineered type II monoclonal antibody (217) and causes an increased secretion of perforin and IFN-γ compared to RTX and ofatumumab. Accordingly, the highest ADCC against B-cell lymphoma cell lines and primary follicular lymphoma cells was found for obinutuzumab (215). Similar to anti-CD20 antibodies, Gertner-Dardenne found alemtuzumab, an anti-CD52 antibody, to increase γδ T-cell dependent ADCC against lymphoma cell lines (214).

Solid Tumors

Breast Cancer

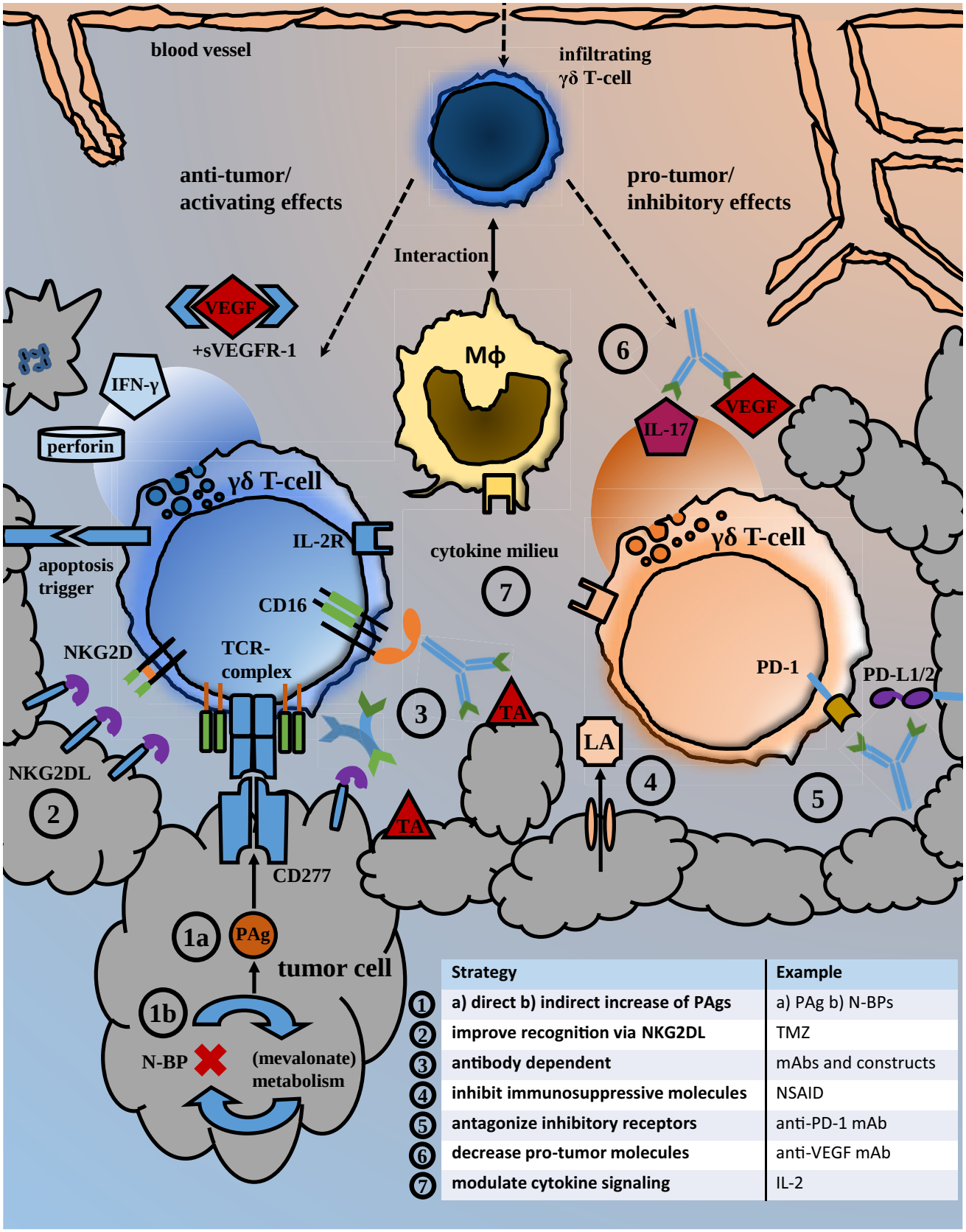
Two groups investigated whether the human epidermal growth factor receptor 2 (HER2/neu) specific antibody trastuzumab enhances γδ T-cell dependent ADCC toward breast cancer cell lines *in vitro* (63, 213). The addition of trastuzumab greatly increased lysis of HER2/neu overexpressing cell lines, whereas there was no change in a HER2/neu negative cell line (213). The extent of ADCC was increased with higher density of HER2/neu expression. Anti-tumor activity was confirmed in an animal model with SCID Beige mice. Here, the tumor growth was more efficiently inhibited by a combination treatment with γδ T-cells and trastuzumab compared to treatment with trastuzumab or γδ T-cells alone (63).

Neuroblastoma and Ewing's Sarcoma

Both in neuroblastoma and in Ewing's sarcoma, the disialoganglioside specific antibody ch14.18/CHO increased γδ T-cell mediated ADCC *in vitro* (124, 218). This finding was confirmed in an advanced immunodeficient mouse model, where *ex vivo* stimulated and adoptively transferred γδ T-cells with simultaneous administration of ch14.18/CHO antibody impaired tumor growth more efficiently than single antibody or sole γδ T-cells treatment (124).

Antibody Constructs and Nanobodies

Antibody constructs have been studied in both lymphoma and solid tumor models. Seidel et al. used the Fc modified CD19 antibody 4G7SDIE as a backbone for bispecific CD19-CD16 and CD19-CD3 antibody constructs (211). Although no direct comparison between unaltered antibodies and the antibody constructs was made, the constructs proofed active in inducing cytotoxic reactions by γδ T-cells. Schiller et al. went one step further and engineered a so called "single chain triplebody," called SPM-1, that consists of three single chain antibody fragments (CD19-CD19-CD16) (219). Indeed, SPM-1 induced a higher lysis compared to 4G7SDIE. A comparable approach is a recombinant construct consisting of a CD20 single-chain fragment variable (scFV) linked to MICA or ULBP2 which enhances cytotoxicity of stimulated γδ T-cells against CD20+ lymphoma cell lines and primary CLL cells *via* NKG2D (220). Oberg et al. designed two bispecific antibodies that bind either CD3 or the Vγ9 TCR-chain on γδ T-cells and Her2/neu expressed



	Strategy	Example
1	a) direct b) indirect increase of PAgs	a) PAg b) N-BPs
2	improve recognition via NKG2DL	TMZ
3	antibody dependent	mAbs and constructs
4	inhibit immunosuppressive molecules	NSAID
5	antagonize inhibitory receptors	anti-PD-1 mAb
6	decrease pro-tumor molecules	anti-VEGF mAb
7	modulate cytokine signaling	IL-2

FIGURE 1 | Continued

FIGURE 1 | Strategies for the inhibition of pro-tumor and the enhancement of anti-tumor effects. Overview of the local tumor microenvironment that illustrates important immune cell interactions and exemplary types of therapeutic interventions facilitating anti-tumor activity. Following their migration from blood to tissue, γδ T-cells may interact with macrophages and exhibit local pro- but also anti-tumor effects. Possible therapeutic strategies aiming to improve the recognition and killing of cancer cells by γδ T-cells as well as those intended to antagonize immunosuppressive receptor signaling and molecules are listed under points 1–7. Abbreviations: BrHPP, bromohydrin pyrophosphate; DCA, dichloroacetate; (G)M-CSFR, (granulocyte-)macrophage colony-stimulating factor receptor; IFN-γ, interferon-γ; IL-2R, interleukin-2 receptor; LA, lactic acid; mAb, monoclonal antibody; Mφ, macrophage/monocyte lineage cell; N-BP, amino-bisphosphonates; NKG2DL, NKG2D ligands; NSAID, nonsteroidal anti-inflammatory drugs; PAg, phosphoantigens; PD-1, programmed cell death protein 1; PD-L1/2, programmed death-ligand 1/2; sVEGFR-1, soluble vascular endothelial growth factor receptor; TA, tumor antigen; TCR-complex, T-cell receptor complex; TMZ, temozolomide; VEGF, vascular endothelial growth factor.

by pancreatic adenocarcinoma cells (221). Both antibodies enhanced γδ T-cell mediated cytotoxicity and adoptive transfer of γδ T-cells combined with [(HER2)2xVγ9] antibody therapy inhibited growth of pancreatic cancer in a SCID Beige mouse model (221). Furthermore, Hoh et al. demonstrated improved anti-tumor effects against hepatocellular carcinoma and hepatoblastoma cells with MT110, an epithelial cell adhesion molecule EpCAM/CD3 bispecific T-cell engager antibody, compared to the anti-EpCAM antibody adecatumumab (222). Zhang et al. utilized a bifunctional fusion protein (anti-CD3 single-scFV/-NKG2D) that binds NKG2DL+ tumor cells and recruits and stimulates T-cells *via* CD3 (223). This fusion protein was able to stimulate IFN-γ production by T-cells, increased cytotoxic reaction against NKG2DL+ tumor cells *in vitro* and promoted survival in a murine lymphoma model (223).

Another innovative approach is the use of so called nanobodies, a single heavy chain fragment. They bind highly selective to the Vγ9Vδ2 chain and elicited either inhibiting or activating reactions from γδ T-cells (224, 225). Although no data on cytotoxic features against tumor cells are available, it seems to be a promising approach to a selective modulation of Vγ9Vδ2 T-cell activity.

Summary

Monoclonal antibodies combine high target specificity with a favorable toxicity profile, but often depict limited activity when used as single agents. Therefore, combination with γδ T-cells is a promising concept for cancer immunotherapy. There are many mAbs for various hematological and non-hematological malignancies in clinical use already and more are currently in pre-clinical or early clinical development. Several such mAbs are promising combination partners as they show a uniformly strong enhancement in γδ T-cell mediated cytotoxicity. However, results of the only clinical study in this regard, which used RTX plus *in vivo* stimulation of γδ T-cells fell short of expectations. With the advent of new and Fc optimized antibodies and more specifically stimulated γδ T-cells, a higher effectivity might be achievable.

COUNTERACTING PRO-TUMOR EFFECTS

The local interplay of malignant, immune and stroma cells *via* direct cellular interactions and soluble factors characterizes the tumor microenvironment. Under these conditions, infiltrating immune cells can be suppressed and therapeutic activation may even unfold unintended tumor-promoting effects. Beside macrophages and regulatory T-cells (70, 96), IL-17-producing γδ T-cells (γδ T17 cells) are often suggested as important local mediators of tumor progression as repetitively demonstrated in animal models (226–228). It is possible to induce IL-17 production in human cells γδ T-cells *in vitro* (229) and γδ T17 cells were described in the human tumor microenvironments (7, 230) where they have been found inversely correlated with survival and associated with increased stage in breast (6) and colorectal cancer (7). It is important to note that not all studies differentiated between Vδ2 and non-Vδ2 cells or other γδ T-cell subclasses but it seems likely that both, Vδ2 but mainly the non-Vδ2 cells produce IL-17 (7). Direct proof is lacking, but it has been suggested that γδ T-cells can be changed toward an IL-17 producing phenotype by means of the tumor microenvironment (229, 231). Beside IL-17, vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor are predominately recognized as pro-tumor factors in the microenvironment, but it may not be reasonable to attribute an exclusive pro- or anti-tumor effect to any signal protein, cytokine, cell type or receptor-ligand interaction. For example VEGF facilitates neo-angiogenesis and immunosuppressive effects (232, 233) but also promotes tissue trafficking of different leukocytes (234, 235). The use of immunostimulatory drugs can induce unexpected changes in VEGF levels, as we observed an increase in VEGF serum levels following treatment with ZOL plus low-dose IL-2 in cancer patients (72). Pro-angiogenic factors like VEGF play an important pro-tumor role and predict poor clinical response to certain types of immunotherapy (72, 236). We recently described that following stimulation with IL-2 local lymphocyte-monocyte interactions regulate VEGF homeostasis *via* release of VEGF and soluble VEGF receptor 1 in a time-dependent manner *in vitro* (237). Potential pro-tumor factors and cells could be additionally targeted in combination with γδ T-cell therapy, for example *via* VEGF or IL-17 antagonists. VEGF antibodies are already widely used as cancer therapeutics making clinical studies investigating such a combination therapy feasible. The modest clinical effects of anti-angiogenic strategies call for a more fundamental analysis of VEGF signaling in the tumor microenvironment and the contribution of immune cells to these processes. The same also applies for other factors like IL-17.

Finally, both pro- and anti-tumor effects are mediated locally, as a consequence the *in vivo* efficacy of Vγ9Vδ2 T-cells will depend on their ability to infiltrate into the relevant tissues. Unfortunately we have little information concerning the capacity of activated γδ T-cells to reach the tumor in humans. One single clinical study demonstrated that autologous, *ex vivo* stimulated γδ T-cells predominately migrate to lung, liver and

spleen and could also be detected in individual tumor sites (84). Whether or not an effector cell is capable of tissue homing might be predicted by expression of chemokine receptors, selectins and other cell adhesion molecules. Expression of these molecules however depends on γδ T-cells subpopulation and differentiation status (43, 238, 239).

CONCLUSION

The results from pre-clinical research and individual clinical responses to γδ T-cell therapy encourage to carry on studying γδ-T-cell biology and aim to improve γδ T-cell related anti-cancer therapies. The question is, how the manifold observations on cellular mechanisms can help to establish better anti-cancer strategies and which drugs have an actual translational perspective. An overview on current γδ T-cell dependent therapeutic strategies and immune cell interactions in the tumor microenvironment is given in **Figure 1**. The use of mAb in combination with activated γδ T-cells is strikingly effective *in vitro*. Still the results from *in vivo* experiments did not always keep up with such expectations and the results of the only clinical trial did not proof superior to mAb monotherapy. We will need a thorough understanding of Vγ9Vδ2 T-cell subpopulations and their functional differences and must learn how to influence differentiation and prevent exhaustion. Our knowledge regarding the migration and tissue infiltration of Vγ9Vδ2 T-cells *in vivo* is still sparse, as is the understanding of pro- and anti-tumor mechanisms and cellular interactions in the tumor microenvironment. The establishment of better models could help deciphering those local and time-dependent processes. While the relevance of metabolic changes for immune and cancer cell function is now increasingly acknowledged, we need to learn how immune cells detect and respond to such changes. Reactivity to PAg by Vγ9Vδ2 T-cell may serve as an example, but we should be able to target even more specific tumor characteristics with cellular or combination therapy in the future.

AUTHOR CONTRIBUTIONS

TH wrote the manuscript and prepared the figure. MS and DP drafted sections and edited the manuscript. MW structured and edited the manuscript. All authors read and approved the submitted version.

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Whodunit? The Contribution of Interleukin (IL)-17/IL-22-Producing γδ T Cells, αβ T Cells, and Innate Lymphoid Cells to the Pathogenesis of Spondyloarthritis

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γδ T cells, αβ T cells, and innate lymphoid cells (ILCs) are capable of producing interleukin (IL)-17A, IL-17F, and IL-22. Among these three families of lymphocytes, it is emerging that γδ T cells are, at least in rodents, the main source of these key pro-inflammatory cytokines. γδ T cells were implicated in multiple inflammatory and autoimmune diseases, including psoriasis, experimental autoimmune encephalomyelitis and uveitis, colitis, and rheumatoid arthritis. Recent findings pointed toward a central role of γδ T cells in the pathogenesis of spondyloarthritis (SpA), a group of inflammatory rheumatic diseases affecting the axial skeleton. SpA primarily manifests as inflammation and new bone formation at the entheses, which are connecting tendons or ligaments with bone. In SpA patients, joint inflammation is frequently accompanied by extra-articular manifestations, such as inflammatory bowel disease or psoriasis. In humans, genome-wide association studies could link the IL-23/IL-17 cytokine axis to SpA. Accordingly, antibodies targeting IL-23/IL-17 for SpA treatment already showed promising results in clinical studies. However, the contribution of IL-17-producing γδ T cells to SpA pathogenesis is certainly not an open-and-shut case. Indeed, the cell types that are chiefly involved in local inflammation in human SpA still remain largely unclear. Some studies focusing on blood or synovium from SpA patients reported augmented IL-17-producing and IL-23 receptor-expressing γδ T cells, but other cell types might contribute as well. Here, we summarize the current understanding of how γδ T cells, αβ T cells, and ILCs contribute to the pathogenesis of human and experimental SpA.

Keywords: interleukin-23, interleukin-17, γδ T cells, T_H17 cells, innate lymphoid cells, spondyloarthritis

INTRODUCTION

Spondyloarthritis (SpA) encompasses a group of human rheumatic diseases that typically manifest as inflammation and new bone formation at axial joints, leading to severe lower back pain and impaired spinal mobility. Thereby, inflammation starts from entheses, the tendon to bone attachment sites. The family of SpA includes ankylosing spondylitis (AS), the SpA prototype (1), reactive arthritis, axial and undifferentiated SpA as well as psoriatic arthritis (PsA) and inflammatory bowel disease (IBD)-associated arthritis. Different SpA pathologies demonstrate similar disease patterns and similar

genetic associations. First, the MHC class I molecule HLA-B27 was identified to confer susceptibility to SpA, and HLA-B27 is present in approximately 90% of AS patients in Europe (2). Over the last few years, more and more genome-wide association studies revealed a link between the interleukin (IL)-23/IL-17 axis and SpA susceptibility (3–6). Newly identified susceptibility genes comprise *IL-12B*, *IL-1R*, *CARD9*, *TYK2, STAT3*, and *IL-23R*, the gene encoding for the IL-23 receptor (IL-23R). The latter is particularly interesting, because single nucleotide polymorphisms in *IL-23R* were associated not only with AS (7) or PsA (8) but also with psoriasis (9) and IBD (10), hence pathologies that frequently accompany articular inflammation in SpA.

Nonsteroidal anti-inflammatory drugs and TNF inhibitors serve as first-line treatment for SpA. However, new treatment strategies emerged with the identification of the IL-23/IL-17 axis as putative key pathway associated with SpA. Most prominently, anti-IL-17A (receptor) treatment improved SpA disease symptoms (11–17). By contrast, IL-23 inhibition presented ambiguous results (18–21) (ClinicalTrials.gov number NCT02437162). If these drugs should completely replace old treatment modalities in the future, it still needs to be validated further (22–24).

Enthesitis (25), thus entheseal inflammation, represents a main characteristic of SpA. It was suggested that mechanical stress and local microdamage might initiate entheseal inflammation (26, 27), proposing the enthesis as primary lesion in SpA-associated joint inflammation (28–30). However, the link between host genetics, e.g., the IL-23/IL-17 axis, and local inflammation as well as new bone formation is not entirely clear. Strikingly, several SpA-focused studies suggested that the IL-23/IL-17 cytokine axis and innate immune activation might be of greater importance than classical autoreactivity of B or T cell receptors (6, 31, 32). Indeed, several albeit not all SpA patients demonstrated an increase in IL-23/IL-17 serum or synovial fluid levels (33–37). IL-17 cytokines are usually produced by lymphocytes, although earlier studies observed IL-17-producing mast cells (38), neutrophils, and myeloperoxidase-expressing cells (39) in SpA synovia. So, who does it? In the following, we summarize and discuss current data about human and experimental SpA and the three prime suspects of the IL-23/IL-17 axis: γδ T cells, αβ T cells, and innate lymphoid cells (ILCs).

γδ T Cells

Although pre-committed effector γδ T cells represent a major source of IL-17/IL-22 under steady-state conditions in rodents (40–42), data reporting IL-17/IL-22-producing γδ T cells in healthy human individuals are rare (42–44). However, γδ T cells are clearly associated with different infections and tumors as well as autoinflammatory and autoimmune diseases in humans (45, 46). First studies suggesting a possible connection between γδ T cells and SpA were published approximately 30 years ago, just shortly after the discovery of γδ T cells (47, 48). By now, a number of studies demonstrated a decrease of γδ T cells in blood (49–51), while others showed that γδ T cells were frequently present in SpA patients' synovial fluid (52, 53), suggesting that γδ T cells might play a role in disease induction and/or persistence in humans.

In fact, a direct association of γδ T cells and IL-17/IL-22 secretion in human SpA was first described by Kenna and colleagues, demonstrating an enrichment of IL-23R$^+$ IL-17-producing γδ T cells in blood of AS patients (54). Strikingly, this phenotype was absent in rheumatoid arthritis patients (54), suggesting specific involvement of IL-17-producing γδ T cells in SpA pathogenesis rather than in arthritic inflammation in general. Along the same line, the analysis of tissue samples from enthesitis-related arthritis (55), reactive arthritis or undifferentiated SpA (56) as well as juvenile idiopathic arthritis (JIA) patients (57) revealed an increase in blood and synovial fluid IL-17-producing γδ T cells. Notably, such increased numbers of IL-17-producing γδ T cells might be driven by a defined arthritic cytokine environment (57). Although IL-23 certainly represents the main driver cytokine inducing enhanced IL-17 secretion by different cell types, also IL-9-driven expansion of IL-17-producing γδ T cells in PsA synovial fluid was recently demonstrated (58).

γδ T cells were implicated not only in SpA and related diseases in humans but also in mice. In various mouse models for non-autoimmune arthritis, including non-autoimmune antigen-induced arthritis (59), mannan-induced arthritis (60), or CFA-injected IFN-$\gamma^{-/-}$ mice (61), γδ T cells were increased in numbers and were the main source of pathogenic IL-17 in inflamed tissues.

So, how do SpA-associated IL-17-producing γδ T cells get into inflamed sites in humans and mice? It is tempting to speculate that circulating and/or γδ T cells from distant tissues might leave their sites of origin and gather at the sites of crime, the inflamed joints. Accordingly, blood isopentenyl pyrophosphate-responsive Vγ9$^+$ (53) or α4β7$^+$ mucosal (γδ) T cells (52) might preferentially accumulate in JIA joints during an acute flare or a low acute-phase response, respectively. Likewise, CCR2$^+$Vγ6$^+$ IL-17-producing γδ T cells were recruited to joints by CCL2-inducing CD4$^+$ T cells in arthritic *Il-1rn*$^{-/-}$ mice (62).

However, there is more to be considered than migration of γδ T cells into inflamed tissues when trying to solve the case of idiopathic local entheseal inflammation in SpA. Applying an IL-23-dependent mouse model resembling inflammation-driven bone destruction (63) and most features of human SpA (64), tissue-resident IL-23R$^+$RORγt$^+$CD3$^+$CD4$^-$CD8$^-$ lymphocytes were discovered in mouse entheses (64). Systemic IL-23 overexpression induced local inflammation in the enthesis by triggering resident IL-23R$^+$RORγt$^+$CD3$^+$CD4$^-$CD8$^-$ lymphocytes to secrete IL-17 and IL-22, ultimately leading to IL-17-dependent enthesitis, IL-22-dependent bone remodeling as well as aortic root inflammation and psoriasis (64). Based on this study, we could recently demonstrate that Vγ6$^+$ γδ T cells reside within mouse entheses, where they constitute the large majority of IL-23R$^+$RORγt$^+$CD3$^+$CD4$^-$CD8$^-$ lymphocytes in steady state and increase in numbers during inflammation (65). Whether *Tcrd*$^{-/-}$ mice would thus be protected from IL-23-dependent entheseal inflammation is still a matter of investigation. Notably, aging male DBA mice still develop severe enthesitis and new bone formation in the absence of γδ T cells (66). However, whether this phenotype results from entheseal γδ T cell redundancy or an increased presence of enthesis-resident lymphocytes other than γδ T cells that functionally refills their empty niche (67) still remains an open question.

Strikingly, resident γδ T cells have just recently also been identified in human entheses (68). Thus, it appears likely that

under steady-state conditions, entheseal γδ T cells reside in this very specific anatomical niche to control tissue homeostasis and possibly physiological bone remodeling after injury and exercise, while upon the elevation of IL-23 serum levels, they are driven to increase IL-17/IL-22 production and thus promote SpA (**Figure 1**).

Innate Lymphoid Cells

Innate lymphoid cells were identified around 10 years ago (69), but might have played important roles in joint inflammation even before adaptive immune cells developed over 450 million years ago. By now, it is clear that ILCs are crucially involved in the pathogenesis of a variety of inflammatory diseases, but also in tissue homeostasis (70, 71). For example, different ILC subsets were implicated in human and experimental rheumatic diseases (72). Recently, elevated numbers of ILC1s, ILC2s, and ILC3s were measured in blood samples from PsA patients, while only ILC3 numbers positively correlated with disease activity (73). However, circulating ILC3s in PsA patients displayed an immature phenotype, only produced moderate amounts of IL-17/IL-22, and did not express NKp44 (73). Interestingly, this further implies that final ILC3 maturation might only occur directly at the site of inflammation, presumably directly in the joints. However, as ILCs possess a high degree of plasticity (74), the transformation of inflammatory ILC1s or ILC2s into ILC3-like cells might also be possible (75, 76). In addition to an enrichment of NKp44$^-$ ILC3s in PsA blood, NKp44$^+$CCR6$^+$ IL-17- (77) or GM-CSF-producing (78) ILC3s were abundantly present in SpA synovial fluid. Here, circulating NKp44$^+$ILC3 numbers inversely correlated with disease activity (77), possibly resulting from the migration of circulating (immature) ILC3s to target tissues.

Association between SpA and intestinal inflammation is well established (79), and at least 50% of SpA patients suffer from (sub)clinical gut inflammation (80). A connection between ILCs and SpA-associated gut inflammation was described by different studies. In blood from entheropathic SpA patients, levels of IL-17-producing ILC3s were significantly higher as compared to IBD patients and healthy controls (81). While intestinal CD4$^+$ T cells represented the main source of local IL-22 in Crohn's disease patients, IL-22-producing NKp44$^+$ ILCs were predominant in the gut of AS patients (82), highlighting differences between the etiology of IBD and SpA-associated intestinal inflammation. Surprisingly, particularly α4β7$^+$IL-23R$^+$ IL-17/IL-22-producing ILC3s were increased in gut, blood, bone marrow, and synovial fluid from AS patients with intestinal inflammation, suggesting that gut-derived functionally mature ILC3s might emigrate from intestinal tissues to α4β7 ligand-expressing joints, promoting local SpA-associated inflammation (83). SpA-associated α4β7$^+$ synovial T cells were also already described before (84). Based on these results, ILC3s were proposed to function as "cytokine shuttles from gut" to extra-intestinal tissues (85). Strikingly, IL-17/IL-22-producing ILC3s appeared to be RORγt$^-$ but Tbet$^+$ (83), possibly reflecting a particular developmental stage (86).

Although tissue residency is not well established for human ILCs, there is good evidence that ILCs in mice are generally tissue-resident (87, 88). Thus, the migration of gut-derived ILCs into SpA joints appears surprising at first glance. However,

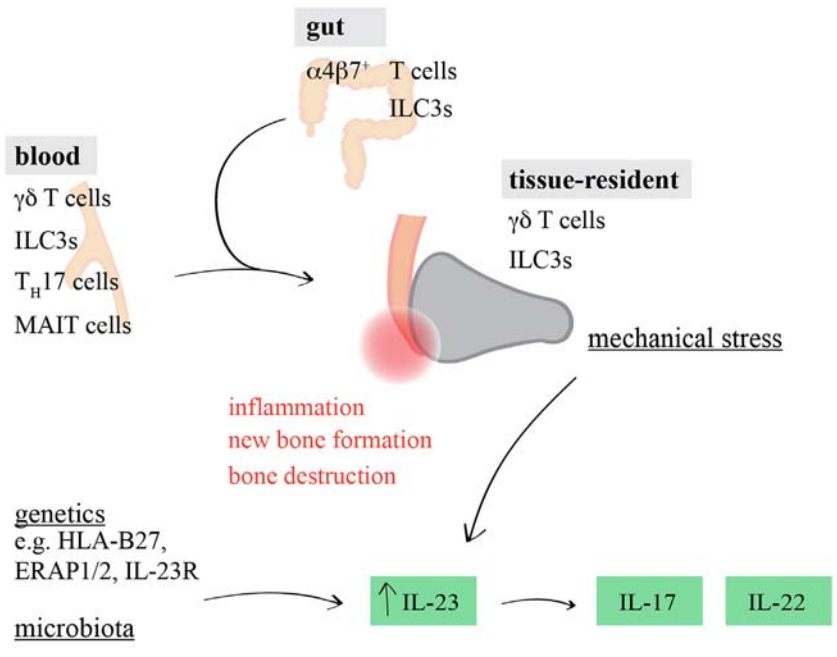

FIGURE 1 | Involvement of interleukin (IL)-17/IL-22-producing lymphocytes in spondyloarthritis (SpA)-associated inflammation. Genetic and epigenetic predisposition, altered microbial composition, and entheseal microdamage can influence the induction and progression of tissue inflammation in SpA. In addition to tissue-resident γδ T cells and ILC3s, circulating and/or gut-derived γδ T cells, ILC3s, T_H17 cells, or mucosal-associated invariant T (MAIT) cells might promote IL-23-driven joint inflammation by producing increased amounts of IL-17 and IL-22.

photoconversion experiments in mice could reveal CCR7-dependent ILC3 migration from gut to mesenteric lymph nodes (89), supporting the notion that intestinal ILCs can, at least to some extent, traffic to distant sites. The expression of a particular chemokine/cytokine signature, homing receptors, and respective ligands might promote their traveling. For instance, NKp44 ligand was shown to be expressed by chondrocytes, even in non-inflamed joints (90).

The migration of peripheral ILC3s into inflamed tissues in the context of SpA appears to be an interesting hint. However, the presence of resident NKp44$^+$ ILC3s in non-inflamed human spinal entheses has just been reported (68). Further, that study demonstrated that, in consistence with mouse entheseal tissues (64, 65), human entheses responded to stimulation with IL-23/IL-1β by increasing IL-17/IL-22 production (68). Thus, in addition to γδ T cells, enthesis-resident ILC3s represent another candidate of innate lymphocytes that might be involved in the induction and/or progression of SpA (**Figure 1**).

αβ T Cells

Although recent studies point toward a chief contribution of innate lymphocytes to SpA, it is inevitable to include αβ T cells into the inner circle of prime suspects.

The "arthritogenic peptide theory" suggested that CD8$^+$ T cells specific for HLA-B27-presented peptides might be involved in SpA disease pathogenesis (91, 92). Some immunohistological analyses indeed demonstrated that CD8$^+$ T cells were predominant in human entheseal infiltrates (93, 94). However, studies in an experimental SpA model, HLA-B27-transgenic rats, did not support this theory (95, 96). Notably, HLA-B27 molecules can also be recognized as B27 β2-microglobulin-free heavy chains by killer immunoglobulin-like receptors (KIRs) (97, 98). HLA-B27/KIR3DL2 binding can induce RORγt expression in CD4$^+$ αβ T cells and thus a T helper 17 (T_H17) cell phenotype (99). Accordingly, although not confirmed in early axial SpA (100), KIR3DL2$^+$ T_H17 cells were increased in AS patients, suggesting that these cells might represent a therapeutic target for SpA treatment (101). HLA-B27 also appears to be associated with dysbiosis (102–105), possibly resulting from HLA-B27 misfolding-induced (106, 107) upregulation of the IL-23/IL-17 axis (108), innate immune activation, and intestinal T_H17 cell expansion (109) early in life (110).

In fact, several albeit not all (39, 54, 111) SpA studies observed increased amounts of T_H17 cells in blood and/or synovial fluid (100, 112–117), possibly in a sex-dependent manner (118). While IL-17 is the most representative cytokine ascribed to T_H17 cells, GM-CSF-producing T_H17 cells were also elevated in SpA patients (78). However, the increase in GM-CSF-producing lymphocytes was not specific for T_H17 cells, as IL-17-producing CD8$^+$ T cells, γδ T cells, and ILC3s co-producing GM-CSF were similarly expanded (78).

Supposing that increased numbers of T_H17 cells promote SpA—how to keep these cells in check? In fact, miR-10b-5p (119) and IL-10-producing B cells (120) were recently proposed as putative negative regulators trying to control T_H17 cells from SpA patients. Although increases in regulatory T cells in gut (121), blood, and synovial fluid (122, 123) from SpA patients also hinted toward an unsuccessful reaction to suppress autoinflammation, these cells might be functionally defect (124), demonstrating an imbalance in IL-10/IL-17 production (125).

Finally, unconventional/innate IL-17/IL-22-producing αβ T cell subsets might be associated with SpA. While neither human nor experimental SpA-associated IL-17-producing invariant natural killer T cells were identified so far, IL-17-producing CD8$^+$ T cells (78, 126) or mucosal-associated invariant T (MAIT) cells (127, 128) were described. Surprisingly, increased IL-17 production by MAIT cells derived from AS patients was IL-23-independent, but rather promoted by IL-7 (128)—similar findings were recently reported in multiple sclerosis patients (129). Indeed, *IL7R* polymorphisms are associated with AS (4), and it was proposed that mechanical stress-induced IL-7 secretion by synovial fibroblasts could induce MAIT cell activation and thus IL-17 secretion during SpA pathogenesis (130). However, enthesis-resident MAIT cells have not been described so far.

Although seemingly plenty of studies described an association of T_H17 cells and SpA, CD4$^+$ cell depletion did not protect from IL-23-dependent inflammation (64). Along the same line, aging male DBA *Tcrb*$^{-/-}$ mice did still develop enthesitis and new bone formation (66), and γδ T cells, but not αβ T cells, dominated among pathogenic IL-17-producing enthesis-resident lymphocytes in mice (65). Together, this indicates that T_H17 cells might rather not mediate the first line of action when local entheseal immune cells are provoked by the various environmental triggers, such as mechanical stress, dysbiosis, or genetic and epigenetic predisposition (131) (**Figure 1**).

CONCLUDING REMARKS: WHODUNIT?

After lining up the usual and a few unusual suspects, it seems that although strong arguments point toward an important contribution of the IL-23/IL-17 axis mediating SpA, it still remains difficult to pinpoint which of the above-described cell types are major players (132). Overall, recent studies collectively favor innate and innate-like immune cell involvement rather than adaptive T cells (6, 31, 32). As opposed to conventional B and T cells, innate and innate-like lymphocytes are commonly enriched in non-lymphoid tissues, and thus association with autoinflammatory diseases affecting particular tissues appears feasible (133). In this respect, it is worth considering the differential effects that genetics and environmental factors might elicit in innate versus adaptive immune traits (134).

Still, the relative contributions of tissue-resident cells, i.e., γδ T cells and ILC3s, versus recruited cells to SpA pathologies are not entirely clear. The "mechanical stress and entheseal microdamage hypothesis" (26, 27) supports the idea of inflammation-promoting enthesis-resident cells. In this regard, one might hypothesize that mechanical stress triggers resident immune cell activation—either directly or indirectly *via* stromal cell activation. Indeed, mechanical stress was shown to support enthesitis and new bone formation in TNF$^{\Delta ARE}$ and aging male DBA mice, whereas remarkable experiments involving hind limb unloading significantly reduced disease symptoms (135). Since human tissues are generally difficult to obtain, various animal

studies experimentally addressed immune pathways associated with SpA. However, it should be noted that many animal models only work in a specific genetic background, and vast differences exist between individual SpA models. While some models strongly depend on IL-23, others are based on TNF dysregulation (136, 137). Consequently, experimental data might be controversial: while Rag2$^{-/-}$ mice did not develop pathologies upon IL-23 overexpression (64), arguing against a role for innate lymphocytes in disease induction, enthesitis in Rag1$^{-/-}$ TNF$^{\Delta ARE}$ mice was unaffected (135).

In the human system, many traits originate from analysis of circulating immune cell populations. However, such data remain inherently difficult to interpret: an increase in a particular circulating cell population does not unequivocally suggest their increased migration to joint tissues, while a decrease cannot unambiguously imply these cells already relocated from blood into distant sites. And why should otherwise tissue-resident ILC3s, T_H17 cells, MAIT cells, or γδ T cells, leave the intestine and migrate into axial sites and distant tissues? Indeed, increased levels of CCL20, the chemokine attracting CCR6$^+$ cells, were detected in SpA joints, albeit not as prominent as in rheumatoid arthritis (138). Altered gut epithelial and vascular barrier integrity in SpA patients might further promote intestinal immune cell emigration (139). Importantly, the trafficking of intestinal IL-17-producing γδ T cells to an entirely different tissue, the leptomeninges, was described in a mouse model for stroke (140, 141). Notably, a gut/joint axis in SpA might also exist for antigen-presenting cells carrying bacterial antigens from gut to axial sites, thus contributing to the induction of a local immune response (142). Whether SpA-associated joint-infiltrating lymphocytes enter tissues as already activated and functionally mature cells also remains an open question. Relatedly, CXCL4 was recently identified as a novel potent inducer of human T_H17 cells enriched in PsA joints, and CXCL4 levels also positively correlated with disease severity, thus suggesting a CXCL4-driven local boost of T_H17 cells (143).

In conclusion, there is growing evidence that innate and tissue-resident IL-17-producing γδ T cells and perhaps also ILC3s might be locally primed by genetic and epigenetic predisposition, mechanical stress as well as by increased systemic inflammation caused by intestinal dysbiosis. However, future studies will need to elaborate prevailing theories about the SpA-associated sequence of events. No matter whether ILC3s, γδ T cells, or any other innate lymphocytes primarily promote inflammation in SpA, in the end, the pathogenic action of all these cell types can be collectively targeted *via* the IL-23/IL-17 axis.

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All the authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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Molecular Determinants of Target Cell Recognition by Human γδ T Cells

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The unique capabilities of gamma-delta (γδ) T cells to recognize cells under stressed conditions, particularly infected or transformed cells, and killing them or regulating the immune response against them, paved the way to the development of promising therapeutic strategies for cancer and infectious diseases. From a mechanistic standpoint, numerous studies have unveiled a remarkable flexibility of γδ T cells in employing their T cell receptor and/or NK cell receptors for target cell recognition, even if the relevant ligands often remain uncertain. Here, we review the accumulated knowledge on the diverse mechanisms of target cell recognition by γδ T cells, focusing on human γδ T cells, to provide an integrated perspective of their therapeutic potential in cancer and infectious diseases.

Keywords: gamma-delta T cell, T cell receptor, NK cell receptor, NKG2D, tumor immunology

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INTRODUCTION

More than three decades after the discovery of gamma-delta (γδ) T cells (1), the research community is still missing a compelling picture about their mechanisms of activation and target cell recognition. Despite the relatively small abundance of γδ T cells in the human blood, it is clear that this lymphocyte population plays an important role at the interface between the innate and the adaptive immune systems. These cells share T cell receptor (TCR) rearrangements and memory functions (2) with their αβ T cell counterparts, but differ in their response kinetics and mechanisms of target cell recognition. Thus, γδ T cell activation is typically independent of antigen presentation by major histocompatibility complex (MHC) molecules. Furthermore, γδ T cells bear a plethora of NK cell receptors (NKRs) on their surface, which allow for very fast responses against infected or transformed cells (3), thus contributing to a first line of defense that precedes antigen-specific αβ T-cell responses (4).

Unlike αβ T cells, there is little evidence of thymic negative selection of self-reactive γδ T cells. Vγ9Vδ2 T cells, which constitute the major (60–95%) γδ T cell subtype in humans, seemingly expand in the periphery in response to microbial or stress-induced phosphorylated antigens (2) while displaying preferential Vγ9-JP TCR rearrangements (5). Other human γδ T cell subsets, namely Vδ1$^+$ and Vδ3$^+$ T cells that are highly reactive to cytomegalovirus (CMV) infection (6), display TCR repertoires biased toward sequences recognizing CMV-infected cells (7). But while Vγ9Vδ2 TCR recognition has been well characterized and discussed (5, 8), it remains less clear how other γδ T cell subsets are activated to participate in lymphoid stress surveillance (9).

The purpose of this review is to discuss the current knowledge on target cell recognition by human γδ T cells (**Table 1**), emphasizing the role of the TCR as well as NKRs and their ligands, in the context of cancer and infectious diseases.

TUMOR CELL RECOGNITION

Early research on the molecular mechanisms of γδ T cell recognition in the 1990s led to the realization of its unusual independence of peptide processing and MHC-restricted presentation, in marked contrast with αβ T lymphocytes (42–44). One of the first lines of evidence came from non-peptidic

TABLE 1 | Tumor- or infected cell-associated ligands recognized by gamma-delta (γδ) T cells.

Ligand	Receptor	γδ subset	Infection/cancer	Reference
CD1 proteins + endogenous or exogenous lipids	T cell receptor (TCR)	Duodenal	Infection	(10, 11)
BTN3A1 + phosphoantigens	TCR	Vγ9Vδ2	Infection	(5)
Endothelial protein C receptor	TCR	Vγ4Vδ5	Both	(12)
Annexin A2	TCR	Vγ8Vδ3	Both	(13)
Heat shock protein 60	TCR		Both	(14–17)
F1-ATPase	TCR		Cancer	(18)
SEA and SEE	TCR		Infection	(19)
OXYS	TCR		Infection	(20)
DXS2	TCR		Infection	(21)
Glycoprotein I	TCR		Infection	(21)
MSH2	TCR		Both	(14, 22)
	NKG2D			(22)
HLA-E	NKG2C		Infection	(23)
HA	Sialic acid receptor		Infection	(24)
CD48	2B4		Cancer	(25–27)
MICA/MICB	TCR	Vδ1	Both	(28–30)
	NKG2D			(29–32)
MICA	NKG2D	Vγ9Vδ2	Cancer	(33)
UL16 binding protein (ULBP)1	NKG2D	Vγ9Vδ2	Cancer	(34)
ULBP2	NKG2D	Vδ1	Cancer	(35, 36)
ULBP3	NKG2D	Vδ1	Cancer	(35–37)
ULBP4	TCR and NKG2D	Vδ2	Cancer	(38)
?	NKp30	Vδ1	Both	(39, 40)
PVR/Nectin-2	DNAX accessory molecule 1	Vγ9Vδ2	Cancer	(41)

"?" means undescribed/unknown in the referenced studies.

prenyl pyrophosphates ["phosphoantigens" (PAg)] recognized by Vγ9Vδ2 TCRs (45, 46). Initially, bacteria and parasites were shown to produce strong PAg agonists for Vγ9Vδ2 TCRs (47), but later it became clear that these could also be activated by weaker agonists, such as isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate, that are natural intermediates of the mevalonate pathway of isoprenoid and steroid synthesis in eukaryotic cells (48). Importantly, the dysregulation of the mevalonate pathway in some tumor cells allows for the accumulation of these (weaker) PAgs, thus promoting Vγ9Vδ2 TCR-mediated recognition (49). Furthermore, treatment with zoledronate or pamidronate (which are approved drugs) was shown to be very effective at inducing the accumulation of intracellular PAgs like IPP, and thus potentiate TCR-dependent Vγ9Vδ2 T cell cytotoxicity against tumor cell targets, including cancer stem cells (50).

A key recent breakthrough was the discovery of butyrophilin-related proteins, especially BTN3A1, as major molecular determinants of Vγ9Vδ2TCR-mediated recognition of PAgs, even if the underlying mechanism has gathered some controversy. A model supporting extracellular PAg presentation to the Vγ9Vδ2 T cell (in a MHC-like manner) was first proposed, with biophysical and structural data in support (51). However, following reports demonstrated that PAgs interact directly with the intracellular B30.2 domain of BTN3A1 through a positively charged surface pocket; and that charge reversal of pocket residues abrogates PAg binding and Vγ9Vδ2 T cell activation, with no detectable association with the extracellular domain of BTN3A1 (13, 52, 53). More recently, it has been shown that changes in the juxtamembrane domain of BTN3A1, which is located close to the start of the B30.2 domain, induced marked alterations in Vγ9Vδ2 T cell reactivity, thus highlighting the importance of the intracellular domain for the correct Vγ9Vδ2 T cell function and activation (54). Because of its location between the intracellular and the extracellular domains, the B30.2 domain seems critical in translating the pAg-induced conformational change of BTN3A1 from the inside to the outside of the target cells (55, 56).

Besides sensing PAgs, γδ T cells seemingly recognize transformed cells through proteins that are expressed at the cell surface in a stress-induced manner. Some examples are typically endogenous proteins, such heat shock protein 60 (14–17) or FI-ATPase (18), that can be ectopically expressed on the cell membrane upon transformation and recognized by Vγ9Vδ2 TCRs to promote tumor cell lysis. More recently, endothelial protein C receptor (EPCR), which acts on the coagulation cascade, was shown to be exposed on the cell surface during transformation and recognized by a non-Vδ2 (Vγ4Vδ5) TCR (12). Similarly, Annexin A2, expressed on tumor cells in response to increasing quantities of reactive oxygen species, engaged directly with a Vγ8Vδ3 TCR (13). The identification of these rather different ligands highlights the complexity of tumor cell recognition *via* γδ TCRs. This notwithstanding, it is clear that γδ T cells also rely on "NK-like" mechanisms for tumor cell recognition, using receptors such as 2B4 and NKG2D, originally thought to be specific to NK cells.

The first indication of an NK-like recognition mechanism was unveiled upon the ability of stimulated murine γδ T cells to recognize CD48 (25, 26), a well-known 2B4 ligand, suggested to work as an accessory molecule that strengthens effector–target interactions (27). Surprisingly, only the $2B4^+$ γδ T cells were able to develop non-MHC-restricted cytotoxicity against lymphoma cells (57, 58). Although 2B4 is also expressed on activated human γδ T cells, its relevance is still unclear as 2B4 engagement failed to promote proliferation or cytokine production (59).

Much more consensual is the role of NKG2D, which is widely expressed not only in NK cells but also in most γδ and some αβ

T cells (31, 60, 61). In human γδ T cells, both Vδ1$^+$ and Vδ2$^+$ subsets, NKG2D was shown to be responsible for recognition of tumor cells expressing MHC class I chain-related (MIC) A/B (28, 29, 31–33, 62) or UL16 binding protein (ULBP) 1/2/3/4 (34–38, 50, 63) ligands. In fact, human carcinoma samples from lung, breast, kidney, ovary, and prostate cancers expressing MICA or MICB presented higher levels of infiltrating Vδ1$^+$ T cells, which in turn were able to target and kill autologous and heterologous tumor cells (25, 59). Our group's work revealed that ULBP1 was particularly important for leukemia and lymphoma cell recognition by PAg-activated Vγ9Vδ2 T cells (34). Notwithstanding, one should note the relevance of a synergistic TCR engagement for an efficient cytotoxic response (37, 38). In fact, some works suggested that MIC or ULBP recognition by γδ T cells is not only restricted to NKG2D but also involves the γδ TCR (26, 31). A similar recognition pattern was also observed against human MutS homolog 2 (hMSH2) ectopically expressed in epithelial tumor cell lines. Both TCRγδ and NKG2D were able to interact with hMSH2 and contribute to Vδ2$^+$ γδ T cell-mediated cytotoxicity and interferon γ (IFN-γ) production (14, 22).

Besides 2B4 and NKG2D, DNAX accessory molecule 1 (DNAM-1) was also shown to be widely expressed in Vδ1$^+$, Vδ2$^+$, and Vδ1$^-$Vδ2$^-$ γδ T cell subsets (64); and masking DNAM-1 on γδ T cells significantly inhibited tumor cell killing (64, 65). DNAM-1-dependent γδ T cell recognition was reported for hepatocellular carcinoma (41), acute (65) and chronic (64) myeloid leukemia, and multiple myeloma (66) cell lines. More specifically, Vγ9Vδ2 T cells were shown to use DNAM-1 to interact with Nectin-2 and PVR that are widely expressed in the tumor cell targets (41, 65). Curiously, PVR engagement potentiated γδ T cell cytotoxicity, whereas Nectin-2 blocking did not affect it (41). Tumor targets that expressed both DNAM-1 and NKG2D ligands were able to engage both receptors on γδ T cells, having a synergistic effect on their cytolytic activity (41, 64, 66). Moreover, therapeutic strategies that enhanced the expression of NKG2D or DNAM-1 ligands, such as MICA/B and ULBP1/2, or Nectin-2 and PVR, respectively, potentiated γδ T cell recognition of colon cancer, glioblastoma, multiple myeloma, and lymphoma cells (67–70).

From a therapeutic perspective, γδ T cell recognition of tumor cells may also rely on the induced expression of natural cytotoxicity receptors (NCRs) that recognize a distinct set of tumor-associated ligands, such as B7-H6 or BAT3 (71). Thus, our group has shown that NKp30 and NKp44 can be reproducibly induced *in vitro* in Vδ1$^+$ (but not Vδ2$^+$) γδ T cells (39). A very mild expression of NKp44 on expanded γδ T cells had been reported before (72); and shown to contribute γδ T cell cytotoxicity against myeloma cells (61). In our studies, we observed not only a robust expression of NKp44 but also NKp30, in Vδ1$^+$ T cells activated with TCR agonists and IL-15 (or IL-2); and both receptors enhanced γδ T cytotoxicity against tumor target cells (39, 73). Among the various known ligands for NCRs, it is still unclear which are most relevant for NCR$^+$ Vδ1$^+$ T cell recognition of tumor cells. While the NKp30 ligand, B7-H6, is an obvious candidate (74), a very recent report identified an unanticipated ligand for NKp44 in the form of platelet-derived growth factor (PDGF)-DD (75), known for its capacity to promote of tumor cell proliferation, epithelial–mesenchymal transition, and angiogenesis. PDGF-DD ligation to NKp44 enhanced IFN-γ and TNF-α secretion (by NK cells), which in turn induced tumor cell growth arrest (75). Additional investigation will be needed to elucidate the relative importance of NCR, NKG2D, DNAM-1, or TCR ligands in tumor cell recognition by γδ T cells, aiming to maximize their potential in cancer immunotherapy.

INFECTED CELL RECOGNITION

Multiple lines of evidence since the late 1980s have shown that γδ T cells display strong activities against bacteria, including *Mycobacterium tuberculosis* (76–81); parasites, such as *Plasmodium falciparum* (82–86); and viruses (87, 88), most notably CMV (89–91).

Vγ9Vδ2 T cells can be specifically and potently activated by PAgs like (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, an intermediate of the 2-C-methyl-D-erythritol 4-phosphate pathway employed by eubacteria and apicomplexan protozoa but not by eukaryotes (48, 92, 93). This likely underlies the striking expansions of Vγ9Vδ2 T cells in individuals infected with *M. tuberculosis* (76–81) or *P. falciparum* (83). Besides PAgs, several other molecules of microbial origin have been proposed as γδ T cell antigens accounting for the specific recognition of infected cells. These candidates include the bacterial superantigens SEA (and to a lesser extent SEE) (19); OXYS and DXS2, two mycobacterial proteins found to activate γδ T cells from BCG-infected human subjects but not from healthy donors (20, 21); and HSV-1 glycoprotein I, specifically recognized by a Vγ1.2Vδ8 TCR independently from antigen processing and MHC presentation (20, 21).

Subsequent reports demonstrated that γδ T cells also recognize stress antigens of cellular origin, either in antibody-like or antigen-presentation-like fashion. γδ T cells can indeed directly recognize stress proteins like hMSH2, a nuclear protein ectopically expressed on the cell surface of different epithelial tumor cells and induced by EBV transformation (22); and Annexin A2 whose expression was induced by CMV infection and recognized specifically by a Vγ8Vδ3 T cell clone (13). On the other hand, γδ T cells can recognize nonpolymorphic MHC-like (class Ib) proteins presenting lipids, such as CD1 proteins, in a similar way to other unconventional T cells like NKT or MAIT cells (11, 94–96). In particular, a subpopulation of Vδ1$^+$ T cells has been clearly shown to bind CD1d loaded with the self-lipid sulfatide (97) but any concrete link to the recognition of infected (or transformed) cells remains to be established. Of note, another CD1-like protein, EPCR, was shown to bind directly (independently of lipid cargo) the TCR of a Vγ4Vδ5 T cell clone (expanded from a CMV$^+$ individual), thus allowing it to recognize endothelial cells infected with CMV (12).

In addition to the TCR, γδ T cells can also use NKG2D to recognize cells infected with various viruses and intracellular bacteria (32, 98–102). More specifically, the stress-inducible molecule, MICA, was induced on the surface of dendritic and epithelial cells by *M. tuberculosis* infection *in vitro* and *in vivo*; and its binding to NKG2D, substantially enhanced the TCR-dependent Vγ9Vδ2 T cell response to PAgs (28). Furthermore, in the case of *Brucella*, ULBP1 was the main NKG2D ligand upregulated on infected macrophages, and its engagement promoted Vγ9Vδ2 T cell cytotoxicity and cytokine production, which contributed to the inhibition of bacterium development (100).

A few other receptors have implicated in γδ T cell recognition of infected cells. Thus, another NKR, NKG2C, constitutively expressed on Vδ1$^+$ T cells, induced a cytolytic response against HIV-infected CD4$^+$ T cells expressing its ligand, HLA-E (23). On the other hand, we found that NKp30 can also play an important role in HIV-1 infection upon its induced expression in Vδ1$^+$ T cells; NKp30 ligation triggered the production of CCL3, CCL4, and CCL5 chemokines that suppressed the replication of a CCR5 tropic strain of HIV-1 (40). Finally, in the case of avian influenza (H5N1), γδ T cells were reported to use sialic acid receptors for the recognition of viral hemagglutinin (24). To understand how different microorganisms may elicit distinct pathways of γδ T cell recognition of pathogen-associated or stress-induced antigens remains a challenge for future research.

CONCLUDING REMARKS

In contrast with the well-established paradigm of MHC-restricted recognition of peptides by conventional αβ T cells, or even MHC class Ib-dependent recognition of lipids by unconventional αβ T cells, the molecular mechanisms of target cell recognition by γδ T cells remain poorly understood. A notable exception is the BTN3A1-mediated sensing of PAgs by Vγ9Vδ2 T cells, which underlies their responses to tumors and infections like TB or malaria. For most other γδ T cell subsets, however, TCR specificities are either unknown, not generalizable or of unclear physiological relevance. Therefore, the identification of relevant, non-Vγ9Vδ2 TCR ligands remains a major challenge in the γδ T cell field.

On the other hand, while NKRs are also clearly involved in γδ T cell recognition of tumor or infected cells, we still lack appropriate understanding how the multiple signals derived from all the expressed NKRs are integrated, also with those coming from the TCR itself. This likely depends on the relative expression levels of the various putative NKR and TCR ligands in each target cell, which adds significant complexity to the process of γδ T cell recognition.

The broad spectrum of MHC-unrestricted recognition of infected or transformed cells by γδ T makes them attractive candidates for adoptive cell therapy (ACT). All clinical trials have thus far concentrated on Vγ9Vδ2 T cells, probably due to their relative abundance in the peripheral blood and especially the availability of FDA-approved drugs, such as zoledronate and pamidronate, that allow their activation and expansion *in vivo* (103). Vγ9Vδ2 ACT has shown promising pre-clinical results against TB (104) and has already been tested in various cancer clinical trials [reviewed in Ref. (105)] that documented its safety and some (albeit still sub-optimal) efficacy (106–108). This could be maybe explained by Vγ9Vδ2 T cell susceptibility to exhaustion and activation-induced cell death (AICD). Nonetheless, improvements in Vγ9Vδ2 ACT protocols may still increase their efficacy, as indicated by some studies with exogenous provision of IL-2, importantly without the need for lymphodepleting preconditioning (109, 110). As for Vδ1$^+$ γδ T cells, they are less susceptible to AICD and exhaustion when compared to Vγ9Vδ2 T cells (111). However, no clinical trial has yet focused on this γδ T cell subset, mostly due to the lack of clinical-grade protocols allowing their successful expansion. Importantly, we have recently developed a clinical-grade process to effectively expand Vδ1$^+$ T cells while also inducing NCR (and augmenting NKG2D) expression; and established the proof-of-concept in leukemia xenograft models (73). We further anticipate NCR$^+$ Vδ1$^+$ ACT to be a promising therapeutic strategy also for solid tumors and chronic viral infections.

AUTHOR CONTRIBUTIONS

AS, BL, and BS-S conceived and wrote the manuscript. AS and BL contributed equally to the manuscript.

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CD3ε Expression Defines Functionally Distinct Subsets of Vδ1 T Cells in Patients With Human Immunodeficiency Virus Infection

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Human γδ T cells expressing the Vδ1 T cell receptor (TCR) recognize self and microbial antigens and stress-inducible molecules in a major histocompatibility complex-unrestricted manner and are an important source of innate interleukin (IL)-17. Vδ1 T cells are expanded in the circulation and intestines of patients with human immunodeficiency virus (HIV) infection. In this study, we show that patients with HIV have elevated frequencies, but not absolute numbers, of circulating Vδ1 T cells compared to control subjects. This increase was most striking in the patients with *Candida albicans* co-infection. Using flow cytometry and confocal microscopy, we identify two populations of Vδ1 T cells, based on low and high expression of the ε chain of the CD3 protein complex responsible for transducing TCR-mediated signals (denoted $CD3\varepsilon^{lo}$ and $CD3\varepsilon^{hi}$ Vδ1 T cells). Both Vδ1 T cell populations expressed the CD3 ζ-chain, also used for TCR signaling. Using lines of Vδ1 T cells generated from healthy donors, we show that CD3ε can be transiently downregulated by activation but that its expression is restored over time in culture in the presence of exogenous IL-2. Compared to $CD3\varepsilon^{hi}$ Vδ1 T cells, $CD3\varepsilon^{lo}$ Vδ1 T cells more frequently expressed terminally differentiated phenotypes and the negative regulator of T cell activation, programmed death-1 (PD-1), but not lymphocyte-activation gene 3, and upon stimulation *in vitro*, only the $CD3\varepsilon^{hi}$ subset of Vδ1 T cells, produced IL-17. Thus, while HIV can infect and kill IL-17-producing $CD4^+$ T cells, Vδ1 T cells are another source of IL-17, but many of them exist in a state of exhaustion, mediated either by the induction of PD-1 or by downregulation of CD3ε expression.

Keywords: human immunodeficiency virus, Vδ1 T cells, CD3ε, interleukin-17, programmed death-1, flow cytometry

Abbreviations: ART, antiretroviral therapy; DC, dendritic cell; FITC, fluorescein isothiocyanate; FMO, fluorescence minus one; HIV, human immunodeficiency virus; IL-17, interleukin-17; IFN-γ, interferon-γ; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PD-1, programmed death-1; PMA/I, phorbol myristate acetate with ionomycin; TCR, T cell receptor; TD, terminally differentiated.

INTRODUCTION

T cells expressing the γδ T cell receptor (TCR) represent a minor population of lymphocytes that expands in blood and peripheral tissues upon exposure to bacteria (1, 2), fungi (3), yeast (4, 5), and viruses (6–8). γδ TCRs bind non-peptide antigens in a major histocompatibility complex (MHC) unrestricted manner, leading to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) on the CD3 γ, δ, ε, ζ, and sometimes FcRγ proteins (9, 10). They respond rapidly by killing target cells, releasing cytokines, and providing ligands that mediate the activation and differentiation of other cells of the immune system (11, 12, 13).

Human γδ T cells comprise three predominant cell populations (Vδ1, Vγ9Vδ2, and Vδ3) based upon differences in the δ chain of the TCR (14, 15). Vδ1 TCRs are diverse and can recognize the stress-inducible proteins MICA and MICB, which are expressed by some tumor and virus-infected cells (16), glycolipid antigens presented by CD1c (17) and CD1d (18, 19) and the algal protein phycoerythrin (20). In addition to the TCR, Vδ1 T cells can be activated *via* ligation of other stimulatory receptors, including NKG2C, NKG2D, NKp30, toll-like receptors, and the β-glucan receptor, dectin 1 (5, 21–24). Upon activation, Vδ1 T cells proliferate, release cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor-α, and interleukin-17 (IL-17), chemokines, such as CCL3, CCL4, and CCL5, and they can kill $CD4^+$ T cells *in vitro* (4, 21, 23, 25–27).

Vδ1 T cells are found at higher frequencies in the blood, intestinal mucosa, and bronchoalveolar fluid of patients with human immunodeficiency virus (HIV) compared with healthy subjects (28, 29, 30, 31, 32, 33). We have examined the frequencies, phenotypes, and functions of circulating Vδ1 T cells in a cohort of untreated and antiretroviral therapy (ART)-treated patients with HIV and healthy control subjects. We find that percentage frequencies, but not absolute numbers of Vδ1 T cell are higher in the untreated patients compared to ART-treated patients and control subjects. We also have identified two subsets of Vδ1 T cells based on low and high levels of expression of the CD3ε polypeptide, denoted $CD3\varepsilon^{lo}$ and $CD3\varepsilon^{hi}$ Vδ1 T cells. Both were expanded in patients with HIV and, in particular, in the patients with *Candida albicans* co-infection. Phenotypic and functional analysis of these Vδ1 T cell subsets indicated that the $CD3\varepsilon^{lo}$ cells frequently express terminally differentiated (TD) and exhausted phenotypes and are unable to produce IL-17. These results suggest that HIV may induce a state of Vδ1 T cell inactivation.

MATERIALS AND METHODS

Study Population

Venous blood was obtained from 36 patients with HIV infection (21 males and 15 females) attending the Genitourinary Infectious Diseases Department at St. James's Hospital, Dublin. At the time of blood sample collection, 22 patients were receiving ART and 14 were not. The $CD4^+$ T cell count ranged from 55 to 1,857 (median 529) cells/μl of blood in the treated patients and 261–1,115 (median 578) cell/μl in the untreated patients. The viral load ranged from <50 to 72,796 (median < 50) copies/ml in the treated patients and <50–51,000 (median 578) copies/ml in the untreated patients. Three patients were positive for hepatitis B virus and three were positive for hepatitis C. As controls, blood samples were obtained from 23 healthy age- and gender-matched control subjects. Ethical approval for this study was obtained from the Joint Research Ethics Committee of St. James's Hospital and Tallaght Hospitals, Dublin, and all participants gave written, informed consent. Buffy coat packs from healthy blood donors were kindly provided by the Irish Blood Transfusion Service. Whole blood was used for enumerating T cells, as described below. Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway) and used immediately in all procedures.

Antibodies and Flow Cytometry

Fluorochrome-conjugated monoclonal antibodies (mAbs) specific for the human Vδ1 TCR (clone TS-1), CD3ε (clones MEM-1 and HIT-3a), CD3ζ (clone 6B10.2), CD27 (clone 0323), CD45RA (clone HI100), programmed death-1 (PD-1) (clone EH12.1), lymphocyte-activation gene 3 (LAG-3) (clone 11C3C65), and CD31 (clone WM59) were obtained from Thermo Fisher Scientific (Dublin, Ireland), BioLegend (San Diego, CA, USA), and Beckman Coulter (High Wycombe, UK) and used according to the manufacturers' recommendations. The CD3ε mAb (clone SP4) was kindly provided by Dr. Balbino Alcarón (Severo Ochoa Center for Molecular Biology, Madrid, Spain). Up to 10^6 PBMC, γδ T cell-enriched PBMC or expanded Vδ1 T cell lines were labeled with mAbs and analyzed using a CyAN ADP (Beckman Coulter) or FACSCanto (Becton Dickinson, Oxford, UK) flow cytometer. Data were analyzed with FlowJo v7.6 (Tree Star, Ashland, OR, USA) software. Single-stained OneComp Beads (Becton Dickinson) were used to set compensation parameters; fluorescence minus one (FMO) and isotype-matched Ab controls were used to set analysis gates. Fixable viability dye (eBioscience) was used to determine cell viability. The gating strategy for enumerating Vδ1 T cells is shown in **Figure 1A**. Total PBMC were analyzed for the enumeration of γδ T cell subsets. γδ T cell-enriched PBMC, prepared by negative selection using magnetic beads (Miltenyi Biotec, Bergische Gladbach, Germany), were used as a source of Vδ1 T cells for subsequent phenotypic and functional analysis.

Enumeration of Vδ1 T Cells

Absolute numbers of T cells per μl of blood were determined using Trucount tubes (BD Biosciences) according to the manufacturer's protocol. The percentages of $CD3^+$ cells that expressed Vδ1 TCRs, were determined by flow cytometry, as described above, allowing us to calculate the absolute counts of Vδ1 T cells (per μl of blood).

Vδ1 T Cell Sorting and Expansion

Lines of Vδ1 T cells were generated from healthy blood donors as described previously (5). Briefly, PBMC were prepared from buffy coat packs and monocytes were isolated by positive selection using CD14 Microbeads (Miltenyi Biotec, Gladbach Bergische, Germany). Monocytes were allowed to differentiate into immature dendritic cells (DCs) by culturing them for 6 days in the presence

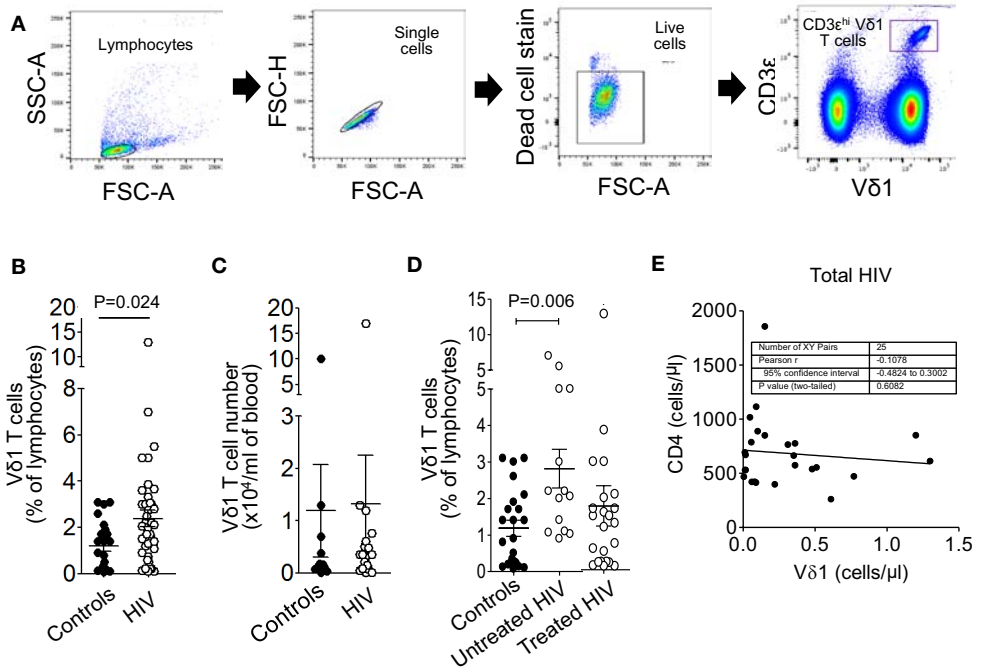

FIGURE 1 | Circulating Vδ1 T cell frequencies but not numbers are higher in patients with untreated human immunodeficiency virus (HIV) infection. Peripheral blood mononuclear cells were prepared from blood samples of 36 patients with HIV infection and 23 healthy donors, stained with monoclonal antibodies specific for CD3 and the Vδ1 T cell receptor and analyzed by flow cytometry. **(A)** Gating strategy for the enumeration of Vδ1 T cells, showing sequential gates on the lymphocytes, single cells, live cells, and Vδ1 T cells. **(B,C)** Scatter plots showing circulating Vδ1 T cell frequencies **(B)** and absolute numbers **(C)**. **(D)** Scatter plots showing Vδ1 T cell frequencies in control subjects (n = 23) and HIV patients divided into untreated (n = 14) and antiretroviral therapy-treated (n = 22) groups. Groups were compared using the Mann–Whitney U test. **(E)** Correlation between absolute counts of Vδ1 T cells and total $CD4^+$ T cells in the blood of 25 patients with HIV infection.

of granulocyte–monocyte colony-stimulating factor and IL-4 as described (34). Immature DC were plated at densities of 100,000 cells/ml and stimulated overnight with medium only, with heat- or ethanol-killed *C. albicans* (5×10^6 cells/ml) (5). *C. albicans* strain 10231 was obtained from the American Type Culture Collection and cultured for 24 h on malt extract agar. Fungi were cultured for 24 h, isolated, counted, and then inactivated by heating at 96°C for 60 min. Samples were then centrifuged at $5{,}000 \times g$ for 10 min, the supernatants discarded, and the pellets washed with phosphate buffered saline (PBS). Inactivation was confirmed by plating an aliquot onto malt extract agar and incubating for 7 days to check for growth.

Total γδ T cells were enriched from PBMCs using human anti-TCR γ/δ Microbeads (Miltenyi Biotec). γδ-enriched cells (200,000 cells/ml) were cultured in the absence or presence of *C. albicans*- or curdlan-treated DCs at 2:1 ratios in complete serum-free AIM-V medium (AIM-V containing 0.05 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.02 M HEPES, 55 μM β-mercaptoethanol, 1× essential amino acids, 1× nonessential amino acids, and 1 mM sodium pyruvate). Co-cultures were challenged with phytohemagglutinin (1 μg/ml; Sigma-Aldrich, Dublin, Ireland) and cultured with rIL-2 (40 U/ml; Miltenyi Biotec), which was added in fresh medium every 2–3 days. Cultures were restimulated every 2 weeks with activated DCs and phytohemagglutinin, which resulted in yields of >10 million Vδ1 T cells by day 28.

Confocal Microscopy

Expanded Vδ1 T cells were sorted into cells with high and low surface expression of CD3ε using a MoFlo XDP Cell Sorter (Beckman Coulter). The cell populations were subsequently incubated on poly L-lysine-coated 8-well Lab-Tek glass chamber slides (Nunc; Thermo Fisher Scientific) for 30 min at 37°C. The cells were fixed with an equal volume of 8% paraformaldehyde for 15 min at 37°C, permeabilized with 0.3% triton X-100 in PBS for 5 min at room temperature and then blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. The samples were incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3ε antibody (clone SK7, BioLegend, 1/50 dilution in 3% BSA/PBS) and incubated overnight at 4°C. After two washes in PBS, the slides were counter-stained with Hoechst 33258 (Molecular Probes) for 30 min at room temperature to visualize the nuclei. The slides were then imaged under 63× oil immersion with a Zeiss laser scanning confocal 510 microscope (Carl Zeiss, Hertfordshire, UK). The mean fluorescence intensity (MFI) of CD3 staining and Hoechst staining in individual cells was quantified using Zen 2009 imaging software (Carl Zeiss). The MFI of Hoechst served as an internal reference control between the different populations.

Analysis of Intracellular Cytokine Production

Interleukin-17 expression by fresh, unexpanded Vδ1 T cells within γδ T cell-enriched PBMCs was examined by flow cytometry after stimulation of the cells for 6 h with medium alone or with 1 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin (PMA/I) in the presence of brefeldin A to prevent cytokine release from the cells (5, 34).

Statistical Analysis

Prism GraphPad software (San Diego, CA, USA) was used for data analysis. Cell frequencies and numbers determined by flow cytometry in subject groups and cytokine levels in treatment groups were compared using the Mann–Whitney *U* test. *P* values <0.05 were considered significant. Correlations were defined using Pearson's correlation coefficient.

RESULTS

Vδ1 T Cell Frequencies but Not Numbers Are Higher in Patients With Untreated HIV Infection

Peripheral blood mononuclear cells were prepared from blood samples of 36 patients with HIV infection and 23 healthy donors, stained with mAbs specific for CD3ε and the Vδ1 TCR and analyzed by flow cytometry (**Figure 1A**). **Figure 1B** shows that the frequencies, as percentages of lymphocytes, of Vδ1 T cells were significantly higher in the HIV patient samples. Absolute counts of Vδ1 T cells were not significantly different between patients and controls (**Figure 1C**), suggesting that the percentage increases in Vδ1 T cells are a result of the depletions of $CD4^+$ T cells by HIV. When the patients were divided into untreated ($n = 14$) and ART-experienced ($n = 22$) groups, the frequencies of Vδ1 T cells were found to be higher only in the untreated patients (**Figure 1D**). Vδ1 T cell numbers did not correlate significantly with total $CD4^+$ T cell counts (**Figure 1E**), suggesting that the increases in Vδ1 T cells in patients with HIV do not simply compensate for the depletions of $CD4^+$ T cells. These data confirm and extend previous observations of altered Vδ1 T cell frequencies in patients with HIV.

Significant Numbers of Vδ1 T Cells Do Not Appear to Express CD3ε

A surprising observation made, while determining the frequencies of Vδ1 T cells in patients and control subjects, was that significant numbers of Vδ1 T cells do not appear to express CD3ε. CD3ε-negative Vδ1 T cells were detected in PBMC and in γδ T cell-enriched PBMC from both patients and control subjects using three different anti-CD3ε mAbs (clones MEM-1, SP4, and HIT-3a) after gating out dead cells, doublets and using FMO controls (**Figure 2A**). This allowed us to subdivide Vδ1 T cells into two groups on the basis of low and high expression of the TCR co-receptor, denoted $CD3\varepsilon^{lo}$ and $CD3\varepsilon^{hi}$ Vδ1 T cells, respectively. The levels of Vδ1 TCR expression were slightly higher in $CD3\varepsilon^{hi}$ compared to $CD3\varepsilon^{lo}$ Vδ1 T cells in both HIV patients and control subjects, although these differences did not

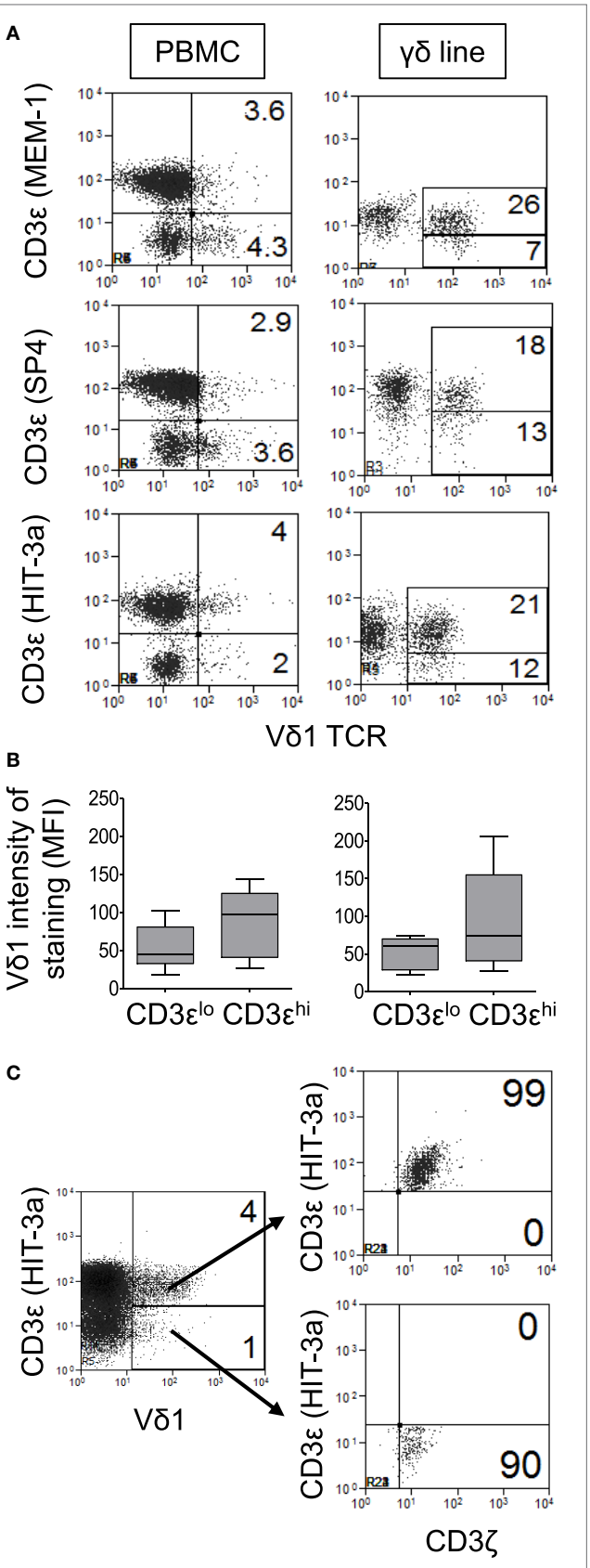

FIGURE 2 | Continued

FIGURE 2 | Significant numbers of Vδ1 T cells do not appear to express CD3ε. Peripheral blood mononuclear cell (PBMC) and γδ T cell-enriched PBMC were stained with antibodies specific for Vδ1 and CD3ζ and three different anti-CD3ε monoclonal antibodies (clones MEM-1, SP4, and HIT-3a) in separate tubes and analyzed by flow cytometry. **(A)** Representative flow cytometry dot plots of PBMC (left panels) and expanded Vδ1 T cells (right panels) from a patient with human immunodeficiency virus (HIV) infection showing the expression of CD3ε by Vδ1 T cells. **(B)** Box plots showing mean fluorescence intensities of staining for Vδ1 T cells in CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from six healthy donors (left) and nine HIV patients (right). **(C)** Representative flow cytometry dot plot showing CD3ζ expression by gated CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells. Results are representative of PBMC or γδ T cell-enriched PBMC from four different donors.

reach statistical significance (**Figure 2B**). Further flow cytometric analysis revealed that both CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells express the CD3ζ polypeptide (**Figure 2C**).

CD3ε^{lo} Vδ1 T Cells Express Very Low Levels or No Intracellular CD3ε

The low levels of CD3ε expression by some Vδ1 T cells may be due to internalization of the CD3ε chain. To investigate if CD3ε^{lo} Vδ1 T cells express intracellular CD3ε, Vδ1 T cells were purified from two healthy donors and sorted by flow cytometry into cells with high and low surface expression of CD3ε (**Figure 3A**). The cell populations were then bound to slides, fixed, permeabilized, blocked with bovine serum albumin, and stained with a FITC-conjugated mouse anti-human CD3ε antibody and Hoechst 33258. Cells were imaged by confocal microscopy and MFIs were quantified. **Figures 3B,C** show that CD3ε^{hi} Vδ1 T cells express high levels of cell surface CD3ε and low levels of intracellular CD3ε. By contrast, CD3ε^{lo} Vδ1 T cells express very low levels of cell surface or intracellular CD3ε, indicating that the CD3ε^{lo} Vδ1 T cell phenotype is not the result of internalization CD3ε.

CD3ε^{lo} and CD3ε^{hi} Vδ1 T Cells Are Both Preserved in Patients With HIV and Especially in Patients With *C. albicans* Co-Infection

We next investigated if the percentage frequencies of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells correlated with the presence of HIV infection in untreated and ART-treated patients. PBMCs were prepared from 14 patients with untreated HIV infection, 22 patients receiving ART and 23 healthy donors, stained with mAbs specific for CD3ε and Vδ1 and analyzed by flow cytometry. **Figure 4A** shows that both subsets of Vδ1 T cells are expanded in the untreated patients, whereas CD3ε^{hi} Vδ1 T cells, only, are expanded in treated patients. There were no significant differences in the frequencies of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells in patients with HIV. We previously reported that Vδ1 T cells expand and release IL-17 in response to *C. albicans*, a common co-infection in patients with HIV (5). **Figure 4B** shows that the frequencies of both subsets of Vδ1 T cells were significantly higher in patients with *Candida* co-infection ($n = 13$) compared to patients with no evidence of fungal infection ($n = 19$), indicating that fungal infection makes a significant contribution to the increased frequencies of Vδ1 T cells reported in patients with HIV infection (28–33).

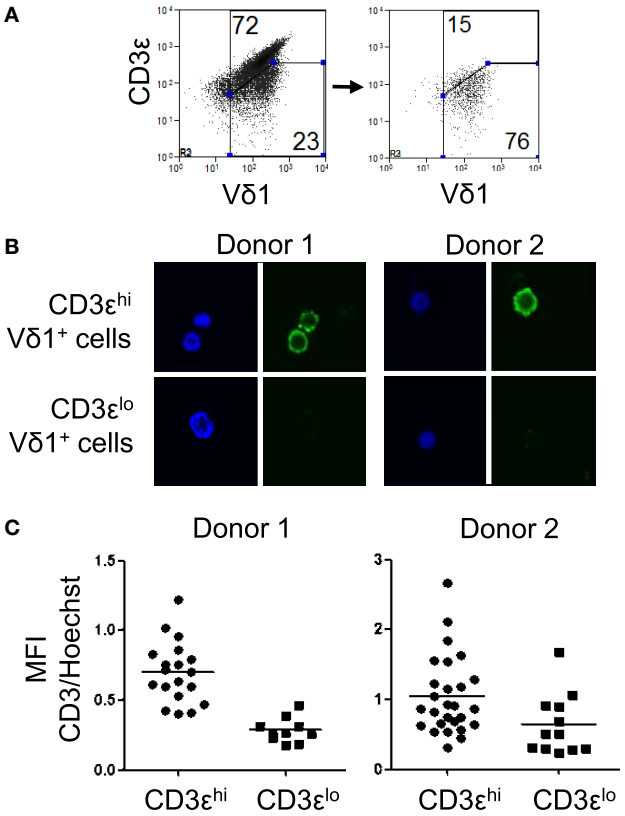

FIGURE 3 | CD3ε^{lo} Vδ1 T cells express very low levels of intracellular CD3ε. Vδ1 T cells were purified from two independent blood donors and sorted using a flow cytometric cell sorter into cells with high and low surface expression of CD3ε. The cell populations were incubated on poly L-lysine-coated glass chamber slides, fixed, permeabilized, and blocked with BSA. The samples were then incubated overnight with a FITC-conjugated mouse anti-human CD3ε antibody, washed twice, and counter-stained with Hoechst 33258 to visualize the nuclei. The slides were then imaged under oil immersion with a laser scanning confocal microscope and mean fluorescence intensity (MFI) was quantified. **(A)** Flow cytometry dot plots showing CD3ε and Vδ1 T cell receptor expression by a line of expanded Vδ1 T cells (left) and sorted CD3ε^{lo} Vδ1 T cells (right). **(B)** Confocal micrographs showing CD3ε (green) and nuclei (blue) in sorted CD3ε^{hi} (top) and CD3ε^{lo} (bottom) Vδ1 T cells from two donors. **(C)** Scatter plots showing the ratios of MFI of CD3 to Hoechst staining in individual cells from the sorted populations of CD3ε^{hi} and CD3ε^{lo} Vδ1 T cells from two donors.

CD3ε Expression by Vδ1 T Cells Can Be Modulated by Activation

We next investigated if CD3ε expression by Vδ1 T cells is stable or if it can be modulated by activation. CD3ε^{hi} and CD3ε^{lo} Vδ1 T cells were sorted from lines of Vδ1 T cells that were expanded from three donors. Cells were restimulated with PMA/I (**Figure 5A**) or DC pulsed with heat-killed *C. albicans* and PHA (**Figure 5B**) and cultured in the presence of IL-2. The expression of CD3ε by gated Vδ1 T cells was examined at times 0, 1, 7, and 14 days by flow cytometry. CD3ε expression by sorted CD3ε^{hi} Vδ1 T cells was transiently downregulated by activation with PMA/I (**Figure 5A**). **Figures 5B,C** show that CD3ε expression by CD3ε^{lo} Vδ1 T cells can be upregulated over time post reactivation with

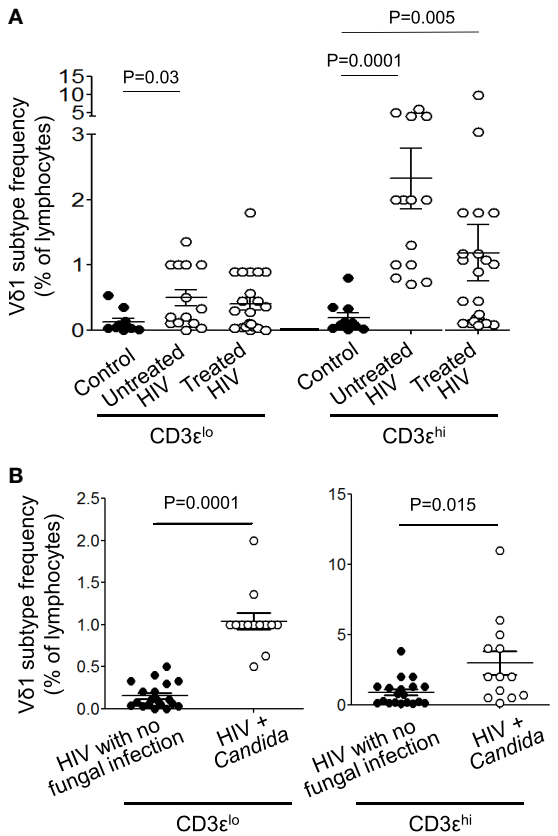

FIGURE 4 | Both CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells are expanded in untreated patients with human immunodeficiency virus (HIV) infection and especially in patients with *Candida* co-infection. Peripheral blood mononuclear cells were prepared from blood samples of 14 patients with untreated HIV infection, 22 patients receiving antiretroviral therapy and 23 healthy donors, stained with monoclonal antibodies specific for CD3ε and Vδ1 and analyzed by flow cytometry. **(A)** Scatter plots showing the frequencies of circulating CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells in untreated and treated patients with HIV infection and healthy control subjects. **(B)** Scatter plots showing the frequencies of circulating CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells in patients with HIV divided according to the absence (n = 19) or presence (n = 13) of *Candida albicans* co-infection. Groups were compared using the Mann–Whitney *U* test.

antigen. Thus, CD3ε can be transiently downregulated in CD3ε^{hi} Vδ1 T cells and upregulated in the CD3ε^{lo} population following activation, indicating that CD3ε expression is not fixed and that its downregulation is reversible.

CD3ε^{lo} Vδ1 T Cells More Frequently Have TD Phenotypes Than CD3ε^{hi} Vδ1 T Cells

The differentiation status of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells in 19 patients with HIV infection and 18 control subjects was examined by flow cytometric analysis of CD45RA and CD27 co-expression (34, 35). **Figure 6A** shows that significant proportions of total lymphocytes and gated CD3ε^{hi} Vδ1 T cells within γδ T cell-enriched PBMC from patients and controls expressed naïve (CD45RA^{+}CD27^{+}), central memory (CD45RA^{-}CD27^{+}), effector memory (CD45RA^{-}CD27^{-}), and TD (CD45RA^{+}CD27^{-}) phenotypes. By contrast, CD3ε^{lo} Vδ1 T cells from control subjects exhibited significantly higher frequencies of TD cells compared to CD3ε^{hi} Vδ1 T cells (**Figures 6A,B**). A similar increase in TD cells among CD3ε^{lo} Vδ1 T cells was found in the patients with HIV, with 90–100% of these cells being CD45RA^{+}CD27^{+} in some patients, but this did not reach statistical significance. Interestingly, the proportions of CD3ε^{hi} Vδ1 T cells that expressed TD phenotypes were higher in the HIV patients compared to control subjects. When the HIV-infected patients were divided into untreated (n = 13) and ART-treated (n = 14) subjects, the proportions of CD3ε^{lo} Vδ1 T cells expressing TD phenotypes was only marginally higher than those of CD3ε^{hi} Vδ1 T cells (**Figure 6B**). These results show that significant proportions of CD3ε^{lo} Vδ1 T cells express TD phenotypes, suggesting that they are exhausted as a result of HIV infection.

CD3ε^{lo} Vδ1 T Cells More Frequently Express PD-1, but Not LAG-3 or CD31, Than CD3ε^{hi} Vδ1 T Cells

Human immunodeficiency virus can induce the expression of the inhibitory receptors PD-1 and LAG-3 on HIV-specific T cells leading to their inactivation (36–42). Since Vδ1 T cells with TD phenotypes are preserved in patients with HIV infection, we investigated if CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from five untreated patients with HIV infection and eight control subjects express PD-1 or LAG-3. We also investigated if these cells express the naïve T cell marker CD31 (43). **Figure 7** shows that PD-1 is expressed at higher levels on CD3ε^{lo} Vδ1 T cells compared to CD3ε^{hi} Vδ1 T cells from eight healthy donors. A similar trend, although not statistically significant was found in five untreated HIV patients (**Figure 7B**). PD-1 expression by CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells was similar in patients and control subjects. By contrast, neither CD3ε^{lo} nor CD3ε^{hi} Vδ1 T cells from patients or controls expressed LAG-3. CD31 was expressed by variable proportions of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells and its expression was not altered in patients with HIV (**Figure 7**).

CD3ε^{lo} Vδ1 T Cells Exhibit Impaired IL-17 Production

The increased expression of PD-1 and TD phenotypes of CD3ε^{lo} Vδ1 T cells suggest that these cells are in a state of exhaustion. We and others have shown that Vδ1 T cells are rapid and potent producers of IL-17 (4, 5). We investigated if CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from patients with HIV infection and control subjects differ in their ability to produce IL-17. γδ T cell-enriched PBMC from 13 healthy donors and 11 patients with HIV were stimulated for 6 h with PMA/I or incubated in medium alone and IL-17A expression by gated CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells was examined by flow cytometry (**Figure 8A**). **Figures 8B,C** show that PMA/I treatment induced the production of IL-17 by significant numbers of CD3ε^{hi} Vδ1 T cells from both control subjects and HIV patients. However, stimulation of CD3ε^{lo} Vδ1 T cells with PMA/I did not lead to IL-17 production, suggesting that these cells are at least partially inactivated (**Figure 8**).

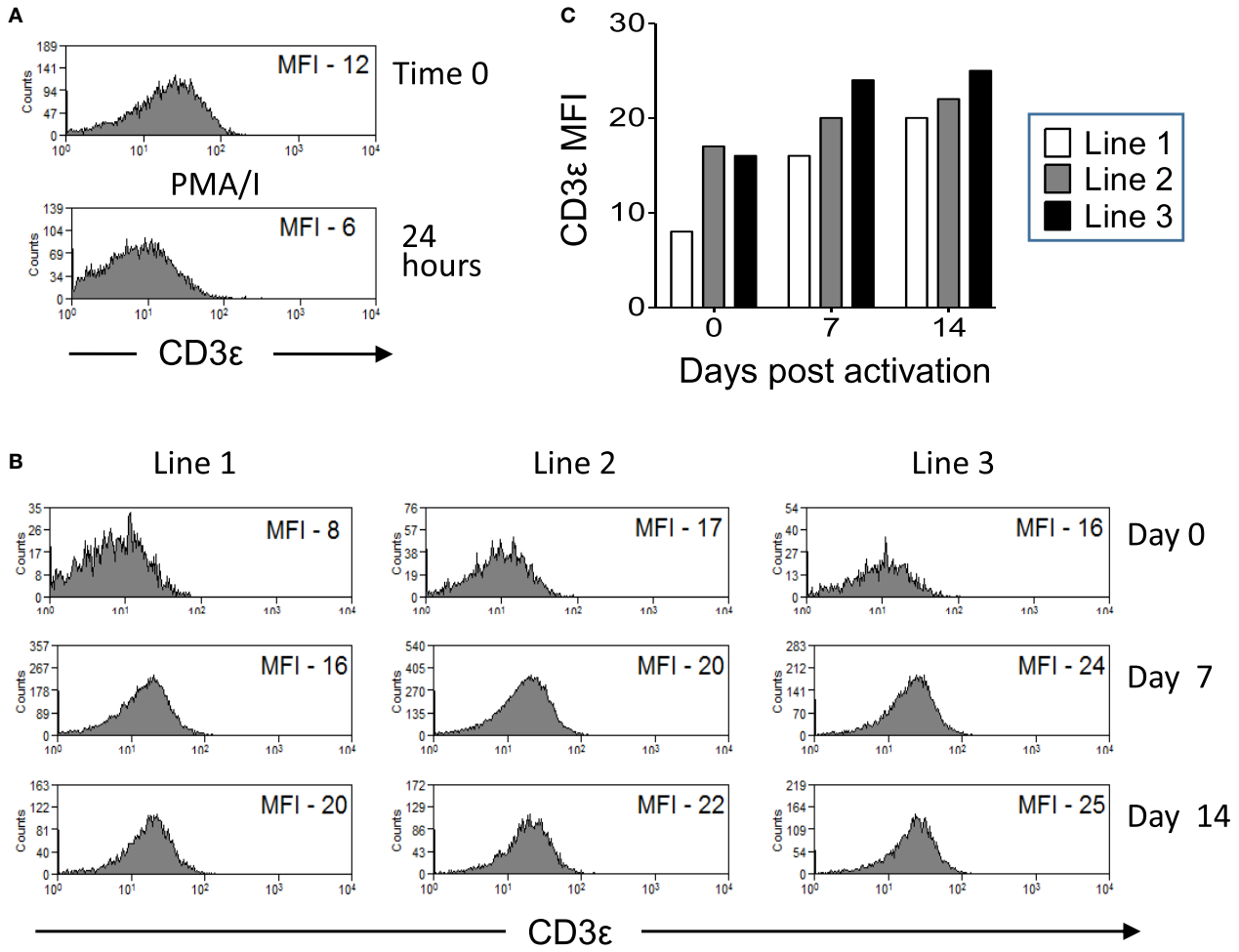

FIGURE 5 | CD3ε expression by Vδ1 T cells is modulated over time post activation. CD3ε^{hi} and CD3ε^{lo} Vδ1 T cells were sorted from lines of expanded Vδ1 T cells from three donors. Cells were restimulated with phorbol myristate acetate with ionomycin (PMA/I) or heat-killed *Candida albicans* presented by monocyte-derived dendritic cell and PHA and cultured in the presence of interleukin (IL)-2. The expression of CD3ε by gated Vδ1 T cells was examined at times 0, 1, 7, and 14 days by flow cytometry. **(A)** Flow cytometry histograms showing downregulation of CD3ε by sorted CD3ε^{hi} Vδ1 T cells stimulated for 24 h with PMA/I. **(B)** Flow cytometry histograms showing the upregulation of CD3ε expression by sorted CD3ε^{lo} Vδ1 T cells after stimulation with *C. albicans* and culture with IL-2. **(C)** Mean fluorescence intensities (MFIs) of CD3ε expression by Vδ1 T cells from three donors over time after *C. albicans* stimulation.

DISCUSSION

Numerous studies have shown that Vδ1 T cells are proportionally expanded in patients with HIV (28–33). Vδ1 T cells may contribute to immunity against HIV by killing infected CD4$^+$ T cells (21, 25), releasing antiviral cytokines (4, 25, 27) and chemokines (23). They may also contribute to the immunodeficiency associated with HIV infection, by depleting CD4$^+$ T cells (26). In this study, we have shown that Vδ1 T cells are not expanded in our patients with HIV infection, but their overall percentages are increased, suggesting that these cells are merely preserved in patients with HIV, while other cells are depleted. Since Vδ1 T cells are an important source of innate IL-17 (4, 5), it is also possible that their main role in patients with HIV is to stimulate immunity against co-infecting bacteria and fungi (1–5). Consistent with this hypothesis, we and others have found that Vδ1 T cells expand and produce IL-17 in response to *C. albicans* and that their frequencies are highest in HIV-positive patients with *Candida* co-infection (4, 5). Vδ1 T cells are also thought to be major producers of IL-17 in patients with colorectal cancer, in whom they have reduced IFN-γ production (44, 45). However, very few of these cells from healthy donors and patients with primary immunodeficiencies were reported to produce IL-17 (46), suggesting that IL-17 production by Vδ1 T cells is dependent on environmental factors, such as infection.

The TCR consists of a clonotypic αβ or a γδ glycoprotein heterodimer, generated by somatic recombination of germline gene segments, that recognizes antigens associated with antigen-presenting molecules, such as MHC, MR1, or CD1 (10). The TCR polypeptides associate with the CD3 complex, formed by the CD3 γ, δ (not to be confused with the TCR γ and δ polypeptides), ε and ζ subunits, which are invariable and mediate signal transduction. CD3ε can form heterodimers with CD3γ and CD3δ, while

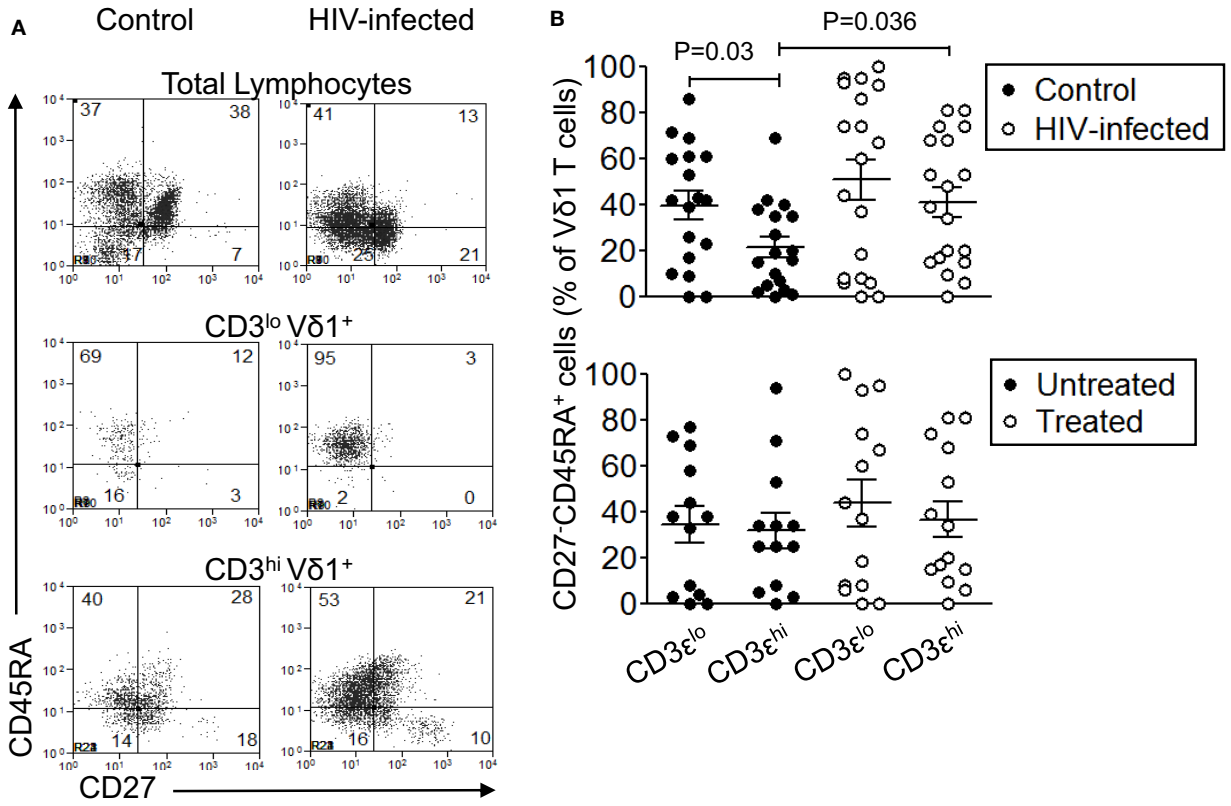

FIGURE 6 | Significant proportions of CD3ε^{lo} Vδ1 T cells have terminally differentiated (TD) phenotypes. Peripheral blood mononuclear cell (PBMC) from 19 patients with human immunodeficiency virus (HIV) infection and 18 control subjects were enriched for γδ T cells using magnetic bead separation, stained with monoclonal antibodies specific for CD3ε, Vδ1, CD45RA, and CD27, and analyzed by flow cytometry. **(A)** Representative flow cytometry dot plots showing CD45RA and CD27 expression by total lymphocytes (upper panels), CD3ε^{lo} Vδ1 T cells (center panels), and CD3ε^{hi} Vδ1 T cells (bottom panels) in a control subject (left panels) and a patient with HIV infection (right panels). **(B)** Scatter plots showing the frequencies of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from the patients and controls (upper graph) that expressed TD (CD45RA^{+}CD27^{-}) phenotypes. The lower graph shows the frequencies of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from untreated (n = 13) and antiretroviral therapy-treated (n = 14) patients who expressed TD phenotypes. Groups were compared using the Mann–Whitney U test.

CD3ζ frequently exists as a homodimer, and CD3δε, CD3γε, and CD3ζζ are all capable of transducing activating signals in response to TCR ligation (9, 10). The CD3 γ, δ, ε, and ζ polypeptides all contain ITAMs in their cytoplasmic domains, which are required for intracellular assembly and surface expression of the TCR and signal transduction events that mediate thymocyte maturation and mature αβ T cell activation (47–50). Humans and mice lacking CD3ε have no αβ or γδ T cells (49, 51), indicating an absolute requirement for CD3ε in early T cell development. However, unlike in αβ T cells, γδ TCR rearrangement can occur in the absence of CD3ε (50) and some mature γδ T cells do not express CD3ε (52). γδ TCRs can also signal through FcRγ homodimers and CD3ζ-FcRγ heterodimers (52).

In this study, we have identified two populations of Vδ1 T cells, one of which expresses normal levels of CD3ε and the other which appears to express no or low levels of CD3ε, but normal levels of CD3ζ. CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells were present in PBMC from patients with HIV and in control subjects and in expanded lines of Vδ1 T cells. Using confocal microscopy of sorted CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells, we show that the absence of CD3ε is unlikely to be due to internalization of the polypeptide, since intracellular CD3ε was not detected. To investigate the stability of CD3ε expression, CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells were sorted from lines of Vδ1 T cells and restimulated with *C. albicans* and cultured in the presence of IL-2. We found that CD3ε^{hi} Vδ1 T cells could downregulate CD3ε and CD3ε^{lo} Vδ1 T cells could upregulate CD3ε expression, suggesting that the expression of this component of CD3 can be modulated by activation and that its downregulation is reversible. CD3ε expression is required for progression of thymocyte maturation from the double positive CD4^{+}CD8^{+} stage to the single positive CD4^{+} or CD8^{+} stage and for assembly of the pre-TCR (49, 50, 53, 54), but appears to be dispensible in mature T cells, where it may act to amplify weak signals from the TCR (55, 56). Thus, it is possible that CD3ε^{lo} Vδ1 T cells have a lower responsiveness to antigenic stimulation than CD3ε^{hi} Vδ1 T cells. Interestingly, Vδ1 TCR expression was slightly lower in CD3ε^{lo} compared to CD3ε^{hi} Vδ1 T cells in HIV patients and control subjects, adding further support to this idea. CD3ε contains endocytosis determinants that may contribute to the up- and downregulation of CD3ε on T cells (57) and recent studies have provided evidence that CD3ε expression can be downregulated by tumor-educated tolerogenic DC (58) and

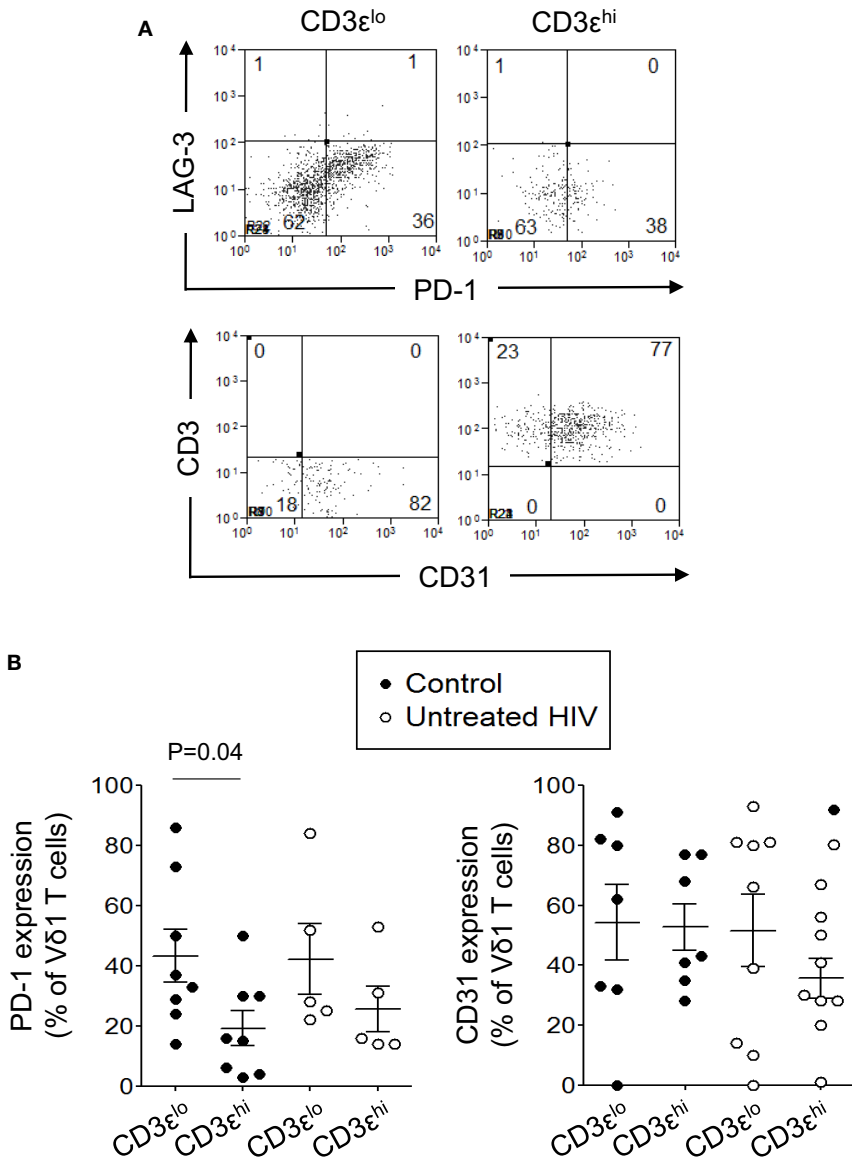

FIGURE 7 | CD3ε^{lo} Vδ1 T cells more frequently express programmed death-1 (PD-1), but not lymphocyte-activation gene 3 (LAG-3) or CD31, than CD3ε^{hi} Vδ1 T cells. Peripheral blood mononuclear cell prepared from eight healthy donors and five untreated patients with human immunodeficiency virus (HIV) were enriched for γδ T cells using magnetic bead separation, stained with monoclonal antibodies specific for CD3ε, Vδ1, PD-1, LAG-3, and CD31 and analyzed by flow cytometry. **(A)** Flow cytometry dot plots showing PD-1, LAG-3, and CD31 expression by gated CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from a patient with HIV. **(B)** Scatter plots showing the frequencies of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from the patients with HIV and control subjects that expressed PD-1 and CD31. Groups were compared using the Mann–Whitney *U* test.

possibly by HIV (59, 60). We found that both CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells are expanded in patients with untreated HIV infection compared to control subjects, but especially in patients with *C. albicans* co-infection. Thus, CD3ε^{lo} Vδ1 T cells accounted for 0.1% of lymphocytes in controls, compared to 0.5% in untreated HIV patients ($P = 0.03$) and >1% in patients with HIV and *Candida* infection ($P = 0.0001$). Likewise, CD3ε^{hi} Vδ1 T cells accounted for 0.2% of controls, compared to 2.3% of untreated HIV patients ($P = 0.0001$) and >3% of patients with HIV and *Candida* infection ($P = 0.015$). Future studies are required to identify the antigenic specificities of the Vδ1 TCR and to ascertain if Vδ1 T cell numbers or the ratios of CD3ε^{hi} to CD3ε^{lo} Vδ1 T cells can be used as a prognostic marker of *Candida* co-infection.

To determine if CD3ε^{lo} Vδ1 T cells display phenotypic or functional differences from CD3ε^{hi} Vδ1 T cells, PBMC freshly isolated from healthy donors were enriched for γδ T cells and further analyzed by flow cytometry. We found that CD3ε^{lo} Vδ1 T cells more frequently have TD phenotypes and express PD-1, but not LAG-3, compared to CD3ε^{hi} Vδ1 T cells, suggesting that they have previously been activated and exist in a state of inactivation. PD-1 and LAG-3 expression by HIV-specific CD4^{+} and CD8^{+} T cells is a feature of HIV infection, is associated with

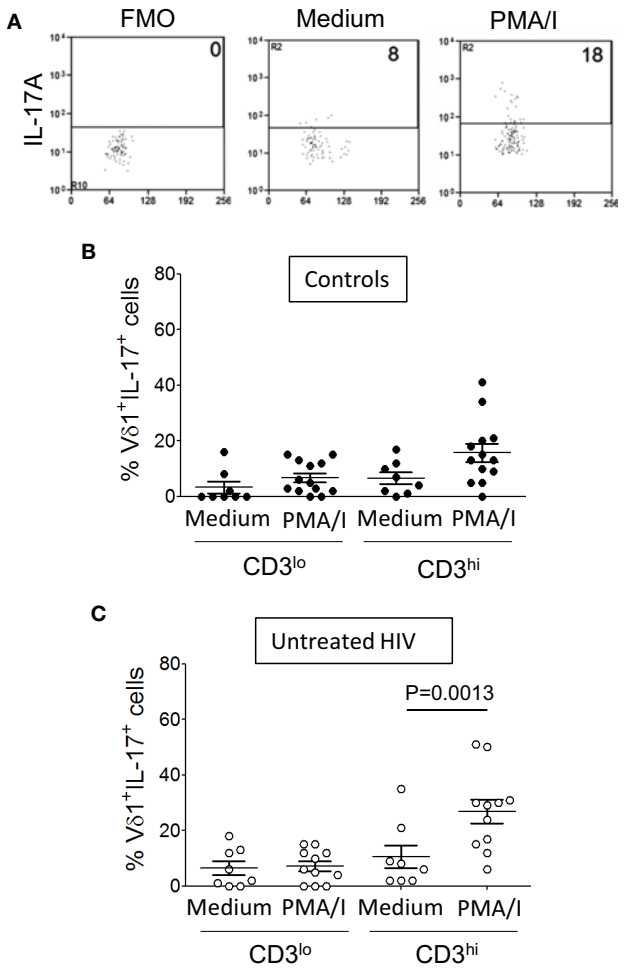

FIGURE 8 | CD3ε^{lo} Vδ1 T cells exhibit impaired interleukin (IL)-17 production. Peripheral blood mononuclear cell (PBMC) from 13 healthy donors and 11 patients with untreated human immunodeficiency virus (HIV) infection were enriched for γδ T cells using magnetic bead separation. The cells were stimulated for 6 h with phorbol myristate acetate with ionomycin (PMA/I) or incubated in medium alone in the presence of brefeldin A. **(A)** Flow cytometry dot plots showing IL-17A expression by gated CD3ε^{hi} Vδ1 T cells within unstimulated PBMC (center panel) and PMA/ionomycin-stimulated PBMC (right panel) from a patient with HIV. The left panel shows a fluorescence minus one (FMO) control dot plot. **(B,C)** Scatter plots show the frequencies of CD3ε^{lo} and CD3ε^{hi} Vδ1 T cells from healthy donors **(B)** and HIV patients **(C)** that produced IL-17. Groups were compared using the Mann–Whitney *U* test.

T-cell exhaustion and disease progression, and is thought to promote viral persistence (36–42). Our finding that Vδ1 T cells, and especially the CD3ε^{lo} subset of Vδ1 T cells, frequently express PD-1 indicates that this induction of exhaustion in HIV infection extends to γδ T cells and suggests that mAb blocking of PD-1 may benefit patients with HIV (61). Previous workers have reported a skewing of Vγ9Vδ2 T cells toward TD in patients with HIV (32, 62), which is associated with impaired IFN-γ production (63). We tested if CD3ε^{lo} Vδ1 T cells display properties of exhaustion by testing their ability to produce IL-17, a cardinal function of Vδ1 T cells (4, 5). We found that significant proportions of CD3ε^{hi} Vδ1 T cells, but not CD3ε^{lo} Vδ1 T cells, produced IL-17 in response to PMA/I stimulation *ex vivo*. Therefore, CD3ε^{lo} Vδ1 T cells may represent a population of inactive, TD T cells. Since IL-17 production is only one of multiple effector activities of Vδ1 T cells, future studies are required to determine if other activities, such as IFN-γ production, are deficient in CD3ε^{lo} Vδ1 T cells. Vδ1 and Vγ9Vδ2 T cells expressing low levels of CD3 and exhibiting impaired responses to stimulation have been reported to accumulate in sites of active *Mycobacterium tuberculosis* infection (64, 65) and Paget et al. (66) reported that murine Vγ6Vδ1^{+} T cells with low levels of CD3 predominantly produce IFN-γ whereas the same cells with high levels of CD3 produce IL-17. Thus, modulation of CD3ε expression may be a general mechanism for the regulation of γδ T cell activity.

The results of this study indicate that Vδ1 T cells persist in the blood of patients with untreated HIV infection, and especially in patients with *Candida* co-infection, while other T cells are depleted. Although it is not known if Vδ1 T cells can directly recognize HIV or HIV-infected cells, a recent study has shown that γδ TCR exposure to viruses can promote the expansion of virus-reactive T cells, providing strong evidence that γδ T cells mediate adaptive immune responses to viruses (67). Previous studies have demonstrated that Vδ1 T cells proliferate and release IL-17 in response to *C. albicans*, by a mechanism that requires IL-23 release from DC (4, 5). The preservation of Vδ1 T cells in patients whose IL-17-producing CD4^{+} T cells may be depleted by HIV, identifies Vδ1 T cells as an alternative potential source of IL-17. However, it appears that significant proportions of Vδ1 T cells in patients with HIV have been driven to a state of inactivation, expressing TD phenotypes and the inhibitory receptor PD-1 and failing to produce IL-17 upon stimulation. Downregulation of CD3ε, a signaling molecule known to augment TCR-mediated responses (55, 56), may represent another mechanism by which the effector functions of Vδ1 T cells can be inhibited.

ETHICS STATEMENT

Ethical approval for this study was obtained from the Joint Research Ethics Committee of St. James's Hospital and Tallaght Hospitals, Dublin, and all participants gave written, informed consent.

AUTHOR CONTRIBUTIONS

PD, TR, and DD: conceived the study. PD, CM, MF, KD, AP, JO, A Long, and MD: performed experiments, acquired and analyzed data. DR, SO, and FM: directed and coordinated sample collection. A Loy, JW, and FM: performed sample collection. DD: wrote the manuscript.

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Human γδ T-Cell Control of Mucosal Immunity and Inflammation

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Human γδ T-cells include some of the most common "antigen-specific" cell types in peripheral blood and are enriched yet further at mucosal barrier sites where microbial infection and tumors often originate. While the γδ T-cell compartment includes multiple subsets with highly flexible effector functions, human mucosal tissues are dominated by host stress-responsive Vδ1$^+$ T-cells and microbe-responsive Vδ2$^+$ T-cells. Widely recognized for their potent cytotoxicity, emerging data suggest that γδ T-cells also exert strong influences on downstream adaptive immunity to pathogens and tumors, in particular *via* activation of antigen-presenting cells and/or direct stimulation of other mucosal leukocytes. These unique functional attributes and lack of MHC restriction have prompted considerable interest in therapeutic targeting of γδ T-cells. Indeed, several drugs already in clinical use, including vedolizumab, infliximab, and azathioprine, likely owe their efficacy in part to modulation of γδ T-cell function. Recent clinical trials of Vδ2$^+$ T-cell-selective treatments indicate a good safety profile in human patients, and efficacy is set to increase as more potent/targeted drugs continue to be developed. Key advances will include identifying methods of directing γδ T-cell recruitment to specific tissues to enhance host protection against invading pathogens, or alternatively, retaining these cells in the circulation to limit peripheral inflammation and/or improve responses to blood malignancies. Human γδ T-cell control of mucosal immunity is likely exerted *via* multiple mechanisms that induce diverse responses in other types of tissue-resident leukocytes. Understanding the microenvironmental signals that regulate these functions will be critical to the development of new γδ T-cell-based therapies.

Keywords: human, mucosal, gammadelta T-cells, Vdelta1, Vdelta2

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INTRODUCTION

The γδ T-cell compartment includes some of the most numerous "antigen-specific" cell types in peripheral blood and is enriched yet further in mucosal tissues including the lung and intestine (1, 2). In humans, γδ T-cells are typically divided into distinct subsets based on δ-chain usage, each being specialized to detect a different class of common antigen or host molecule generated by microbial infection, stress, and/or malignant transformation. Pathobionts frequently invade the body *via* epithelial barriers, which are also major sites of tumorigenesis, hence γδ T-cell function in mucosal tissues represents a critical component of host protection against a range of major diseases. While the ability of human γδ T-cells to lyse infected or transformed host cells has been well documented, less is known about their influence on downstream antimicrobial immunity and mucosal inflammation,

which must be carefully regulated in order to prevent autoimmune pathology, tissue damage, and cancer. Indeed, a recent analysis of tumor transcriptome data identified γδ T-cell infiltration as the best prognostic marker of survival (1), indicating that γδ T-cell responses can significantly influence clinical outcomes in human patients, but the mucosal functions of these cells and their impact on barrier protection remain poorly understood. This mini-review focuses on the potential roles of γδ T-cells in human mucosal tissues, with an emphasis on their ability to influence conventional leukocyte responses at these sites. We consider that γδ T-cell detection of stress molecules and microbial signals can significantly alter adaptive immunity and inflammation at mucosal barrier sites, consistent with the increasing recognition that tissue-resident T-cells play essential roles in human immunity. Where useful context has been drawn from studies performed in animal models, the non-human origins of these data have been clearly indicated.

γδ T-CELLS MEDIATE EPITHELIAL BARRIER PROTECTION

Epithelial cells are exposed to a variety of microbial and environmental signals that induce distinct patterns of cytokine and chemokine secretion, as well as rapid changes in cell surface expression of host stress molecules. Acting in concert, these factors can stimulate a range of leukocyte responses as complex as those imparted by myeloid antigen-presenting cells (3). Innate-like lymphocytes residing in the epithelial layer and underlying mucosa are key responders to these barrier stress signals, and γδ T-cells comprise a major component of this "unconventional" lymphocyte pool. It is well-established that epithelial signaling to γδ T-cells begins early, in the thymus, where these cells are imparted with greater gut-homing potential (integrin α4β7 expression) than conventional lymphocytes, and exhibit more efficient proliferation upon subsequent recruitment to the murine mucosa (4). Less clear is how far epithelial cells continue to shape γδ T-cell function upon their arrival in mucosal tissues, although an intimate functional relationship controlled by a variety of different signals seems increasingly likely (5). Indeed, the γδ T-cell repertoire in human intestine undergoes major changes with age and becomes oligoclonal in adults (6), suggesting strong local selection by site-specific signals that include host butyrophilin-like molecules (5, 7), dietary and microbial ligands for the aryl hydrocarbon receptor (8), and common pathogen products and stress antigens. Accordingly, studies in parabiotic mice have demonstrated that the frequency of γδ T-cell mixing between animals is low in the gut epithelium, whereas up to 50% cell exchange between animals can be observed in the lamina propria (9). These data suggest that Vδ1$^+$ intraepithelial lymphocytes (γδ-IEL) may develop *in situ*, whereas lamina propria γδ T-cells depend both on recruitment from the peripheral blood and local proliferation in order to maintain the local pool. In mice, it is widely accepted that the majority of γδ T-cells are pre-programmed with cytokine potential and effector functions within the thymus (10). However, recent data suggest that γδ T-cell function outside the thymus is more plastic than originally thought (11), and the murine Vγ7$^+$ subset appears to require Btnl1 expression by the gut epithelium to develop IFNγ-expressing capacity (5). In humans, the closely related proteins BTNL3 and BTNL8 may similarly cooperate to promote colonic expansion of the analogous Vγ4$^+$ subset (5), although the functional impact of this mechanism remains unclear, and populations expressing alternative γ-chains also reside in this tissue. Nonetheless, human γδ T-cell function does not appear to be "hard-wired" in the thymus and remains receptive to site-specific cues that likely induce distinct functional profiles in different tissues and organs. Intriguingly, BTNL2 is primarily expressed in the small intestinal epithelium and appears to function as a negative regulator of T-cell activation (12), with mutations in this protein conferring increased risk of inflammatory bowel disease (IBD) (13). It is possible, therefore, that therapeutic strategies targeting BTNL molecules and/or γδ T-cell activation in the human gut may yield new treatment options for patients with IBD.

Consistent with a role for γδ-IEL in monitoring gut barrier function, recent data indicate that these cells are highly motile in the mouse intestine and actively scan the epithelium for signs of cellular stress, with pro-inflammatory cytokines and/or pathogen encounter significantly modulating this behavior (14, 15). Indeed, while γδ-IEL numbers appear largely unaffected in germ-free mice (16), epithelial cell contact with gut bacteria can induce γδ-IEL expression of antimicrobial peptides (17), confirming that exposure to the microbiota can significantly alter their function. It is likely that human gut γδ-IELs scan the epithelium for expression of MHC I-related genes MICA and MICB, which function as stress-inducible triggers for γδ T-cell cytotoxicity (18, 19). MICA/B expression has already been identified in carcinomas of the lung and colon, where these molecules are associated with enhanced tumor infiltration by cytotoxic γδ T-cells (20). Accordingly, γδ T-cells isolated from human lung tumors can selectively lyse autologous malignant cells *ex vivo* (21). Vδ1$^+$ γδ T-cells also seem to be expanded in many transplant recipients, where they express gut-homing receptors and are strongly activated by intestinal tumor cells but not healthy epithelial cell lines (22).

MICA/B is recognized with high affinity by the natural killer (NK) cell receptor NKG2D (23), which is expressed by human γδ-IELs under the control of IL-15 (24). This cytokine appears to play an important role in steady-state maintenance of the murine γδ-IEL compartment (25), and thymic expression of IL-15 is required to modulate histone acetylation of the Vγ5 gene segment, which is preferentially used by mouse gut γδ-IELs (26). Consistent with these data is the observation that epithelial supply of IL-15 cytokine plays a crucial role in γδ T-cell control of mucosal inflammation in murine colitis (27). Similarly, human intestinal Vδ1$^+$ T-cells are significantly expanded in both celiac disease and IBD (28, 29), which are characterized by high mucosal levels of the tissue damage-associated cytokine IL-15 (30–32). Intriguingly, patients with celiac disease exhibit upregulated activity of cytotoxic lymphocytes (24), but a subset of NKG2A$^+$ γδ T-cells can reportedly decrease IFNγ expression by cocultured gut αβ T-cells (33). Similarly, transfer of γδ-IELs into mice that lack these cells can protect against chemical colitis by decreasing host lymphocyte expression of pro-inflammatory cytokines and modulating epithelial production of IL-15 (27). These data strongly suggest that γδ-IELs help maintain the integrity of the epithelium by altering

the local activity of other gut leukocyte subsets, and that IL-15 may alert these cells to tissue stress, including the need to remove infected/malignant epithelial cells from the barrier. Consequently, when intestinal γδ T-cells are deleted in murine models, the gut epithelium displays uncontrolled IFNγ expression, chronic inflammation, and impaired barrier regeneration (34).

Vδ1$^+$ T-cell influences on other leukocyte populations have previously been reported in various settings of relevance to mucosal barrier protection. For example, maturation of CD1c$^+$ myeloid dendritic cells (DC) can be induced by direct contact with CD1c-restricted Vδ1$^+$ T-cells *in vitro* (35), suggesting that similar interactions may also occur at mucosal sites *in vivo*. The resultant mature, CD1c$^+$ DC are endocytic, can efficiently present novel protein antigens, and are more potent stimulators of naïve T-cell proliferation than DC activated with cytokines alone. Intriguingly, these characteristics can also be observed in human lung DC isolated from patients with atopic asthma and may represent genuine features of mucosal inflammatory disorders (36). Vδ1$^+$ T-cell-induced maturation of CD1$^+$ myeloid DC does not rely on foreign antigen and is chiefly mediated by TNFα (35), which also triggers rapid activation of γδ T-cells (37–39), and likely enables timely immune responses to a barrier breach. Indeed, full DC maturation has long been known to require interaction with T-cells (40), but αβ T-cell clones with fine antigen specificity are rare in the periphery. Tissue-resident γδ T-cells may therefore accelerate DC maturation in the mucosa by relying on non-polymorphic molecules to mediate this interaction (41). Indeed, increased CD1 expression by APC has already been reported to enhance T-cell stimulation in a murine model (42). It is also important to note that this model does not preclude a role for the microbiota, since microbial antigen can enhance APC presentation of self-antigens to CD1-restricted T-cells (43). Indeed, CD1$^+$ DC can also present pollen-derived lipid antigens to Vδ1$^+$ T-cells derived from the blood of allergic donors (44), suggesting that similar interactions can also occur in the human lung in allergic asthma. While it is unclear to what extent laboratory mice can accurately model asthma pathology (45), previous studies have observed a major influence of lung γδ T-cells on allergen-induced airway hyperreactivity and excess production of IgE (46, 47), which are cardinal features of the human disease. Intriguingly, these effects were again associated with a shift in cytokine production by pulmonary αβ T-cells, further suggesting that γδ T-cells can exert complex effects *via* their influence on other mucosal leukocyte populations. Indeed, gut-tropic γδ T-cells can promote Th1/Th17 differentiation of CD4$^+$ T-cells *in vivo* to exacerbate colitis in murine models (48, 49), and a Vδ2$^+$ subset expressing the PD1 isoform Δ42 promotes gut inflammation in humanized mice *via* putative effects on DC (50). Together, these data suggest that γδ T-cells may exert similarly potent influences on adaptive immunity and inflammation in human mucosal tissues.

γδ T-CELLS STIMULATE COMPLEX MUCOSAL LEUKOCYTE RESPONSES

Often referred to as rare cells, recent estimates suggest that the phosphoantigen-responsive Vδ2$^+$ population in fact accounts for ~1 in 40 memory T-cells in healthy adults and may represent the single largest recall response in the human body (2, 51). Indeed, Vδ2$^+$ T-cells are capable of expanding yet further to dominate the blood lymphocyte pool in a wide range of infections (52, 53), which has led to extensive study of these cells in the circulation as well as the common misconception that they are restricted to the blood. However, several reports have now identified that the majority of blood Vδ2$^+$ T-cells express homing receptors for epithelial barrier sites including the skin (CLA) and intestine (integrin α4β7 and CCR9) (50, 54, 55). This tissue-tropic phenotype is consistent with the role of Vδ2$^+$ T-cells in host protection against pathogens that colonize epithelial barriers and produce the metabolite (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) (56). Indeed, while circulating Vδ2$^+$ T-cells are well situated to detect host cell accumulation of phosphoantigen in blood malignancies (57), the majority of non-hematological cancers are epithelial in origin, such that Vδ2$^+$ T-cell recruitment to these sites is likely to enhance tumor surveillance as well as antimicrobial immunity. Vδ2$^+$ T-cells express high levels of α4β7 in humans (22, 55, 58), are rapidly recruited to mucosal tissues in higher primates *in vivo* (59, 60), and mediate effective host protection against bacteria in humanized mice (61). Recent work in a macaque model also demonstrated that injection of HMB-PP or related compounds stimulates Vδ2$^+$ T-cell expansion in the blood and accumulation of a CD27$^+$ IFNγ-producing subset in the lungs (59). Intriguingly, lung accumulation of Vδ2$^+$ T-cells persisted for several months and was associated with a corresponding increase in CD4$^+$ and CD8$^+$ conventional T-cell numbers, suggesting that activation of mucosal Vδ2$^+$ T-cells exerts multiple downstream effects on other leukocyte compartments. Indeed, Vδ2$^+$ T-cell activation *in vivo* has since been shown to enhance conventional Th1 responses in the lung and promote mucosal release of growth factors that confer protection against a range of different pathogens (including *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Yersinia pestis*) (60, 62, 63). Since HMB-PP injection into macaques promotes Vδ2$^+$ T-cell expansion and recruitment to the intestinal mucosa as well as the lung (59, 60), it is likely that these cells exert similar effects on αβ T-cell responses and antimicrobial immunity in the primate gut. Consistent with this concept, human gut tissue contains Vδ2$^+$ T-cells that express the tissue-resident memory T-cell marker CD103, exhibit distinct patterns of cytokine production, and modify IFNγ expression by autologous gut CD4$^+$ T-cells (55, 64). In mice, T-cell entry into the epithelium combined with local IL-15 and TGF-β signaling is required for the formation of long-lived memory cells that express CD103 (65). Whether or not CD103$^+$ Vδ2$^+$ T-cells in human tissues represent a long-lived population with distinct roles in mucosal immunity is currently unclear.

We have previously demonstrated that activation of Vδ2$^+$ T-cells in human intestine modulates cytokine production by colonic αβ T-cells in the same piece of gut tissue (55), indicating that these cells are present in sufficient numbers to exert potent effects on downstream mucosal immunity. Moreover, like Vδ1$^+$ T-cells the Vδ2$^+$ population can promote generation of mature DC *via* a TNF-dependent mechanism (66, 67), illustrating a marked ability of these "innate-like" cells to trigger adaptive immune responses. In the case of the Vδ2$^+$ subset, this process

also confers potent APC capacity on the γδ T-cells, likely allowing rapid amplification of immune responses at sites of barrier breach and microbial invasion. Early work in this area demonstrated that microbial activation induced human Vδ2$^+$ T-cells to process and present antigens as efficiently as DC, as well as provide co-stimulatory signals that stimulated naïve αβ T-cell proliferation and differentiation (68, 69). It is now widely recognized that blood Vδ2$^+$ T-cells can display remarkably flexible APC functions, while the nature of the αβ T-cell responses they induce in tissues is likely directed by the stimuli encountered at specific anatomical sites. Indeed, "Vδ2-APC" function appears to be optimally induced by microenvironmental signals known to be highly enriched in the human gut, namely microbe-derived HMB-PP (70), pro-inflammatory mediator TNFα (71), and epithelial cytokine IL-15 (32). It is perhaps unsurprising then that human intestinal Vδ2-APC are efficient inducers of the barrier defense mediator IL-22 (72), whereas conventional myeloid APC in this tissue are instead specialized to induce "pro-symbiotic" IL-17 responses (73). Intriguingly, therapeutic antibody-mediated disruption of Th17 biology led to increased mucosal inflammation in patients with Crohn's disease during randomized controlled trials (74, 75), suggesting that Vδ2$^+$ T-cell-directed immunotherapies might prove to be an effective method of enhancing barrier protection without impacting on mucosal levels of IL-17. Indeed, recruitment of circulating Vδ2$^+$ T-cells to inflamed skin lesions has already been identified in patients with psoriasis (54), and this population can also infiltrate the peritoneal cavity in patients with bacterial infections (39). In both cases, local Vδ2$^+$ T-cell numbers and activation state were significantly correlated with therapeutic/patient outcomes, suggesting that these cells significantly impact on the clinical course of both inflammatory and infectious pathologies affecting multiple human tissues.

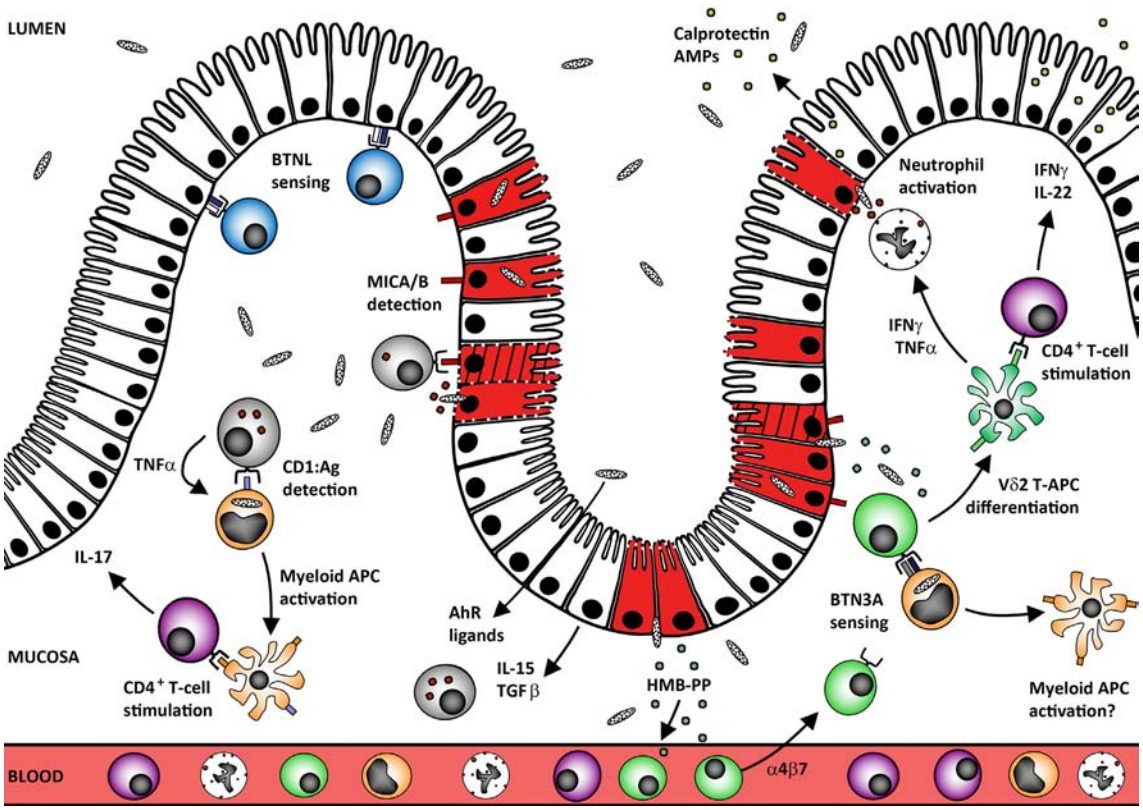

FIGURE 1 | Human mucosal γδ T-cells protect the epithelial barrier against microbes and tumors. Tissue-resident γδ T-cells may develop *in situ* under the control of site-specific BTNL heterodimers that maintain these cells in a primed but inactivate state (blue Vδ1$^+$ cells). Human mucosal barrier sites are also enriched in CD1$^+$ myeloid APC (orange cells) that capture microbes and may undergo local TNF-induced maturation *via* self-antigen presentation to CD1-restricted γδ T-cells. The resultant mature APC can stimulate conventional αβ T-cell responses at the site of infection without the need to migrate through the draining lymphatics. Loss of BTNL signaling or upregulation of MICA/B expression by the infected/transformed/stressed epithelium (red/hatched/membrane-damaged cells) also triggers γδ T-cell cytotoxic responses that rapidly lyse the compromised cells (gray cells; both Vδ1$^+$ and Vδ2$^+$ subsets). Maintenance of these "epithelial surveillance" γδ T-cell populations is regulated by a complex variety of signals including local provision of AhR ligands, epithelial cytokine IL-15, and growth factor TGF-β. These factors likely also play critical roles in promoting tissue residence of recruited γδ T-cell populations. In the case of Vδ2$^+$ T-cells (green cells), recruitment from the blood could be driven by (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) translocation across the defective mucosal barrier. Accumulation of microbial HMB-PP in the mucosa can then trigger BTN3A-mediated activation of Vδ2$^+$ T-cells in the presence of IL-15 to promote differentiation into potent APC (and perhaps also reciprocal activation of local myeloid cell populations). This process supports rapid local generation of presenting cells that can stimulate CD4$^+$ T-cell expression of barrier protectant cytokines, including IFNγ and IL-22 (purple CD4$^+$ T-cells). These mediators promote epithelial release of antimicrobial peptides (AMPs) including calprotectin and cooperate with TNFα to promote neutrophil activation and survival.

FUTURE CONSIDERATIONS FOR THERAPEUTIC TARGETING OF MUCOSAL γδ T-CELLS

Human γδ T-cells display potent effector functions when exposed to microbial antigens and/or host molecules commonly encountered at barrier sites, but accumulating evidence suggests an additional ability to modulate downstream mucosal leukocyte responses (**Figure 1**). These features may be shared by multiple γδ T-cell subsets in human tissues, since even the little-studied Vδ3$^+$ subset appears capable of complex patterns of cytokine expression and promoting DC maturation mediated by CD1/TNFα (76). Together, these data suggest that tissue-resident γδ T-cells play important roles in activating host immunity to microbes across multiple mucosal sites, not just the lung and intestine. Indeed, recent findings indicate that commensals residing in the ocular mucosa can induce γδ T-cell expression of IL-17 to drive neutrophil recruitment and protect the mouse eye against bacterial and fungal pathogens (77). Human γδ T-cells in mucosal tissues may be similarly specialized to detect local microbial and host stress molecules and respond not only with rapid effector functions, but also by relaying critical information to other mucosal leukocyte populations. Data from our own laboratories indicate that human Vδ2$^+$ T-cells can significantly modify intestinal immune responses *via* direct antigen presentation *in vitro* (72), and influence the clinical outcome of microbial infections *in vivo* (39), hence these cells should be a high priority for the development of novel immunotherapies. Indeed, a recent transcriptome analysis of 585 human colorectal cancer samples revealed that tumor infiltration by IFNγ-producing Vδ2$^+$ T-cells in particular was associated with higher probability of 5-year disease-free survival (78). Given that γδ T-cells also exhibit potent activity in non-malignant settings, it seems likely that therapies targeting these cells could prove effective in a range of different pathologies. Multiple drugs already in widespread clinical use likely owe their therapeutic efficacy in part to modulation of γδ T-cell function, including the anti-α4β7 antibody vedolizumab (79–81), anti-TNF agents including infliximab (38, 82–85), and immunosuppressant drug azathioprine (86). The aminobisphosphonate drug zoledronate has also been shown to promote Vδ2$^+$ T-cell activation *in vivo* by inhibiting the farnesyl pyrophosphate synthase enzyme to allow host cell accumulation of isopentenylpyrophosphate (87). Work is ongoing to identify optimal strategies for zoledronate adjunctive therapy, which has so far displayed variable patient benefit in clinical trials (88, 89). However, the continuing development of aminobisphosphonate pro-drugs and γδ-selective nanobody agonists/antagonists will soon yield more potent therapies for a variety of major human disorders (90–92). Given that γδ T-cell biology is closely associated with epithelial barriers, a key consideration for future treatment strategies will be ensuring the effective targeting of γδ T-cells to tissues of interest (55, 58, 93, 94). Limiting γδ T-cell egress from the blood may prove beneficial when treating blood malignancies and mucosal inflammatory disorders such as IBD (e.g., with anti-integrin antibodies), whereas enhancing cell migration to barrier sites will likely be key to enhancing protection against mucosal infection and epithelial tumors. The extent to which therapeutic outcomes are influenced by the mechanisms that promote γδ T-cell tissue residency will also need to be explored.

AUTHOR CONTRIBUTIONS

NM and ME drafted the manuscript, revised the content, and approved the final version.

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Human γδ T-Cells: From Surface Receptors to the Therapy of High-Risk Leukemias

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γδ T lymphocytes are potent effector cells, capable of efficiently killing tumor and leukemia cells. Their activation is mediated by γδ T-cell receptor (TCR) and by activating receptors shared with NK cells (e.g., NKG2D and DNAM-1). γδ T-cell triggering occurs upon interaction with specific ligands, including phosphoantigens (for Vγ9Vδ2 TCR), MICA-B and UL16 binding protein (for NKG2D), and PVR and Nectin-2 (for DNAM-1). They also respond to cytokines undergoing proliferation and release of cytokines/chemokines. Although at the genomic level γδ T-cells have the potential of an extraordinary TCR diversification, in tissues they display a restricted repertoire. Recent studies have identified various γδ TCR rearrangements following either hematopoietic stem cell transplantation (HSCT) or cytomegalovirus infection, accounting for their "adaptive" potential. In humans, peripheral blood γδ T-cells are primarily composed of Vγ9Vδ2 chains, while a minor proportion express Vδ1. They do not recognize antigens in the context of MHC molecules, thus bypassing tumor escape based on MHC class I downregulation. In view of their potent antileukemia activity and absence of any relevant graft-versus-host disease-inducing effect, γδ T-cells may play an important role in the successful clinical outcome of patients undergoing HLA-haploidentical HSCT depleted of TCR αβ T/CD19$^+$ B lymphocytes to cure high-risk acute leukemias. In this setting, high numbers of both γδ T-cells (Vδ1 and Vδ2) and NK cells are infused together with CD34$^+$ HSC and may contribute to rapid control of infections and leukemia relapse. Notably, zoledronic acid potentiates the cytolytic activity of γδ T-cells *in vitro* and its infusion in patients strongly promotes γδ T-cell differentiation and cytolytic activity; thus, treatment with this agent may contribute to further improve the patient clinical outcome after HLA-haploidentical HSCT depleted of TCR αβ T/CD19$^+$ B lymphocytes.

Keywords: γδ T-cells, receptors, hematopoietic stem cells, HLA-haploidentical transplantation, αβ T-cell, B-cell depletion

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GENERAL FEATURES OF HUMAN γδ T-CELLS

γδ T-cells straddle the innate and adaptive arms of the immune system and are involved in response to pathogens [e.g., mycobacteria; cytomegalovirus (CMV)] and tumors. Similar to αβ T-cells, γδ T-cells develop in the thymus, but express a rearranged T-cell receptor (TCR) consisting of a TCR-γ and a TCR-δ chain (1). Although important information has been obtained by studies in mice, this review will be focalized on human γδ T-cells (2). Four human γδ T-cell populations can be identified

by the TCR Vδ expression (Vδ1, Vδ2, Vδ3, and Vδ5) (3). Vδ1, Vδ2, Vδ3, and Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9, and Vγ11 are the most frequently gene segments used in rearrangement of δ and γ chains, respectively (4). In humans, most peripheral blood γδ T-cells express Vδ2 TCR chain paired with Vγ9 chain (5), while γδ T-cells expressing Vδ1 or Vδ3 TCR chain can be paired with various Vγ chains (6) and they are predominant in epithelial tissues of skin, lungs, intestine and reproductive tract (7), liver, spleen, and thymus (8). γδ T-cells with Vγ4Vδ5 TCR are able to bind the endothelial protein C receptor (9). Moreover, four subsets of γδ T-cells were detected (Vδ4, Vδ6, Vδ7, and Vδ8) in peripheral blood of patients with B-cell non-Hodgkin lymphoma, but the γ chain pairings are still unknown. Although the majority of γδ T-cells do not express either CD4 or CD8, there is a small percentage of γδ T-cells that are CD8 positive (8). Different mechanisms of TCR rearrangement occur in mouse immune system (2).

The major pathways of γδ T-cell activation involve triggering of the γδ TCR that, at variance with αβ T-cells, does not recognize peptides presented by antigen-presenting cells (APCs) in the context of the MHC. The γδ TCR may bind soluble or membrane proteins, such as tetanus toxoid (10), bacterial proteins (11), viral proteins (2), and heat shock proteins. Moreover, the γδ TCR may bind CD1d expressed by professional antigen presenting cells (APCs), presenting glycolipids and microbial lipids (12).

In adult human, γδ T-cell population represents 1–5% of all $CD3^+$ cells. In peripheral blood of healthy human subjects, T-cells expressing Vγ9Vδ2 TCR can account for up 95% of γδ T-cells (10) and render between 1 and 10% of all blood T-cells (2). Conversely, Vδ1 T-cells represents only 10–30% of γδ T-cells in peripheral blood of healthy human (10). In the lymphoid tissue and in the gut- and skin- associated lymphoid systems, γδ T-cells show a frequency similar to that detected in peripheral blood (11). Vγ9Vδ2 T-cells are activated (13, 14) by natural metabolites known as phosphoantigens (PhAgs), such as isopentenyl pyrophosphate (IPP), produced in eukaryotes through the mevalonate pathway involved in cholesterol synthesis and protein prenylation (15). A dysregulated mevalonate pathway leading to overproduction of endogenous IPP occurs in transformed cells (16, 17). The endogenous production of IPP and related pyrophosphates and the consequent ability of a given cell type to activate γδ T-cells can be pharmacologically manipulated. A critical enzyme in the mevalonate pathway is farnesyl pyrophosphate synthase (FPPS), which acts downstream of IPP production. Targeted knockdown of FPPS leads to accumulation of IPP and subsequent activation of γδ T-cells (18). Treatment of tumor cells or monocytes with the bisphosphonate zoledronic acid (ZOL), which blocks FPPS function, leads to increased IPP production, and thereby induces selective activation of Vγ9Vδ2 T-cells (17, 19, 20). Until recently, it was unclear how the Vγ9Vδ2 TCR could recognize PhAgs. This enigma has been clarified by the discovery that butyrophilin 3A1 (also known as CD277) plays an essential role in the interaction of PhAgs with the Vγ9Vδ2 TCR, although the fine mechanisms of the phenomenon are still to be fully elucidated (4, 21).

Upon activation, γδ T-cells can produce large amounts of Th1 cytokines, such as IFNγ and TNFα, and directly induce monocyte-derived dendritic cell maturation and activation, suggesting a potential adjuvant role of this cross-talk in enhancing antigen-specific αβ T-cell response (12, 13). In this respect, it has been reported that γδ T-cells may take up and process soluble proteins inducing proliferation, cytokine production and cytotoxicity by $CD8^+$ $\alpha\beta^+$ T-cells (22).

The ability of γδ T-cells to kill hematological and solid tumors and to release Th1-type cytokines, combined with the possibility of growing these cells in culture, has attracted great interest for their use as adoptive cell therapy of cancer. Emphasis has been placed on Vγ9Vδ2 T-cells, which are easily expanded *in vitro* by PhAg stimulation (induced by exposure of cells to ZOL) and can be further boosted *in vivo* with ZOL or other synthetic PhAgs. Several clinical trials of Vγ9Vδ2 T-cell-based immunotherapy for both hematological malignancies (23–26) and solid tumors (27–32) have been conducted with promising results. A note of caution on the efficacy of these approaches comes from the plasticity of γδ T-cells controlled by the signals from the microenvironment, which can switch the antitumor profile of these cells to a tumor-promoting one, for example through induction of IL-17 production (33).

γδ T-CELLS: RECEPTORS AND LIGANDS

A feature typical of NK cells shared by γδ T-cells is the ability to kill malignant and infected cells in the absence of any prior exposure. Moreover, γδ T-cells share with NK cells the expression of different NK receptors (NKRs), such as the NK activating receptor DNAM-1, the Fc receptor CD16, and the C-type lectin-like receptor NKG2D (34). Tumor cell recognition and the associated γδ T-cells activation require the engagement of the TCR and/or NKRs, mostly NKG2D. NKG2D binds MHC class I polypeptide-related sequence MICA, MICB, and UL16 binding proteins (ULBPs) expressed on stressed and tumor cells. Overexpression of the NKG2D ligands ULBP1 and ULBP4 (35) by hematological and epithelial tumors, respectively, drives efficient cytotoxic responses by Vγ9Vδ2 T-cells. The proteins that can induce Vδ1 activation are incompletely known, although CD1c and CD1d, members of CD1 family, can activate Vδ1 T-cells through TCR binding (36). Vδ1 T-cells of the human intestinal epithelium are able to recognize MICA and MICB ligands, by the synergistic actions of TCR and NKG2D. Moreover, in Vδ1 T-cells subset, the interaction of NKp30 with B7-H6, expressed on tumor cells, allows a specific antitumor activity (9). Both TCR and NKG2D bound overlapping fragments of MICA, with different affinity and kinetics, the affinity of NKG2D being by far superior to that of TCR (37). The TCR–MICA complex was particularly stable, suggesting a sequential model, whereby the initial binding of NKG2D is followed by the formation of the more stable TCR–MICA complex. MICA engagement by TCR was found to be indispensable for γδ T-cell-mediated cytotoxicity, while NKG2D played a co-stimulatory role (38). ULBP molecules may be recognized in a similar manner, as it has been shown that ULBP4 engages both NKG2D, and Vγ9Vδ2 TCR. DNAM-1, another NKR involved in activation of Vγ9Vδ2 T-cells, binds its ligand nectin-like 5 on tumor cells rapidly triggering the cytotoxic activity of Vγ9Vδ2 T-cells (39). Controversial results have been reported regarding the expression and function of NKp44 on a minor subset (less than 10%) of γδ T-cells after culture in the presence of IL-15 (40). In addition, some γδ T-cells may express the HLA-E-specific CD94/NKG2A

inhibitory receptor. Thus, following interaction with HLA-E$^+$ cells, the functional activity of these cells may be modulated, as reported in the case of γδ T-cells interacting with enterocytes (41). The sequential recognition of different targets by γδ T-cells could play an important role in immunosurveillance, as it allows the latter cells to rapidly scan target cells for stress markers indicative of possible infection or malignant transformation. The requirement for a multicomponent stress context for full γδ T-cell activation could then provide fail-safe protection against autoimmunity. The apparent co-existence of diverse co-stimulatory axes decreases the chances of immune evasion. The main interactions between γδ T-cells and tumor cells are shown in **Figure 1**.

γδ T-CELLS AND HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

The role of γδ T-cells in HSCT has been the subject of numerous studies in the last three decades (**Figure 2**). After initial reports with contrasting results (42–44), it was demonstrated that 5-year disease-free and overall survival of leukemia patients who received HLA-mismatched allo-HSCT depleted of TCR αβ T-cells correlated significantly with high number of γδ T-cells circulating in patient peripheral blood after transplantation (45–47). It was proposed that γδ T-cells, recovering after the allograft, play a relevant role in the graft-versus-leukemia (GvL) (46), albeit other studies have highlighted the prominent GvL activity of NK cells in T-cell-depleted HSCT (48, 49). Analysis of the TCR Vδ repertoire revealed that circulating Vδ1 cells are predominant in patients with high γδ T-cells counts, whereas patients with low γδ T-cells counts and healthy individuals display mostly Vδ2 cells (46).

A study carried out at a single institution investigated the clinical impact of γδ T-cell reconstitution in 102 consecutive pediatric patients with acute leukemia given allogeneic HSCT from different donors and employing different cell sources (50). A major finding was that the probability of infections in patients with high counts of circulating γδ T-cells after the allograft was significantly lower than that in patients with low or normal counts of γδ T-cells. In particular, no bacterial infection occurred in the former patient group. Furthermore, also event-free survival of patients with high numbers of circulating γδ T-cells after HSCT was significantly better than that of patients with low or normal numbers of γδ T-cells (50).

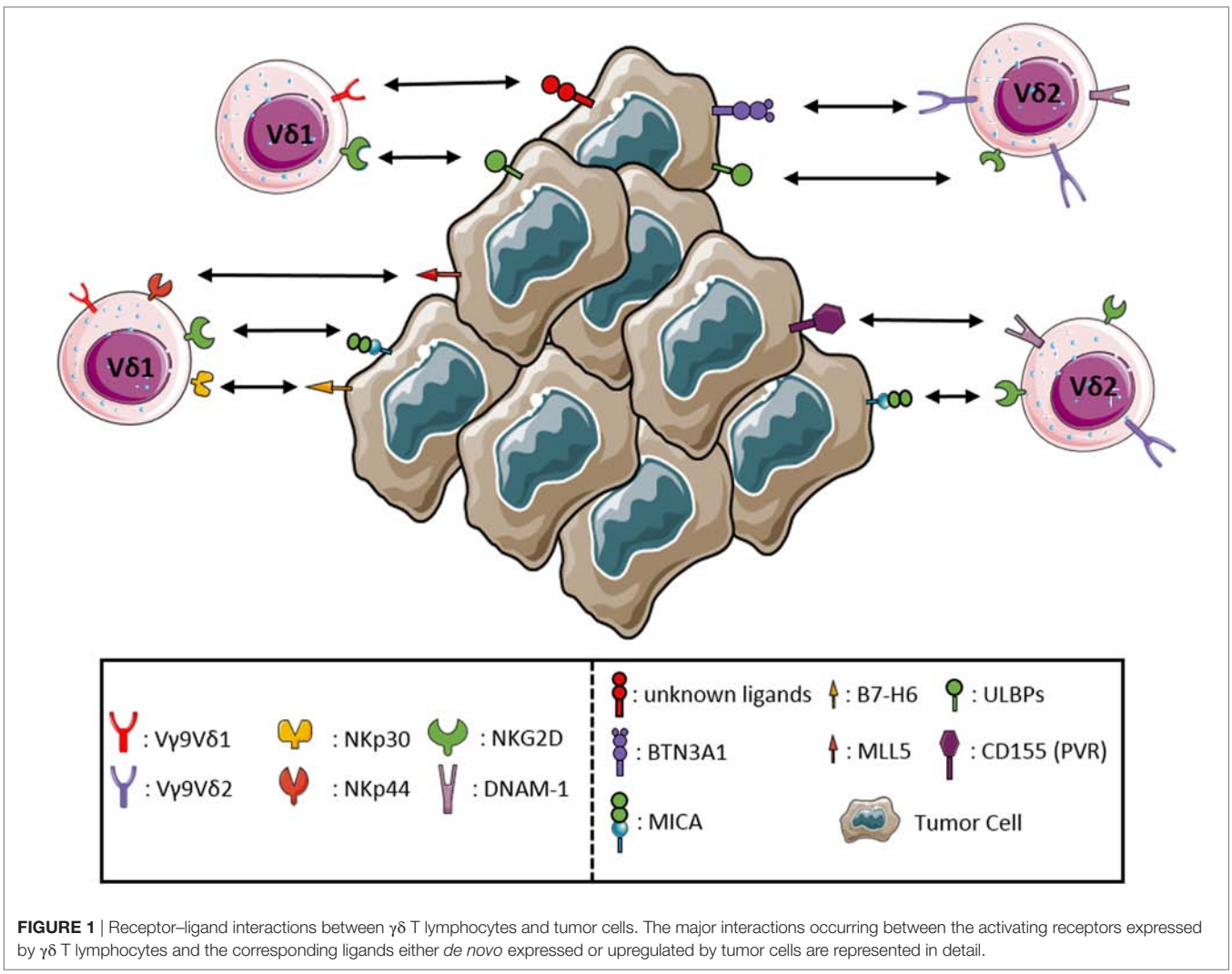

FIGURE 1 | Receptor–ligand interactions between γδ T lymphocytes and tumor cells. The major interactions occurring between the activating receptors expressed by γδ T lymphocytes and the corresponding ligands either *de novo* expressed or upregulated by tumor cells are represented in detail.

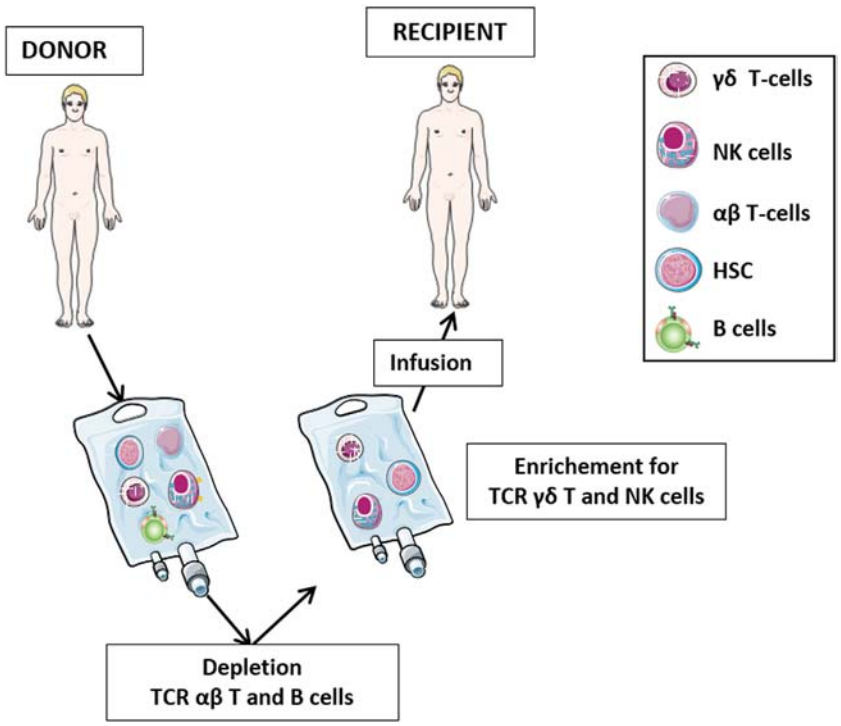

FIGURE 2 | The protocol of T-cell receptor (TCR) αβ T-cell and B-cell depletion recently developed in haplo-HSC transplantation. The protocol for haplo-HSCT allows infusion of donor HSC, together with high number of γδ T lymphocytes and NK cells. This novel strategy permits, during the early posttransplant period (6–8 weeks), a better control of leukemia cells escaping the preparative regimen, thus preventing disease relapses. In this transplantation setting, in addition to γδ T-cells and NK cells developing late from donor HSC, these promptly available effectors transferred with the graft may immediately exert their graft-versus-leukemia effect and contribute to the control of infections.

It is still a matter of debate whether γδ T-cell regeneration in HSCT recipients occurs either *via* the peripheral expansion of mature donor T-cells present in the graft or through a differentiation process from donor HSC. Using polymerase chain reaction-based complementarity-determining region (CDR)3 spectratyping and DNA sequencing for TCR δ chains, it was initially shown that the size distribution patterns of CDR3 were recovered a few months after allo-HSCT and that such recovery was faster than that of αβ T-cells (51). Clonal predominance of TCR Vδ1$^+$ cells occurred after transplantation in a few patients, and follow-up of a donor-recipient pair supported the hypothesis that peripheral expansion of mature donor T-cells contained in the graft was the main pathway of γδ T-cell regeneration after allo-HSCT (51). More recently, a study has evaluated human γδ T-cell reconstitution using an RNA and cDNA-based next generation sequencing (NGS) approach that has allowed the investigation at the clonal level of TCR γ and δ chain (TRG and TRD) repertoires in sorted γδ T-cells before and after allo-HSCT (52). In the absence of CMV reactivation, such repertoires developed after 30–60 days from allo-HSCT and remained stable over at least 6 months. TRG and TRD repertoires after transplantation were qualitatively comparable to those present before transplantation, but contained clonotypes different from those found in the donor, suggesting that they were generated *de novo* from donor HSC through a process of cell maturation. In addition, reactivation of CMV caused massive perturbations of TRG and TRD repertoires, being associated with preferential proliferation and expansion of a few individual Vδ1 and Vδ3 T-cell clones. Taken together, these studies indicate that γδ T-cells are capable of adaptive responses generating different TRG and TRD repertoires and different clonal expansions (51, 52).

γδ T-CELLS AND αβ T-CELL-DEPLETED HLA-HAPLOIDENTICAL HSCT

Hematopoietic stem cell transplantation from an HLA-haploidentical relative (haplo-HSCT) provides most patients lacking a suitable matched donor with the chance of undergoing transplantation. Clinical development of haplo-HSCT has been boosted by the demonstration that extensive T-cell depletion from the graft efficiently prevents both acute and chronic graft-versus-host disease (GvHD), even when the donor and the recipient were mismatched for an entire HLA haplotype (53, 54). The therapeutic efficacy of T-cell-depleted haplo-HSCT largely depends on donor NK cells either generated from HSC or infused with the graft mediating a potent GvL effect (55–58). Such effect is delayed in patients transplanted with positively selected donor CD34$^+$ cells, since the first wave of killer-immunoglobulin-like receptor-positive, alloreactive NK cells, differentiating from infused HSC appears after a minimum time interval of 6–8 weeks (49, 56, 59, 60). The delayed availability of mature NK cells,

mainly responsible of GvL effect, explains, at least in part, the transplant-related mortality and the early leukemic relapses, particularly in acute myeloid leukemia (AML). To circumvent this problem, a novel strategy of graft manipulation has been set up, whereby both T-cells bearing the αβ T-cell receptor (TCR) and CD19$^+$ B lymphocytes are depleted from the graft before infusion (61, 62). This approach abates TCR αβ T-cell-mediated GvHD, prevents Epstein–Barr virus-driven B cell lymphoproliferative disorders occurring in immunocompromised patients, and allows to transfer to the recipient high numbers of haploidentical CD34$^+$ cells and of mature NK cells and TCR γδ T-cells, which can readily exert protective functions against leukemia cell regrowth and life-threatening infections (63–66). Notably, TCR γδ T-cells and NK cells share a number of features that are relevant in the haplo-HSCT setting. Both cell types: (i) kill tumor cells in a MHC-independent manner (34), (ii) are involved in anti-CMV immune responses (67), (iii) do not mediate GvHD, since they do not recognize peptide antigens presented in the context of MHC (12), (iv) interact with each other and with additional immune cells, such as αβ T-cells and dendritic cells (12), and (v) following activation, are cytotoxic to mesenchymal stromal cells, a major component of tumor microenviroment (68).

We have recently investigated γδ T-cell reconstitution after haplo-HSCT depleted of TCR αβ$^+$ T/CD19$^+$ B cells (**Figure 2**) in 27 children, 15 of whom had leukemia and 12 primary immune-deficiencies or bone marrow failure syndromes (63). Immunophenotypic characterization of peripheral blood mononuclear cells performed at 1, 3, and 6 months after transplantation showed an initial predominance of γδ over αβ T-cells, followed by progressive recovery of the latter cells. γδ T-cells included three different populations, i.e., Vδ2, Vδ1 and, at a lower extent, Vδ2$^-$, Vδ1$^-$ (63). Four subsets of human γδ T-cells have been identified based upon the expression of the CD45 and CD27 surface markers: *naïve* (CD45RA$^+$, CD27$^+$), central memory (CM: CD45RA$^-$, CD27$^+$), effector memory (EM: CD45RA$^-$, CD27$^-$), and terminally differentiated (EMRA: CD45RA$^+$, CD27$^-$) (69, 70). Similar to the corresponding αβ T-cell subsets, *naïve* and CM γδ T-cells express lymph-node homing receptors and are devoid of immediate effector functions. In contrast, EM and EMRA γδ T-cells express receptors for migration to inflamed tissue where they mediate effector functions, such as cytotoxicity and cytokine production (70). Studies carried out on Vγ9Vδ2 T-cells have demonstrated that PhAg-stimulated *naïve* cells generate TCM cells, while cytokine-stimulated TCM cells differentiate into TEM or TEMRA in the absence of antigen (69). Notably, TEMRA Vγ9Vδ2 T-cells are the major subset endowed with potent antitumor and antibacterial activity (69). Analysis of the differentiation status of γδ T-cells in our patients given haplo-HSCT showed that TCM cells were predominant in both Vδ2 and Vδ1 cells. The relative proportions of the different Vδ2 and Vδ1 subsets remained stable over time and were similar to those detected in the donor. *Naïve* Vδ2 cells increased significantly between 20 days and 3 months after haplo-HSCT, suggesting that circulating γδ T-cells in transplanted patients consisted of not only mature cells derived from the graft, but also of cells differentiating from donor HSC (63). Investigation of TRG and TRD repertoires in recipients of haplo-HSCT depleted of TCRαβ$^+$ T and CD19$^+$ cells using powerful NGS techniques will shed new light on the origin of γδ T-cells in this setting.

Studies performed in solid organ transplantation and HSCT recipients have demonstrated that a remarkable expansion of Vδ2$^-$ γδ T-cells displaying a TEMRA immunophenotype and exerting cytotoxic function takes place in the course of CMV infection (67). The investigation of the γδ TCR junctional diversity revealed the expansion of Vδ1 and Vδ3 T-cells with a restricted repertoire during CMV infection (67). The mechanism whereby Vδ2- γδ T-cells recognize CMV-infected cells involves γδ TCR, still incompletely defined co-stimulatory molecules including LFA-1, and different γδ TCR ligands expressed by virus-infected cells (67). One of such ligands is the recently identified MHC-related molecule endothelial protein C receptor (71). CMV-induced Vδ2 γδ T-cells are able to recognize and kill hematological tumor cell lines and primary AML blasts (72, 73). Consistently with these notions, our patients who experienced CMV reactivation displayed a significant expansion of the Vδ1 T-cell subset with a cytotoxic TEMRA phenotype, which was absent in patients without CMV reactivation. These CMV-driven Vδ1 T-cells killed *in vitro* primary acute lymphoblastic leukemia and AML blasts more efficiently than Vδ1 T-cells from patients that did not reactivate CMV infection, suggesting that CMV infection promotes both expansion and activation of Vδ1 T-cells (63).

EFFECT OF ZOLEDRONIC ACID ON γδ T-CELLS RECOVERING AFTER αβ T-CELL-DEPLETED HLA-HAPLOIDENTICAL HSCT

We demonstrated that Vδ2 T-cells from patients who received haplo-HSCT depleted of TCRαβ$^+$ and CD19$^+$ cells expanded *in vitro* upon incubation with ZOL, which promoted the acquisition of an EM phenotype and potentiated the cytotoxic activity against primary leukemic blasts. Such activity was dependent on the levels of PhAgs expressed by leukemia cells and on TCR Vγ9 mediated recognition of the latter cells (63). Indeed, the lytic capacity of γδ T-cells was strongly enhanced by sensitizing leukemic target cells with ZOL. These *in vitro* results provided the rationale to investigate in a subsequent clinical study the effect of ZOL infusion in 43 pediatric recipients of haplo-HSCT depleted of TCRαβ$^+$ and CD19$^+$ cells (74). ZOL was infused every 28 days at least twice in most patients. Such treatment was safe and well tolerated, and, when administered three or more times, reduced GvHD occurrence and improved overall survival. The first treatment with ZOL induced the differentiation of Vδ2 T-cells, which switched from a CM to an EM/EMRA phenotype. Such maturation correlated with increased Vδ2 cell-mediated cytotoxicity against primary leukemia cells irrespective of their PhAg expression. Proteomic analyses identified an anti-proliferative effect of infused ZOL on total γδ T-cells that was consistent with the decrease of Vδ2 T-cells starting 3 months after HSCT. Such effect was already evident after the first ZOL infusion and it was further boosted by the subsequent infusions. In contrast, the percentage of Vδ1 T-cells increased during ZOL infusions irrespective of CMV reactivation (74). Altogether, these results suggest that haplo-HSCT transplanted pediatric patients may benefit from ZOL treatment.

CONCLUSION

Similar to NK cells, γδ T-cells are endowed with antileukemia and anti-infection potential and do not mediate GvHD. These features are particularly useful in the setting of haplo-HSCT depleted of TCR $\alpha\beta^+$ T and $CD19^+$ B cells, since the graft infused into the patient is highly enriched in mature γδ and NK cells ready to exert their effector functions. Both Vδ2 and Vδ1 γδ T-cells are cytotoxic toward primary acute leukemia cells, while Vδ1 and Vδ3 cells undergo adaptive clonal expansions driven by CMV reactivation that are reminiscent of antigen-specific αβ T-cells responses. Pharmacological manipulation, for example, through ZOL administration, may potentiate the anti-leukemic activity of endogenous Vδ2Vγ9 T-cells; if this effect translates into a significant benefit for the patients awaits to be definitively proved in prospective controlled clinical trials. Future studies aimed at deconvoluting the fine mechanisms whereby γδ T-cells recognize malignant and virus-infected cells will help improve the therapeutic potential of γδ T-cells in the setting of haplo-HSCT.

AUTHOR CONTRIBUTIONS

All authors discussed together the general outline of the article. VP, NT, and LM wrote the first draft that was subsequently reviewed by PV, IV, AM, and FL. Thereafter, all authors contributed to the elaboration of the final version of the manuscript.

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While this manuscript was under revision, AM passed away on February 17, 2018. His seminal discoveries of KIRs and NCR, as well as important studies on human NK cell pathophysiology, represent true milestones not only in Immunology but, more generally, in Medicine. Indeed, the unthinkable clinical outcome of patients with otherwise fatal high-risk leukemia in the haploidentical HSCT setting stem mostly from AM's discoveries. We are missing his invaluable scientific insight and, even more, his uncommon humanity, irony, and smile. We are dedicating this contribution to the memory of AM, an unforgettable friend and outstanding scientist.

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METHODS
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Zoledronate Triggers Vδ2 T Cells to Destroy and Kill Spheroids of Colon Carcinoma: Quantitative Image Analysis of Three-Dimensional Cultures

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New successful anti-cancer strategies are based on the stimulation of immune reaction against tumors: however, preclinical testing of such treatments is still a challenge. To improve the screening of anti-cancer drugs, three-dimensional (3D) culture systems, including spheroids, have been validated as preclinical models. We propose the spheroid 3D system to test anti-tumor drug-induced immune responses. We show that colorectal carcinoma (CRC) spheroids, generated with the epithelial growth factor (EGF), can be co-cultured with Vδ2 T cells to evaluate the anti-tumor activity of these effector lymphocytes. By computerized image analysis, the precise and unbiased measure of perimeters and areas of tumor spheroids is achievable, beside the calculation of their volume. CRC spheroid size is related to ATP content and cell number, as parameters for cell metabolism and proliferation; in turn, crystal violet staining can check the viability of cells inside the spheroids to detect tumor killing by Vδ2 T cells. In this 3D cultures, we tested (a) zoledronate that is known to activate Vδ2 T cells and (b) the therapeutic anti-EGF receptor humanized antibody cetuximab that can elicit the antibody-dependent cytotoxicity of tumor cells by effector lymphocytes. Zoledronate triggers Vδ2 T cells to kill and degrade CRC spheroids; we detected the T-cell receptor dependency of zoledronate effect, conceivably due to the recognition of phosphoantigens produced as a drug effect on target cell metabolism. In addition, cetuximab triggered Vδ2 T lymphocytes to exert the antibody-dependent cellular cytotoxicity of CRC spheroids. Finally, the system reveals differences in the sensitivity of CRC cell lines to the action of Vδ2 T lymphocytes and in the efficiency of anti-tumor effectors from distinct donors. A limitation of this model is the absence of cells, including fibroblasts, that compose tumor microenvironment and influence drug response. Nevertheless, the system can be improved by setting mixed spheroids, made of stromal and cancer cells. We conclude that this type of spheroid 3D culture is a feasible and reliable system to evaluate and measure anti-tumor drug-induced immune responses beside direct anti-cancer drug effect.

Keywords: CRC, spheroid, γδ T cells, epidermal growth factor receptor, antibody-dependent cellular cytotoxicity, zoledronate

INTRODUCTION

It is known that the immune system can control both survival and proliferation of tumors (1–3). Among anti-tumor lymphocytes, γδ T cells can be triggered by multiple stimuli elicited by transforming cells; for this reason they represent a category of effector cells employable in immunotherapy (4, 5). T lymphocytes of the Vδ2 subset that express the Vγ9 chain (Vγ9Vδ2) represent the majority of γδ T cells (6). At variance with αβ T cells, Vγ9Vδ2 T lymphocytes recognize non-peptide phosphorylated small molecules, namely phosphoantigens (PAg), that allow their *in vivo* and *in vitro* activation and expansion (7–10). In mammalian cells, a physiologic PAg recognized by Vγ9Vδ2 T lymphocytes is the isopentenylpyrophosphate (IPP), one of the mevalonate pathway products (8–10). The ability of IPP to trigger Vγ9Vδ2 T lymphocytes is thought to be mediated by the recognition *via* T cell receptor (TCR) (11–13). Pharmacological treatment with amino bisphosphonates (N-BPs), such as zoledronate (Zol), blocking the farnesyl pyrophosphate synthase of the mevalonate pathway, leads to IPP accumulation in tumor cells and, as a consequence, to the activation and expansion of Vγ9Vδ2 T lymphocytes (11–13). As final outcome, the expanded γδ T cell population is able to produce anti-cancer cytokines and exert anti-tumor cytotoxicity. N-BPs have been used in the treatment of many kind of bone diseases, including osteoporosis and bone tumors (14). Besides, due to their role as potent activators of γδ T lymphocytes, these compounds have been proposed for cancer immunotherapy (5, 14, 15). In addition, γδ T cells expressing the FcγRIIIA (CD16) can kill tumor cells by antibody-dependent cellular cytotoxicity (ADCC); this mechanism can be exploited using therapeutic antibodies (16).

On the basis of the promising potential of γδ T lymphocytes as anti-cancer agents *in vitro*, several clinical trials have been launched in the last 10 years for patients with hematological and non-hematological malignancies (5). Despite several encouraging results in multiple myeloma and non-Hodgkin lymphoma, γδ T cell-based immunotherapy gave disappointing results in different solid tumors, raising the question of whether tissue characteristics and/or microenvironment deeply contribute to determine the outcome of treatments (17). This indicates that preclinical models that allow the definition of drug safety and efficacy are needed. However, to test immunosurveillance in animal models is difficult; to this purpose humanized mice, EBV-infected animals with activated T cells, or the NOG, NSG engrafted with $CD34^+$ cells or peripheral blood lymphocytes and patient-derived tumor xenografts have been used (18–20). This approach, mostly used in studies on the pharmacological control of immunocheckpoints, is very expensive and requires a long time to be set up and defined for each tumor. Also, there is increasing evidence that tumor development in humans is not always reproducible and predictable in other animals, in particular when extremely artificial models are used (21–24). As an alternative, several three-dimensional (3D) culture systems, including spheroids, have been validated by the European Union Reference Laboratories for Alternatives to Animal Testing (EURL ECVAM) as preclinical models, to overcome these inconveniences (25–28).

In this context, we propose the spheroid 3D culture system to evaluate anti-tumor drug-induced immune response; in particular, we analyzed the anti-tumor effects of Vδ2 T lymphocytes triggered by Zol and/or the anti-epidermal growth factor receptor (EGFR) humanized antibody (huAb) cetuximab (Cet) on different CRC cell lines. We show that 1. Spheroids can be a reliable 3D culture system that allows co-culture of tumor cells and effector lymphocytes. 2. Zol and/or Cet triggers Vδ2 T cells to kill and degrade spheroids of colorectal carcinoma (CRC) cells. 3. The effects of drugs and effector cells can be measured by computerized imaging.

MATERIALS AND METHODS

Cell Cultures

The human CRC cell lines Caco2, HT29, HCT15, SW480, DLD1, HCT116, LS180, WiDr, LoVo, Colo205, Colo320 DMF, SW620, T84, and SW48, were from the cell bank of the Policlinico San Martino (kind gift of Blood Transfusion Centre, Dr. Barbara Parodi). CRC cell lines in adherent cultures were maintained in RPMI-1640 (Gibco, Life Technologies Italy, Monza) medium supplemented with 10% fetal serum (FBS, Gibco™ One Shot™ Fetal Bovine Serum, Thermo Fisher Scientific Italy, Monza, Italy), penicillin/streptomycin and L-glutamine (BioWhittaker® Reagents, Lonza, Basel, Switzerland) in a humidified incubator at 37°C with 5% CO_2.

Immunofluorescence Assay and Cytofluorimetric Analysis

Immunofluorescence assay was performed as described (29) with the anti-ICAM1 monoclonal antibody (mAb) (14D12D2, IgG1) (29), or anti-CD133-specific mAb (W6B3C1, IgG1, Miltenyi Biotec, Bergish Gladbach, Germany) on CRC cell lines or with anti-Vδ2 mAb (BB3, IgG1) (30) or anti-CD3 mAb (289/10/F11, IgG2a) or anti-CD16 mAb (VD4, IgG1) (29) on Zol-stimulated lymphocytes, followed by Alexafluor647 anti-IgG2a or PE-anti-IgG1 goat anti-mouse antiserum (GAM) (Life Technologies, Milan, Italy). The Fc chimeras (soluble receptors fused with the Fc of human immunoglobulins): Fc-NKG2D and Fc-DNAM1 were purchased from R&D System (Minneapolis, MN, USA) and used on CRC cell lines in immunofluorescence assay followed by Alexafluor647 goat anti-human antiserum (Life Technologies). At least 5,000 cells/sample were run on a CyAn ADP cytofluorimeter (Beckman-Coulter Italia, Milan, Italy) and results analyzed with the Summit 4.3 software.

Tumor Spheroid Generation

Optimal experimental conditions for the generation of tumor cell spheroids were selected starting from decreasing number of each tumor cell line (2×10^4–1×10^4–5×10^3 per well) in flat-bottom 96-well plates (Ultra-Low attachment multiwell plates, Corning®Costar®, NY, USA) with DMEM-F12 (BioWhittaker® Reagents, Lonza) in serum free medium (SFM), supplemented with epithelial growth factor (EGF) (Peprotech Europe, London, UK) at 10 ng/ml final concentration ($\geq 1 \times 10^6$ U/mg). EGF was selected, among three other natural ligands of EGFR [transforming growth

factor-α (TGFα) ($\geq 5 \times 10^6$ U/mg); amphiregulin (AREG), ED_{50} for proliferative effect is 5–10 ng/m; and epiregulin (EPN), $\geq 5 \times 10^5$U/mg; all from Peprotech], to obtain proliferation of CRC cells as spheroids, compared to cell growth determined in two-dimensional (2D) conventional cell culture conditions, evaluated by measurement of ATP content along time. Generation of spheroids was monitored till day 14 and proliferation and dimension (perimeter, area, and volume) were analyzed in each culture well. At least triplicates were analyzed for each culture condition and the number of spheroids analyzed is indicated in each figure (a minimum of 150 single spheroids for each independent experiment). Experiments were performed on day 6 of spheroids formation (Figures S1A,B in Supplementary Material). This day was chosen as at this time all cells in culture were alive and the diameter of spheroids was of about 250 μm.

Ex Vivo Expansion of Vδ2 T Cells

Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult donor's buffy coat (institutional informed consent signed at the time of donation and EC approval PR163REG2014) by density gradient centrifugation using Lymphocyte Separating Medium (Pancoll human, Density: 1.077 g/ml, PAN-Biotech, Munich, Germany) as described (29). To obtain Vδ2 T lymphocyte populations, 10^5 PBMC were cultured in 96 W U-bottomed plates in 200 μl of RPMI-1640 (Gibco Life Technologies) medium supplemented with 10% FBS (Gibco™), penicillin/streptomycin and L-glutamine (BioWhittaker® Reagents) and with 1.0 μM zoledronic acid as zoledronate (Zol, Selleckem, Munich, Germany) at 37°C humidified cell incubator with 5% of CO_2. After 24 h, and on day 5 and 7, 100 μl of culture medium were discarded and 100 μl of recombinant human IL-2 (30 IU/10 ng/ml final concentration, Miltenyi Biotec Italia, Bologna) were added to the cultures (Figures S1A,B in Supplementary Material). The percentage of Vδ2 T lymphocyte was determined at different time points (day 0, 7, 10, 14, 21) by indirect immunofluorescence and cytofluorimetric analysis using the anti-Vδ2 TCR-specific mAb BB3 (30) followed by isotype-specific Alexafluor647 conjugated goat anti-mouse antiserum (GAM, Life Technologies) as described (Figure S1C in Supplementary Material, left plot) (29). Lymphocyte populations were used as effector cells in co-cultures experiment with tumor cell spheroids after day 21, when the percentage of Vδ2 lymphocytes were more than 96% of total cells (Figure S1C in Supplementary Material, central graph). At that time, the majority of cells expressed the FcγRIII CD16 (Figure S1C in Supplementary Material, right histogram). In some experiments, Vδ2 lymphocytes were separated from PBMC using the positive Easy-Sep Do-It-Yourself Selection Kit (Stemcell Technologies, Vancouver, BC, Canada): the purity of separation was always more than 96% and the recovery about 75%. Vδ2 T lymphocytes were cultured in 200 μl in U-bottomed plates with 10 μg/ml of phytoemoagglutinin A (PHA, Sigma Chemical Co., St. Louis, MO, USA) and 30 IU/ml (10 ng/ml) of IL-2 (Miltenyi Biotech).

CRC Spheroids and Vδ2 T Cell Co-Cultures

On day 6, spheroids with a maximal diameter of 250 μm were composed of living cells, as assessed by culturing a sample under adherent conventional conditions for 12 h and subsequent identification of living cells with propidium iodide (PI, Sigma) staining, ATP content, and crystal violet assay. In preliminary experiments, the number of CRC cells present in a culture well containing spheroids was determined measuring the ATP content referred to the ATP content of the same cell line at a known cell concentration in conventional culture system. The number of cells present in a given tumor spheroid was determined calculating the ratio between spheroid volume and the volume of a single cell, measuring the diameter of a cell and assuming that this cell displayed a spherical shape. Co-cultures were set up by adding decreasing amount of Vδ2 T cells (1.5×10^5, 0.75×10^5, and 0.35×10^5) to spheroids of CRC cells on day 6 and further culture in RPMI SFM for different periods of time (4, 12, 24, and 48 h). The optimal amount of Vδ2 T cells to detect the cytotoxic effect, determined in preliminary experiments by crystal violet assay or by image analysis, was 0.75×10^5 cells/well and it corresponded approximately to a 1:1 effector to target (E:T) ratio. The time point selected was 24 h based on CRC spheroid damage evaluated by microscopy and image analysis. In some samples, the anti-Vδ2 mAb BB3 (5 μg/ml) was added. In other experiments, the anti-EGFR therapeutic antibody cetuximab (Cet, obtained as Erbitux left over from the Antiblastic Unit of Policlinico San Martino) was added at the concentration of 2 μg/ml to evaluate the ADCC exerted by Vδ2 T cells. CRC cell lines used in these experiments were selected on the basis of expression of EGFR (reactivity with Cet) and the ability of forming spheroids.

PI Staining, ATP Content, and Crystal Violet Assay

To determine cell membrane permeability, cells were stained with a PI solution (50 μg/ml) and incubated 15 min at RT. After extensive washing, at least 5,000 cells/sample were run on a CyAn ADP cytofluorimeter (Beckman-Coulter) and results analyzed with the Summit 4.3 software. ATP content was determined using the CellTiter-Glo® Luminescent Cell Viability Kit (Promega Italia Srl, Milan, Italy) following manufacturer's instruction using the luciferase reaction consisting in mono-oxygenation of luciferin catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxigen. Luminescence was detected with the VICTORX5 multilabel plate reader (Perkin Elmer, Milan, Italy) expressed as relative light units (RLU); in some instances the, RLU were converted in μM of ATP according to a standard curve. The crystal violet assay was performed with the Crystal Violet Cell Cytotoxicity Assay Kit (Biovision, Milpitas, CA, USA). Briefly, CRC spheroids alone or co-cultured with Vδ2 T cells in the presence or not of Zol were transferred in conventional adherent plates and after 48 h were stained with crystal violet following manufacturer's instruction. After extensive washing, adherent cells were solubilized and the amount of crystal violet proportional to the amount of living cells was measured with the VICTORX5 multilabel plate reader (Perkin Elmer) at the optical density (OD) of 595 nm.

Images and Measurement of Spheroid Size

Cell cultures were analyzed with Olympus IX70 bright field inverted microscope equipped with a CCD camera (ORCA-ER,

C4742-80-12AG, Hamamatsu, Japan), associated with the CellSens software (version 1.12, Olympus, Tokyo, Japan). Images were taken with 10× objective NA 0.30 or 20× objective NA 0.40 (100× or 200× magnification). After calibration of the plate, a grid corresponding to each well was created and analyzed; at least nine focal points were taken for each well of interest to obtain optimal images to be measured. After this operation through an automated *x–y* axis motorized table SCAN IM 120 × 100-2 mm (Marzhauser Wetzlar GmBH, 35579 Wetzlar, Germany) images of the entire well were taken. The dimension of the camera chip was 2/3-inch format, thus images were partly overlapped (from 15 to 35%) by the software to obtain appropriate stitched images (usually 50 images for each well were taken and 3–6 replicates performed for each experimental condition). The final result was an image at 16-bit gray scale with a size of 1,102 × 9,626 pixel (7.1 mm × 6.2 mm) in .csi format that can be analyzed with the CellSens software. The measurement of spheroids was performed manually by applying the "Count and Measure" tool of the CellSens software. This tool allows to get circular region of interest (ROI) that can represent the perimeter of the spheroid. Through calibration of the microscope, the number of pixel of the image were converted into μm, the diameter of each spheroid was calculated and the data were inserted in an Excel sheet with a defined color corresponding to a ROI and to a spheroid. Data were then transferred to GraphPad Prism computer program (version 5.03) for subsequent statistical analysis and generation of graphs (see Results). To avoid counting and measuring very small spheroids or single cells or small cell/debris aggregates, only spheroids with a diameter greater than 50 μm were selected. The perimeter of spheroids was identified by the different image contrast with the bottom of the well. Spheroids that did not show an approximately spherical shape, but appeared as the fusion/overlap of several spheroids, were considered as composed of different single spheroids depicting partly overlapping ROI.

Statistical Analysis

Data are presented as mean ± SEM or ±SD. Statistical analysis was performed using two-tailed unpaired Student's *t* test. The cutoff value of significance is indicated in each figure legend.

RESULTS

Generation and Measurement of CRC Spheroids

First, we determined which CRC cell lines can grow and give rise to spheroids under controlled culture conditions. To this aim, CRC cells were cultured in SFM, without or with EGF (10 ng/ml), in ultra low adherent plates and analyzed microscopically from day 1 to 7 of culture. We found that not all the CRC cell lines tested give rise to spheroids under these experimental conditions. Indeed, HCT15, SW480, HT29, Caco2, DLD1, HCT116, WiDr, SW620, LoVo, Colo205, Colo741, and T84 (**Figures 1a–n**) could form spheroids, while SW48, Colo320DMF, and LS180 cell lines simply led to cell aggregates where single cells were still distinguishable on day 7 of culture (**Figures 1o–q**). HCT15, SW480, DLD1, HCT116, SW620, and T84 spheroids displayed a smooth surface (**Figures 1a,b,e,f,h,n**), while other cell lines, including HT29, Caco2, LoVo, Colo205, and Colo741, spheroids showed a rough surface (**Figures 1c,d,i,l,m**) or smooth and rough surface such as WiDr (**Figure 1g**). It is of note that spheroids obtained with Caco2 cells appeared with a central portion darker than the periphery, suggesting that the culture conditions used can generate crypts-like structures (**Figure 1d**). The ability to form spheroids was not related to the expression of CD133 marker (Figure S2A in Supplementary Material); indeed, some cell lines able to form spheroids were completely negative for this marker (HCT15, SW480, DLD1, Colo205, Colo741, and T84) while SW48 cell line was $CD133^+$ but did not give rise to spheroids.

The expression on tumor cells of surface molecules involved in the interaction with effector lymphocytes was comparable to that displayed by the same cell line in conventional cultures in adherent plates. As an example, the expression of ICAM1, the ligand for LFA1, was similar in SW480 cells from 2D cultures or disaggregated spheroids (Figure S2B in Supplementary Material for SW480, not shown HCT15, HT29, and WiDr). Likewise the chimeric Fc-NKG2D and Fc-DNAM1 activating receptors could equally bind CRC cells derived from spheroids or conventional 2D cultures (Figure S2B in Supplementary Material for SW480; not shown HCT15, HT29, and WiDr).

In order to determine the size of spheroids present in a culture well, we applied a manual method using a microscope equipped with a motorized *x–y* table and a CCD camera (see Materials and Methods). As shown in **Figure 2**, to determine spheroid size, the whole area of a given culture well was reconstructed by CellSens software using single images then partly overlapped (**Figure 2A**, final 100× magnification of the image). The *z*-focus was predetermined manually, focusing random from 3 to 5 individual points for each well, to avoid interferences due to the irregular surface of culture wells. Each well image was subjected to computerized analysis of single spheroids and their measurement. The ROI were manually depicted with the CellSens software tool that uses three points to get a circle, assuming that the 3D spheroids displayed a circular perimeter when observed in 2D (**Figure 2B**). The perimeter and area of each spheroid could be determined by a measure tool of the software; **Figures 2C,D** show these measures for spheroids and single cells.

The evaluation of these parameters in one representative experiment using the CRC cell line HCT15 is shown in **Figure 3**. Addition of EGF to cell cultures triggered a strong increment in the size of spheroids and this effect was already detectable and statistically significant on day 3. The growth of spheroids was much more evident at day 7 and at this time point spheroids were of heterogeneous size, ranging from a perimeter of 150 μm to more than 500 μm (**Figure 3A**). Likewise, area and volume (**Figures 3B,C**) were significantly increased by day 3 and more evident on day 7. It is of note that it was possible to calculate mathematically the number of cells in a given tumor spheroid, assuming that the spheroid volume is the sum of volumes of the single cells (data not shown). This allowed to calculate that the number of cells for each spheroids was less than 500 to more than 3,000 (data not shown). To define whether this analysis was objective and reproducible, avoiding biases due to the operator, results of independent measurement have been compared. As shown in

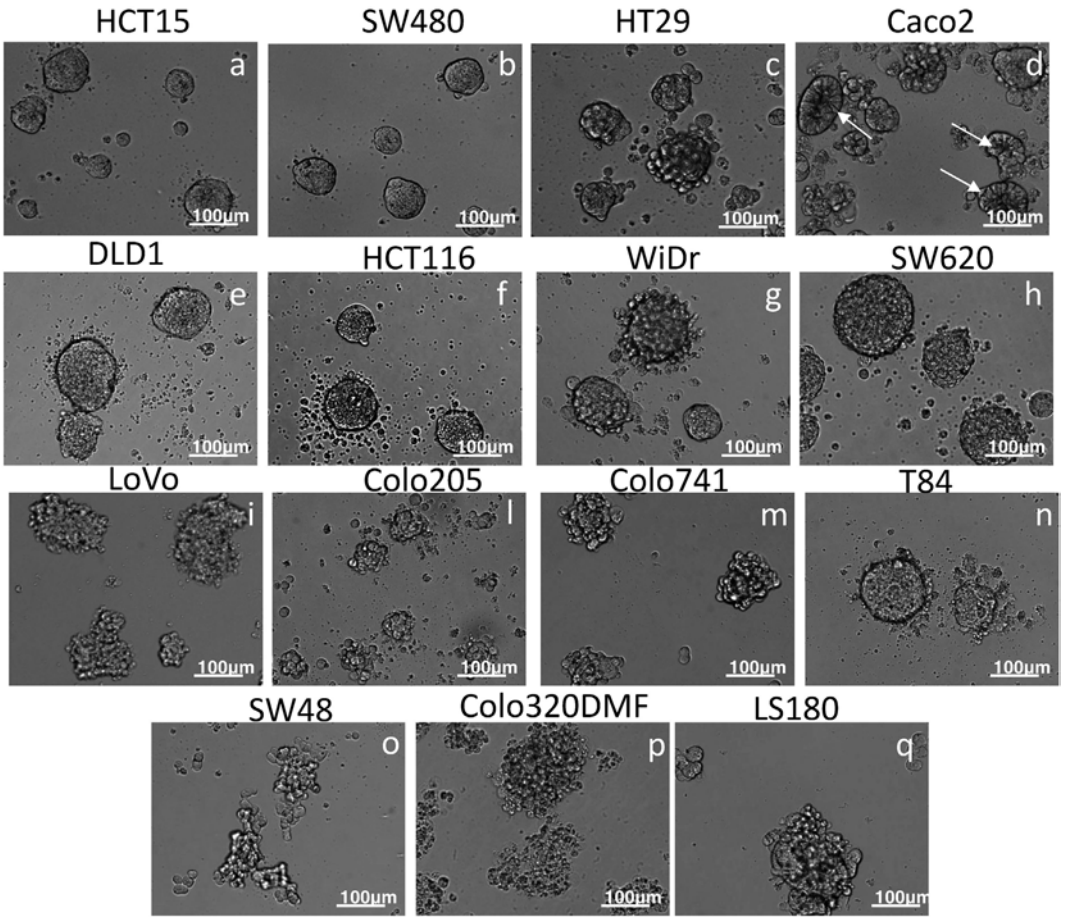


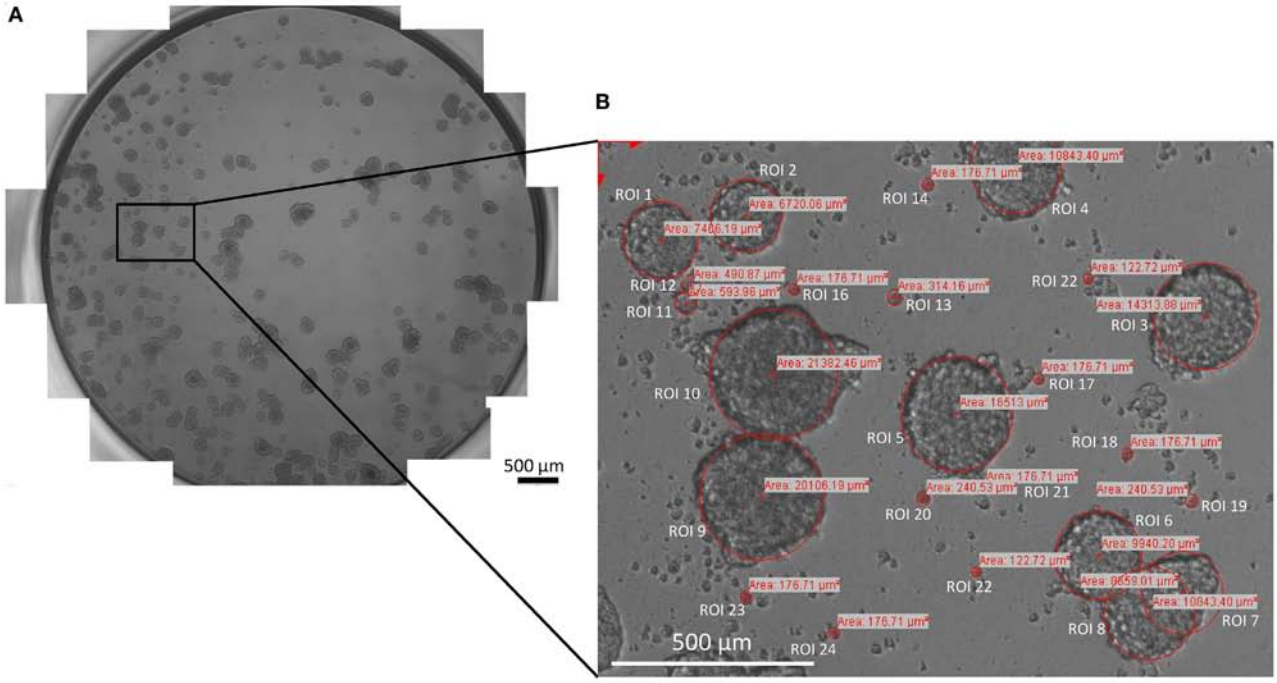
FIGURE 1 | Generation of CRC spheroids. The indicated CRC cell lines were cultured in serum free medium supplemented with 10 ng/ml of epithelial growth factor in very low adherent 96-well flat-bottomed microplates and analyzed on day 6 by inverted IX70 microscope (Olympus); images were taken with 20× objective NA 0.40 (200× magnification). Bar in each panel: 100 µm. Some spheroids were characterized by a spherical-like appearance with smooth surface **(a,b,e,f,h,n)**, others displayed rough surface **(c,d,g,i–m)**. In some instances (HT29, Colo205, Colo741, and T84), on the outer surface or next to the spheroid single cells are present. SW48, Colo320DMF, and LS180 **(o–q)** formed unshaped cell aggregates. In the case of Caco2, cell line the central portion of the spheroid appeared darker (arrows) than the peripheral region, suggesting a crypt-like morphology.

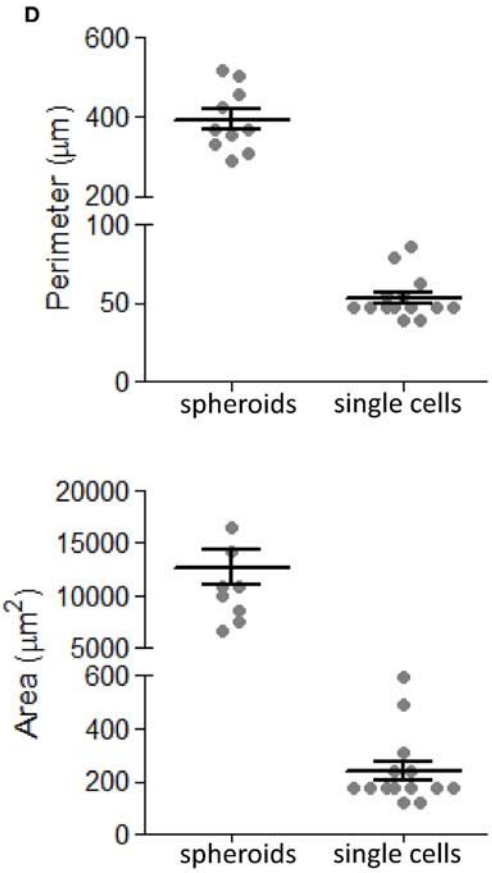
Figure S3 in Supplementary Material, no significant differences were found in the evaluation of SW620 or HCT15 spheroid perimeters (Figure S3A in Supplementary Material) and areas (Figure S3B in Supplementary Material) by three operators.

In another series of experiments, we assessed whether the evaluation of spheroids size can allow to identify differences among the efficiency in spheroid generation of EGFR ligands other than EGF, including AREG, EPN, and TGFα. We observed that all these ligands could lead to spheroids of WiDr (**Figure 4A**), HT29 (**Figure 4B**), SW620, and Caco2 (data not shown) cell lines. Indeed, a significant increase of spheroid volume was detectable with all the EGFR ligands used compared to spheroids size in SFM not supplemented with these factors (**Figures 4A,B**). The fold of size increment was related to the amount of the EGFR ligand used. For instance, with TGFα the volume of spheroids of WiDr cell line was sixfold, fivefold, and threefold larger at 100, 10, and 1 ng/ml than that of spheroids in medium without TGFα (**Figure 4A**). Furthermore, a stronger effect on the increase of spheroids volume was found with EGF and TGFα, compared to AREG and EPN (**Figures 4A,B**, $p < 0.001$). Importantly, an increase in ATP cell content corresponded to the increase of spheroids size (**Figures 4C,D**) supporting the hypothesis that spheroid volume is related to an increment of cell metabolism and proliferation. Indeed, experiments with HCT15, HT29, Caco2, and SW480 CRC cell lines showed a direct relationship between ATP content and cell number (Figure S4A in Supplementary Material).

Analysis of the Effect of Zol and Vδ2 T Lymphocytes on CRC Spheroids

Tumor spheroids are currently used to assess the effect of cytotoxic drugs as models for *in vitro* therapeutic screening (25–28). Indeed, 3D tumor cell cultures show several *in vivo* features of tumors, including drug response and resistance (25–28). To our knowledge, the analysis of effector lymphocyte activity on tumor cell spheroids has not been performed yet. Some reports have analyzed the effect of anti-cancer lymphocytes on the killing of





C

	ROI Name	Area µm²	Perimeter µm
spheroids	ROI 1	7466,19	306,31
	ROI 2	6720,06	290,60
	ROI 3	14313,88	424,12
	ROI 4	10843,40	369,14
	ROI 5	16513,00	455,53
	ROI 6	9940,20	353,43
	ROI 7	8659,01	329,87
	ROI 8	10843,40	369,14
	ROI 9	20106,19	502,65
	ROI 10	21382,46	518,36
single cells	ROI 11	593,96	86,39
	ROI 12	490,87	78,54
	ROI 13	314,16	62,83
	ROI 14	176,71	47,12
	ROI 15	122,72	39,27
	ROI 16	176,71	47,12
	ROI 17	176,71	47,12
	ROI 18	176,71	47,12
	ROI 19	240,53	54,98
	ROI 20	240,53	54,98
	ROI 21	176,71	47,12
	ROI 22	122,72	39,27
	ROI 23	176,71	47,12
	ROI 24	176,71	47,12

FIGURE 2 | Continued

FIGURE 2 | Procedure of CRC spheroid image analysis and measurement. CRC spheroids (this example is referred to SW620 cell line) were analyzed under an inverted IX70 microscope (Olympus) with 10× objective NA 0.30 (100× magnification), equipped with an automated *x–y* axis motorized table SCAN IM (Marzhauser Wetzlar) and image taken under control of the CellSens computer program. **(A)** The entire well was reconstructed taking sequential and partially overlapping images, stitched one to each other to give rise to the image of the well. **(B)** Region of interest (ROI) that identify tumor cell spheroids and areas (μm^2) calculated by CellSens software after calibration of the image. **(C)** Data inserted by the CellSens software in an Excel sheet were analyzed with Graph Pad PRISM (version 5.03) software **(D)**. **(D)** Perimeters (upper graph) and areas (lower graph) of spheroids and single cells. Mean ± SEM of the ROI shown in panel **(B)** and measured in panel **(C)**.

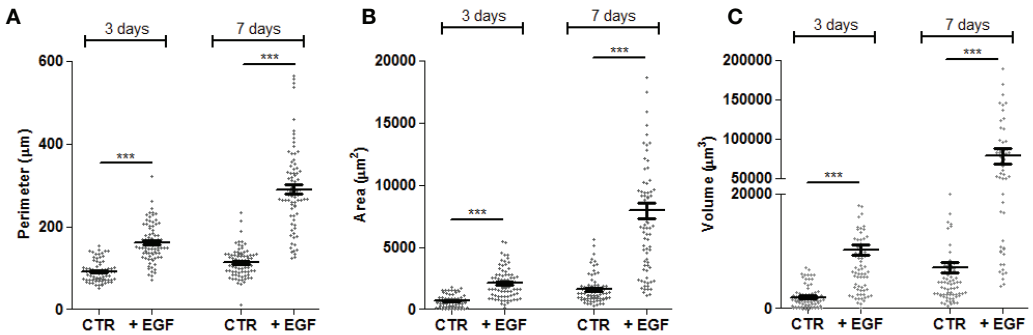


FIGURE 3 | Measurements of perimeter, area, and volume of CRC spheroids. The perimeter **(A)**, area **(B)**, and volume **(C)** of each spheroid was calculated as explained in **Figure 2** and data were plotted with Graph Pad PRISM software. This example is referred to the HCT15 cell line cultured as indicated in **Figure 1**. Spheroid dimensions were evaluated after 3 and 7 days of culture without epithelial growth factor (EGF) (CTR) or with 10 ng/ml EGF. Each symbol indicates a region of interest which in turn corresponds to a single spheroid. Bar: mean ± SEM of that group of measures. ***$p < 0.001$.

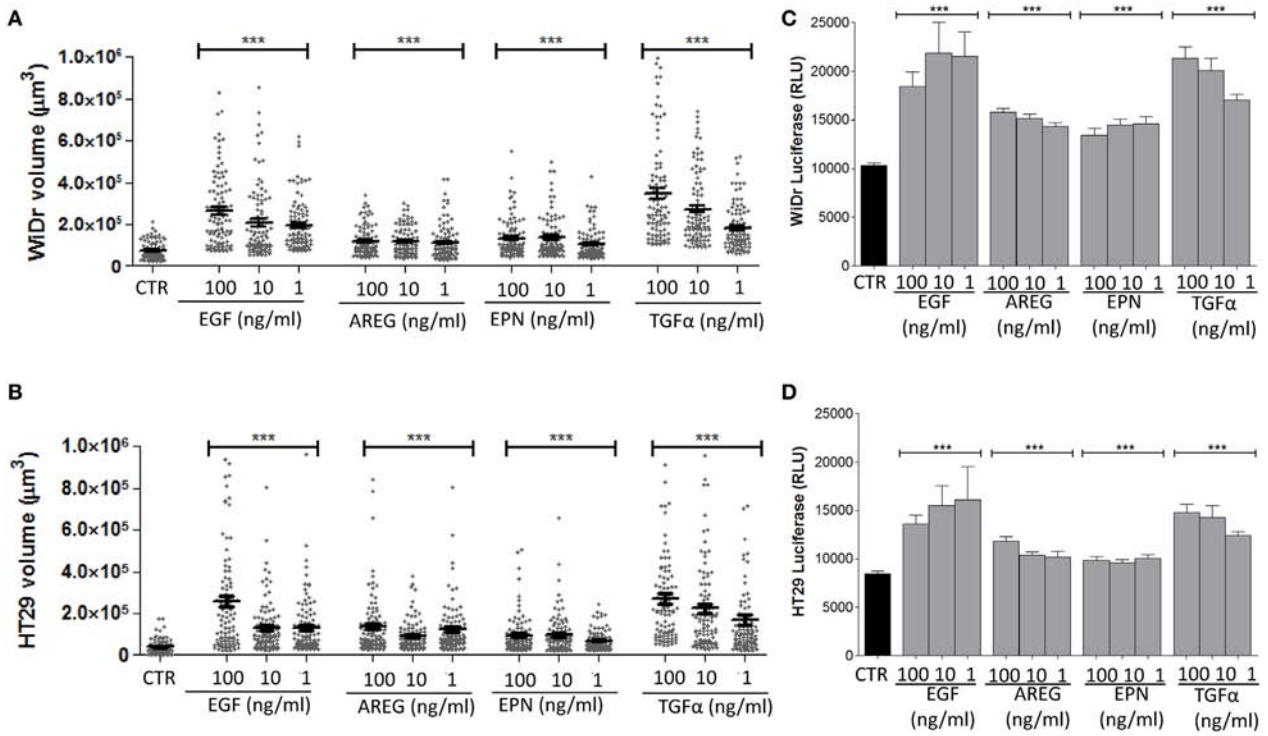


FIGURE 4 | Effect on spheroid size of different epidermal growth factor receptor ligands. **(A,B)** CRC cell lines [WiDr **(A)**; HT29 **(B)**] were cultured in serum free medium (SFM) without (CTR) or with 100-10-1 ng/ml of epithelial growth factor (EGF), amphiregulin (AREG), epiregulin (EPN), or transforming growth factor-α (TGFα), in very low adherent 96-well flat-bottomed microplates. Cell cultures were analyzed on day 6 by inverted IX70 microscope (Olympus) and images were taken with 10× objective NA 0.30 (100× magnification). Each symbol in the different plots indicates a spheroid. Data are shown as volume (μm^3). Bar: mean ± SEM of that group of measures. ***$p < 0.0001$. **(C,D)** ATP content of the same cultures, expressed as luminescence of luciferase activity. Black columns indicate the ATP content of cells cultured in SFM and gray columns the ATP content of cell cultured in the presence of the indicated factors. Data are the mean ± SD of 6-well replicates for each culture condition. ***$p < 0.001$.

CRC cells isolated from tumor spheroids (31). This means that spheroids were first disaggregated and a single cell suspension was then used in conventional cytolytic experiments. In our hands, a single tumor cell suspension could be obtained by gently harvesting and transferring spheroids in phosphate buffered saline or culture medium. However, we observed that in some CRC cell lines, the percentage of tumor cells stained with PI was higher among cells derived from disaggregated spheroids than from adherent cultures (Figure S4B in Supplementary Material, central vs left subpanels). This strongly suggests that cell suspensions obtained from spheroids may have an altered membrane permeability. Conversely, intact CRC spheroids transferred into adherent plates showed a percentage of PI^+ cells comparable to that of CRC cells cultured under conventional conditions (Figure S4B in Supplementary Material, right vs left panels). Thus, to evaluate the effect of Zol or Vδ2 T cells, preformed spheroids of CRC cell lines were used. In any case, in each experiment we assessed the vitality of CRC spheroids, besides evaluating their size. To this aim, in parallel with image analysis, measurement of ATP content and staining with crystal violet were performed. Both these tests provide information about the vitality of cells in a spheroid: indeed, ATP content is directly related to cell metabolism and proliferation, while crystal violet works as an intercalating compound in DNA, thus staining the nuclei and allowing the quantitative determination of living cells that are able to adhere to the culture plate.

The spheroids size of the three representative CRC cell lines HCT15, SW620, and DLD1 was analyzed upon incubation for 24 h with either Zol (5 μM), or Vδ2 T lymphocytes or with Zol and Vδ2 T cells. Data from independent experiments, performed with different bulk Vδ2 T cell populations from six healthy donors, have been pooled together providing a very large number of spheroids evaluated for each cell line analyzed. **Figure 5A** shows that the combination of Vδ2 T cells and Zol led to a strong decrease of spheroid size in all CRC cell lines. This effect was striking with HCT15 cell line, where the size of tumor spheroids was reduced to one/third (**Figure 5A**, left graph). Zol alone can slightly reduce the size of HCT15, SW620 and DLD1 cell lines, while the reductive effect due to Vδ2 T cells, in the absence of Zol, was evident only using HCT15 as target cells (**Figure 5A**, left graph). When the Vδ2 T cell populations obtained from the six different donors were analyzed independently (Figure S5 in Supplementary Material), we found that Zol could trigger anti-tumor Vδ2 T lymphocytes in four donors (donors 1–4), while in one donor Vδ2 T cells were efficient also in the absence of Zol (donor 5), and in one case (donor 6) were not effective even in the presence of Zol.

The parallel evaluation of crystal violet assay (**Figure 5B**) and measurement of ATP content (**Figure 5C**) complete the analysis of the effect of Zol and/or Vδ2 T cells on tumor cell spheroids. Crystal violet assay was performed after gently harvesting spheroids and subsequent culture in conventional adherent plates for 48 h; this allowed living tumor cells to adhere to plastic before staining. Non-adherent cells (dying tumor cells and Vδ2 T cells present in culture) were discarded upon extensive washing, and plates were read on a spectrophotometer. As shown in **Figure 5B**, a strong reduction of OD in cultures of HCT15, SW620, and DLD1 spheroids with Vδ2 T cells was detected, indicating that most CRC cells have been killed; this effect was significantly enhanced by Zol.

ATP content was measured at 24 h (at the same time point of the evaluation of spheroids size): the ATP detected in the cultures with Zol (5 μM) and Vδ2 T cells (**Figure 5C**, +Vδ2+Z5) was lower than that of either CRC spheroids (CTR) or co-cultures of Vδ2 T cells and CRC spheroids (+Vδ2). Zol alone determined a slight decrease of ATP when added to cell spheroids of any CRC cell line tested. These data suggest a reduced metabolism and/or proliferation in cultures with low ATP content, conceivably due to Vδ2 T anti-CRC activity. However, the addition of Vδ2 T cells to HCT15 spheroids led to an increment of ATP content (**Figure 5C**, left graph), making difficult to discriminate the ATP of effector lymphocytes and of tumor cells.

CRC Spheroids to Evaluate TCR Involvement in Vδ2 T Cell Killing and ADCC Triggered With Cetuximab

It is known that the recognition of IPP produced as a consequence of Zol effect on target cell metabolism is TCR dependent (4, 6–8). Thus, we assessed whether the anti-Vδ2 TCR-specific mAb BB3 can impair this recognition. As shown in **Figure 6**, the area (**Figure 6A**) and volume (**Figure 6B**) of HCT15 spheroids was markedly reduced when Zol and Vδ2 T cell populations were added to cell cultures. The addition of the anti-Vδ2-specific mAb significantly inhibited the reduction of spheroid size found in the presence of Vδ2 T cells and Zol (**Figures 6A,B**). This indicates that the 3D spheroid culture system allows the evaluation of the Zol-triggered Vδ2 T cell anti-tumor effect and of TCR involvement.

Vδ2 T cells can also be elicited to develop anti-tumor activity through ADCC; this can be obtained using therapeutic antibodies, such as the anti-EGFR huAb Cet (32, 33). Thus, we assessed whether the activation of ADCC triggered by Cet can reduce the size of tumor spheroids of HCT15 CRC. Vδ2 T cells were incubated with HCT15 spheroids and Cet added at the onset of the period of incubation; the size of spheroids was analyzed after 24 h. We found that when Cet and Vδ2 T cell populations were co-incubated with tumor spheroids, a strong reduction of spheroid size (**Figure 7A**) and cell number/spheroid (**Figure 7B**) was detected in experiments performed with Vδ2 T lymphocytes, enriched in $CD16^+$ cells (Figure S1 in Supplementary Material, right histogram), from three different donors. Cet slightly reduced the spheroid size when used alone. These data suggest that Cet can trigger Vδ2 T lymphocytes to exert anti-CRC ADCC also in this 3D culture system.

DISCUSSION

In this paper, we analyzed the anti-tumor effect of Vδ2 T cells on different CRC cell lines organized in spheroid 3D structures. We show that 1. Spheroids can be a feasible and reliable system that allows the study of tumor cell interaction with effector lymphocytes in 3D cultures. 2. Zoledronate and cetuximab trigger Vδ2 T cells to kill and degrade spheroids of CRC cells. 3. The effect of

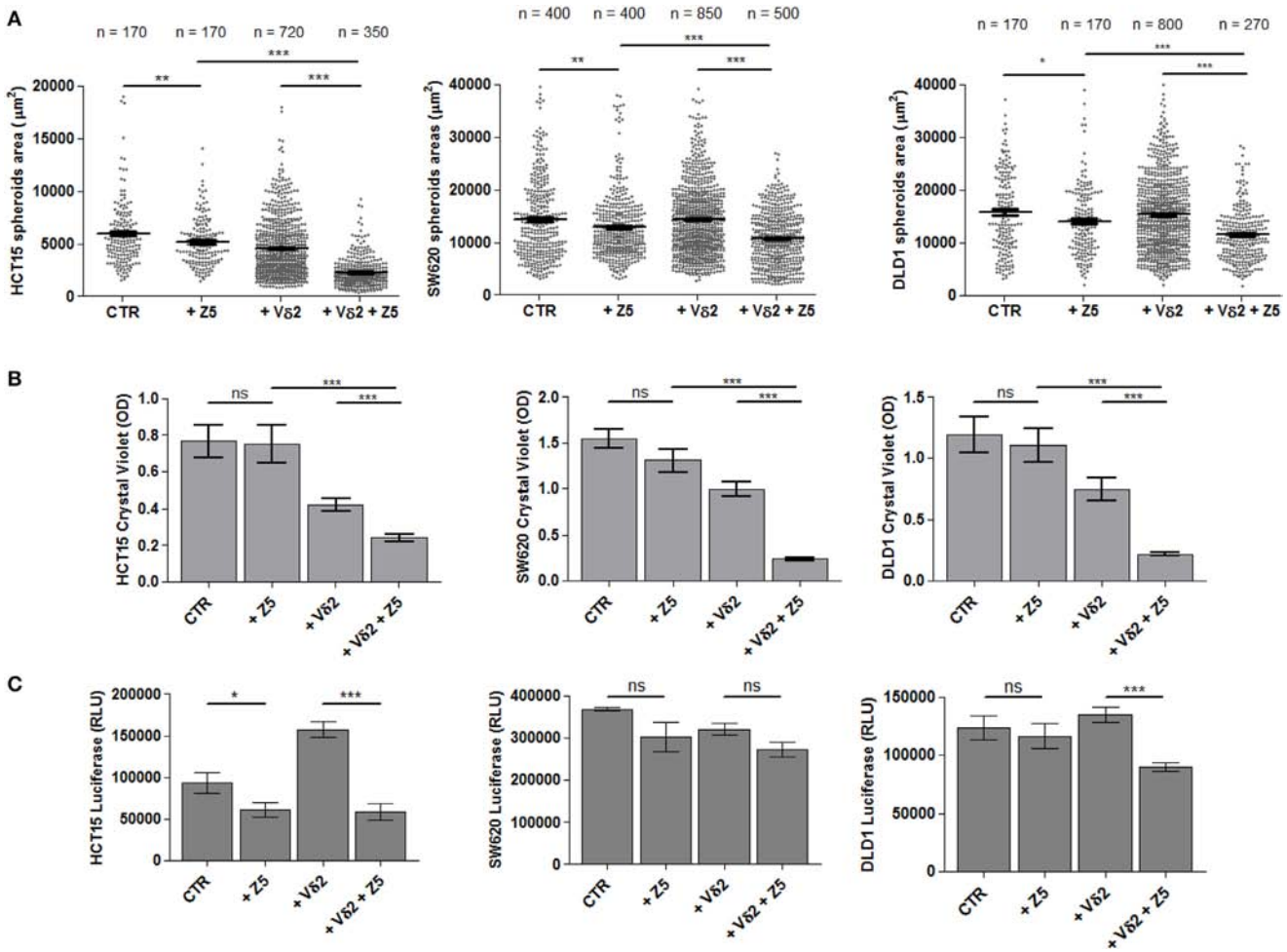


FIGURE 5 | Effect of Zol and Vδ2 T cell populations on CRC spheroid size and viability. **(A)** Spheroids from different CRC cell lines (HCT15, left; SW620, middle; and DLD1, right) were generated in serum free medium supplemented with epithelial growth factor (10 ng/ml). On day 6, culture wells were incubated for additional 24 h without (CTR) or with 5 µM Zol (+Z5) or Vδ2 T cells (+Vδ2) or Vδ2 T cells + 5 µM Zol (+Vδ2+Z5). Then, each culture well was analyzed for the measurement of spheroids. The number of spheroids analyzed is indicated in the upper part of each plot; data are from experiments performed with six different Vδ2 T cell populations in triplicate wells for each donor. Each symbol in the plot indicates a single spheroid. Bars: mean ± SEM of that group of measures. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. **(B)** Cells from the different cultures defined in panel **(A)**, were gently harvested and transferred to conventional 96-well flat-bottomed plates to allow the adhesion of CRC epithelial cells. After 48 h, cell cultures were labeled with crystal violet followed by extensive washes (to remove non-adherent cells) and lysis of adherent cells. Optical density (OD) at 595 nm was evaluated with a VICTORX5 analyzer. Bars: mean ± SD of three experiments. $^{***}p < 0.0001$; ns, not significant. **(C)** Cell cultures obtained as indicated in panel **(A)** were analyzed for ATP content with luciferase assay. Data are shown as luminescence in relative light units (RLU) arbitrary units determined with a VICTORX5 analyzer. Bars: mean ± SD of three experiments.

the drug and of effector Vδ2 T cells can be evaluated and measured by computerized imaging.

The success rate of many new anti-cancer drugs and immunotherapies in clinical trials is surprisingly low, despite the promising effects obtained in preclinical studies (34). This might be due to the fact that 2D cultures poorly reflect the real tumor microenvironment, while in animals metabolism is not necessarily comparable to humans. This is particularly true in the case of CRC, where an appropriate animal model is lacking. The spatial organization, with different mechano-structural and physico-chemical features, has been proposed as the main factor responsible for the failure of conventional 2D culture systems (35, 36). Moreover, there is increasing evidence that human tumor development is not always reproducible and predictable in other animals, in particular when extremely artificial models are used, including humanized mice, needed to test anti-cancer immune response (18–20).

Among the 3D culture systems validated by EURL ECVAM as preclinical models, we used the simplest one, that is tumor cell spheroids (21–24, 37). Although tissue architecture is not entirely reproduced, the system is reminiscent of the small tumor cell clusters that may appear in the first stages of cancer development. Our present data indicate that we can count and measure the size of a large number of spheroids in replicate culture wells and different experimental conditions. Many CRC cell lines can be used in this assay (HCT15, SW480, HT29, Caco2, DLD1, HCT116, WiDr,

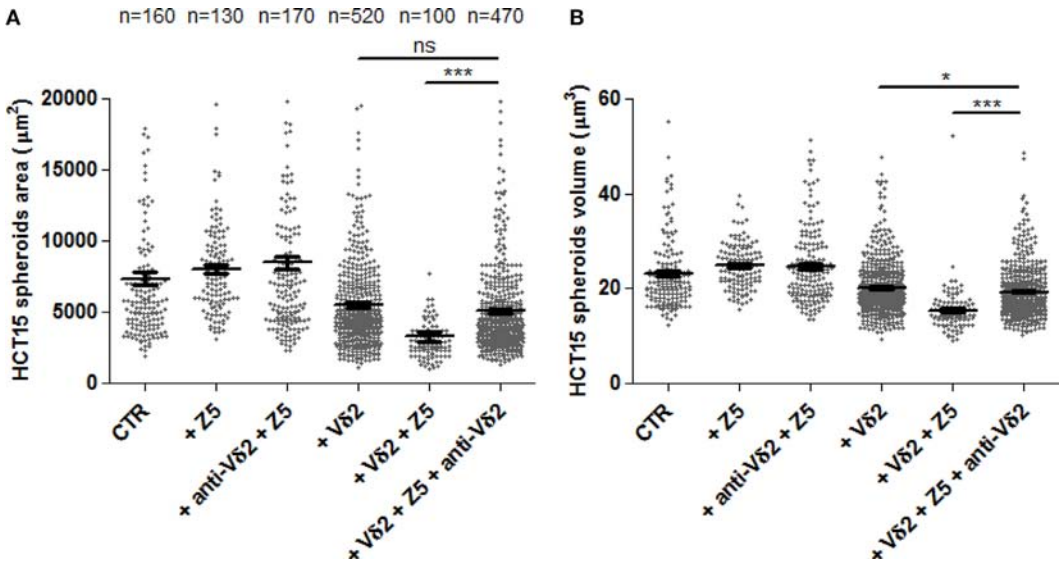


FIGURE 6 | The effects of Zol and Vδ2 T cells on CRC spheroids are dependent on Vδ2TCR. HCT15 spheroids were generated in serum free medium supplemented with epithelial growth factor (10 ng/ml) and incubated on day 6 for additional 24 h in medium without (CTR) or with 5 µM Zol (+Z5) or Vδ2 T cells (+Vδ2) or Vδ2 T cell populations + 5µM Zol (+Vδ2+Z5). In some experiments, saturating amounts of the anti-Vδ2-specific monoclonal antibody BB3 (5 µg/ml) were added at the onset of the 24 h incubation (+anti-Vδ2+Z5, +Vδ2+Z5+anti-Vδ2). Then, each culture well was analyzed for the identification and measurement of spheroids as area **(A)** or volume **(B)**. The number of spheroids analyzed is indicated in the upper part of panel **(A)**; data are from experiments performed using six different Vδ2 T cell populations in triplicate wells for each donor. Each symbol in the plot indicates a single tumor cell spheroid. Bar: mean ± SEM of each group of measures. $*p < 0.01$; $***p < 0.0001$; ns, not significant.

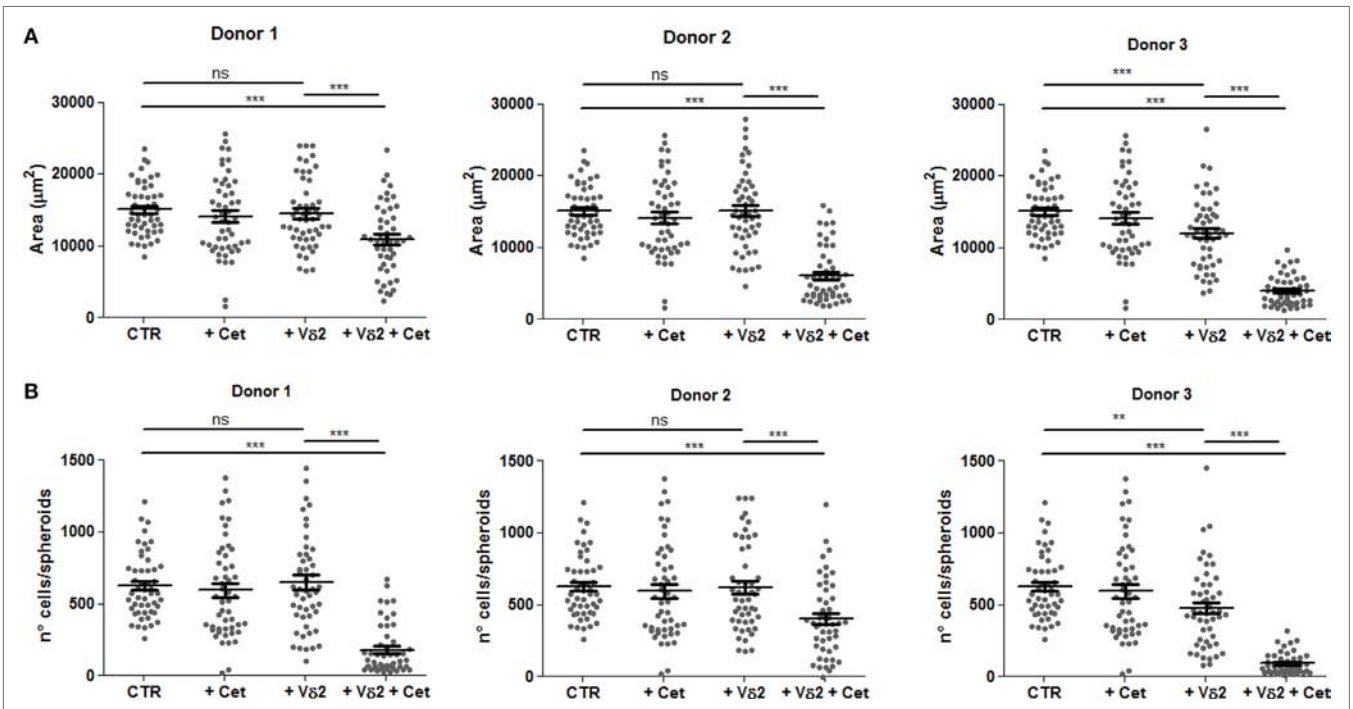


FIGURE 7 | Vδ2 T cells can be triggered by the anti-epidermal growth factor receptor (EGFR) therapeutic antibody cetuximab to reduce CRC spheroids. HCT15 spheroids were generated in serum free medium supplemented with epithelial growth factor (10 ng/ml). On day 6, cultures were incubated for additional 24 h in medium without (CTR) or with the anti-EGFR antibody cetuximab (2 µg/ml, +Cet) or Vδ2 T cells (+Vδ2) or Vδ2 T cells and cetuximab (2 µg/ml, +Vδ2+Cet). Then, each culture well was analyzed for the identification and measurement of spheroids. Data are representative of experiments obtained with three different Vδ2 T cell populations in triplicate wells for each donor. Each symbol in the plot indicates a single tumor cell spheroid. Data are expressed as area of spheroids [**(A)**; μm^2] or cell number in each spheroid **(B)**. Bar: mean ± SEM of that group of measures. $**p < 0.01$; $***p < 0.001$; ns, not significant.

SW620, LoVo, Colo205, Colo741, and T84), with the exception of some (SW48, Colo320DMF, and LS180) that may raise some difficulties in size measurement due to incomplete spheroid formation. Caco2 cells are even able to generate crypts-like structures that are more representative of CRC tissue. Acquisition of images, reconstruction of the whole culture well, computerized analysis of single spheroids and their measurement are easily performed with the CellSens computer system and manual definition of the ROI was reproducible without significant differences among operators; nevertheless, we are working on the construction of the macro of the ImageJ open source image processing program suitable for automated ROI definition, to achieve more precise, reliable, and quick sample analysis. Automated image analyses would also allow the evaluation of multiple *z*-planes and 3D reconstruction of spheroids. Nevertheless, in our system spheroid volume is calculated by the software starting from the maximal radius of the ROIs selected during the calibration procedure, thus giving a bona-fide 3D measure. Perimeter and area definition are already automatically determined by a measure tool of the CellSens software. Also, it is possible to calculate mathematically the number of cells in a given tumor spheroid, providing further information on tumor growth. Image analysis permits to evaluate the effects exerted by different EGFR ligands on CRC spheroid size, showing that EGF and TGFα are very efficient factors in increasing spheroid volume. Moreover, CRC spheroid size is related to ATP cell content and a direct relationship between ATP content and cell number can be defined, as parameter for cell metabolism and proliferation. Under these experimental conditions, several drugs, including Zol, can be tested for their anti-tumor efficiency: moreover, the system allows to distinguish among the response elicited in different donors as well.

Of note, we could analyze Vδ2 effector T lymphocyte activity on CRC cell spheroids. Some effects of anti-cancer lymphocytes on the killing of CRC cells isolated from tumor spheroids has been previously shown (31). However, in that report spheroids were first disaggregated and single cell suspensions of colon stem cells used in conventional cytolytic experiments. In our hands, under these experimental conditions, in some CRC cell lines a high percentage of cells show altered membrane permeability and this would advise against their use as target cells. Indeed, the large majority of assays designed to evaluate cytotoxicity are based on the different ability of cells with altered membrane permeability and cells with intact membrane to release an intracellular probe. Conversely, intact CRC spheroids displayed a membrane permeability superimposable to that of the original adherent cell line and are suitable for cytolytic assays. Moreover, crystal violet staining was used to check the viability of CRC cells of the whole spheroid and ATP measurement to evaluate cell metabolism and proliferation.

In this setting, a strong and reproducible reduction of spheroid size in all CRC cell lines was observed using Vδ2 T cells and Zol. The pharmacologic effect of Zol alone in reducing spheroid size was detected on HCT15, SW620, and DLD1 cell lines, while the reductive effect due to Vδ2 T cells, in the absence of Zol, was evident only using HCT15 as target cells. In any case, this 3D spheroid culture system allows the evaluation of drug and/or Vδ2 T cell anti-tumor effects; also, the system reveals any difference in the sensitivity of CRC cell types to drug or lymphocyte effects, and in the efficiency of anti-tumor effectors from distinct donors. Data from crystal violet staining were in line with image analysis of spheroid size; at variance, ATP measurement was reliable and reproducible only in CRC spheroids without Vδ2 T lymphocytes, conceivably due to the fact that in co-cultures the ATP of both cell types is measured, impairing the distinction between the fate of effectors and targets. Thus, we would advise crystal violet assay to check and support image analysis of tumor spheroid size to detect tumor killing exerted by Vδ2 T cells, alone or in combination of drugs, including Zol, at variance with ATP content that is useful only to test drug effects.

Using a specific mAb in inhibition experiments, we also show that Vδ2 TCR is involved in the spheroid reduction and killing elicited by Zol. Another important application of this 3D culture system is the evaluation of anti-CRC ADCC exerted by Vδ2 T lymphocytes triggered by the therapeutic anti-EGFR huAb cetuximab (Cet). Indeed, when Cet and Vδ2 T cell populations were co-incubated with CRC spheroids a strong reduction of their size and cell number/spheroid was detected in experiments performed with Vδ2 T lymphocytes, enriched in $CD16^+$ cells. Cet slightly reduced the spheroid size when used alone. This suggests that also therapeutic mAbs other than Cet can be evaluated for their efficacy in producing a direct anti-tumor cytotoxic effect or ADCC in this 3D culture system. A limitation of this model is represented by the absence of stromal or myeloid or other types of neighboring cells that compose the tumor microenvironment; however, the system potentially allows the setting of mixed spheroids, e.g., using a core of stromal cells and an envelope of cancer cells. Mixed spheroids with different degree of complexity, made of CRC cell lines and cancer-associated fibroblasts (CAF), with or without PBMC, have been reported (38). In this mixed spheroid system, CAF conferred to co-cultured CRC cells reduced chemosensitivity, supporting that tumor microenvironment deeply influences the outcome of drug treatments. Also, it is known that stromal cells can down-regulate the function of immune effector cells, although we have reported that zoledronate can prevent this inhibition, allowing the occurrence of effector lymphocyte cell function (1, 29).

In conclusion, mixed lymphocyte-CRC spheroids represent a reliable, reproducible, and cheap 3D culture system that allows the evaluation and measurement of anti-cancer drugs that exploit the immune response. Image computerized analysis guarantees the precise and unbiased analysis of data, although some efforts are still needed to reach a good automated ROI definition and image assessment of the whole spheroid.

ETHICS STATEMENT

Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult donor's buffy coat upon institutional informed consent signed at the time of donation and EC approval PR163-REG2014 of the Regional Ethic Committee.

AUTHOR CONTRIBUTIONS

SV carried out cell isolation, generation, and culture of spheroids, and functional assays; SV, MZ, and AP performed immunofluorescence

and image analyses, designed the work, and wrote the paper. AP takes primary responsibility for the paper content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00998/full#supplementary-material.

FIGURE S1 | Tumor cell spheroids formation and three-dimensional (3D) co-culture system with Vδ2 T cells. **(A)** Schematic representation and time schedule of the 3D culture system. CRC cell lines were cultured in serum free medium supplemented with 10 ng/ml of epithelial growth factor in very low-adherent 96-well flat-bottomed microplates (10^4 cells/well). Cell cultures were checked every day (a) by inverted IX70 microscope. On day 6 (b) either Zol (5 μM) or Vδ2 T cells or Zol and Vδ2 T cells were added and the spheroids analyzed after additional 24 h of incubation (c). Then, image analysis, ATP content measurement, and crystal violet assay were performed. **(B)** Bright field microscopic images of the cultures described in panel **(A)**. (a) CRC spheroid. (b) Vδ2 T cells added to CRC spheroids. (c) Zol and Vδ2 T cells added to CRC spheroids. **(C)** Phenotype of the Vδ2 T cell populations obtained culturing peripheral blood mononuclear cell with Zol and then used in the assay described in panel **(A)**. Left dot plot: double staining and FACS analysis of Vδ2 T cells with anti-CD3 and anti-Vδ2 mAbs at 21 days of culture; in each quadrant, percentage of cells. Central graph: percentages of Vδ2 T cells at the indicated time points; each dot represents a single donor. Bars: mean ± SD. Right graph: expression of CD16 antigen on Vδ2 T cells (day 21) assessed by immunofluorescence and FACS analysis. Dark grey: negative control with unrekated APC-Ig. Log red fluorescence intensity (a.u.) vs cell number. The percentage of positive cells is indicated. One representative donor out of six.

FIGURE S2 | Phenotype of CRC cell lines and CRC spheroids. **(A)** Immunofluorescence performed on the indicated CRC cell lines with the anti-CD133-specific monoclonal antibody (mAb) followed by Alexafluor647 GAM isotype specific antiserum. Dark gray histogram in each panel: fluorescence of cells stained with the second reagent alone. Light gray histogram: fluorescence of cells stained with anti-CD133 mAb. **(B)** Immunofluorescence performed on SW480 cell line cultured under conventional conditions (upper row) or as spheroids (lower row), with the anti-ICAM1 mAb, followed by Alexafluor647 GAM isotype specific antiserum, or the Fc-NKG2D or the Fc-DNAM1 chimeras, followed by Alexafluor647 anti-human specific antiserum. Data are expressed as log far red MFI in arbitrary units (a.u.).

FIGURE S3 | Measurement of perimeter and area of CRC spheroids by different operators. **(A,B)** The perimeter **(A)** and area **(B)** of SW620 (left measures in each panel) or HCT15 (right measures in each panel) spheroids were analyzed independently by three operators (OP1, OP2, and OP3), calculated as in **Figure 2** and data plotted with Graph Pad PRISM software. Each symbol indicates a region of interest (ROI) which in turn corresponds to a single spheroid. Bar in each plot shows the mean ± SD of that group of measures.

FIGURE S4 | ATP content and propidium iodide (PI) staining in CRC spheroids. **(A)** ATP content, expressed as μM calculated referring to luminescence of a standard curve, in the CRC cell lines HCT15, HT29, Caco2, and SW480, seeded at the indicated number of cells/well. Data are the mean of 6-well replicates for each culture condition. **(B)** PI staining of HCT15 (upper row), SW620 (central row), and SW480 (lower row) CRC cell lines. Left histograms: adherent cells in conventional cultures; middle histograms: disaggregated spheroids; and right histograms: spheroids recovered and cultured in adherent plates. The percentages of PI positive cells are indicated in each histogram.

FIGURE S5 | Effect of Vδ2 T cell populations from different donors on spheroid size. HCT15 spheroids were obtained after culture for 6 days in serum free medium supplemented with epithelial growth factor (10 ng/ml). Cultures were incubated for additional 24 h in medium without (CTR) or with 5 μM Zol (+Z5) or Vδ2 T cells (+Vδ2) or Vδ2 T cells + 5μM Zol (+Vδ2+Z5). Then, each culture well was analyzed for the identification and measurement of spheroids. Data are from experiments performed using six different Vδ2 T cell populations (donors 1, 2, 3, 4, 5, and 6) in triplicate wells for each donor and are expressed in μm^2. Each symbol in the plot indicates a single tumor cell spheroid. Bars: mean ± SEM. *$p < 0.01$; **$p < 0.001$, ***$p < 0.0001$; ns, not significant.

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Corrigendum: Zoledronate Triggers Vδ2 T Cells to Destroy and Kill Spheroids of Colon Carcinoma: Quantitative Image Analysis of Three-Dimensional Cultures

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Origin and Evolution of Dendritic Epidermal T Cells

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Dendritic epidermal T cells (DETCs) expressing invariant Vγ5Vδ1 T-cell receptors (TCRs) play a crucial role in maintaining skin homeostasis in mice. When activated, they secrete cytokines, which recruit various immune cells to sites of infection and promote wound healing. Recently, a member of the butyrophilin family, *Skint1*, expressed specifically in the skin and thymus was identified as a gene required for DETC development in mice. *Skint1* is a gene that arose by rodent-specific gene duplication. Consequently, a gene orthologs to mouse *Skint1* exists only in rodents, indicating that *Skint1*-dependent DETCs are unique to rodents. However, dendritic-shaped epidermal γδ T cells with limited antigen receptor diversity appear to occur in other mammals. Even lampreys, a member of the most primitive class of vertebrates that even lacks TCRs, have γδ T-like lymphocytes that resemble DETCs. This indicates that species as divergent as mice and lampreys share the needs to have innate-like T cells at their body surface, and that the origin of DETC-like cells is as ancient as that of lymphocytes.

Keywords: dendritic epidermal T cell, γδ T cell, *Skint1*, skin, epidermis, intraepithelial lymphocyte

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INTRODUCTION

In the mouse epidermis, ~95% of cells are keratinocytes, and the remaining 5% are immune cells such as Langerhans cells and T cells. The majority of epidermis-resident T cells are γδ T cells with a highly dendritic shape, extending dendrites both basally and apically; along with Langerhans cells, these γδ T cells form an interdigitating network within the layers of the epidermis (1). Therefore, they are called dendritic epidermal T cells (DETCs) (2). Interestingly, ~90% of DETCs express an invariant Vγ5Vδ1 T-cell receptor (TCR) without any junctional diversity [also called Vγ3Vδ1 TCR according to the nomenclature of Garman et al. (3)]. It is thought that DETCs recognize a limited set of "stress antigens" induced on damaged or dysregulated keratinocytes through their invariant TCRs in a major histocompatibility complex (MHC)-independent manner (4). Although the molecular identity of "stress antigens" recognized by Vγ5Vδ1 TCR remains unknown, co-stimulatory molecules on DETCs, which synergistically amplify TCR signals, have been identified. The most important among them are the junctional adhesion molecule-like protein JAML (5), CD100 (also known as semaphorin 4D) (6), and NKG2D receptors (7, 8), which interact with the coxsackie and adenovirus receptor, plexin B2, and a group of stress-inducible MHC class I-like molecules known as NKG2D ligands (9, 10), respectively.

Once activated, DETCs retract their dendrites, adopt a rounded shape, and secrete a range of cytokines, chemokines, and tissue-specific growth factors, leading to increased keratinocyte proliferation and recruitment of infiltrating leukocytes, thereby promoting wound healing and immune surveillance in the skin. Among the cytokines secreted by DETCs is insulin-like growth factor 1, which aids wound healing by preventing apoptosis of keratinocytes and DETCs themselves in an

autocrine manner (11). Although controversial, one recent study has shown that a subset of DETCs secretes interleukin-17A that induces production of antimicrobial peptides such as β-defensin 3 and regenerating islet-derived protein 3γ (12). The latter peptide also induces keratinocyte proliferation and differentiation after skin injury (13), indicating that interleukin-17 plays a role in both infection control and epithelial proliferation at wound sites.

In the thymus of fetal mice, γδ T cells with particular γ and δ chains appear sequentially in discrete waves. DETC progenitors bearing Vγ5Vδ1 TCRs appear at embryonal days 14–16 (14, 15), after which they home to the epidermis. On the other hand, terminal deoxynucleotidyl transferase (TdT), which generates junctional diversity in V(D)J recombination by attaching additional nucleotides (so-called N nucleotides) at the 3′-end of gene segments in a template-independent way (15), is not expressed in fetal thymus and starts to be expressed 4 days after birth (14). Forced expression of TdT in fetal thymus produces DETCs expressing Vγ5Vδ1 TCRs with junctional diversity, which populate the epidermis of newborn mice (16). These DETCs, however, gradually disappear after birth, unlike normal DETC. Therefore, it appears that TCR specificity is not required for epidermal migration of DETC progenitors, but important for renewing and sustaining DETCs in the epidermis. Likewise, epidermis-resident T cells in TCRδ-deficient mice, which mainly express variable αβ TCRs, are gradually lost and not retained over a lifetime (17), again indicating the importance of TCR specificity for the maintenance of epidermal T cells.

Dendritic epidermal T cells have been identified in rodents such as mice (18) and rats (19, 20). However, little is known about the origin and evolution of DETCs. Recently, a gene essential for DETC development, named *Skint1* (selection and upkeep of intraepithelial T cells protein 1), was identified in mice (21, 22). Interestingly, the *Skint1*-like (*SKINT1L*) gene is inactivated in humans, raising the possibility that this inactivation might be responsible for the deficiency of DETCs in humans. In this review, we summarize the evolution of the *SKINT* gene family and its implications for the origin and evolution of DETCs. Available evidence indicates that *Skint1*-dependent DETCs are unique to rodents. However, if we define DETCs more broadly as dendritic-shaped epidermal γδ T cells with limited antigen receptor diversity, they seem to occur in other mammals. Indeed, even lampreys, a member of the most primitive class of vertebrates equipped with lymphocytes, have DETC-like cells, suggesting that DETCs also exist in jawed vertebrates other than mammals.

SKINT1 AND THE *SKINT* GENE FAMILY

The epidermis of FVB/N mice from Taconic Farms (FVB/N Tac) lacks Vγ5Vδ1 DETCs, while γδ T-cell repertoires in other tissues are normal (23). *Skint1* was identified as a gene responsible for this depletion of canonical DETCs (21). SKINT1 is a membrane-bound immunoglobulin (Ig) superfamily protein made up of an Ig variable (IgV) domain, an Ig constant domain and three transmembrane regions. It is specifically expressed by thymic epithelial cells and skin keratinocytes. The *Skint1* gene of FVB/N Tac mice contains a premature termination codon in the region coding for the segment between the second and third transmembrane regions. In *Skint1*-deficient mice, Vγ5Vδ1 T cells are present in fetal thymus in comparable numbers to wild-type FVB/N mice at embryonic days 14–16.5, but the production of mature Vγ5Vδ1 T cells migrating to the epidermis is impaired because of defective thymic selection of Vγ5Vδ1 T cells (23). The complementarity determining region 3-like loop in the IgV domain of SKINT1 molecules appears important for this selection (24). However, it is not known whether SKINT1 or the SKINT1 molecular complex interacts with Vγ5Vδ1 TCR itself or an as yet uncharacterized molecule expressed uniquely on DETC progenitors (22).

Skint1 is a member of the *Skint* gene family. Mice have 11 *Skint* genes (paralogs) designated *Skint1* to *Skint11*, coding for structurally related proteins with similar, though not identical, expression patterns (21). These paralogs appear to have distinct functions. Thus, neither *Skint2* nor *Skint7* can compensate for the loss of *Skint1* function in reaggregate fetal thymic organ culture (22). Furthermore, mice selectively deficient in epidermal *Skint1* expression show only a minor delay in wound healing compared to mice deficient in *Skint3* or *Skint9*, suggesting that *Skint1* is mainly involved in the maturation of DETC progenitors in the thymus, and that *Skint3* and *Skint9* play a more important role in mediating DETC activation in the epidermis (25). These observations suggest that *Skint* paralogs have undergone functional specialization, with only *Skint1*serving as a selecting component for Vγ5Vδ1 T cells.

EVOLUTION OF THE *SKINT* GENE FAMILY

The *SKINT* gene family, which occurs only in placental mammals, is a member of a larger gene family known as the butyrophilin family (26–28). It comprises three subfamilies, *SKINT1*, *SKINT7*, and *SKINT9* (29). **Figure 1** shows the phylogenetic tree of *SKINT1* subfamily genes in placental mammals. Whereas mice and rats have multiple copies of these subfamily genes, most mammals have either a single copy of *SKINT1* genes known as *SKINT1L* or altogether lack this subfamily. The branching pattern of the phylogenetic tree indicates that mouse *Skint1* to *Skint6* genes emerged by rodent-specific gene duplication from an *SKINT1L* gene. Therefore, non-rodents do not have an *SKINT* gene orthologs to mouse *Skint1*. Actually, a gene ortholog to mouse *Skint1* exists only in some rodents, specifically family Muridae or murids such as mice, rats, hamsters, and Mongolian gerbils. Coupled with the finding that *Skint* paralogs in mice have undergone functional specialization and have distinct functions (22, 25), these observations indicate that authentic *Skint1* genes are unique to rodents, more precisely murids.

A notable feature of *SKINT1L* genes is that they are absent in a number of species such as elephants, sloths, armadillos, alpacas, horses, cats, dogs, and ferrets. They are also inactivated in several mammalian species. Thus, all the hominoids including humans, great apes (chimpanzees, gorillas, and orangutans), and lesser apes (gibbons) have *SKINT1L* genes inactivated by multiple mutations (29). One of the mutations, the stop codon located at the ninth residue of the IgV domain, is shared by all the hominoid sequences. Because Old World monkeys such as olive baboons, green monkeys, cynomolgus macaques, and rhesus macaques do not have this mutation, and their *SKINT1L* genes are apparently functional, this stop codon was most likely responsible for the initial inactivation of the hominoid *SKINT1L* gene (29). Tarsiers, pigs,

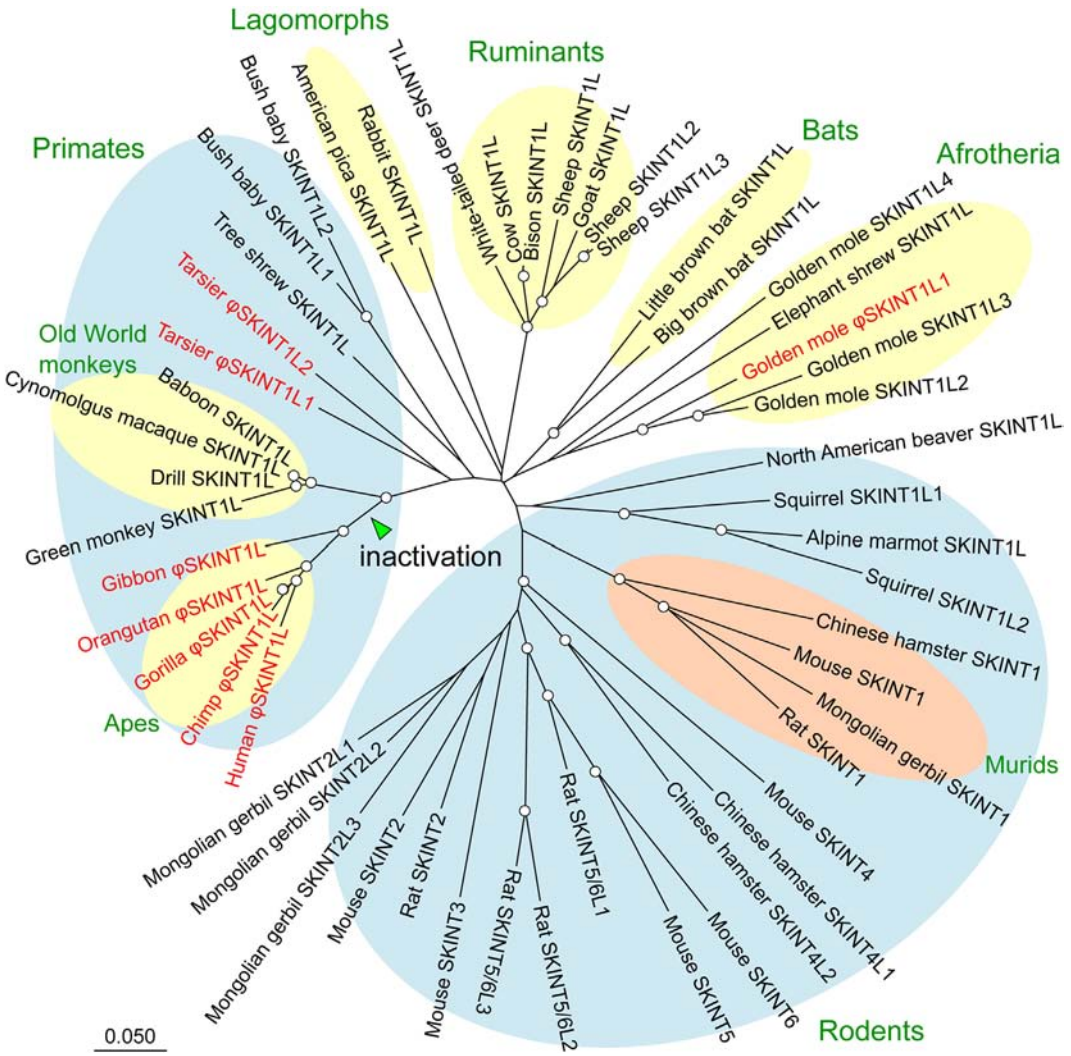

FIGURE 1 | Phylogenetic tree of the SKINT1 subfamily. SKINT1 subfamily sequences were retrieved from the NCBI database (WGS and nr databases) using the mouse Skint1 sequence as a query. Deduced amino acid sequences of SKINT1 subfamily proteins were aligned, and the phylogenetic tree was constructed using the MUSCLE and neighbor-joining programs implemented in MEGA7, respectively. The distance matrix was obtained by calculating p-distances for all pairs of sequences. Gaps were excluded using the pairwise-deletion option. The reliability of branching patterns was assessed by bootstrap analysis (1,000 replications). Pseudogenes are indicated by φ and shown in red. Nodes supported by bootstrap values over 80% are indicated by open circles. *Skint7* to *Skint11* genes of mice are not shown as they are the members of the *SKINT7* or *SKINT9* subfamily.

and whales also have inactivated *SKINT1L* genes. Therefore, *SKINT1L* appears to have been lost or inactivated independently in multiple mammalian lineages.

DETCs IN OTHER MAMMALS

The observation that the *Skint1* gene essential for DETC development exists only in rodents (**Figure 1**) indicates that *Skint1*-dependent DETCs are unique to rodents. Indeed, rats are the only species in which the presence of cells quite similar to mouse DETCs has been unambiguously demonstrated (20). In the rat epidermis, αβ T cells occupy only 0.03–0.24% of $CD3^+$ cells, indicating that the vast majority of T cells are γδ T cells (30). Immunostaining with a specific antibody against γδ TCR revealed that the rat epidermis abundantly contains γδ T cells with dendritic morphology (19). Vγ and Vδ chains expressed on these γδ T cells are very similar to mouse Vγ5 and Vδ1, with 92 and 95% amino acids sequence identity, respectively, and lack junctional diversity.

In cattle, a representative member of γδ-high species, more than 80% of skin T cells, of which at least 44% are γδ T cells, occur in the superficial 0.5 mm of the dermis, with only 3% in the epidermis (31). Thus, distribution of skin T cells differs from that in mice. Skin-resident bovine T cells are irregular in shape and frequently have a flattened outline with wavy cytoplasmic projections. Furthermore, although the information on TCR γ- and δ-chain usage in epidermal γδ T cells is not available, skin-resident γδ T cells as a whole predominantly use Vγ3 and Vγ7 while the Vδ usage is diverse (32). Therefore, it is possible that

cattle have DETCs broadly defined as dendritic-shaped epidermal γδ T cells with limited antigen receptor diversity.

In humans, Vδ1 T cells, which express different Vγ elements, are the major γδ T cell subset preferentially homing to epithelial tissues such as skin and intestine (33, 34). In the skin, Vδ1 T cells reside mainly in the dermis but are also found in the epidermis. Like mouse DETCs, activated Vδ1 T cells produce insulin-like growth factor 1 and promote wound healing (33). They also exert cytotoxic responses against tumors. Therefore, they seem to perform functions similar to those of rodent DETCs. However, because human epidermal Vδ1 T cells do not have a distinctive dendritic shape, it seems inappropriate to call them DETCs.

In non-human primates, the possibility of the existence of DETCs was examined in cynomolgus macaques (crab-eating macaques) because, unlike humans which have inactivated *SKINT1L*, macaques have a single copy of structurally intact *SKINT1L* (29). Like its mouse counterpart, macaque *SKINT1L* is expressed in the thymus and skin, and the basal and suprabasal layers of the macaque epidermis contain a population of dendritic-shaped γδ T cells. Macaque epidermal T cells predominantly expressed Vγ10Vδ1 TCRs, but both Vγ and Vδ chains displayed junctional diversity. Also, expression of macaque Vγ10 was not restricted to epidermal lymphocytes. Therefore, it was concluded that macaques do not have rodent-type DETCs (29), but it is possible that they have DETCs defined as dendritic-shaped epidermal γδ T cells with limited antigen receptor diversity.

In summary, DETCs that are selected by SKINT1 molecules and display an invariant γδ TCR are unique to rodents, but DETCs in a broad sense appear to occur in other mammals, although more detailed investigation is required to draw definitive conclusions.

ORIGIN AND EVOLUTION OF DETCs

Recent work has uncovered that the epidermis of lampreys, a member of jawless vertebrates, contains dendritic-shaped γδ T-like cells with limited antigen receptor diversity reminiscent of DETCs (35).

Jawless vertebrates represented by lampreys and hagfish are the most primitive class of vertebrates equipped with adaptive immunity; accumulated evidence indicates that lymphocytes forming the cornerstone of adaptive immunity emerged in a common ancestor of jawed and jawless vertebrates. Interestingly, instead of TCRs and B-cell receptors (BCRs), jawless vertebrates use members of the leucine-rich repeat (LRR) family of proteins known as variable lymphocyte receptors (VLRs) for antigen recognition (36–39). Like gnathostome antigen receptors, VLRs are clonally expressed on lymphocytes. The diversity of VLRs, which is assumed to be comparable to that of TCRs and BCRs, is generated during lymphocyte development by assembly of multiple LRR modules with highly variable sequences through a gene-conversion-like mechanism. Jawless vertebrates have three major populations of lymphocytes distinguished by expression of distinct types of VLRs known as VLRA, VLRB, and VLRC. $VLRB^+$ cells resemble gnathostome B cells; when activated by specific antigen, they undergo clonal proliferation and secrete VLRB molecules as antibodies. On the other hand, $VLRA^+$ and $VLRC^+$ cells develop in lympho-epithelial thymus-like structures named thymoids, do not secrete VLR molecules, and resemble gnathostome T cells in gene expression profiles and responses to mitogens. Of the two T-like lymphocyte populations, $VLRC^+$ cells resemble γδ T cells in gene expression profiles in that they express the SRY-box containing gene 13 encoding a fate-determining transcription factor important for γδ T-cell lineage determination and interleukin-17. They also resemble gnathostome γδ T cells in tissue localization; $VLRC^+$ cells in lampreys are distributed predominantly in the epithelium of skin, intestine, and gill (35). In the epidermis, $VLRC^+$ cells are ~8 times more abundant than $VLRA^+$ cells and display dendritic morphology. Furthermore, the diversity of VLRC receptors in epidermal lymphocytes is markedly reduced compared to that in kidneys and blood.

The existence of DETC-like lymphocytes in the epidermis of lampreys indicates that the strategy of deploying γδ-like T cells to epithelia was adopted in a vertebrate ancestor and has been maintained in many vertebrate animals.

CONCLUDING REMARKS

Skint1-dependent DETCs appear unique to rodents such as mice and rats. However, DETCs broadly defined as dendritic-shaped epidermal γδ T cells with limited antigen receptor diversity appear to exist in other mammals. The presence of DETC-like cells in lampreys suggests that DETCs also occur in jawed vertebrates other than mammals. In animals in which *Skint1* is absent, other members of the butyrophilin family may perform equivalent functions. Also, "stress antigens" recognized by DETCs most likely differ from species to species. This difference, along with the difference in the butyrophilin members used for selection, may account for the observation that γδ TCRs on DETCs are invariant in rodents, whereas those on putative DETCs in other animals are not invariant, but display limited diversity.

AUTHOR CONTRIBUTIONS

YS and RM conducted experiments which formed part of the arguments made in this paper. YS and MK wrote the paper.

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GMP-Grade Manufacturing of T Cells Engineered to Express a Defined γδTCR

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γ9δ2T cells play a critical role in daily cancer immune surveillance by sensing cancer-mediated metabolic changes. However, a major limitation of the therapeutic application of γ9δ2T cells is their diversity and regulation through innate co-receptors. In order to overcome natural obstacles of γ9δ2T cells, we have developed the concept of T cells engineered to express a defined γδT cell receptor (TEGs). This next generation of chimeric antigen receptor engineered T (CAR-T) cells not only allows for targeting of hematological but also of solid tumors and, therefore, overcomes major limitations of many CAR-T and γδT cell strategies. Here, we report on the development of a robust manufacturing procedure of T cells engineered to express the high affinity Vγ9Vδ2T cell receptor (TCR) clone 5 (TEG001). We determined the best concentration of anti-CD3/CD28 activation and expansion beads, optimal virus titer, and cell density for retroviral transduction, and validated a Good Manufacturing Practice (GMP)-grade purification procedure by utilizing the CliniMACS system to deplete non- and poorly-engineered T cells. To the best of our knowledge, we have developed the very first GMP manufacturing procedure in which αβTCR depletion is used as a purification method, thereby delivering untouched clinical grade engineered immune cells. This enrichment method is applicable to any engineered T cell product with a reduced expression of endogenous αβTCRs. We report on release criteria and the stability of TEG001 drug substance and TEG001 drug product. The GMP-grade production procedure is now approved by Dutch authorities and allows TEG001 to be generated in cell numbers sufficient to treat patients within the approved clinical trial NTR6541. NTR6541 will investigate the safety and tolerability of TEG001 in patients with relapsed/refractory acute myeloid leukemia, high-risk myelodysplastic syndrome, and relapsed/refractory multiple myeloma.

Keywords: γδTCR, cancer immunotherapy, GMP-manufacturing, T cell engineering, cancer, TEG

INTRODUCTION

Chimeric antigen receptor engineered T (CAR-T) cells are currently entering clinical practice with remarkable response rates resulting in multiple FDA approvals in 2017 (1). Major limitations of current clinical strategies are, however, that CAR-T cells rarely offer solutions to solid tumors. Another restriction of current CAR-T approaches is that target antigens are often present on healthy

tissues. Therefore, we introduced the concept of metabolic cancer targeting through a defined high-affinity Vγ9Vδ2T cell receptor (TCR) (2) and proposed to utilize T cells engineered to express a defined γδT cell receptor (TEGs) as the next generation of CAR-T. Vγ9Vδ2TCRs sense spatial and conformational changes of butyrophilin 3A1 (CD277) and RhoB mediated by intracellular phosphoantigen accumulation (PAg). Transformed cells often have accumulated PAg due to a dysregulated mevalonate pathway, enabling γ9δ2T cells to recognize them (3, 4). The TEG concept allows for selecting the most potent Vγ9Vδ2TCR and targeting of liquid and solid tumors (5). TEGs also overcome the diversity of natural γ9δ2T cells (6) and avoid negative regulation of the Vγ9Vδ2TCR through innate receptors of γ9δ2T cells (7). In addition, as Vγ9Vδ2TCR are introduced in both CD8 effector and CD4 helper cells, TEGs can deliver professional help through, e.g., maturing dendritic cells (5). For clinical testing of the TEG concept, we recently selected a highly tumor reactive Vγ9Vδ2TCR clone (clone 5) from the natural repertoire of a healthy individual (2). This particular Vγ9Vδ2TCR showed a strong reactivity toward a broad range of tumor cells within the TEG format, including primary leukemic blasts (8) as well as primary multiple myeloma cells (9). Due to the selection of a high-affinity Vγ9Vδ2TCR, TEGs also outperform natural γ9δ2T cells in terms of direct tumor recognition (2). For administration of TEGs in human, we recently proposed a purification step of TEGs by depletion of non- and poorly-engineered cells in order to further increase activity and definition of the product (8). However, a Good Manufacturing Practice (GMP)-grade procedure for a TEG drug product has not yet been defined. In this article, we describe the developmental process from a "research method" (8) to a manufacturing procedure that is fully compliant with GMP. Given that this process requires connecting two completely different worlds, a flexible research environment with a rigid GMP environment, the reported developmental process can be of high interest to researchers who aim at translating research findings to the clinic.

MATERIALS AND METHODS

Production of Master Cell Bank (MCB) and Viral Vector Stock

The retroviral vector supernatant was produced in 293Vec-RD114 cells, a 293SF-based packaging cell clone producing RD114 pseudotyped viral particles containing MP71:TCRγ5-T2A-TCRδ5 transgene cassette, by BioNTech (Idar-Oberstein, Germany) (10, 11). To establish this packaging clone, first, a primary seed clone was established in a two-step transfection–transduction protocol. Candidate monoclonal cells were tested for the presence of the TCR transgene using qPCR. Transgene-positive clones were expanded to 14 cm Petri dishes in order to harvest supernatant. Primary seed clones were screened for virus titer production and the most productive cell clone (the producer cell line) was selected to grow a MCB. The MCB was released according to predefined criteria and stored in liquid nitrogen. Sequence integrity of the transgene was confirmed by sequence analysis of the MCB and TEG001 drug product samples.

Preparation of Leukapheresis Material

Patient-derived mononuclear cells obtained by leukapheresis were cryopreserved in freezing medium [sodium chloride (NaCl) 0.9% with 10% dimethylsulfoxide and 5% human albumin (HA)]. The material was thawed at 37°C and mixed with five volumes of leukapheresis thaw medium [X-VIVO 15 chemically defined medium without gentamicin and phenol red (Lonza, Breda, The Netherlands), hereafter, called X-VIVO 15, supplemented with 10% HA]. After washing, cells were resuspended in culture medium with cytokines (X-VIVO 15 medium with 5% human serum), 1.7×10^3 IU/ml of MACS GMP Recombinant Human interleukin (IL)-7 (Miltenyi Biotec, Bergisch Gladbach, Germany), and 1.5×10^2 IU/ml MACS GMP Recombinant Human IL-15 (Miltenyi Biotec).

Activation of T Cells

The cell suspension was diluted to a concentration of 1×10^6 T cells/ml with culture medium containing cytokines. T cells were activated by adding anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific, Etten-Leur, the Netherlands) to the cell suspension at a bead to cell ratio of 1:5 or otherwise indicated, homogenizing for 30 min at room temperature (RT) under rocking conditions, and subsequently incubating for 40–50 h at 37°C/5% CO_2.

At Day 2, activated T cells were harvested by centrifugation and subsequently resuspended in culture medium containing cytokines. Manual cell count using trypan blue exclusion was performed and the cell suspension was further diluted with culture medium containing cytokines to a target concentration of 0.5×10^6 viable cells/ml.

Transduction

Non-tissue culture treated 24-well plates (Thermo Fisher Scientific) were coated with Retronectin (Takara Bio, Saint-Germain-en-Laye, France) at saturating conditions and incubated for 40–50 h at 2–8°C. At the day of transduction, the coated plates were incubated for 30 min at 37°C with 0.4% HA in NaCl, 0.9% to block unspecific binding. Next, viral supernatant was thawed at RT and diluted 1:1 with X-VIVO 15 medium, or as described in the relevant figure. RetroNectin-coated plates were coated with 2.0 ml/well diluted viral supernatant by spinning for 90 min at $500 \times g$ at RT (one-spin hit transduction). The remaining supernatant was aspirated and discarded. Subsequently, 1×10^6 activated cells were added per well of the viral-supernatant-coated plates (2.0 ml cell suspension of 0.5×10^6 cells/ml) and incubated for 16–24 h at 37°C/5% CO_2.

At Day 3, transduced cells were harvested from the 24-well plate, centrifuged, and subsequently resuspended in culture medium with cytokines. Manual cell count was performed and the cell suspension was further diluted with culture medium with cytokines to a final target concentration of 0.25×10^6 viable cells/ml. The cell suspension was transferred to MACS GMP Cell Differentiation Bag(s) (Miltenyi Biotec) and incubated for 60–80 h at 37°C/5% CO_2.

Expansion of Transduced Cells

Transduced cells were cultured from Day 3 to Day 13. At Day 6, samples from cell suspension were taken to determine the

concentration of viable cells by trypan blue exclusion. Transduction efficiency was determined by flow cytometry (% γδTCR positive T cells). The cell suspension was centrifuged and cultured in fresh culture medium supplemented with cytokines to a target concentration of 0.25×10^6 viable cells/ml and incubated for 36–48 h at 37°C/5% CO_2.

At Day 8, manual cell count was performed to determine the concentration of viable cells by trypan blue exclusion. The cell suspension, if applicable, was diluted to a target viable cell concentration of 1×10^6 cells/ml with fresh culture medium without cytokines. The total volume of cell suspension was then supplemented with half the cytokine concentration. The cell suspension was incubated for 36–48 h at 37°C/5% CO_2.

At Day 10, manual cell count was performed to determine the concentration of viable cells by trypan blue exclusion. The cell suspension was centrifuged and further diluted with fresh culture medium supplemented with cytokines to a target viable cell concentration of about 1×10^6 cells/ml. The cell suspension was incubated for 60–80 h at 37°C/5% CO_2.

Purification of TEG001 by Research MACS Depletion of Non- and Poorly-Engineered Immune Cells

pMP71: γTCR-T2A-δTCR-transduced T cells were incubated with biotin-labeled anti-αβTCR antibody (clone BW242/412; Miltenyi Biotec), followed by incubation with an anti-biotin antibody coupled to magnetic beads (anti-biotin MicroBeads; Miltenyi Biotec). Next, the cell suspension was applied to an LD column in a QuadroMACS™ Separator. αβTCR-positive T cells were depleted by MACS cell separation according to the manufacturer's protocol (Miltenyi Biotec).

Purification of TEG001 by CliniMACS Depletion of Non- and Poorly-Engineered Immune Cells

At Day 13, the cell suspension volume was reduced, when necessary, to 150–200 ml by removing supernatant after centrifugation. Anti-CD3/CD28 beads were removed from the cell suspension of transduced T cells using a magnet (Dynamag Cell Therapy Systems magnet).

The cell suspension was processed as follows:

a) Washed with phosphate buffered saline/ethylenediaminetetraacetic Acid/HA buffer (PBS/EDTA buffer with 0.5% HA) and adjusted to a volume of 95 ml with PBS/EDTA/HA buffer.
b) Incubated with 7.5 ml of TCRαβ-Biotin reagent (biotin-labeled anti αβTCR antibody (clone BW242/412; Miltenyi Biotec)) for 30 min on a swivel plate.
c) Washed with 600 ml PBS/EDTA/HA buffer and after centrifugation, the volume was adjusted to 190 ml with PBS/EDTA/HA buffer.
d) Incubated with 15 ml of anti-Biotin reagent (anti biotin antibody coupled to magnetic beads) for 30 min on a swivel plate.
e) Washed by adding PBS/EDTA/HA buffer to a volume of about 600 ml and removing supernatant after centrifugation. Subsequently, PBS/EDTA/HA buffer was added to a volume of about 200 ml and the αβTCR-expressing T cells (non- and poorly-engineered cells) were depleted using a CliniMACS Plus instrument (Magnetic Activated Cell Sorting) cell separation, program "depletion 3.1."
f) Washed twice with infusion medium (NaCl 0.9% for infusion with 4% HA) and resuspended in infusion medium to obtain 25 ml TEG001 drug substance.

Cells and Cell Lines

Daudi (CCL-213) was obtained from the American Type Culture Collection and ML-1 was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Cell lines were authenticated by short tandem repeat profiling/karyotyping/isoenzyme analysis. All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. In addition, all cell lines were routinely verified by growth rate, morphology, and/or flow cytometry and tested negative for mycoplasma using MycoAlert Mycoplasma Kit. Daudi and ML-1 were cultured in RPMI + 1% Pen/Strep + 10% FCS (Bodinco, Alkmaar, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or apheresis material obtained from the Sanquin Blood Bank (Amsterdam, the Netherlands).

Flow Cytometry

Antibodies used for flow cytometry include: pan-γδTCR-PE (clone IMMU510; Beckman Coulter, Woerden, the Netherlands), pan-αβTCR-APC (clone IP26; eBioscience, Thermo Fisher Scientific), CD4-V450 (clone RPA-T4; BD Biosciences), CD8α-PerCP-Cy5.5 (RPA-T8; Biolegend), CD3-eFluor 450 (OKT-3; eBioscience), CD45-FITC (2D1; BD Biosciences), CD16-FITC (3G8; BD Biosciences), CD56-FITC (MY31; BD Biosciences), CD27-APC-eFluor780 (O323; eBioscience), CD45RO-PE-Cy7 (UCHL-1; BD Biosciences). All samples were analyzed on a BD LSRFortessa using FACSdiva software (BD Biosciences).

ELISPOT and ELISA Assays

IFNγ ELISPOT was performed as previously described (5, 6). Briefly, 15,000 TCR-transduced or mock-transduced (TEG-LM1) T cells and 50,000 target cells (ratio 0.3:1) were cocultured for 24 h in nitrocellulose-bottomed 96-well plates (Merck, Schiphol-Rijk, the Netherlands), pre-coated with anti-IFNγ antibody (clone 1-D1K) (Mabtech, Nacka Strand, Sweden). Plates were washed and incubated with a second biotinylated anti-IFNγ antibody (clone 7-B6-1) (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were visualized with tetramethylbenzidine substrate (Sanquin) and the number of spots was quantified using ELISPOT Analysis Software (Aelvis, Hannover, Germany). IFNγ ELISA was performed using ELISA-ready-go! Kit (eBioscience) following manufacturer's instructions. Effector and target cells (E:T 15,000:15,000) were incubated for 24 h in the presence of pamidronate when indicated.

Statistical Analyses

Differences were analyzed using indicated statistical tests in GraphPad Prism 7 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Defining Optimal Activation of Primary T Cells With Anti-CD3/CD28 Coated Beads

Stimulation of T cells with immobilized anti-CD3 and anti-CD28 antibodies provides both an antigen stimulus and co-stimulation for optimal T cell activation and expansion (12). Consequently, anti-CD3/CD28-coated beads are widely applied to engineer cellular products for different types of adoptive αβT cell therapies (13–16). The optimal anti-CD3/28 bead to CD3+ T cell ratio for activation, transduction with viral supernatant, and expansion of engineered immune cells is, however, frequently dependent on the specific transgene and production process. In order to define the best anti-CD3/28 bead to CD3+ T cell number for engineering TEGs, PBMCs were first analyzed for CD3+ T cell content by flow cytometry, and then incubated with various ratios of anti-CD3/CD28 beads in the presence of the cytokines interleukin (IL)-7 and IL-15. As a control stimulus, soluble OKT-3 and IL-2 was used. Next, T cells were transduced with non-GMP grade retroviral supernatant and expanded as described in the Section "Materials and Methods." After 10 days, the total number of TEGs, defined as double positive TEGs when expressing γδTCRs and αβTCRs or single positive TEGs when expressing γδTCRs only, were assessed by flow cytometry. The mean total cell number of single and double positive TEGs ranged from 2 to 6 × 10^6 cells when stimulated with anti-CD3/28 beads, and peaked at an anti-CD3/28 bead to T cell ratio of 1:5, while our standard OKT3 research protocol delivered 3 × 10^6 cells (**Figure 1**). Due to the limited number of replicates, when performing a Mann–Whitney *U*-test, the difference between none of the conditions was significant ($p > 0.05$). A 1:5 bead to T cell ratio was chosen for the activation of T cells in all following TEG manufacturing procedures. This ratio is sufficient to activate T cells and is substantially lower in numbers than advised by the manufacturer and, therefore, saves costs during the future production procedures.

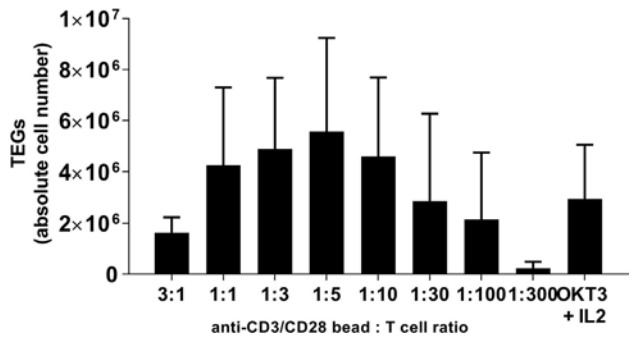

FIGURE 1 | TEG yield depends on the optimal anti-CD3/CD28 bead to T cell ratio at the day of T cell activation. Multiple anti-CD3/CD28 bead to T cell ratios were tested and compared with respect to total TEG yield at day 10. After activation, transduction and expansion TEG numbers were defined by combining viable cell count with flow cytometry for γδTCR+ T cell percentage. OKT3 + IL2 served as a control activation and expansion stimulus. Mean absolute cell number + SD is shown, $n = 2–3$. The differences according to Mann–Whitney *U* tests are not significant ($p > 0.05$).

Selection of a GMP-Grade Retroviral Producer Cell Clone

One of the most critical raw materials of the TEG manufacturing process is the viral vector supernatant used to transduce the T cells. Selection of a potent GMP-grade cell clone that produces the retroviral vector encoding the γδTCR is, therefore, critical for the success of the GMP-grade transduction process. The retroviral vector supernatant was produced using 293SF-based packaging cells, 293Vec-RD114, by BioNTech (10, 11). A large number of engineered producer cell clones were generated and the virus titer in the supernatant was assessed by titration experiments on Jurkat cells and analysis of γδTCR positive cells by flow cytometry (**Figure 2A**). The 8 best clones were selected for the second round of testing. Two additional clones from the upper midfield (#8, #62) were added to confirm the ranking (**Figure 2B**). Clone #73 was selected as the best GMP-grade retroviral producer cell line and was, therefore, further expanded and the titer was assessed before and after 0.45 μm filtration of the supernatant from different harvesting runs (**Figure 2C**). Filtration was performed in order to eliminate cell debris, a key step for generating GMP grade viral supernatant. This associated, however, with an up to sixfold reduction in viral particles in different harvesting runs (**Figure 2C**).

Virus Titer Impacts Transduction Efficiency of Primary T Cells

In the manufacturing of genetically modified cellular medicines, there is a strong relationship between transduction efficiency and the ability to produce sufficient cell numbers that meet predefined quality criteria (17). To optimize the production process we, therefore, assessed the amount of virus needed for optimal efficiency in a one-hit transduction. Viral supernatant generated in different pre-GMP proof runs from producer cell line clone #73 was used with virus titers ranging from 8.7 × 10^3 to 2.7 × 10^6 infectious particles (ip) per milliliters. Transduction efficiency was evaluated after 7 and 10 days of expansion with the optimized 1:5 anti-CD3/28 bead to T cell ratio. The percentage of TEGs was determined by flow cytometry using a pan-γδTCR antibody. The total number of both single and double positive TEGs was determined. Viral supernatant containing 1 × 10^6 ip/ml provided transduction efficiencies of 60–70% TEGs (**Figure 3A**). The majority of TEGs showed high expression levels of γδTCR while being negative for αβTCR due to the successful competition of the introduced γδTCR chains against endogenous αβTCRs for components of the CD3 complex as reported (8). Thus, virus titers around 1 × 10^6 ip/ml are sufficient for the generation of TEGs.

Impact of T Cell Density on Transduction and Expansion Efficiency

It has been suggested that the density of activated T cells during the transduction procedure influences the transduction efficiency (18). Therefore, the optimal cell concentration during transduction was investigated within the context of four different virus titers (range 0.03–1.5 × 10^6 ip/ml), and five different T cell densities (range 0.1–2.0 × 10^6/ml). During the expansion phase, T cell densities were adjusted to defined concentrations at day 3

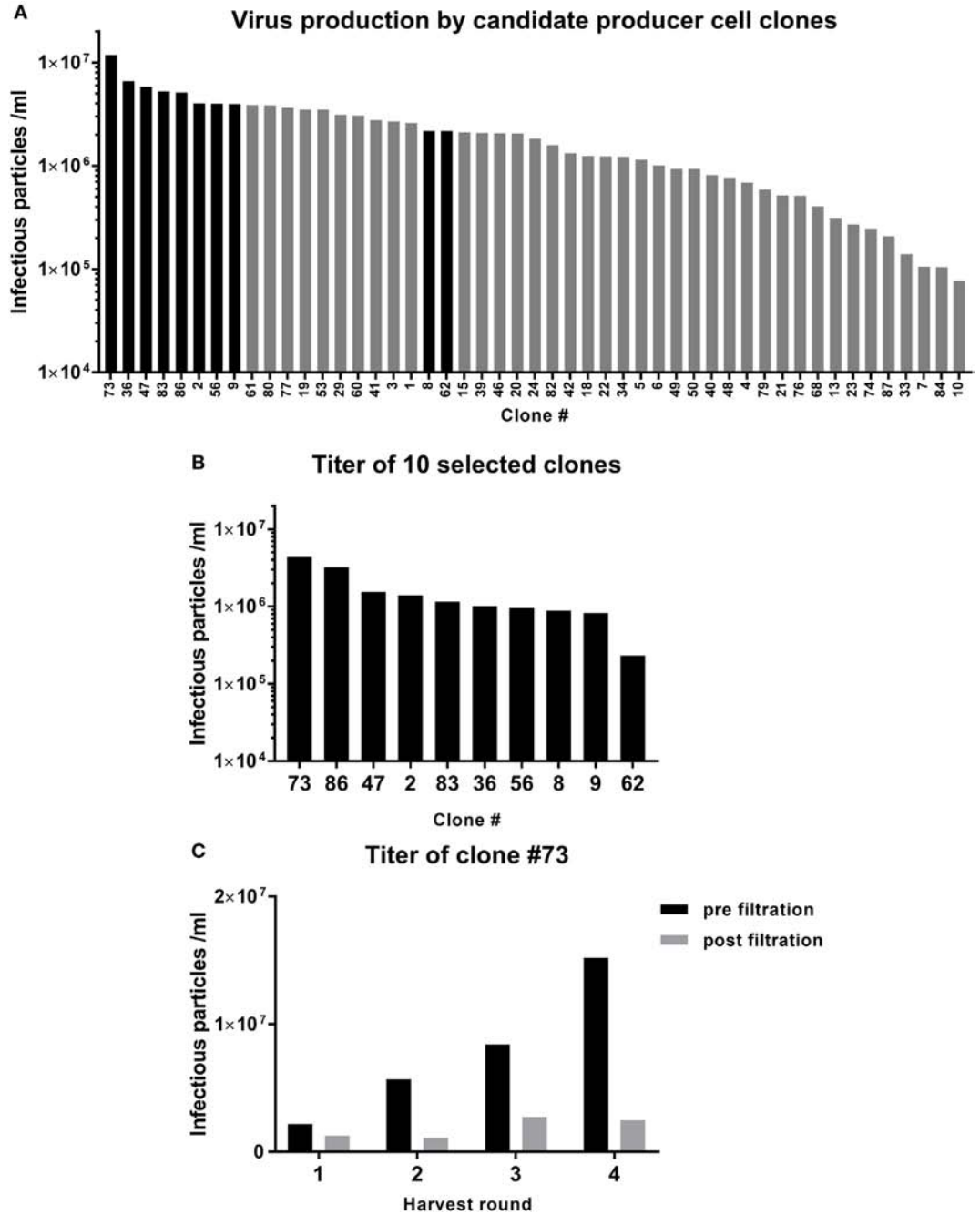

FIGURE 2 | Selection of a viral vector producer cell clone. Retroviral supernatant was produced in 293vec-RD114 packaging cells. **(A)** The titer produced by the different clones was assessed in Jurkat cells. The clones depicted by the black bars were selected for a second round of testing **(B)**. **(C)** Clone #73 was picked for production of the TEG001 Good Manufacturing Practice retroviral supernatant. The titer was assessed after four rounds of harvest, before, and after filtration.

and 6 (both 0.25×10^6/ml), and day 8 and 10 (both 1.0×10^6/ml). The percentage of γδTCR-positive T cells was determined after 7 and 10 days by flow cytometry using a pan-γδTCR antibody (**Figure 3B**). Differences in TEG transduction efficiencies were only observed for very low virus titer conditions (0.03×10^6 ip/ml). A cell concentration of 0.5×10^6/ml was selected as the standard cell density during transduction for TEG001 manufacturing process.

Impact of GMP-Grade Virus Titer From Clone #73 on Transduction Efficiency

Due to procedural differences between the manufacturing of research-grade and GMP-grade retroviral supernatant, the relationship between virus titer and transduction efficiency for the final GMP viral supernatant batch, which will be used for the production of TEGs for the clinical trial, was further investigated.

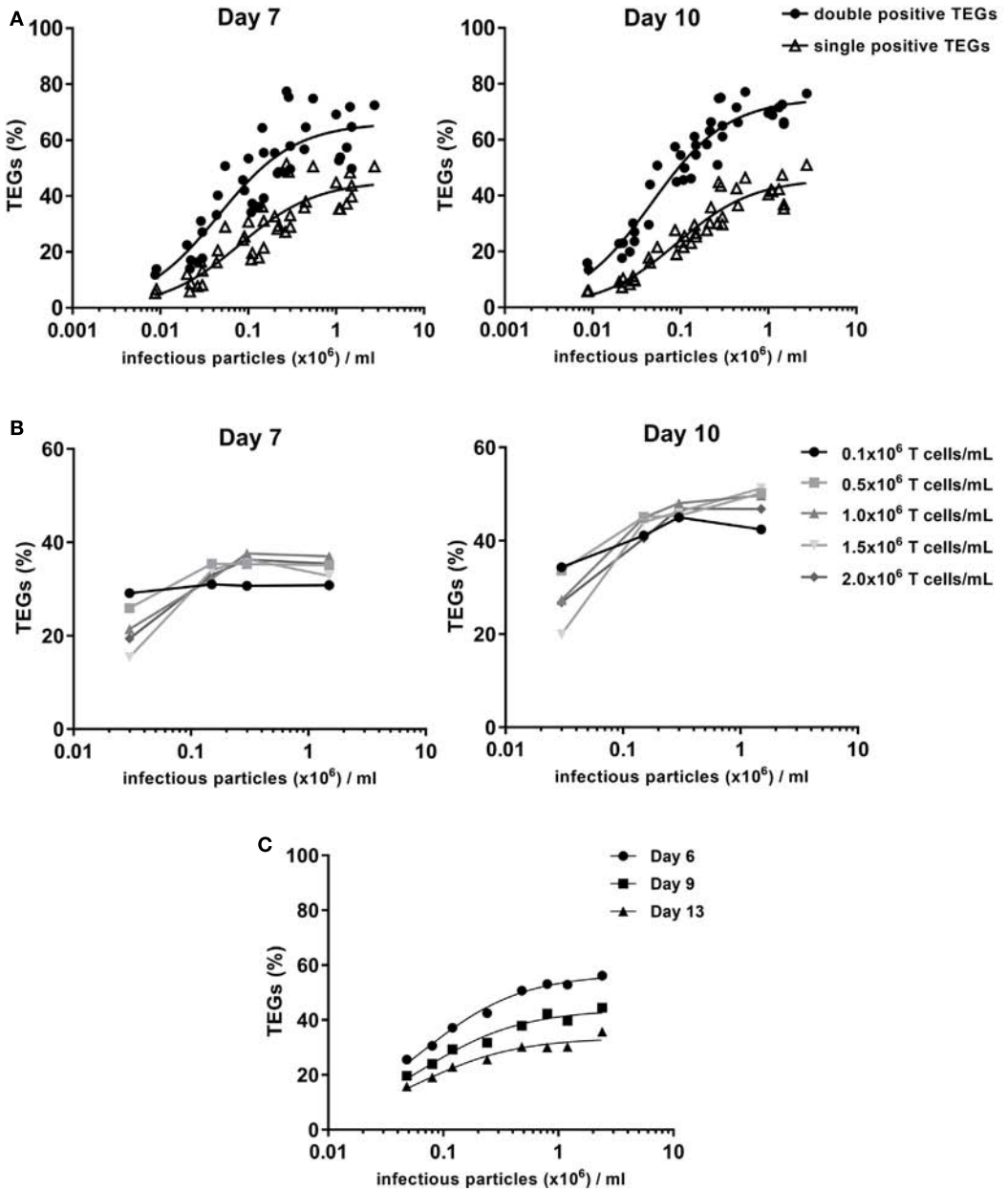

FIGURE 3 | Transduction efficiency depends on the virus titer. T cells were activated with 1:5 CD3/CD28 bead to cell ratio followed by retroviral transduction with the γδTCR and expansion. **(A)** 0.5×10^6 cells per ml were transduced with different concentrations of pre-Good Manufacturing Practice (GMP) viral supernatant. The % TEGs are shown as total double positive TEGs (black circles) and single positive TEGs (open triangles) at day 7 and day 10 after activation. **(B)** The relationship between T cell concentration and transduction efficiency was investigated. Transduction efficiency was evaluated after a 7- and 10-day expansion period, for a range of T cell concentrations during transduction and four different virus titers. **(C)** 0.5×10^6 cells/ml were transduced with different concentrations of GMP viral supernatant to determine the relationship between virus titer and transduction efficiency after a 6-, 9-, and 13-day expansion period.

T cells were transduced at a density of 0.5×10^6 cells/ml with different dilutions of GMP-grade viral supernatant in a one-spin hit transduction procedure. The transduction efficiency and total γδTCR positive cell numbers were evaluated after 6, 9, and 13 days by flow cytometry using a pan-γδTCR antibody (**Figure 3C**). The percentage of TEGs with the different pre-GMP titers was highest between day 6 (**Figure 3C**) and day 10 (data not shown) followed by a small decrease, most likely in line with our previous observation that engineered immune cells have a slight disadvantage in proliferative capacity early after transduction when compared to non engineered immune cells (19). In line with the observation for pre-GMP-grade viral supernatant, the final GMP-grade viral supernatant that will be used for the clinical study provided transduction efficiencies of up to 60% TEGs when utilizing a titer of 1.2×10^6 ip/ml (**Figure 3C**).

Higher γδTCR Expression Increases Antitumor Activity

Defining a potency assay for a medicinal product is critical for assessing whether the final product is biologically active. Previous reports suggested that γδTCR expression levels correlate with activity of TEGs (5, 8). To formally confirm that indeed γδTCR expression is key for TEG activity, we used a defined CD4+ T cell clone (20), which underwent the transduction procedure but remained untransduced (T cells A) or was transduced with the MP71:TCRγ5-T2A-TCRδ5 retroviral vector, resulting in low and intermediate γδTCR single positive cell lines (T cells B and C, respectively). Primary T cells from a GMP proof run were used to generate the cell line with a high single positive γδTCR fraction that was further purified for CD4+ T cells by CD4 MACS selection after transduction (T cells D) (**Figure 4A**). Next, activity of theses γδTCR cell lines with different amounts of single γδTCR-positive T cells was compared side by side in the presence of pamidronate against Daudi as a positive, or ML1 as a negative tumor target (**Figure 4B**). CD4+ TEGs with higher γδTCR expression had a higher activity in terms of IFNγ cytokine secretion when compared to CD4+ T cell clones with lower or literally absent γδTCR expression. As control, T cells A–C, which express an endogenous allogeneic HLA-DPB1*04:01-reactive αβTCR, were coincubated with an HLA-DPB1*04:01 expressing B cell line. This resulted in cytokine levels equivalent or higher than from T cells D, indicating that T cells A–C were highly functional when triggered by the endogenous TCR (data not shown). These data are in line with previous reports from our group (8) and support the rationale to enrich in the GMP process only for TEGs with highest γδTCR expression. Therefore, we defined γδTCR-positive expression as a potency assay for TEGs and γδTCR single positive TEGs defines the functionally most active population.

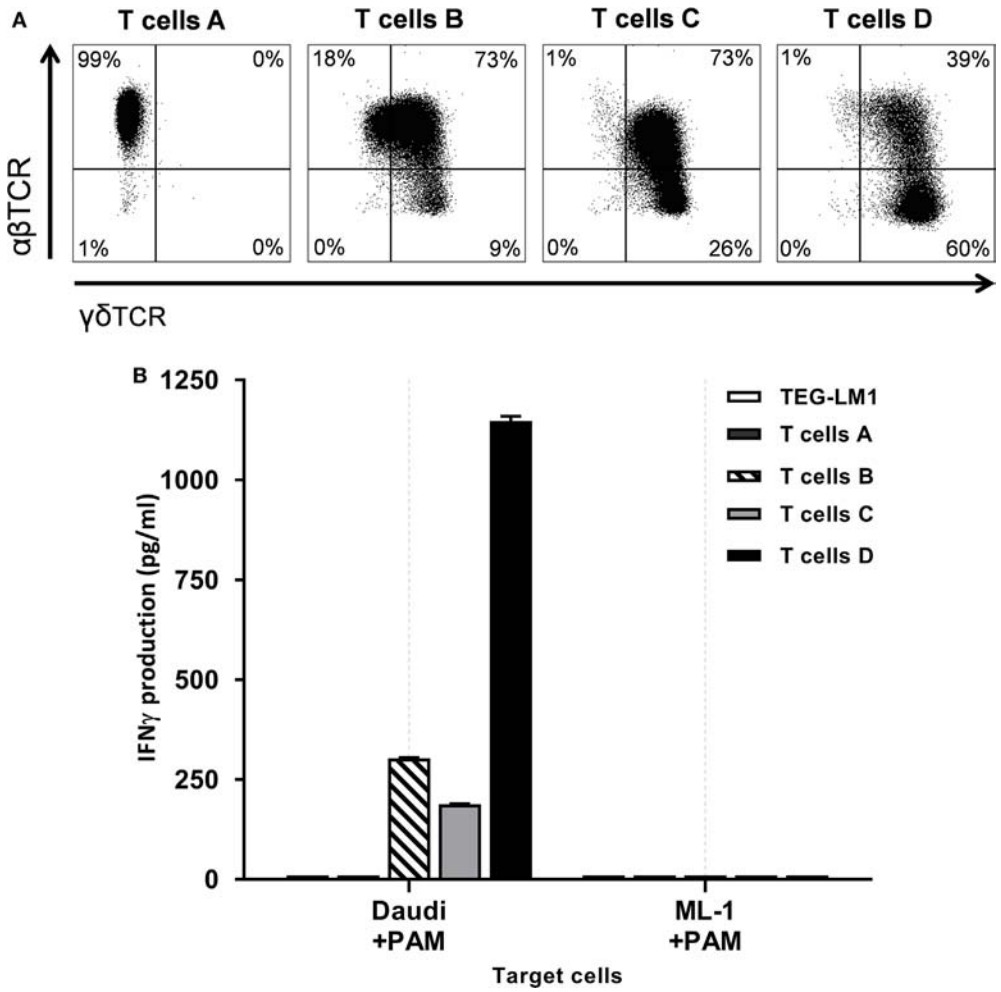

FIGURE 4 | γδTCR expression defines functional activity. **(A)** A defined CD4+ T cell clone (20) underwent the transduction procedure but remained untransduced (T cells A) or was transduced with the MP71:TCRγ5-T2A-TCRδ5 retroviral vector, resulting in low and intermediate γδTCR single positive cell lines (T cells B and C, respectively). Primary T cells were used to generate the cell line with a high single positive γδTCR fraction that was further purified for CD4+ T cells by CD4 MACS selection after transduction (T cells D). In all cases, after one cycle of expansion, γδTCR and αβTCR expression was measured in the viable CD45+ gate by flow cytometry after which the cells were used in a function assay. **(B)** Different T cells were coincubated with the indicated tumor target cell lines in the presence of pamidronate in triplicate. Daudi is the prototypic TEG001 positive target, ML-1 is the negative control target. TEG-LM1 served as the negative control effector. After 20 h at 37°C, supernatant was harvested and analyzed by IFNγ ELISA. Mean IFNγ production + SD is shown.

Enrichment of TEGs by CliniMACS Through Depletion of Non- and Poorly-Engineered Immune Cells

Non- and poorly-engineered cell fractions are usually present in genetically engineered T cell products and associate with little or no activity as shown also for TEG001 (**Figures 4A,B**). In addition, such cell fractions could even be harmful as they might harbor unwanted specificities. To avoid this potential drawback, a procedure for the depletion of non- and poorly-engineered T cells was developed by taking advantage of the observation that upon introduction of a γδTCR, the endogenous αβTCR expression is substantially decreased or even absent (2, 5). First, we defined "in process controls." During six large-scale proof runs, the percentage of γδTCR-positive cells was, therefore, assessed at day 6 (in process control), before and after the CliniMACS depletion procedure. Between 31 and 63% of all cells were positive for γδTCR at day 6, and 73–92% at day 13 after CliniMACS depletion (**Figure 5A**). In addition, we assessed the robustness of the

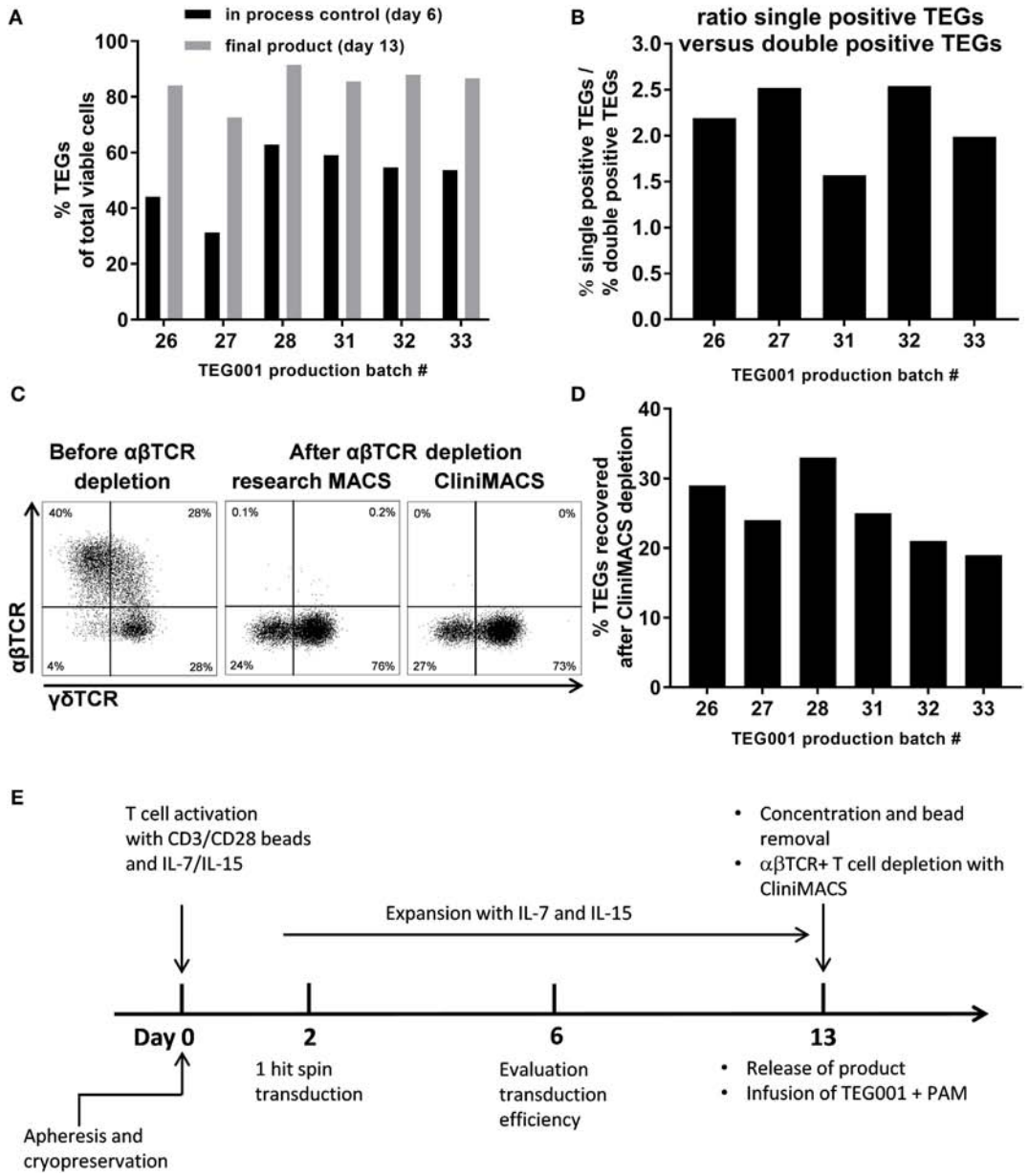

FIGURE 5 | Successful enrichment of TEGs by CliniMACS depletion. **(A)** Comparison of TEGs transduction efficiency during production (in-process control, day 6) and at the end of production (final product) of six different production batches. **(B)** After introduction of pMP71:γTCR-T2A-δTCR and expansion of the T cells both γδTCR+αβTCR− T cells as γδTCR+αβTCR+ T cells are present. **(C)** During one of the research scale production batches, the cells at Day 13 were split and the non-transduced T cells were depleted using the research MACS or CliniMACS cell separation systems. **(D)** γδTCR+ cell recovery as percentage of the γδTCR+ cell input was measured after each αβTCR CliniMACS depletion. **(E)** Overview of the Good Manufacturing Practice TEG001 production process.

observation that introducing γδTCR substantially outcompetes endogenous αβTCRs during different GMP runs. The ratio of single positive to double positive TEGs ranged between 1.6 and 2.5 and was above 1.5 for all runs (**Figure 5B**). Then, we aimed to assess whether a depletion of non- and poorly-engineered TEGs could not only be performed with research devices (8), but also with GMP-grade αβTCR beads on a CliniMACS device. Therefore, we compared side by side the research-scale and large-scale depletion of non- and poorly-engineered TEGs through αβTCR depletion on a CliniMACS. After the depletion procedure, we observed a comparable purity of single positive TEGs after both procedures (research-grade: 73% versus clinical-grade: 76%; **Figure 5C**), while the remaining αβTCR positive T cell fraction was very low for both research-grade and GMP-grade (0.3 and 0.0%, respectively). The αβTCR negative γδTCR negative populations present in both research-grade and GMP-grade depleted products (24 and 27%, respectively) mainly consisted of NK cells (data not shown). This double negative population was present at the end of all manufacturing runs ($n = 6$, range 8–27%), and was donor and batch dependent. The recovery after CliniMACS αβTCR depletion, indicated as percentage of γδTCR + T cell output of the respective input, varied between 19 and 33% ($n = 6$, **Figure 5D**). This procedure allowed us to produce TEGs in numbers up to 2×10^9 cells and is, therefore, sufficient to deliver dosages needed for the planned clinical study. The complete manufacturing schedule is depicted in **Figure 5E**.

Immunological Phenotype of TEG001 Drug Substance

Next, we characterized the immunological phenotype of the drug substance TEG001, as the *in vivo* proliferation capacity and function of genetically modified cell therapy products is not only determined by the introduced receptor but also by the differentiation phenotype of the individual T cells. The differentiation phenotype of TEGs was determined by measuring the expression of CD27 and CD45RO (21) of five pre-GMP production runs. The major subset of TEGs was, after a 2-week expansion period, T cells with an effector (T_{eff}) and effector memory (T_{em}) phenotype (**Figure 6A**). In addition, all products contained engineered central memory T cells (T_{cm}), an immune subset enabling potential long-term persistence of TEG *in vivo* (22).

Release Specifications of TEG001 Drug Substance and TEG001 Drug Product, Batch Analyses, and Stability

Release specifications are an essential component of quality assurance and protect the patient from receiving a suboptimal cellular product. Based on our small-scale runs, we defined, therefore, product release specifications. To avoid overly stringent product definition criteria, which could result in the discarding of a product for clinical use, we defined release criteria for the TEG001 drug substance as ≥50% γδTCR positive T cells, ≥70% viability, and ≤10% αβTCR positive T cells in addition to standard microbiological release criteria (**Table 1**). All six large-scale runs met these release criteria as indicated in **Table 2**. In addition, after formulation of the drug product, stability of TEG001 drug product was assessed. This is essential for clinical practice as products are frequently administered for logistical reasons within 1 day after production is complete. Therefore, a fraction of TEG001 drug product was stored for 16 and 20 h at 4°C and tested for viability over time. All three tested batches remained stable over the tested time period

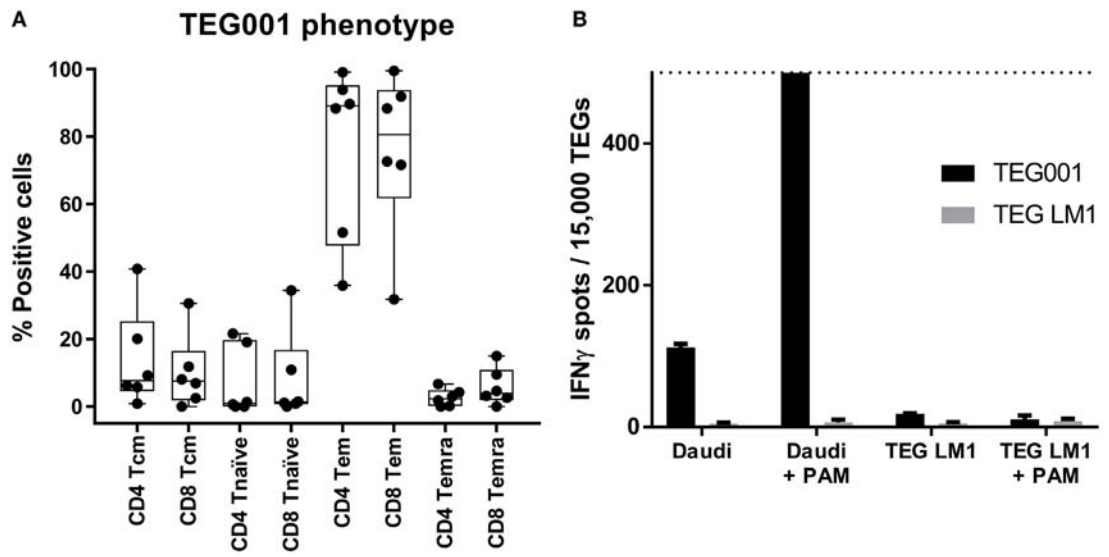

FIGURE 6 | TEGs have a predominant effector-memory/effector phenotype. **(A)** The phenotype of TEGs from four different donors was determined by measuring CD45RO in combination with CD27 expression on day 13, after the CliniMACS depletion. CD45RO+/CD27+ is considered as T_{cm}, CD45RO−/CD27+ as T_n, CD45RO+/CD27− as T_{em}, and CD45RO−/CD27− as T_{emra} (21). **(B)** TEGs were produced according to the described procedure after which they were stored at 2–8°C. After 20 h, the TEGs were coincubated with Daudi in the absence and presence of pamidronate (PAM) as a positive target, or TEG LM1 in the absence and presence of PAM as a negative target. TEG LM1 as effector served as the negative control. The maximum assay sensitivity was set at 500 spots (dashed line).

(**Table 3**). Next, we tested whether GMP-grade TEG001 drug product is functional after storage for 20 h at 4°C, by co-incubation of TEG001 and TEG-LM1 (mock control) with the reference target cell line Daudi, in the presence of pamidronate (2, 8). TEG001 was effective in recognizing Daudi, while there was no recognition by mock TEGs when assessed by IFNγ ELISPOT (**Figure 6B**).

TABLE 1 | Release specifications TEG001 drug substance.

Parameter	Method	Acceptance criteria
Identity		
- TEG001	Flow cytometry	Identity confirmed
Purity		
- % γδTCR-positive T cells	Flow cytometry	≥50%
- Viability	Manual cell count	≥70%
Impurities		
- % αβTCR-positive T cells[a]	Flow cytometry	≤10%
Microbiology		
- Sterility	Ph.Eur	Negative
- Mycoplasma	Ph.Eur	Negative
- Endotoxins	Ph.Eur	<2.0 IU/ml

The TEG001 release specifications as defined in the investigational medicinal product dossier.

[a]Only applicable for patients who previously received an allogeneic hematopoietic stem cell transplantation.

DISCUSSION

We have developed a robust GMP-grade TEG production protocol, which not only includes a conventional transduction and expansion step but also a very stringent CliniMACS enrichment procedure to guarantee high purity of the drug substance. This purification procedure can be used for any engineered immune cell product, which associates with a reduced expression of the αβTCR, like CAR-T introduced in the αTCR locus (23, 24). By utilizing this protocol, we have been able to produce and enrich TEGs in numbers, which are sufficient to reach the highest dose level of our upcoming phase I trial NTR6541. Furthermore, we have shown that γδTCR expression can be used as potency assay for TEG001, and that the TEG001 drug product is stable for at least 20 h at 4°, which allows for provisional release and transportation to the location of the infusion.

In current manufacturing processes of CAR-T cells, purification steps are often not included. As a consequence, final products currently infused into patients harbor only between 15 and 55% of engineered immune cells (18, 25, 26). The lack of purity can become a major clinical obstacle, in particular, when engineering T cells from patients who relapse after allogeneic stem cell transplantation. Re-infusion of CAR-T cells in patients after allogenic stem cell transplantation has been reported to associate

TABLE 2 | TEG001 drug substance batch analysis data of large scale runs.

Parameter	Acceptance criteria	Run					
		26	27	28	31	32	33
Identity							
- TEG001	Identity confirmed	Pass	Pass	Pass	Pass	Pass	Pass
Purity							
- % γδTCR-positive T cells	≥50%	84%	73%	92%	86%	88%	87%
- Viability	≥70%	99%	98%	99%	97%	100%	100%
Impurities							
- % αβTCR-positive T cells[a]	≤10%	0.0%	0.0%	0.2%	0.5%	0.4%	0.1%
Microbiology							
- Sterility	Negative	ND	ND	Negative	Negative	ND	ND
- Mycoplasma	Negative	ND	ND	Negative	Negative	ND	ND
- Endotoxins	<2.0 IU/ml	ND	ND	Pass	Pass	ND	ND

TEG001 was produced using our Good Manufacturing Practice large scale production protocol in multiple large scale manufacturing runs. TEG001 was formulated after which the identity, purity, and viability was assessed using trypan blue exclusion and flow cytometry.

[a]Only applicable for patients who previously received an allogeneic hematopoietic stem cell transplantation.

TABLE 3 | Stability data of TEG001 cell suspension for infusion stored at 2–8°C.

Time point	Parameter	Run (content TEG001 cells per 100 ml drug product)						
		31 69×10^6	31 12×10^7	32 71×10^6	32 14×10^7	32 26×10^7	33 73×10^7	33 31×10^7
Formulation	Viability	97%	97%	100%	100%	100%	100%	99%
T = 0 h	Viable cell number recovery	100%	100%	100%	100%	100%	100%	100%
Storage	Viability	96%	97%	99%	100%	99%	100%	100%
T = 16 h	Viable cell number recovery	93%	94%	99%	94%	90%	100%	97%
Storage	Viability	94%	94%	100%	100%	99%	100%	100%
T = 20 h	Viable cell number recovery	84%	94%	99%	94%	90%	97%	97%

TEG001 was produced using our Good Manufacturing Practice large scale production protocol in multiple large scale manufacturing runs. TEG001 was formulated in NaCl 0.9% for infusion with 4% HA at different cellular concentrations, to study the effect of TEGs density on cell viability and viable cell recovery at T = 0 and after storage at 2–8°C for 16 and 20 h.

with incidences of acute and chronic graft versus host disease (GvHD), up to 10% (27). GvHD after infusion of CAR-T cells is most likely a consequence of endogenous αβTCRs still expressed at physiological levels in CAR-T cells, as well as the presence of non-engineered immune cells within the product. With our TEG concept, we provide comprehensive solutions to these problems. First, as suggested from our previous data, not only in the research environment (8) but also with our presented GMP manufacturing process, endogenous αβTCRs are substantially downregulated in TEGs. Reduced expression of endogenous αβTCRs is most likely due to the efficient competition of the introduced γδTCR against endogenous αβTCR for CD3 components of a T cell. Second, the additional GMP enrichment procedure utilizing the CliniMACS system achieves purity of TEGs, which can exceed 90%. The removal of non- and poorly-engineered cells from the final drug substance has another advantage, in addition to the reduced risk of GvHD and a more potent product due to enrichment of γδTCR-positive cells; the improved competition for homeostatic cytokines (28, 29). Other strategies for the refinement of engineered immune cells have been developed recently. However, alternative purification strategies frequently depend on the introduction of an additional truncated protein such as CD19 or epidermal growth factor receptor, which are normally absent in the T cell lineage (30). Using a transgene cassette with an additional sequence for selection purposes can lead to lower transduction efficiencies and reduced expression of the introduced immune receptor or alternative homing (J. Kuball, unpublished observation) and associates frequently with unwanted T cell activation and immunogenicity (31). To the best of our knowledge, we have developed the very first GMP manufacturing procedure in which αβTCR depletion is used as a purification method, thereby delivering untouched clinical-grade engineered immune cells. Despite an efficient elimination of non- and poorly-engineered αβT cells, our procedure also enriches for NK cells. An additional purification step before T cell engineering might, therefore, be intriguing for the next generation of TEG-manufacturing, such as αβTCR+ or CD3+ cell selection before T cell activation (32), as proposed by others.

Advanced therapy medicinal products (ATMPs), such as TEGs, are individualized and complex biological products that require careful consideration of their nature in order to define adequate "in process" and "release" tests. ATMPs are also frequently freshly prepared and directly infused into patients after production, limiting the possibilities for extensive safety and release testing for an individual product. Despite the limited possibilities, regulatory authorities oblige a potency assay before batch release. Potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" (33). Potency must be measured in a robust and biologically relevant way, which reflects the mechanism of action. Thus, defining valid potency assays can be a major challenge for ATMPs. For CAR019, expression of the introduced receptor as a potency assay has been proposed by vendors and accepted by the FDA (17, 34). We provide now evidence that for TEGs, γδTCR expression levels are an adequate potency assay. However, expression levels of receptors are rather simplified and surrogate methods to assess for activity will not predict efficacy *in vivo*. Therefore, alternative methods are needed. High-throughput characterization of TEGs on single cell levels could be interesting alternatives, as previously also reported for CAR-T (35).

In conclusion, we have developed a GMP-grade manufacturing strategy for TEGs incorporating an αβTCR depletion to obtain a final product substantially enriched for TEGs. The described process can also be valuable for any CAR-T product interfering with endogenous αβTCR expression. We also defined release and potency criteria acceptable for competent authorities. TEG001 will be used for an upcoming phase I dose escalation clinical trial registered as NTR6541. This trial aims to investigate the safety and tolerability of TEG001 in patients with relapsed/refractory acute myeloid leukemia, high-risk myelodysplastic syndrome, and relapsed/refractory multiple myeloma.

AUTHOR CONTRIBUTIONS

JK and TS designed experiments. GK, TS, SH, RD, KJ, and JS performed the experiments, AM reviewed data and submitted documents to authorities; GK, TS, ZS, and JK wrote the manuscript; all authors agreed on the final manuscript.

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Functions of Vγ4 T Cells and Dendritic Epidermal T Cells on Skin Wound Healing

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Wound healing is a complex and dynamic process that progresses through the distinct phases of hemostasis, inflammation, proliferation, and remodeling. Both inflammation and re-epithelialization, in which skin γδ T cells are heavily involved, are required for efficient skin wound healing. Dendritic epidermal T cells (DETCs), which reside in murine epidermis, are activated to secrete epidermal cell growth factors, such as IGF-1 and KGF-1/2, to promote re-epithelialization after skin injury. Epidermal IL-15 is not only required for DETC homeostasis in the intact epidermis but it also facilitates the activation and IGF-1 production of DETC after skin injury. Further, the epidermal expression of IL-15 and IGF-1 constitutes a feedback regulatory loop to promote wound repair. Dermis-resident Vγ4 T cells infiltrate into the epidermis at the wound edges through the CCR6-CCL20 pathway after skin injury and provide a major source of IL-17A, which enhances the production of IL-1β and IL-23 in the epidermis to form a positive feedback loop for the initiation and amplification of local inflammation at the early stages of wound healing. IL-1β and IL-23 suppress the production of IGF-1 by DETCs and, therefore, impede wound healing. A functional loop may exist among Vγ4 T cells, epidermal cells, and DETCs to regulate wound repair.

Keywords: wound healing, dendritic epidermal T cell, Vgamma 4 T cell, IL-17A, IGF-1, re-epithelialization

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SKIN γδ T CELLS ARE HEAVILY INVOLVED IN THE WOUND HEALING PROCESS

Wound healing is a complicated repair process to recover the integrity of skin. This process is orchestrated by four overlapping phases, which are clotting, inflammation, re-epithelialization, and remolding (1). Murine γδ T cells as important components of skin immunity engage in inflammation and re-epithelialization in wound repair (2–4). Several subsets of γδ T cells with distinct functions exist in skin tissue: dendritic epidermal T cells (DETCs), which uniformly express an invariant Vγ5Vδ1 TCR (according to Heilig and Tonegawa's nomenclature) and exclusively reside in the murine epidermis (>90%), primarily provide IGF-1 and KGF-1/2 in the epidermis to enhance re-epithelialization and thereby promote skin wound repair. Vγ4 T cells, a dominant subset of murine peripheral and dermal γδ T cells (approximately 50%), provide an early major source of IL-17A to initiate and amplify local inflammation after skin damage (5, 6). Interestingly, although inflammation is required for efficient skin wound healing, excessive inflammation has a negative impact on skin wound repair. In line with

this notion, IL-17A, a potent pro-inflammation factor, exhibits dual roles in skin wound closure (7–9). It has been reported that dermal Vγ4 T cells infiltrate the epidermis and interact with epidermal cells to form an IL-17A-IL-1/23 positive feedback loop for amplifying local inflammation. Li et al. recently revealed that Vγ4 T cells suppress IGF-1 production by DETCs through the IL-17A-IL-1/23 loop and thus delay skin wound healing (10). This suggests that a potential functional link exists between Vγ4 T cells, epidermal cells, and DETCs in the wound-healing process. This review focuses on the functional diversity of skin-resident γδ T cell subsets in wound repair.

THE DEVELOPMENT OF Vγ5 T CELLS IN THE THYMUS

Vγ5 T cells are the first generated γδ T cells on embryonic day (ED) 13 in the early fetal thymus, but they are no longer produced after ED 18 (11, 12). Their γ and δ chains are identically rearranged to Vγ5-Jγ1Cγ1 and Vδ1-Dδ2-Jδ2Cδ, respectively, with invariant canonical junctional sequences (13). IL-7 signaling is required for the rearrangement of TCRγ but not TCRαβ (14). IL-7 and IL-7 receptors are responsible for the recombination of the TCRγ locus by regulating locus accessibility to the V(D)J recombinase (15). Positive selection is necessary for the maturation of Vγ5 T cells in the fetal thymus, which depends on the engagement of TCR and some ligands expressed by thymic stromal cells (16). Skint-1 is essential for the positive selection of Vγ5 T cells in the murine fetal thymus (17). CD122 (the β chain of IL-2/IL-15 receptor, IL-2/IL-15Rβ) and the skin-homing receptors are induced on Vγ5 T cells after positive selection in the fetal thymus, and are crucial for Vγ5 T cells to migrate into the epidermis (16). Vγ5 T cells gain a "memory-like" pre-activation phenotype of $CD44^+CD122^+CD25^-$ before exiting the thymus (11).

THE MIGRATION AND RESIDENCY OF Vγ5 T CELLS IN THE EPIDERMIS

During ED 15.5–16.5, Vγ5 T cells egress from the thymus and move to the epidermal layer of skin (18). The expression of CCR6 is reduced, whereas the expression of sphingosine-1-phosphate receptor 1 (S1P1) is increased on mature Vγ5 T cells, both of which allow mature Vγ5 T cells to exit but retrain immature cells in the thymus (16). Furthermore, the expressions of skin-homing molecules CCR10, CCR4, E, and P selectin ligands, and integrin α_E are also markedly increased on the surface of Vγ5 T cells to help them migrate and reside in the epidermis (16, 19, 20). CCR9, CCR7, and CD62L have low expression on Vγ5 T cells, indicating that Vγ5 T cells are not able to migrate into secondary lymphoid organs (21).

Vγ5 T cells exclusively reside in the murine epidermis and comprise over 90% of murine epidermal T lymphocytes (22). Since the characteristic feature of epidermal Vγ5 T cells is their highly dynamic dendritic morphology, they are named DETCs. DETCs are anchored in the upper epidermis, and most dendrites are immobilized and apical toward keratinocyte tight junctions, while the remaining dendrites are projected to the basal epidermis and extend and contract in a highly mobile state (23). Keratinocytes predominantly express E-cadherin and DETCs express E-cadherin receptor integrin $\alpha_E\beta_7$, especially at the ends of apical dendrites, which assist in anchoring the dendrites of DETCs in the epidermis (23).

HOMEOSTASIS OF DETCs IN THE EPIDERMIS

Dendritic epidermal T cells slowly expand under a steady state to maintain skin homeostasis (23). Vγ5 TCR and Skint-1 signaling are required for DETC homeostasis in the epidermis (17). Several secreted factors also contribute to the maintenance of DETCs. For example, CD122 expressed on DETCs is essential for their proliferation and survival in both fetal thymus and skin (24). IL-15Rα (CD215) is highly expressed on the surface of DETCs (11). IL-15 helps the survival and proliferation of DETCs upon TCR engagement (25). Furthermore, IL-15 can interact with CD122 of DETCs to maintain their localization and homeostasis in the epidermis (25). Importantly, DETCs secrete a small amount of IGF-1 in a steady state to sustain survival and prevent apoptosis of keratinocytes to maintain epidermis homeostasis (4). Liu et al. and Bai et al. recently reported that DETC-derived IGF-1 is positively correlated with keratinocyte-derived IL-15, which is partially controlled by the mTOR signaling pathway (26, 27). In addition, epidermal IGF-1 and IL-15 cooperate to promote the homeostasis of DETCs in diabetic animals (28). CD122 and CD69 are regarded as activation markers on DETCs (23). Vγ5 TCR signals preserve the expression of CD69 and CD122, which help DETCs stay in a state of pre-activation (23).

γδ T CELLS IN THE HUMAN EPIDERMIS

Murine DETCs lack an exact counterpart in humans. γδ T cells have a TCR that expresses the Vδ1 chain reside in both the epidermis and dermis of human skin (29). γδ T cells that exist in peripheral blood express the Vδ2 TCR (30). Cutaneous leukocyte antigen (CLA), which is the ligand for E-selectin and a skin-homing marker, is also expressed on epidermal- and dermal-resident Vδ1 T cells and αβ T cells, while CLA expression on Vδ2 T cells from the blood is low (29). No significant or distinct differences in CLA expression on Vδ1 T cells or αβ T cells exist between the epidermis and dermis. Vδ1 TCR comprise about 10–20% of T cells in the human epidermis and dermis, respectively, while the ratio of Vδ1 T/αβ T cells is less in the epidermis than in the dermis. Epidermal-resident Vδ1 T cells and αβ T cells are activated after acute injury and produce IGF-1 to promote wound repair. However, both Vδ1 T cells and αβ T cells separated from chronic non-healing wounds do not secrete IGF-1, indicating that their function is impaired in chronic wounds compared with T cells isolated from acute wounds (29). Moreover, human blood Vδ2 T cells can be recruited to the skin inflamed with psoriasis. These Vδ2T cells are CLA- and CCR6-positive, secrete proinflammatory cytokines, such as IL-17A, TNF-α, and IFN-γ, and produce psoriasis chemokines, such as IL-8, CCL3, CCL4, and CCL5 (31).

$CLA^+V\delta2$ T cells not only upregulate the production of IGF-1, but also activate keratinocytes dependent on TNF-α and IFN-γ (31). Skint-1 is absent in humans, which may partially explain why the development of TCR chains is different between humans and mice (17).

THE ACTIVATION OF DETCs UPON SKIN INJURY

The activation of DETCs around wound edges is necessary for their proliferation and the secretion of epidermal growth factor during wound healing (3, 4, 32). Once keratinocytes get stressed or damaged, the morphology of DETCs changes from dendritic to round at the wound edge 1 h later (3). TCR complexes locate at the apical dendrite ends under a steady state, but migrate to the basal epidermis upon wounding (23). Functional changes follow the morphological changes, and the expression of IGF-1 is markedly increased in DETCs (4). Differing from αβ T cells, γδ T cells are directly activated by TCR signaling in a non-major histocompatibility complex (MHC)-restricted manner (33). TCRs on DETCs sense some unidentified ligands expressed on damaged keratinocytes after skin injury (34). Apart from TCR signaling, other co-stimulatory molecules [such as NKG2D, junctional adhesion molecule-like protein (JAML), and 2B4] and T cell growth factors have been recently demonstrated to contribute to the activation of DETCs (35–38).

NKG2D

NKG2D is a C-type lectin-like stimulatory receptor, expressed on activated $CD8^+$T cells, macrophages, $NK1.1^+$T cells, and DETCs (39). NKG2D has two alternative splicing isoforms: NKG2D-S (short) and NKG2D-L (long) (40). DETCs constitutively express NKG2D-S, NKG2D-L, and cell surface protein NKG2D (40). NKG2D signals act as co-stimulatory signals for $CD8^+$T cells, or directly trigger cytotoxicity and cytokine production in activated murine NK cells (39). Without TCR engagement, NKG2D signals are sufficient to trigger cytotoxicity and IFN-γ production in DETCs (40). NKG2D ligands, which belong to MHC-class-I related proteins, are expressed under stress conditions, such as infection, tumorigenesis, and tissue damage (41). Retinoic acid early inducible-1 α-ε, mouse UL16-binding protein-like transcript 1, and histocompatibility a-c (H60 a-c) are known NKG2D ligands in mice, and among them H60c has been detected in skin (42). H60c protein is inductively expressed on keratinocytes at wound margins (43). The interaction between NKG2D and H60c is necessary for KGF secretion by DETCs in wound repair (43).

JUNCTIONAL ADHESION MOLECULE-LIKE PROTEIN (JAML)

Junctional adhesion molecule-like protein is expressed on the surface of DETCs (44). Coxsackie and adenovirus receptor (CAR) is induced on damaged keratinocytes and acts as a functional ligand for JAML (44). The JAML–CAR interaction is necessary for the activation of DETCs and cytokine production of TNF-α and KGF-1 (44). Blocking the interaction between JAML and CAR impedes wound healing, suggesting that JAML–CAR provides a costimulatory signal for the activation of DETCs during wound repair (44).

2B4

2B4, a 66-kD glycoprotein, is expressed on NK and T cells and kills tumor targets by non-MHC-restricted mechanisms (45). 2B4 is also detected on DETCs and helps DETCs to mediate cytotoxic killing against skin-derived tumors (46). IL-2 upregulates 2B4 expression and enhances cytotoxic ability of DETCs (46). Whether 2B4 participates in wound healing has not been clarified.

TOLL-LIKE RECEPTOR (TLR) 4

Toll-like receptor 4 is the primary signaling receptor for lipopolysaccharide (LPS) (47). MD2 assists TLR4 for intracellular distribution and accelerates TLR4 for LPS recognition (48). In the steady state, the expression of TLR4-MD2 is lacking on the surface of DETCs, while during cutaneous inflammation, TLR4-MD2 expression is improved on DETCs when they migrate from the epidermis (47). The roles of TLR4-MD2 in wound healing are not known.

IL-15

IL-2 and IL-15 participate in the survival and activation of γδ T cells (25, 49, 50). Both interact with α, β, or γ_c chains of the receptor complexes (50, 51). Although IL-15 is similar to IL-2 in its biological properties and three-dimensional configuration, IL-15 is more important than IL-2 for the survival and proliferation of DETCs upon TCR engagement, because IL-15 and not IL-2 is expressed in the epidermis under a steady state (25). In addition, mature fetal Vγ5 thymocytes and DETCs express the IL-15Rβ chain (CD122) but not IL-2Rα (CD25) (11). Compared to wild-type controls, the number of mature Vγ5 T cells is reduced in the fetal thymus and DETCs are absent in $IL\text{-}15^{-/-}$ mice, while the number of mature Vγ5 T cells is normal in the fetal thymus and DETCs survive in the adult skin of $IL\text{-}2^{-/-}$ mice (25). Therefore, IL-15 rather than IL-2 seems to be necessary for DETC homeostasis in the skin (25, 35). Moreover, activated DETCs secrete IL-15 but fail to produce IL-2, indicating that IL-15 is more important than IL-2 for the efficient activation of DETCs at the early stages of wound healing. Liu et al. and Wang et al. have demonstrated that IL-15 rescues the insufficient activation of DETCs and increases IGF-1 production by DETCs, and IGF-1 in turn induces keratinocytes to secrete IL-15 in diabetic mice (26, 28). Their work indicates that IL-15 and IGF-1 are positively correlated in the wounded epidermis to promote re-epithelialization (**Figure 1**). Furthermore, the regulation of the IGF-1-IL-15 loop partially depends on the mTOR pathway (26–28).

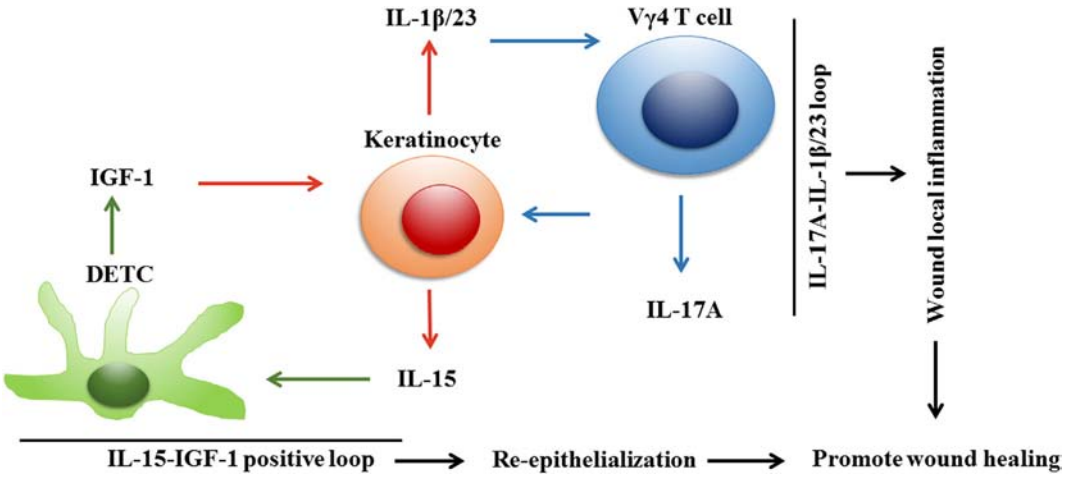

FIGURE 1 | Dendritic epidermal T cells (DETCs), keratinocytes, and Vγ4 T cells constitute two correlated loops to improve wound repair in diabetic mice. Upon skin injury in diabetic mice, keratinocyte-derived IL-15 increases IGF-1 production by DETCs, which in turn enhances keratinocytes to secrete IL-15. A positive correlation between IL-15 and IGF-1 is formed in wounded epidermis, and thereby amplifies IGF-1 production in epidermis for promoting re-epithelialization and wound healing. Meanwhile, keratinocytes could also interact with Vγ4 T cells, which infiltrate in epidermis to form an IL-17A-IL-1β/IL-23 feedback loop to augment local inflammation for efficient skin wound healing. In the wounds of diabetic mice, DETC-mediated IL-15-IGF-1 correlation and Vγ4 T cell-mediated IL-17A-IL-1β/IL-23 loop are coordinated to improve the defects of diabetic wound healing through enhancing re-epithelialization and local inflammation, respectively.

ACTIVATED DETCs PROMOTE RE-EPITHELIALIZATION BY PRODUCING IGF-1 AND KGF-1/2

IGF-1 is primarily produced in the liver, but it is also derived from DETCs in the epidermis. DETCs constitutively generate IGF-1, and keratinocytes express IGF-1R under normal conditions (4). IGF-1 combined with IGF-1R can trigger phosphoinositide 3-kinase and mitogen-activated protein kinase pathways to protect keratinocytes from apoptosis and differentiation (4, 52, 53). Beyond secreting a small amount of IGF-1 in the steady state, DETCs also express IGF-1R to maintain survival in the epidermis *via* an autocrine pathway (4). Phosphorylated IGF-1R is increased at wound margins 24 h after injury, and upregulated IGF-1 protects keratinocytes from apoptosis in damaged areas to assist re-epithelialization (4).

Dendritic epidermal T cells do not secrete KGFs (KGF-1 and KGF-2) in homeostasis conditions, but rapidly produce KGFs upon wounding (3). Keratinocytes constitutively express KGF receptor FGFR2-IIIb, and thus KGFs derived from DETCs can bind FGFR2-IIIb receptor to induce the proliferation and migration of keratinocytes during the re-epithelial phase of wound healing (3, 54). FGFR2-IIIb is not expressed on DETCs, showing that KGFs do not reversely regulate the effector functions of DETCs under stressed conditions (3). DETCs can also secrete TGF-β to aid tissue repair; release GM-CSF XCL1, CCL3, CCL4, CCL5, and hyaluronan to recruit leukocytes to wound sites; and produce IL-17, IFN-γ, and TNF-α to facilitate inflammation (55, 56).

THE DEVELOPMENT OF Vγ4 T CELLS IN THE THYMUS

Vγ4 TCR is rearranged in the late fetal thymus from ED 17 until birth and afterward (57, 58). Vγ4 T cells develop into two main subsets: IL-17A$^+$Vγ4 T cells with the phenotype of CCR6$^+$CD27$^-$, and IFN-γ$^+$Vγ4 T cells with CCR6$^-$CD27$^+$ (59). Certain embryonic thymus conditions are required for γδ T cells to acquire the capacity to produce IL-17A. IL-7 is necessary for the development of γδ T17 cells in the thymus, which can promote the accessibility of the TCR γ locus to V(D)J recombinase and regulate the differentiation of γδ T cells preferentially toward the CD27$^-$IL-17A$^+$ subset (15, 60). CCR6$^+$CD27$^-$γδ T17 cells express the subunit of IL-17A/F receptor IL-17RC, which is not detected on CCR6$^-$CD27$^+$γδ T cells (61). In the absence of IL-17A, CCR6$^+$CD27$^-$γδ T17 cells become overabundant in the thymus and secondary lymphoid organs, indicating that the development and homeostasis of γδ T17 cells is restricted by IL-17A in a negative feedback loop (61). Moreover, transcription factor Sox13 is required for the maturation of IL-17A$^+$Vγ4 T cells in the neonatal thymus, and its mutation is able to protect mice from psoriasis-like dermatitis (62).

Vγ4 T CELLS ARE THE DOMINANT SUBSET OF MURINE DERMAL γδ T CELLS

When exiting the thymus, Vγ4 T cells have obtained stem cell-like properties of self-renewal and are radiation resistant (63). Vγ4 T cells are localized to the secondary lymphoid organs as the dominant subset of murine peripheral γδ T cells, and they are also distributed in the dermal layer of murine skin (63). Vγ4 T cells comprise nearly 50% of dermal γδ T cells, though Vγ1, Vγ5, Vγ6, and Vγ7 T cells also exist in the dermis (64). Vγ4 T cells, as the major γδ T cells in the dermis, are capable of secreting IL-17A and IFN-γ, which play distinctive roles in autoimmune diseases, graft rejection, antiviral immunity, and antitumor responses (6, 10, 33, 65).

Vγ4 T CELLS PROVIDE THE MAJOR SOURCE OF IL-17A AT THE EARLY STAGE OF SKIN INFLAMMATION

Vγ4 T cells have been reported to participate in autoimmune diseases and skin graft rejection at the early stages by producing IL-17A (10, 33, 62, 66). IFN-γ-positive Vγ4 T cells play a protective role in antitumor immunity, but they do not contribute in skin transplantation and wound healing (10, 33, 67). Which cytokine Vγ4 T cells secrete may depend on local circumstances. As it is well-known that Th17 cells are a major source of IL-17A in the adaptive immune response, Vγ4 T cells act as an innate source of IL-17A before Th17 cells play their roles (68). Vγ4 T cells have some features in common with Th17 cells, such as IL-23 receptor, CCR6, and RORγ (68). However, Vγ4 T cells have gained the potent ability to produce IL-17A and express dectin-1 and TLRs when they egress from the thymus and, therefore, they can directly interact with pathogens and secrete IL-17A as the first line of defense against bacterial pathogens (61, 68). Vγ4 T cells also produce IL-17A to induce psoriasis-like skin inflammation, and IL-17A-positive T cells expand promptly in draining lymph nodes when exposed to the inflammatory agent imiquimod (64, 69). Furthermore, we have reported recently that Vγ4 T cells provide a major source of IL-17A in the epidermis at the early stages of wounding. Approximately half of the epidermal IL-17A-positive cells are Vγ4 T cells after skin injury (67). IL-17A production in the epidermis is dramatically decreased after the depletion of Vγ4 T cells in wild-type mice, but it is significantly enhanced in *Tcr*δ$^{-/-}$ animals by the addition of freshly isolated Vγ4 T cells onto wound beds (67). In addition, Vγ4 T cells also migrate to noninflamed skin and peripheral lymph nodes, and respond faster and stronger to a second imiquimod challenge (69). Expanded Vγ4 T cells in lymph nodes can infiltrate back into inflammatory skin *via* S1P1 with similar migratory mechanisms as conventional αβ T cells (70). Of note, we purchased anti-Vγ4 TCR (UC3-10A6) antibody (Ab) from BioXcell to deplete Vγ4 T cells according to previous research (71, 72). However, *in vivo* treatment with both GL3 and UC7-13D5 antibodies against TCR, as identified by Koenecke et al., caused TCR internalization instead of γδ T cell depletion (73). Therefore, we cannot exclude the possibility that Ab treatment cannot eliminate γδ T cells, but instead decreases TCR complexes on the cellular surface.

Vγ4 T CELL-DERIVED IL-17A AND EPIDERMAL IL-1β/IL-23 FORM A POSITIVE FEEDBACK LOOP TO AMPLIFY LOCAL INFLAMMATION AFTER SKIN INJURY

The IL-1β/IL-23-IL-17A axis is critical for the initiation and amplification of inflammatory responses (5, 6, 69, 74, 75). IL-17A has been demonstrated to act upstream to enhance epidermal IL-1β/IL-23 production in a skin graft transplantation model (10). Furthermore, IL-1β and IL-23 production in the epidermis of wound edges is weakened by a deficiency or blockage of IL-17A but enhanced by the addition of rIL-17A (67). Depletion of Vγ4 T cells reduces epidermal IL-1β/IL-23 production, but supplementing wild-type rather than *Il-17a*$^{-/-}$ Vγ4 T cells onto wound beds promotes epidermal IL-1β/IL-23 production in *Tcr*δ$^{-/-}$ mice (67). Therefore, we regard that IL-17A secreted by Vγ4 T cells and IL-1β/IL-23 derived from epidermal cells may form a positive feedback loop in the epidermis around wounds to amplify local inflammation after skin injury. In addition, IL-17A-producing γδ T cells express high levels of CCR6 on their surface and are recruited by CCL20 to inflammatory sites (76). CCL20 neutralization dramatically decreases the infiltration of Vγ4 T cells into the epidermis around wounds and reduces epidermal IL-17A production. Together, IL-1β/IL-23 and CCL20 have the ability to amplify IL-17A production by Vγ4 T cells and thereby exacerbate local inflammation in the epidermis after skin injury (**Figure 2**). However, the capability of Vγ4 T cells to produce IL-17A can be repressed by B and T lymphocyte attenuator, which decreases the accumulation of Vγ4 T cells in imiquimod-induced inflammatory skin and draining lymph nodes (77). Whether IL-17A production by Vγ4 T cells is negatively regulated by BLTA in wound healing needs further investigation.

IL-17A IS REQUIRED FOR EFFICIENT SKIN WOUND HEALING, BUT EXCESSIVE IL-17A RETARDS WOUND REPAIR

IL-17A is an important pro-inflammatory cytokine that plays a critical role in the initiation and amplification of inflammation responses. IL-17A is required for efficient skin wound healing. *Il-17a*$^{-/-}$ mice exhibit defects in wound repair, which can be restored by the addition of rIL-17A and IL-17A-producing DETCs (9). Moreover, IL-17A production is reduced in skin around wounds of diabetic mice, and IL-17A-positive Vγ4 T cells transferred to the wound bed can improve wound healing (78). In addition, DETCs provide a source of epidermal IL-17A after skin injury, which accelerates wound healing by inducing epidermal keratinocytes to express the host-defense molecules β-defensin 3 and RegIIIγ (9). However, Rodero et al. reported a contradictory role for IL-17A in skin wound repair and found that the application of an IL-17A-neutralizing Ab onto the wound bed significantly promoted wound healing (8). To reconcile these conflicting roles of IL-17A in skin wound healing, Li et al. blocked IL-17A with an overdose of neutralizing Ab (200 μg/wound) in wound margins, which led to defective skin wound closure, indicating that IL-17A is essential for efficient wound healing. However, the addition of a moderate dose of anti-IL-17A neutralizing Ab (20 μg/wound) significantly improved skin wound repair. In addition, a high dose of rIL-17A (200 ng/wound) rather than low or medium doses (2 or 20 ng/wound) injected into the wound bed prominently delayed skin wound healing, suggesting that excessive IL-17A has a negative impact on skin wound repair (67). These facts strongly suggest that IL-17A plays dual roles: moderate IL-17A

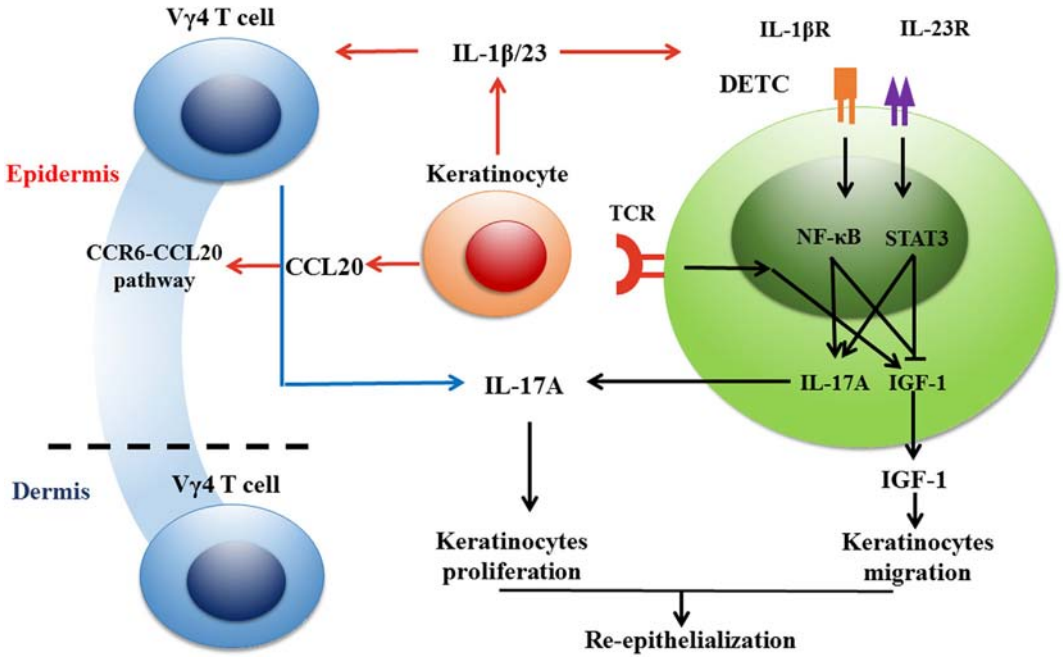

FIGURE 2 | Vγ4 T cells inhibit the production of IGF-1 in dendritic epidermal T cells (DETCs) *via* IL-17A-IL-1β/IL-23 loop and thereby delay wound healing in normal mice. Once keratinocytes get stressed or damaged, DETCs are activated quickly through TCRs by sensing some as-yet-unknown antigens expressed by damaged keratinocytes in a non-major histocompatibility complex restricted manner. Dermal Vγ4 T cells are attracted to the epidermis around wounds by CCL20, which is increased in wounded epidermis, and provide an early source of IL-17A to delay skin wound healing. IL-17 secreted by Vγ4 T cells inhibits IGF-1 production by DETCs in an indirective way where IL-1β and IL-23 produced by keratinocytes act as the bridge between them. IL-1β and IL-23 are key cytokines to suppress IGF-1 production by DETCs. Besides, Vγ4 T cell-derived IL-17A facilitates keratinocytes to secrete IL-1β and IL-23, and thus forms a positive feedback with keratinocyte-derived IL-1β and IL-23. It is worthy to note that IL-1β is more effective than IL-23 in our investigation. NF-κB pathway plays a crucial role in IL-1β-suppressed IGF-1 expression in DETCs. In conclusion, for wounds in normal mice, Vγ4 T cell-mediated IL-17A-IL-1β/IL-23 loop has a negative impact on IGF-1 production by DETCs and thereby delays skin wound closure.

is required for efficient skin wound healing, but excessive IL-17A dampens skin wound closure. We consider that these dual roles do not coexist at the same time, but rather depend on the concentration of IL-17A under the circumstances. Mild amounts of IL-17A at the wound edge re-establish the antimicrobial skin barrier after skin injury by inducing epidermal keratinocytes to express antimicrobial peptides and proteins, but superfluous IL-17A induces the IL-1β/IL-23-IL-17A loop to amplify local inflammation, thus inhibiting wound repair.

Vγ4 T CELLS INHIBIT IGF-1 PRODUCTION OF DETCs TO DELAY SKIN WOUND CLOSURE THROUGH IL-17A

Vγ4 T cells secrete IL-17A to delay wound repair, but IL-17A fails to directly affect the pro-healing function of DETCs, as IGF-1 expression by DETCs is not able to be directly reduced by IL-17A (67). However, epidermal IL-1β and IL-23 are key factors for the suppression of IGF-1 in DETCs. Taken together with the positive loop of IL-17A and IL-1β/IL-23, it is very likely that IL-1β and IL-23 act as the bridge between Vγ4 T cells and DETCs. Furthermore, IL-1β and IL-23 notably promote the phosphorylation of NF-κB and STAT3 and facilitate their translocation from the cytoplasm to the nucleus in DETCs. However, IL-1β shows more significant effects on DETCs than IL-23, which only exhibits synergic inhibition with IL-1β (67). Therefore, we consider that IL-17A produced by Vγ4 T cells indirectly impedes DETCs to secrete IGF-1 and delays skin wound closure with mediators IL-1β and IL-23 (**Figure 2**).

A POTENTIAL FUNCTIONAL LINK BETWEEN Vγ4 T CELLS, EPIDERMAL CELLS, AND DETCs IN SKIN WOUND HEALING

Upon skin injury, epidermal cells interact with DETCs to form an IL-15-IGF-1 loop to amplify IGF-1 production in the epidermis for re-epithelialization (26, 28). Meanwhile, epidermal cells can also interact with epidermis-infiltrating Vγ4 T cells to form an IL-17A-IL-1β/IL-23 loop to augment local inflammation (78). In diabetic wounds, the DETC-mediated IL-15-IGF-1 loop and Vγ4 T cell-mediated IL-17A-IL-1β/IL-23 loop improve the defects of diabetic wound healing by enhancing re-epithelialization and local inflammation, respectively (**Figure 1**). However, in normal wounds, the Vγ4 T cell-mediated IL-17A-IL-1β/IL-23 loop has a negative impact

on IGF-1 production by DETCs and thereby delays skin wound closure (**Figure 2**). This indicates that a balance exists between Vγ4 T cell-derived IL-17A and DETC-derived IGF-1 for optimal skin wound healing.

Some interesting issues need to be further investigated in the near future: the precise underlying mechanisms of IL-1β and IL-23 inhibition of IGF-1 production in DETCs, and the influence of co-stimulatory molecules on the two loops during wound healing. Whether epidermal stem cells are involved in the regulation of IL-17A and IGF-1 during re-epithelialization, and how IL-17A and IGF-1 play roles in the homeostasis, migration, proliferation, and differentiation of epidermal stem cells still remains unknown.

AUTHOR CONTRIBUTIONS

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Myeloid-Derived Suppressor Cells Specifically Suppress IFN-γ Production and Antitumor Cytotoxic Activity of Vδ2 T Cells

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γδ T cells represent less than 5% of circulating T cells; they exert a potent cytotoxic function against tumor or infected cells and secrete cytokines like conventional αβ T cells. As αβ T cells γδ T cells reside in the typical T cell compartments (the lymph nodes and spleen), but are more widely distributed in tissues throughout the body. For these reasons, some investigators are exploring the possibility of immunotherapies aimed to expand and activate Vδ2 T cells, or using them as Chimeric Antigen Receptor carriers. However, the role of immunosuppressive microenvironment on Vδ2 T cells during infections and cancers has not been completely elucidated. In particular, the effects of myeloid-derived suppressor cells (MDSC), largely expanded in such pathologies, were not explored. In the present work, we demonstrated that MDSC may inhibit IFN-γ production and degranulation of phosphoantigen-activated Vδ2 T cells. Moreover, the Vδ2 T cells cytotoxic activity against the Burkitt lymphoma cell line Daudi and Jurkat cell line were impaired by MDSC. The Arginase I seems to be involved in the impairment of Vδ2 T cell function induced by both tumor cells and MDSC. These data open a key issue in the context of Vδ2-targeted immunoteraphy, suggesting the need of combined strategies aimed to boost Vδ2 T cells circumventing tumor- and MDSC-induced Vδ2 T cells suppression.

Keywords: γδ T cells, myeloid-derived suppressor cells, antitumoral activity, immunotherapy, IFN-γ

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INTRODUCTION

γδ T cells represent between 2 and 10% of total circulating CD3+ T lymphocytes (1). Among peripheral CD3+ γδ T cells, those expressing a TCR formed by the Vγ9 and Vδ2 variable regions (hereafter referred to as Vδ2 T cells) constitute up to 90% of γδ T cells (2). These Vδ2 T cells specifically recognize non-peptidic phosphorylated metabolites of isoprenoid biosynthesis, present in most pathogenic bacteria (3), or isopentenyl pyrophosphate, produced also by the human mevalonate biosynthesis pathway (4) that get accumulated in virus-infected and cancer cells due to alterations in the mevalonate pathway. Vδ2 T cells are characterized by high plasticity in their functions: they have the ability to produce pro-inflammatory cytokines, such as IFN-γ, TNF-α (5), and IL-17 (6) and also act as professional antigen-presenting cells (7). Further, Vδ2 T cells act as a bridge between

the innate and adaptive immune response, inducing dendritic cell (DC) maturation and shaping the Th1 and cytotoxic CD8 T cells response (8). Stimulated Vδ2 T cells also display strong cytolytic activity toward allogeneic tumors of various tissue origins. In particular, Vδ2 T cells are able to infiltrate murine and human tumors, to recognize tumor antigens, and to secrete cytotoxic molecules like granzyme and perforin (9, 10). The wide antitumor activity as well as their ability to shape both innate and adaptive immune response in the tumor microenvironment make these cells as good target in immunomodulating strategies in cancer settings. Several clinical trials of Vδ2 T cell-based immunotherapy performed for both hematological malignancies and solid tumors show promise for cellular therapy to control cancer (11); however, the effectiveness of Vδ2 T cell-based therapy is limited, probably due to suppressive function of the tumor microenvironment.

Myeloid-derived suppressor cells (MDSC) are potently immunosuppressive myeloid cells that accumulate in patients with advanced cancers (12), and in various disease settings, including chronic inflammation, viral infections (such as HIV infection), and autoimmune diseases (13–16). At present, it is well known that there are at least two main subsets of MDSC: the monocytic (Mo-MDSC) and the granulocytic (PMN-MDSC) subsets. In particular, in humans PMN-MDSC are identify as CD14− CD11b+ HLA-DRlow/− CD15+ (or CD66+), while Mo-MDSC as CD11b+ CD14+ HLA-DRlow/− CD15− (17), both subsets express CD33 myeloid marker. MDSC suppress T cell activation and cytotoxicity, induce the differentiation and expansion of Tregs, and inhibit NK cell activation by using different mechanisms [reviewed in Ref. (18)]. However, little is known about the suppressive activity of MDSC on Vδ2 T cells. It has been demonstrated that during HIV infection PMN-MDSC are expanded and their frequency is inversely correlated with the capacity of Vδ2 T cells to produce IFN-γ. However, differently from αβ T cells, *in vitro* PMN-MDSC depletion did not completely restore IFN-γ production by Vδ2 T cells from HIV patients (13), suggesting that during HIV infection PMN-MDSC are not the unique player in dampening Vδ2 T cell response. Thus the exact role of MDSC in regulating Vδ2 T cells functions remains to be elucidated. Aim of the present work was to shed light on the effects of the suppressive capacity of MDSC on Vδ2 T cells capabilities.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMC) Separation

PBMC were obtained from buffy coats kindly provided from S. Camillo Hospital. According to NIH definition (https://humansubjects.nih.gov), this study does not require Ethical Committee approval. PBMC were isolated from peripheral blood by density gradient centrifugation (Lympholyte-H; Cederlane). After separation, PBMC were resuspended in RPMI 1640 (EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (EuroClone), 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid) and with 2 mmol/L penicillin and 50 μg/mL streptomycin (EuroClone), hereafter indicated as R10.

Cell Subsets Purification

Vδ2 T cells and PMN-MDSC frequency were analyzed before cell sorting. To obtain a better purification, when Vδ2 T cell and PMN-MDSC percentages were higher than 2 and 3%, respectively, we proceeded to cell sorting as follow: γδ T cells were purified from PBMC using a magnetic negative selection (TCRγδ + T cells isolation kit, Miltenyi Biotec) according to manufacturer's instruction. PMN-MDSC isolation from PBMC using CD15 microbeads (Miltenyi Biotec) according to manufacturer's protocol. The purity of sorted γδ T cells and PMN-MDSC was >85 and 90%, respectively, as verified by flow cytometry (**Figures 1A,B** respectively). Purified cells were rested for 18 h in R10.

Cell Stimulation

Cytokine production and degranulation by γδ T cells was tested by stimulating cells with IPH11 (3 μM, Innate Pharma) in the presence of PMN-MDSC. Brefeldin A was added after 1 h of stimulation. After 18 h, IFN-γ and CD107a were evaluated by flow cytometry. Titration of PMN-MDSC/γδ T cells ratio was performed (2:1, 1:1, 1:2, 1:5) evaluating CD107a expression.

Cytotoxic ability of Vδ2 T cells was analyzed using Daudi and Jurkat cell lines as targets and measuring annexin V binding to apoptotic cells. Briefly, titration of Daudi cells and Jurkat cells/γδ T cells ratios were performed (2:1, 1:1, 1:2, 1:5, 1:10 and 1:1, 1:2, 1:5, 1:10, 1:20, respectively) testing the binding of annexin V (Annexin V-FITC Apoptosis Detection Kit, eBiosciences) to apoptotic Daudi or Jurkat cells by flow cytometry after 18 h of culture. The percentage of killing was calculated as follow: [100% − (% of living Daudi (or Jurkat) cells in the presence of γδ T cells/% of living Daudi (or Jurkat) cells without γδ T cells) × 100]. Where indicated, specific inhibitors of Arginase I (*N*-Hydroxy-nor-L-arginine, nor-NOHA, 1 mM, Calbiochem), indoleamine 2,3-dioxygenase (1-Methyl-D-tryptophan, 1-MTD, 1 mM, Sigma-Aldrich) or nitric oxide synthases (*N*-Monomethyl-L-arginene, Monoacetate Salt, L-NMMA, 1 mM, Calbiochem) were used.

In order to evaluate MDSC capacity to inhibit T cell proliferation, PBMC or purified γδ T cells were labeled with CFDA-SE (Vibrant CFDA SE cell tracer kit, Invitrogen) according with manufacturer's instruction. Labeled PBMC were then cultured with purified PMN-MDSC at 1:1 ratio and stimulated with *Staphylococcus* enterotoxin B (SEB, 200 ng/mL, Sigma-Aldrich). CFDA-SE labeled purified γδ T cells were seeded with PMN-MDSC (1:1 ratio) and activated with IPH 11 (3 μM, Innate Pharma) or with the Burkitt lymphoma cell line Daudi (2:1 ratio effector:target) and IL-2 (100 U/mL, Sigma-Aldrich). Cells were maintained at 37°C in humidified air with 5% CO_2. After 5 days, lymphocytes proliferation was evaluated by flow cytometry.

Flow Cytometry

The Vδ2 T cells and PMN-MDSC frequency and phenotype were evaluated utilizing the following monoclonal antibodies: anti-Vδ1 (Life technology), anti-NKG2A, anti-NKG2D (Beckman Coulter), anti-NKG2C (R&D system), anti-Vδ2, anti-CD3, anti-CD15, anti-CD33, anti-HLA-DR, cocktail of antibodies anti-CD3,

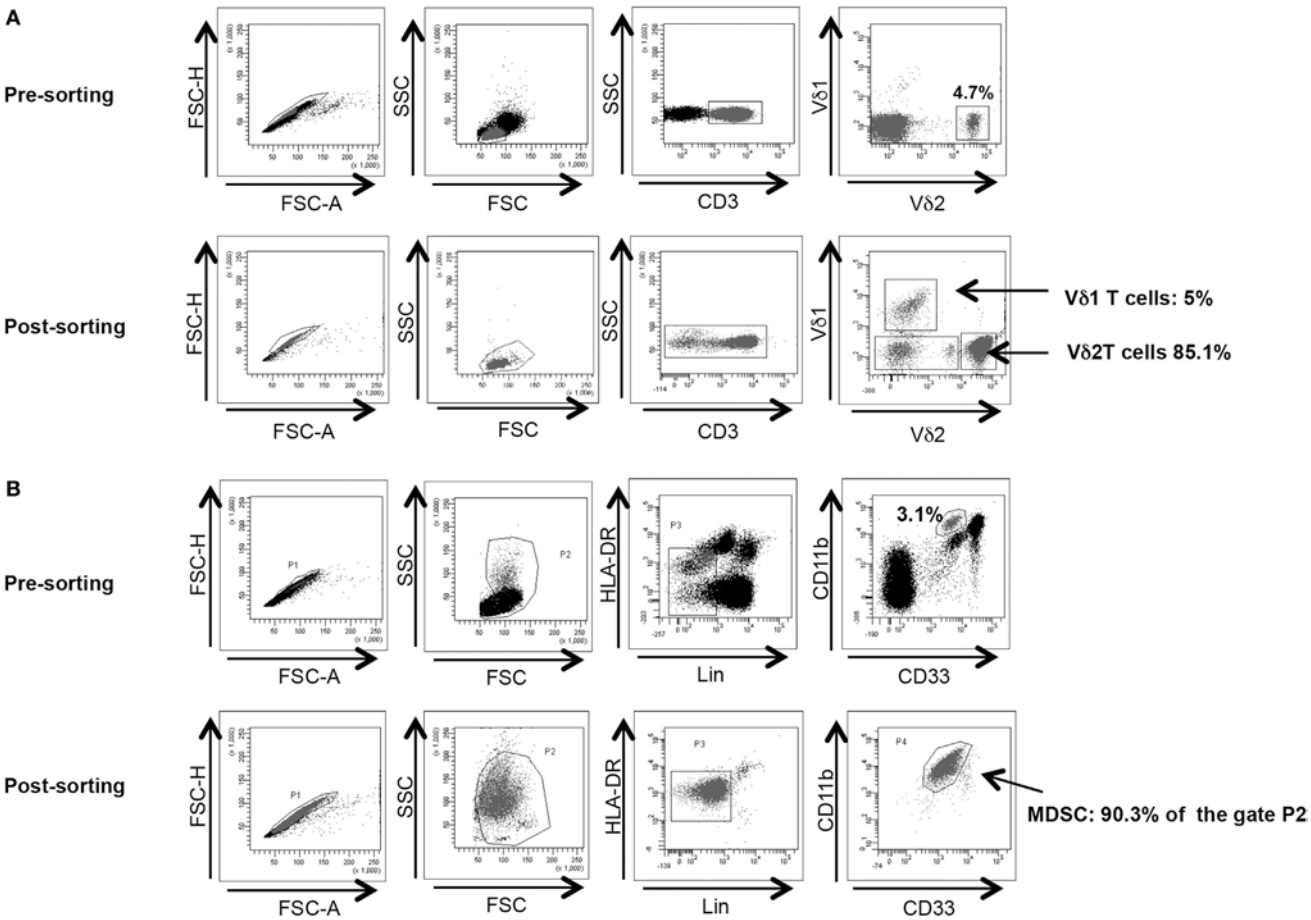

FIGURE 1 | γδ T cell and PMN-myeloid-derived suppressor cells (MDSC) purity after magnetic sorting. **(A)** Representative purity of γδ T cells before and after sorting. Gating strategy is shown. **(B)** Representative purity of PMN-MDSC before and after sorting. Gating strategy is shown.

-CD56, -CD19, anti-CD14, anti-CD11b (BD Biosciences). In brief, the cells were washed twice in PBS, 1% BSA, and 0.1% sodium azide and were stained with the mAbs for 15 min at 4°C. The cells were then washed and fixed with 1% paraformaldehyde and analyzed using a FACS Canto II (Becton Dickinson). For intracellular staining, membrane staining was performed as above described. After fixation cells were incubated with anti-IFNγ (BD Biosciences) for 30 min at room temperature. CD107a detection was accomplished by antibody staining during cell stimulation. After washing cells were analyzed using a FACS Canto II (Becton Dickinson). Apoptosis induction of Daudi cells were accomplished by evaluating Annexin V ligation to Daudi (Annexin V-FITC Apoptosis Detection Kit, eBiosciences) following the manufacturer's instruction. Then cells were stained with anti-CD19, anti-Vδ2, anti-CD3, anti-CD15.

Statistical Analysis

Results were evaluated using a paired *t* test. A p value < 0.05 was considered statistically significant. GraphPad Prism software (version 4.00 for Windows; GraphPad) was used to perform the analysis and graphs.

RESULTS

Vδ2 T Cells Are Partially Inhibited by PMN-MDSC

It has been demonstrated that MDSC are able to inhibit T cell activity, but little is known about MDSC/Vδ2 T cell relationship. To address this issue PMN-MDSC and γδ T cells were magnetically purified (purity >90 and >85%, respectively, **Figures 1A,B**) and were cocultured at different ratios. The ability of MDSC to modulate Vδ2 T cell cytotoxicity and IFN-γ production was evaluated by analyzing the expression of CD107a or IFN-γ on Vδ2 T cells after 18 h. In two preliminary experiments, we optimize the Vδ2/MDSC ratio by looking at CD107a modulation on Vδ2 T cells. As shown in **Figure 2A**, PMN-MDSC partially inhibit the capacity of Vδ2 T cells to express CD107a in response to IPH stimulation at all ratios (**Figure 2A**). Therefore, the γδ T cells/PMN-MDSC 1:1 ratio has been used in subsequent five independent experiments, confirming that PMN-MDSC were able to decrease CD107a expression on Vδ2 T cells (**Figures 2B,C**). We also tested the capability of PMN-MDSC to interfere with IFN-γ production.

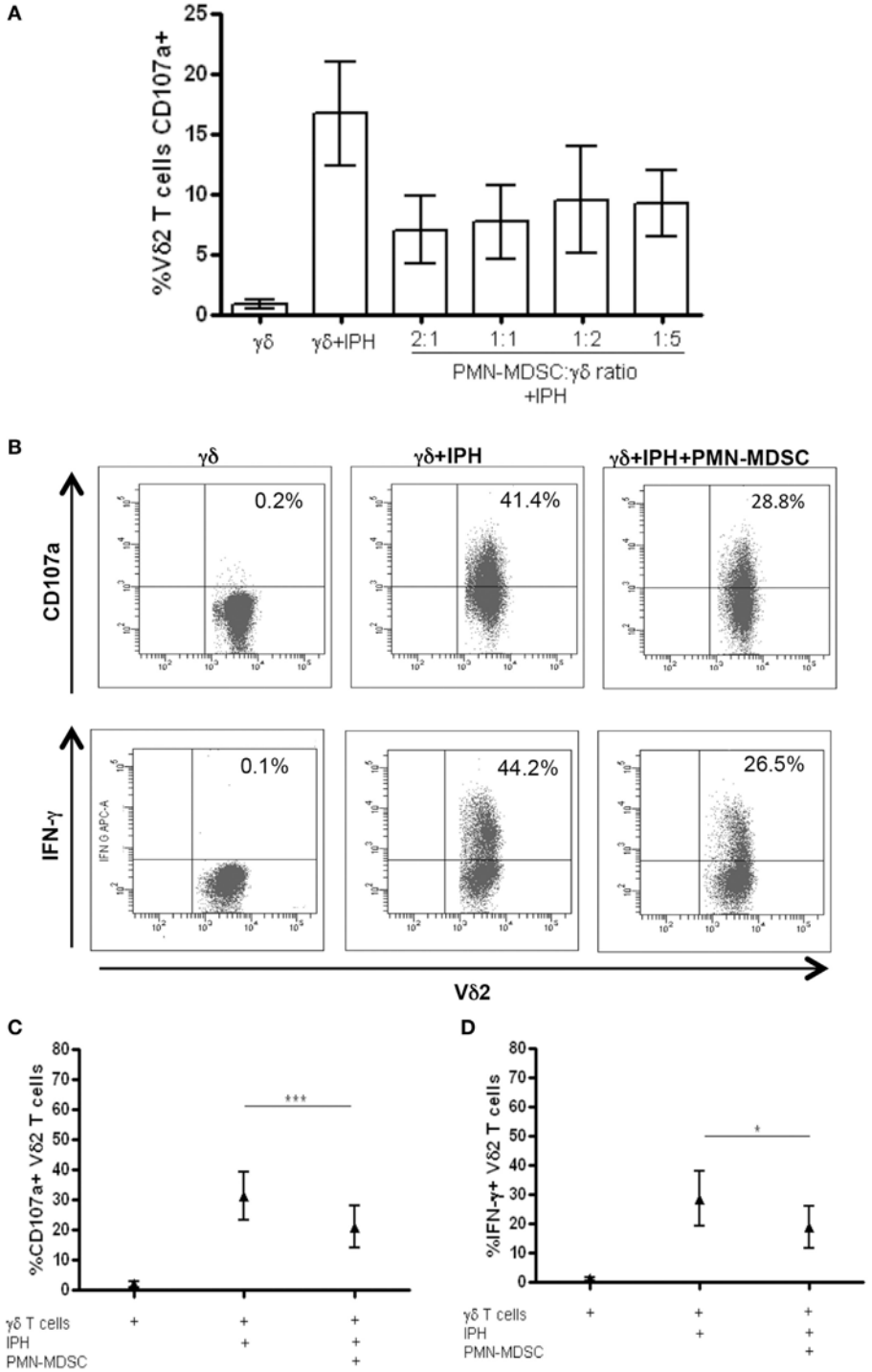

FIGURE 2 | PMN-myeloid-derived suppressor cells (MDSC) inhibit IFN-γ production and CD107a expression by Vδ2 T cells. Purified γδ T cells were stimulated with IPH and IL-2 in the presence of PMN-MDSC and after 18 h IFN-γ and CD107a were evaluated by flowcytometry. **(A)** Titration of PMN-MDSC/γδ T cells ratio tested by measuring CD107a expression on Vδ2+ T cells after IPH stimulation by flowcytometry. Results are shown as mean + SEM of two independent experiments. **(B)** Representative dot plots of IFN-γ production and CD107a expression by Vδ2 T cells. **(C)** Percentage of CD107a + Vδ2 T cells and **(D)** IFN-γ + Vδ2 T cells and in the indicated conditions. Results are shown as Mean + SEM of five independent experiments (*$p < 0.05$, ***$p < 0.0001$).

To this aim, we cultured purified PMN-MDSC and γδ T cells at 1:1 ratio and after 18 h of stimulation with IPH the production of IFN-γ was evaluated by flow cytometry. A decrease of IFN-γ expression was observed in the presence of PMN-MDSC (**Figures 2B,D**), suggesting that PMN-MDSC may also inhibit cytokine production capacity of Vδ2 T cells.

We wondered whether PMN-MDSC may impair the proliferation capability of Vδ2 T cells. To this aim, purified CFDA-SE labeled Vδ2 T cells were stimulated with IPH and IL-2 for 5 days and the proliferation rate was evaluated by flow cytometry. We found that PMN-MDSC did not impact the proliferation of Vδ2 T cells (**Figure 3A**). On the contrary, as previously demonstrated (19) they are able to inhibit the CD3+ T cell proliferation, as SEB-induced proliferation of CD3+ T cells (evaluated using CFDA-SE labeled total PBMC) was decreased by PMN-MDSC (**Figure 3B**).

PMN-MDSC Inhibit Vδ2 T Cells Cytotoxicity Against Tumor Cell Lines

Vδ2 T cells play a pivotal role in controlling tumor cells exerting a potent cytotoxic function. We wondered if the PMN-MDSC-induced down-modulation of CD107a was correlated to a decrease cytolytic capability towards tumor cells. To answer this question, we first cultured Daudi cells, a Burkitt's lymphoma known to be a target for Vδ2 T cell cytotoxicity, with purified γδ T cells at different ratios (to select the best ratio) and the cytotoxic activity of Vδ2 T cells was evaluated by analyzing membrane Annexin V ligation on apoptotic Daudi cells by flow cytometry. We found that 1:2 ratio was sufficient to induce Daudi cell apoptosis (**Figures 4A,B**). Next, Daudi cells were cultured with purified γδ T cells and PMN-MDSC (1:2:2 ratio) for 18 h and apoptotic Daudi cells were analyzed. As expected, we found that Vδ2 T cells are able to kill Daudi cells; however, the presence of PMN-MDSC strongly inhibit their cytotoxic function (**Figure 4C**). We performed the same experiments with a different tumor cells line (Jurkat), and after assessing the ratio sufficient to induce Jurkat cell apoptosis (1:5, **Figure 4C**), a decresed capacity of Vδ2 T cells to kill Jurkat were also observed when PMN-MDSC (1:5:5) were added to the culture (**Figure 4E**). These data indicate that PMN-MDSC inhibition of cytotoxic activity of Vδ2 T cells is not restricted towards Daudi cells. Vδ2 T cell cytotoxicity was deeply controlled by NK receptors expression (20). To evaluate whether MDSC-induced inhibition of Vδ2 T cell cytotoxicity could be mediated by NKRs modulation, the expression of NKG2A, NKG2C, and NKG2D on Vδ2 T cells was evaluated in the absence and in the presence of PMN-MDSC. Results showed that PMN-MDSC did not alter the expression of NKG2A (66.2 ± 8.6 vs 66.3 ± 8.4%), NKG2C (mfi 772 ± 82 vs 748 ± 101), and NKG2D (mfi 1,467 ± 414 vs 1,548 ± 560) on Vδ2 T cells. Finally, Daudi cells were able to induce Vδ2 T cell proliferation in the presence of IL-2, and the proliferation rate of Vδ2 T cells was not affected by PMN-MDSC (**Figure 5**), confirming that PMN-MDSC do not impair Vδ2 T cells proliferating capacity.

Specific Inhibitor of ArgI Restore γδ T Cells Cytotoxic Activity Against Daudi Cells

To understand the mechanisms used by PMN-MDSC to inhibit γδ T cells cytotoxic activity, we cultured Daudi cells with purified γδ T cells and PMN-MDSC (1:2:2 ratio) in the presence of specific inhibitors of Arginase I (nor-NOHA), indoleamine

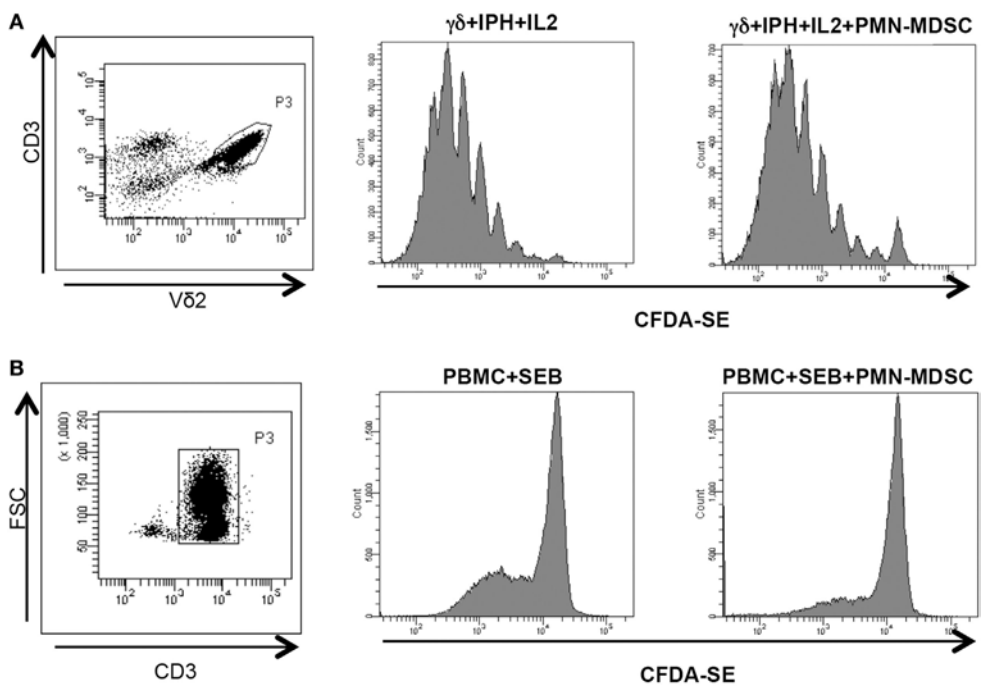

FIGURE 3 | PMN-myeloid-derived suppressor cells (MDSC) does not inhibit Vδ2 T cell proliferation. γδ T cells or peripheral blood mononuclear cell (PBMC) labeled with CFDA-SE were stimulated with IPH in the presence of PMN-MDSC (1:1 ratio). After 5 days, Vδ2+ and CD3+ T cells proliferation was evaluated by flow cytometry. Representative gating strategy and histogram plots of one out of three independent experiments showing Vδ2 T cells **(A)** and CD3+ T cells **(B)** proliferation in the indicated conditions.

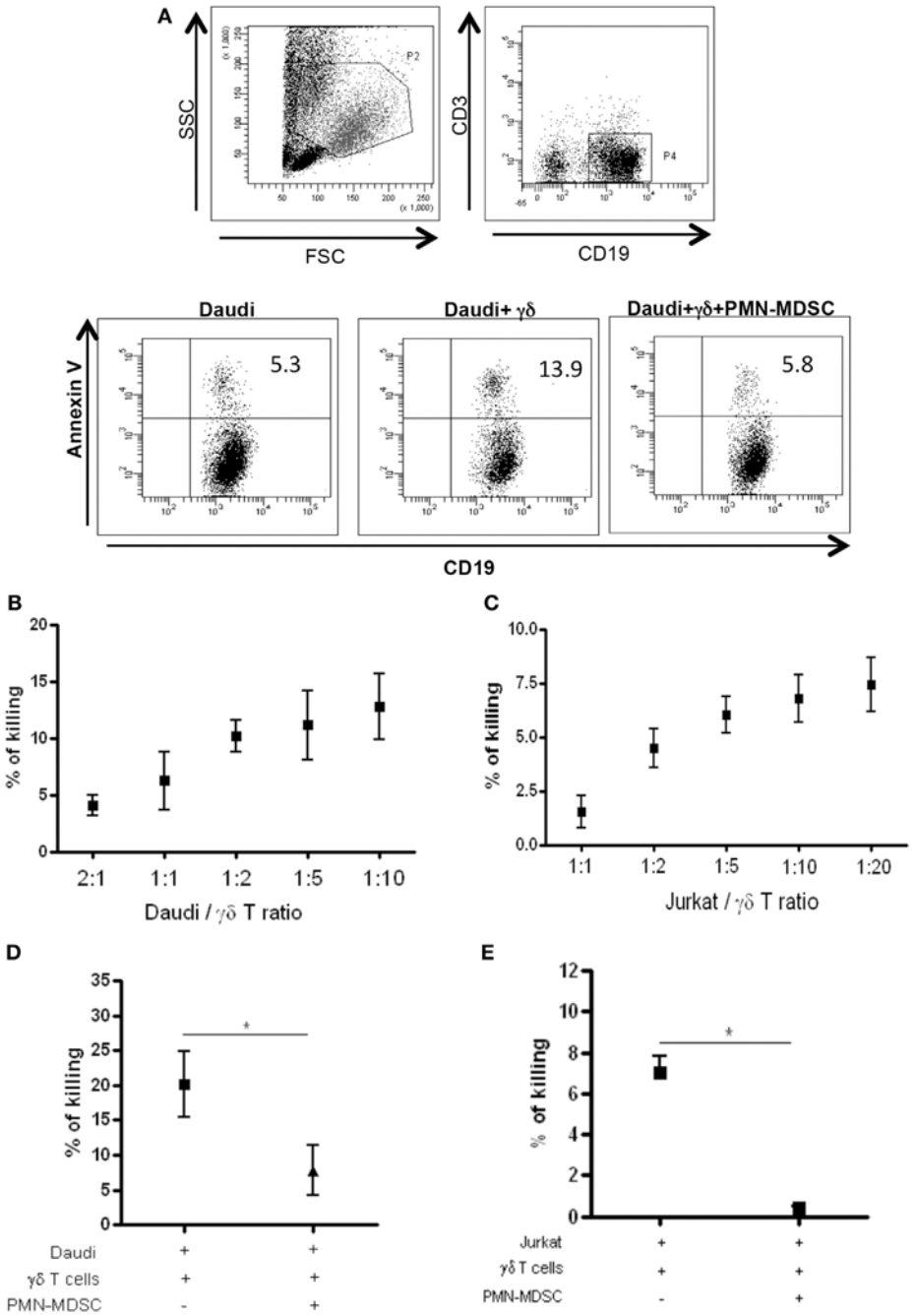

FIGURE 4 | PMN-myeloid-derived suppressor cells (MDSC) inhibit Daudi and Jurkat cells killing by Vδ2 T cells. Daudi and Jurkat cells were cultured with purified γδ T cells and PMN-MDSC, and γδ T cells induced apoptosis were evaluated by measuring annexin V ligation to apoptotic cells. **(A)** Representative dot plots showing gating strategy and Annexin V + Daudi cells in the indicated conditions (1:2:2 ratio). **(B)** and **(C)** Titration of Daudi and Jurkat cells/γδ T cells ratio by measuring the percentage of killing of target cells by flowcytometry. Results are shown as mean + SEM of three independent experiments. **(D)** Percentage of killing of Daudi cells by γδ T cells in the presence of PMN-MDSC (1:2:2 ratio). Results are shown as Mean + SEM of four independent experiments. **(E)** Percentage of killing of Jurkat cells by γδ T cells in the presence of PMN-MDC (1:5:5 ratio). Results are shown as Mean + SEM of three independent experiments (*$p < 0.05$).

2,3-dioxygenase (1-MTD) or nitric oxide sinthases (L-NMMA). We found that 1-MTD and L-NMMA did not affect the capacity of PMN-MDSC to decrease cytotoxic function of γδ T cells (data not shown), suggesting that indoleamine 2,3-dioxygenase and nitric oxide sinthases are not involved. On the contrary, nor-NOHA abrogated the inhibition of cytotoxic function of γδ T cells performed by PMN-MDSC (**Figures 6A,B**). However, an enhancement of of γδ T cells cytotoxicity was also observed in the

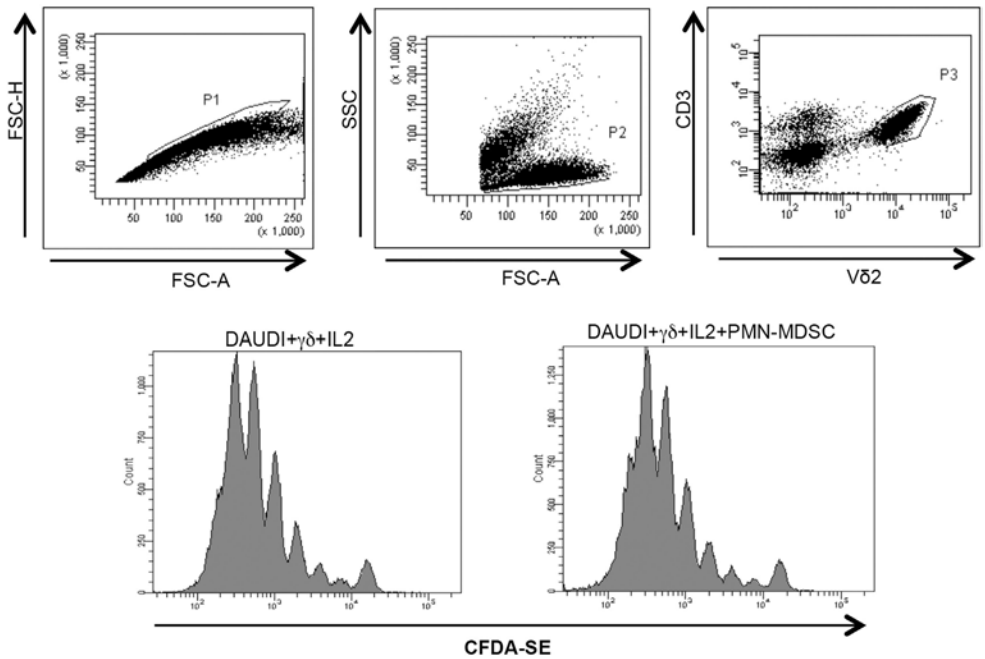

FIGURE 5 | PMN-myeloid-derived suppressor cells (MDSC) does not inhibit Daudi-induced Vδ2 T cell proliferation. Purified γδ T cells labeled with CFDA-SE were stimulated with Daudi cells and IL-2 in the presence of PMN-MDSC. After 5 days, Vδ2+ T cells proliferation was evaluated by flow cytometry. Representative histogram plots of one out of three independent experiments showing Vδ2 T cells proliferation in the indicated conditions.

absence of PMN-MDSC (**Figures 6A,B**), suggesting that Daudi cells may produce ArgI. However, since the addition of PMN-MDSC to nor-NOHA treated γδ T cells cultured with Daudi cells did not decrease the capacity of γδ T cells to kill their targets may suggests that PMN-MDSC used ArgI as well.

DISCUSSION

Vδ2 T cells participate in the early stages of the immune response, in fact they are able to recognize antigens displayed following infection or other forms of stress, and to respond to stress without requiring extensive clonal expansion. Moreover, they are able to shape the subsequent adaptive immunity, representing a bridge between innate and adaptive immune response. Furthermore, Vδ2 T cells crosstalk with different cell types of the innate and adaptive immunity potentiating their activity. Vδ2 T cells are able to give help to B cells in producing antibodies (21), and to induce maturation of DC (22) thus influencing αβ T cell priming. Activated Vδ2 T cells shortly produce huge amount of IFN-γ, TNF-α (5) that play a central role in controlling tumor and infections. A pivotal feature of Vδ2 T cells is the cytotoxic potential against tumor cells (9, 10) and infected cells (23, 24), that participate to control the diseases. However, tumors and microbial pathogens are able to evade the immune system by means different mechanisms. It has been clearly demonstrated that MDSC play a detrimental role during tumor pathologies, and their expansion has been shown during several infections (13, 25, 26). MDSC are able to suppress different cell types [reviewed in Ref. (27)]; however, data on Vδ2 T cells are still lacking. We found that, similarly to αβ T cells (19), PMN-MDSC are able to inhibit IFNγ production by phosphoantigen activated Vδ2 T cells. Differently from αβ T cells, the proliferation of Vδ2 T cells was not affected. It has been suggested that IL-2 may inhibit MDSC functions (28), then we cannot exclude that the use of IL-2 necessary to induce *in vitro* Vδ2 T cell proliferation could alter MDSC suppressive capability. PMN-MDSC induced a decrease of CD107a expression on Vδ2 T cells, indeed the cytotoxic activity against Daudi and Jurkat cells was reduced. The impaired cytotoxicity was independent of the modulation of the major inhibitory and activating NK receptors, suggesting a possible TCR-mediated mechanism.

Myeloid-derived suppressor cell are able to produce different immunosuppressive mediators such as ArgI, iNOS, and IDO, that are all able to induce T cell anergy through different pathways (29). Our data suggest that ArgI may be involved in the inhibition of Vδ2 T cells by PMN-MDSC. Interestingly, ArgI inhibition increased γδ T cells cytotoxity also in the absence of PMN-MDSC, indicating that Daudi cells are able to produce ArgI.

The immunomodulating strategies represent one of the main exciting approaches in the battle against cancer and Vδ2 T cells have been tested as a good target for their wide and potent antitumoral activity. Nevertheless, the suppressive tumor environment could affect these strategies. We showed for the first time that PMN-MDSC inhibit the antitumor activity of Vδ2 T cells, opening a key issue in the context of Vδ2-targeted immunoteraphy. Other studies are mandatory in order to better clarify the molecular mechanisms of these suppression and to propose combined

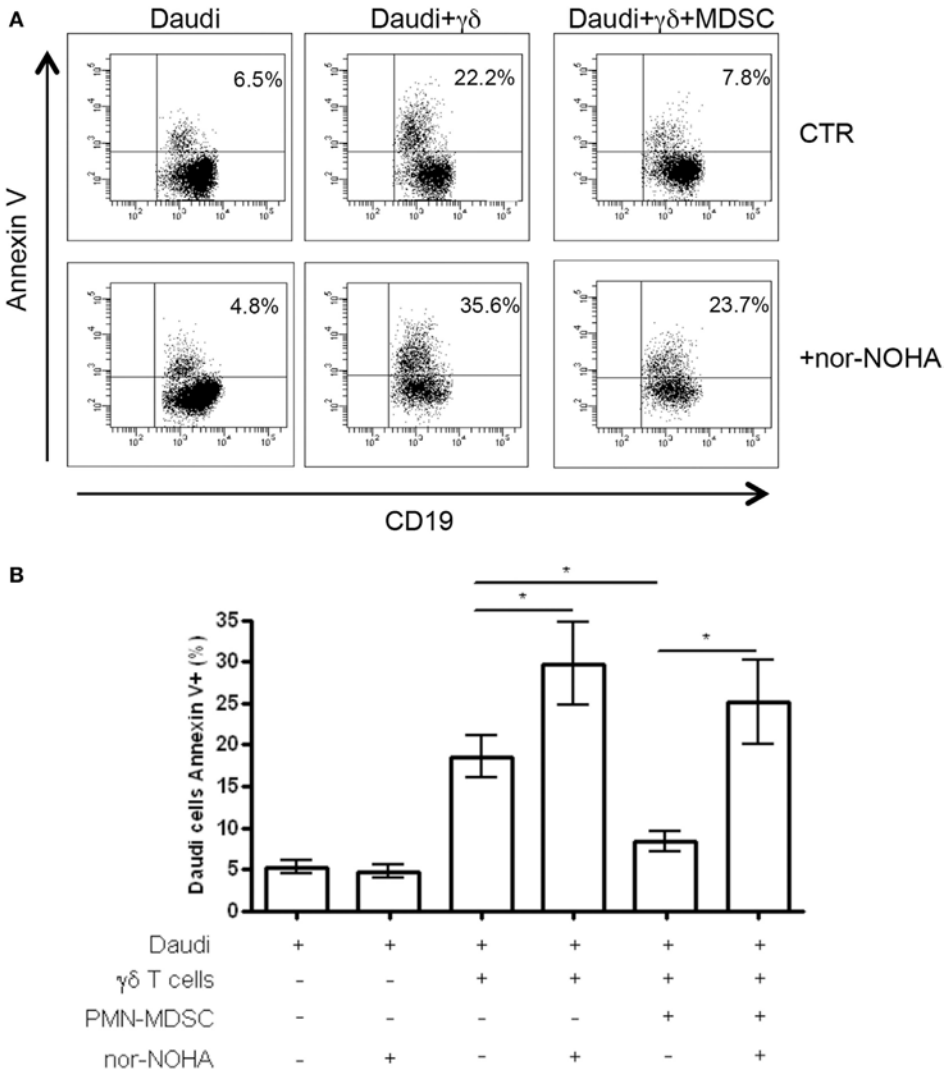

FIGURE 6 | Arg1 is involved in Vδ2 T suppression. Daudi cells were cultured with purified γδ T cells in the presence of PMN-MDSC (1:2:2 ratio) and 1 mM of nor-NOHA. After 18 h apoptotic (Annexin V+) Daudi cells were evaluated by flowcytometry. **(A)** Representative dot plots showing Annexin V + Daudi cells (CD19+) in the indicated conditions. **(B)** Percentage of Annexin V + CD19 + Daudi cells in the indicated conditions. Results are show as mean + SEM of three independent experiments (*$p < 0.05$).

strategies aimed to boost Vδ2T cells circumventing tumor- and MDSC-induced Vδ2 T cells inhibition.

AUTHOR CONTRIBUTIONS

ASacchi and CA designed the study and wrote the paper. NT and ASabatini performed the experiments. ASacchi analyzed the data. EC, VB, RC, and GG contributed to analyze data and revise the paper.

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REVIEW
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Human αβ and γδ T Cells in Skin Immunity and Disease

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γδ T lymphocytes maintain skin homeostasis by balancing keratinocyte differentiation and proliferation with the destruction of infected or malignant cells. An imbalance in skin-resident T cell function can aggravate skin-related autoimmune diseases, impede tumor eradication, or disrupt proper wound healing. Much of the published work on human skin T cells attributes T cell function in the skin to αβ T cells, while γδ T cells are an often overlooked participant. This review details the roles played by both αβ and γδ T cells in healthy human skin and then focuses on their roles in skin diseases, such as psoriasis and alopecia areata. Understanding the contribution of skin-resident and skin-infiltrating T cell populations and cross-talk with other immune cells is leading to the development of novel therapeutics for patients. However, there is still much to be learned in order to effectively modulate T cell function and maintain healthy skin homeostasis.

Keywords: human, γδ T cell, skin immunity, T cell, diabetes, alopecia areata, melanoma, psoriasis

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SKIN AS AN IMMUNOLOGICAL BARRIER

The skin serves as the largest organ in the body and as such provides a barrier against pathogens and regulates physiological changes. This is achieved through the network of cells, extracellular matrix molecules, and accessory organs residing in the complex layers of the skin. Human skin is composed of two compartments that include the epidermis and the dermis. The epidermis is a multilayer barrier composed of differentiating keratinocytes while the dermis is a connective tissue rich in collagen fibers (1). Immune cells including αβ and γδ T cells, and Langerhans cells reside in the epidermis. The dermis hosts a more diverse population of immune cells including: αβ and γδ T cells, dermal dendritic cells (DCs), innate lymphoid cells, plasmacytoid DCs, natural killer T cells, macrophages, mast cells, B cells, and fibroblasts (1). Human skin is estimated to host over 20 billion T cells or one million T cells per square centimeter (2). These T cells are composed of 1–10% γδ T cells with αβ T cells making up the remaining population (2, 3). Together, they mediate processes, such as skin homeostasis, wound repair, and immunity (4–6).

Skin-resident T cells recognize and respond to infected, stressed, or damaged cells by secreting cytokines and growth factors that stimulate cellular proliferation, induce cytolysis, and/or activate other cells to infiltrate the affected region (7–10). While T cells reside in both the epidermis and the dermis, the majority of T cells in normal human skin reside in the dermal–epidermal junction, in appendages, and near blood vessels (2). The epidermal and dermal compartments contain unique phenotypes of resident versus recirculating T cells with the epidermis exhibiting a higher frequency

Abbreviations: DC, dendritic cells; ILCs, innate lymphoid cells; DN, double negative; DETC, dendritic epidermal T cells; LAT, linker activation in T cell; CLA-1, cutaneous lymphocyte antigen-1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion protein 1; MICA, MHC class I related chain A molecules; MICB, MHC class I related chain B molecules; AICD, activation induced cell death; JAML, junctional adhesion molecule-like; B-CLL, lymphocytic leukemia of B-cell type; JAK3, janus kinase 3; T2DM, diabetes mellitus type 2; PD1, programmed cell death protein 1; PDL1, programmed death-ligand 1; LFA-1, lymphocyte function-associated antigen 1.

of CD103$^+$ T resident CD4$^+$ and CD8$^+$ populations (11). CD103 along with β7 integrin bind E-cadherin which is expressed heavily by keratinocytes in the epidermis. Recirculating T central memory cells isolated from the skin express CCR7 and L-selectin, while T migratory memory lack L-selectin (11). Together these receptors provide signals that allow distinct populations of T cells to remain in the skin or recirculate to the blood or lymphatics during normal skin homeostasis.

αβ AND γδ T CELL DEVELOPMENT AND MIGRATION TO THE SKIN

Much of the work to understand T cell development has been performed using mouse models. Epidermal γδ T cells in mice, also known as dendritic epidermal T cells (DETC), seed the epidermis during fetal development in a wave and express a canonical Vγ3Vδ1 TCR (12, 13). It is unclear whether a similar seeding event occurs in humans; however, the majority of skin-resident T cells express the Vδ1 TCR (14, 15). It is important to note that in this review we employ the γδ TCR nomenclature described by Garman et al. (16) and Hayday et al. (17).

Chemokine receptors, cytokines, and adhesion molecules play key roles in T cell homing to the skin. In mice, CCR10 is upregulated on developing γδ T cells in the fetal thymus and required for efficient homing to seed the epidermis (18). In addition, CCR10 is utilized for T cell homing to inflamed skin *via* CCL27 produced by keratinocytes (19). In humans, vitamin D induces T cells to express CCR10 which may play a role in skin retention (20). T cells isolated from human skin also express the chemokine receptor, CCR8. The ligand for CCR8, CCL1, is expressed in the epidermis further suggesting that keratinocytes participate in T cell entry and retention in the skin through the production of chemokines (21). In addition to skin-resident T cells, circulating T cells home to a variety of barrier tissues upon infection and remain there poised for immediate effector functions to protect the organism (22, 23). The CCR6–CCL20 receptor ligand pair plays key roles in activated γδ T cell recruitment to the skin in mice (24). Skin-resident γδ T cells express CCR6, while the ligand, CCL20, can be expressed by keratinocytes, DCs, and endothelial cells. Human epidermal samples normally express low levels of CCL20; however, it is upregulated after an acute injury (25). Thus, CCL20 may act as an indicator of acute injury, initiating recruitment of infiltrating T cells to the epidermis.

The absence of cytokines, such as IL-7, IL-15, and IL-4, in mice results in a reduction/elimination of γδ T cells while IL-10 increases the generation of γδ T cells when present at low concentrations (26–29). These cytokines induce T cell survival and/or proliferation. IL-7R signaling induces rearrangement and transcription of the TCR γ-chain, while IL-15 facilitates γδ epidermal T cell precursor expansion and survival, and IL-4 signaling promotes growth of epidermal γδ T cells (30–33). Thus, critical roles are played by cytokine receptor signaling in γδ T cell development and expansion in sites such as the skin.

Selective recruitment of lymphocytes into human skin is facilitated by the expression of adhesion molecules on the T lymphocytes to ligands in the skin. For example, cutaneous lymphocyte antigen-1 expressed on a subset of human peripheral blood T cells, binds to E-selectin expressed by endothelial cells during inflammation (34). Endothelial cells express other adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1, which also aid in T cell recruitment (35–37). In addition, the integrin CD103 is involved in the recruitment of T cells to the skin and binding to E-cadherin on epidermal cells (38–40). While CD103 is expressed in less than 15% of splenic T cells in mice and less than 3% of T cells in human peripheral blood, it is expressed at much higher rate on murine and human T cells in epithelial tissues (41–43). In mice, CD103 plays key roles in the establishment of γδ epidermal T cell populations as CD103-deficient mice show a significant reduction of γδ epidermal T cells and an impairment in morphology compared to controls (44). Together these chemokine receptors, cytokines and adhesion molecules develop/maintain skin-resident T cell populations and further recruit T cells to sites of inflammation in the skin.

αβ AND γδ T CELL ACTIVATION IN THE SKIN

αβ T cell activation and cytokine production rely on three consecutive signals: TCR ligation, stimulation of costimulatory molecules and cytokine signaling (45–47). These three signals are essential for full functionality of the cell and without proper signaling there is a lack of T cell function, differentiation, proliferation, and survival (48). Co-stimulation is generated through the interaction between costimulatory molecules such as CD28 on the αβ T cell and ligands, such as CD80 and CD86 (46). γδ T cell activation is less understood; however, there are some similarities and differences with αβ T cell activation.

While αβ TCRs rely on MHC presentation of foreign peptides, γδ TCRs recognize some antigens in a manner that is more similar to antibody–antigen interactions (49). The entire repertoire of antigens recognized by γδ T cells is still unknown, yet it is clear that the γδ TCR is required for antigen recognition and the nature of antigen recognition is unique to the TCR expressed by the γδ T cell (49–51). The restricted TCR repertoire of Vγ and Vδ gene segments in both humans and mice leads to speculation that these TCRs recognize conserved self-proteins that become upregulated during stress, damage, or malignancy (52). Human γδ T cells are limited to Vδ1, Vδ2, and Vδ3 expressing populations which are distributed in different locations in the body (**Table 1**). γδ T cell populations have shown the ability to recognize atypical antigens, such as phosphoantigens, stress molecules including MHC class I related chain A molecules and MHC class I related chain B molecule, non-peptide metabolites of isoprenoid biosynthesis, and other unique antigens (53–55). One particular population of Vδ1$^+$ T cells has been shown to recognize CD1 molecules with lipid antigens that are presented by antigen-presenting cells such as DCs (56). Specifically, CD1d is a Vδ1$^+$ T cell ligand in both mice and humans (56, 57). In addition, γδ T cells expressing the Vγ2Vδ2 TCR have the ability to recognize phosphoantigens, which are important products of microbes, such as *Mycobacterium tuberculosis* (58). This suggests that antigen recognition between

TABLE 1 | γδ T cell subsets in humans and their roles in immunopathology.

Subset	Location of highest prevalence	Identified antigen	Features in immunopathology			
			Cancer	Psoriasis	Diabetes	Reference
Vδ1	Barrier tissues	MHC class I related chain A molecule/B, CD1 molecules, sulfatides	Immunosuppressive/ regulatory roles Migrate to tumor site *via* CCR5 and CCR2 Produce IL-17, TNF-α, and IFNγ Kill melanoma cells			(53, 56, 59–63)
Vδ2	Peripheral blood	ULPB4, Phosphoantigens, F1-ATPase, aminoacyl tRNA synthetase	Migrate to tumor site *via* CCR5 and CXCR 3 Express adhesion molecules: lymphocyte function-associated antigen 1, L-selectin, CD44v6	Reduction in circulating CLA+ Vδ2 T cells Migrate to skin Produce TNF-α, IFNγ, IL-17A, and growth factors	Reduction in circulating Vγ2Vδ2 T cells in patients with high BMI Reduced IFN-γ production	(62–66)
Vδ3 (Vδ1−/ Vδ2−)	Peripheral blood, liver	CD1d, CD1c	Increased number in patients with B cell chronic lymphocytic leukemia			(56, 67, 68)

γδ T cells varies among populations and is likely unique to the site, such as the skin, in which they reside.

While co-stimulation is less understood in γδ T cells, CD27 is expressed on most Vγ2Vδ2 T cells and contributes to T cell activation (69). Upon activation, the majority of $CD27^+$ Vγ2Vδ2 T cells produce IFN-γ while IL-17 is rarely produced (69). CD27 signaling also protects against activation induced cell death and increases the expansion of tumor-specific cytotoxic T lymphocytes suggesting a costimulatory role (69). Studies suggest CD2 and ICAM-1 work as costimulatory receptors on $Vδ1^+$ T cells (70). CD2 acts as a cell adhesion and co-stimulatory molecule that binds to CD58, facilitating cell contact and TCR ligation (71). ICAM-1 binds to lymphocyte function-associated antigen 1 inducing intercellular communication and inflammatory responses (72). In mice, the junctional adhesion molecule-like (JAML) is a costimulatory receptor for γδ epidermal T cell activation (73). Resting epidermal T cells express JAML at low levels; however, upon stimulation JAML expression is increased (73). Upon co-stimulation through JAML, epidermal T cells proliferate and produce IL-2, TNFα, and IFNγ (73). In addition another costimulatory receptor, CD100, regulates γδ epidermal T cell responses to keratinocyte damage (74). Ligation of CD100 facilitates the activated "round" morphology of epidermal γδ T cells through ERK kinase and cofilin (74). It will be important to determine whether these costimulatory pathways are also utilized by human skin γδ T cells.

SKIN-RESIDENT T CELL HOMEOSTASIS AND EPIDERMAL MAINTENANCE

Antigen-specific T cells expand during a skin infection and then largely die off leaving a small population of memory cells (11, 75). Recently, these remaining memory T cells have been categorized based on phenotype and function in humans (11). Skin-resident populations include dermal $CD4^+$ $CD103^-$ T cells and epidermal $CD8^+$ or $CD4^+$ T cells that express CD103. The epidermal T cells are less able to proliferate, but exhibit a greater ability to produce IFN-γ and TNF-α (11). Recirculating populations express CCR7 and lack CD69 but breakdown into L-selectin+ central memory cells and L-selectin- migratory memory cells (11). The T migratory memory populations are associated with expanding lesions in patients with cutaneous T cell lymphoma (11).

Memory $CD4^+$, $CCR10^+$, $CCR6^+$, $CCR4^+$ T cells homing to the skin can secrete cytokines that include IL-22, IL-26, and IL-23, which are involved in skin homeostasis (76). IL-22 acts on non-hematopoietic tissue cells of barrier tissues such as keratinocytes and helps regulate cellular differentiation and survival. This suggests IL-22 is involved in maintaining homeostasis of the epithelia (77). IL-23 induces the production of IL-22 and at elevated levels it contributes to a disruption in keratinocyte homeostasis (78). IL-26 has direct and indirect antiviral and antimicrobial properties, yet when not tightly regulated, skin homeostasis can become disrupted causing chronic infections and skin-related diseases (79). It is important to note that in most of these studies the T cell populations were not divided into αβ TCR versus γδ TCR expressing T cells. Thus, future studies will be needed to assess which subset includes the cytokine producing T cells that may be targets for therapeutic interventions.

In the murine epidermis, antigen-specific skin-resident memory $CD8^+$ T cells and DETC adopt a dendritic morphology, increasing the number of cells they contact (75). DETC are distinctive in that they form phosphotyrosine-rich aggregates that keep the cells in a preactivated state (80). This dendritic morphology seems to be particular to the epidermis where receptors such as CD103 and E-cadherin interact, as dermal γδ T cells appear more rounded and are more motile (80, 81). At steady state, epidermal γδ T cells help maintain keratinocyte homeostasis through the production of factors such as insulin-like growth factor-1 (IGF-1) and wound healing through the

production of keratinocyte growth factors (82–84). Mice lacking γδ T cells, TCRδ$^{-/-}$ mice, exhibit delayed wound repair and fewer basal keratinocytes with increased differentiation (84, 85). However, when DETC are added to either the same well as TCRδ$^{-/-}$ skin organ cultures or open wounds of TCRδ$^{-/-}$ mice there is improved wound closure (84, 86).

Human skin-resident T cells have been speculated to also maintain skin homeostasis (5, 87). Both human and murine αβ and γδ skin-resident T cells regulate keratinocytes through the production of IGF-1; demonstrating their ability to influence keratinocyte proliferation and homeostasis (7, 82, 83). Recent work has focused on characterizing skin-resident T cells as compared to CLA$^+$ T cells from blood (88). Results from this study confirm that CLA$^+$ memory cells represent 80–90% of CD3$^+$ cells in the skin and 15% in the blood (88). More interestingly, skin-derived T cells and blood-derived T cells express a different set of genes which are conserved in both mice and humans; these genes are involved in tissue homing and cell activation (88). Some gene signatures were consistent for T cells such as the cell markers *CTLA4*, *CD8A*, and *CD4* (88). T cells residing in human skin express higher levels of a variety of genes including *NR4A2* as compared to T cells in the blood which have elevated expression of genes such as *S1PR1* (88). Thus, T cells in the skin are transcriptionally unique from blood T cells and are comparable to previous gene signatures for T-resident memory cells.

T CELLS HAVE A VARIETY OF FUNCTIONS IN THE SKIN

In human skin, T cells have been shown to perform roles that maintain skin integrity. CD4$^+$ αβ T cells such as Tregs and T-helper cells secrete cytokines in response to infection, tissue damage, and tumors (89). Th1 cells primarily secrete IFN-γ and IL-12 in response to intracellular pathogens that disrupt the skin barrier while Th2 cells fight extracellular pathogens and are involved in atopic diseases by secreting IL-4, IL-13, IL-24, IL-25, and IL-3 (90, 91). CD8$^+$ T cells destroy infected cells by recognizing epitopes of viruses such as the herpes simplex virus, varicella zoster virus, and Epstein–Barr virus (92, 93). While most αβ T cells undergo apoptosis after a pathogen has cleared, a population of αβ T cells become long-lived memory T cells and reside in the skin (94, 95). These memory T cells are involved in inflammation upon viral infection by secreting cytokines, such as IL-22, IL-26, and IL-23 (76). While much of the published research in humans has focused on αβ T cell function in the skin, γδ T cells play key roles in maintaining skin integrity and protecting from malignancy.

γδ T cells monitor skin integrity by recognizing damaged cells and producing IGF-1 (7). Activated skin-resident T cells improve the rate of wound closure in cultured human skin in an IGF-1-dependent manner (7). In addition, human skin-derived γδ T cell clones exert cytotoxic responses against melanoma cell lines (96). Human dermal γδ T cells express the NKG2D receptor, which stimulates cell lysis (97, 98). Once activated, skin-derived γδ T cells produce perforin and induce Fas-mediated cytotoxicity (87). In patients with chronic lymphocytic leukemia of B-cell type, there is an increase in circulating Vδ1$^+$ γδ T cells that respond to autologous leukemic B cells by proliferating and secreting TNF-α and IFN-γ (59). Although T cells play a vital role in providing an effective immune response, they can be harmful if not functioning in a regulated manner.

An imbalance in the number and/or function of skin-resident αβ and γδ T cells has been associated with chronic inflammation and skin-related diseases (99–102). Elevated skin-resident T cells has been reported in individuals with psoriasis and alopecia areata (99, 101). Alternatively, a reduction in T cell infiltration and function has been shown in individuals with type 2 diabetes and melanoma (100, 102). This has highlighted T cells as a promising target for immunotherapeutics. Here, we describe the current findings on T cells in human skin diseases and identify how they are being targeted with immunotherapeutics.

PSORIASIS

2–3% of the US population suffers from psoriasis, which is a chronic inflammatory skin disease (103). It is a multifaceted disease that can occur at any age, has unpredictable onset and remission, and is most commonly characterized by the presence of painful itchy skin lesions (104). Comorbidities of psoriasis include psoriatic arthritis, diabetes, ulcerative colitis, and cardiovascular disease (105, 106). While the mechanism of disease remains unclear, roles for innate immune cells and adaptive immune cells have been identified and participate in the pathogenesis.

Psoriatic lesions are caused by cross-talk between different cell types such as DCs and T cells, along with a number of cytokines including IL-17, IL-12, IFN-γ, TNF-α, and IL-23 (**Figure 1**) (107). Studies show an elevation in IL-23 and IL-12 production by macrophages and DCs in patients with psoriasis (108). Abnormal regulation of the IL-23/IL-17 axis in psoriasis has directed the focus of recent studies on infiltrating CD4$^+$ and/or CD8$^+$ T cells and skin-resident T cells, as contributors to disease pathogenesis (108–110). Dermal T cells are elevated in psoriatic skin compared to healthy skin, increasing from 1% CD3$^+$ T cells to 15% in psoriatic samples (99). The proportion of dermal γδ T cells are also increased with more than 40% of the CD3+ T cells expressing the γδ TCR as compared to 15% in the healthy controls (99). Dermal αβ and γδ T cells also have the ability to secrete IL-17 when stimulated with IL-23, inducing inflammatory cytokines (63, 99, 111). In fact, dermal γδ T cells isolated from psoriasis patients produced more IL-17 upon IL-23 stimulation (99). These cytokines lead to the recruitment of more lymphocytes, neutrophils, and myeloid cells creating a positive feedback loop that maintains cutaneous inflammation and causes epidermal hyperplasia (112). Patients with psoriasis have a reduction in circulating CLA$^+$ Vγ2Vδ2 T cells as compared to healthy patients (63). CLA$^+$ Vγ2Vδ2 T cells are able to home to the skin and are elevated in number in the skin of patients with psoriasis (63). The CDR3 region of TCR genes was recently examined in both psoriatic and healthy patients. Interestingly, skin that has resolved psoriatic lesions retained IL-17-producing pathogenic oligoclonal αβ T cell populations, suggesting a mechanism by which disease can reoccur at the same site (113).

Anti-TNF-α therapies such as etanercept are effective at reducing inflammation and resolving psoriasis. However, the

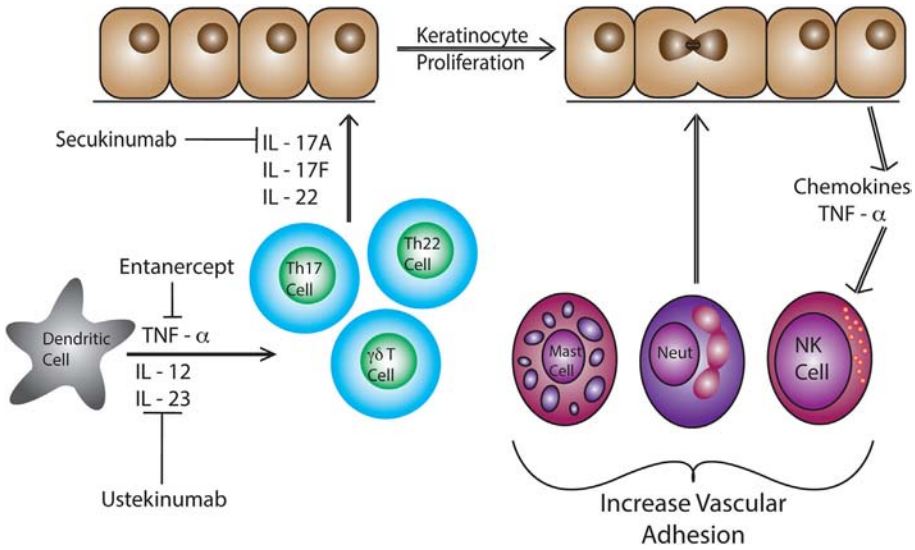

FIGURE 1 | Dendritic cells produce IL-12, IL-23, and TNF- α in response to pathogen-associated molecular pattern activation. These pro-inflammatory cytokines induce differentiation of naïve T cells into Th17 and Th22 cells. These T cells produce IL-22, IL-17A, and IL-17F causing epidermal hyperplasia and induce epidermal chemokine and inflammatory cytokine production. Neutrophils, T cells, mast cells, and NK cells are recruited to the skin and then to the epidermal/dermal junction *via* changes in adhesion molecule expression, such as VLA-1 (CD49a).

understanding of immune mechanisms and the pathogenesis of psoriasis is steadily progressing, allowing for more effective and targeted treatments such as ustekinumab and secukinumab (114). Ustekinumab, a human IgG1k monoclonal antibody, was approved by the FDA in 2009 to treat psoriasis in adults by neutralizing IL-12 and IL-23 (115, 116). Ustekinumab targets IL-12 and IL-23, specifically at the p40 subunit, preventing binding to the IL12Rβ1 and IL-23 receptor complexes on the surface of T cells and NK cells (117). Psoriatic patients treated with ustekinumab show a reduced expression of pro-inflammatory cytokine genes such as cyclin dependent kinase inhibitor 2D, IL-12B, and IL-17A (**Table 2**) (117, 118). IL-17 has been identified as a clinical target for the treatment of psoriasis. Secukinumab binds and blocks the activation of IL-17A which inhibits the production of β-defensin 2 and CXCL8 in human keratinocytes increasing pro-inflammatory cytokine production (119, 120). Elevated levels of IL-17 are a hallmark of psoriasis, therefore, rendering IL-17A inactive reduces inflammation (121, 122). Most recently, the FDA approved brodalumab. This monoclonal antibody binds and blocks the IL-17 receptor A (IL-17A) (123). The inhibition of IL-17R helps regulate and suppress inflammation mediated by IL-17 making it an additional target to treat psoriasis (119). While successful immunotherapies have been developed to treat the pro-inflammatory immune response associated with psoriasis, no drugs have been approved that cure the disease.

ALOPECIA AREATA

Alopecia areata is a polygenic autoimmune disease with a lifetime risk of 1.7% (128). Pathogenesis of alopecia areata involves the dysregulation of immune privilege around the anagen hair follicle (129). While the epithelial bulb of a normal anagen hair follicle does not express MHC Class I or MHC Class II; patients with alopecia areata exhibit increased MHC expression and adhesion molecule upregulation (130, 131). Elevated numbers of Th1 cells in the skin of alopecia patients produce IFN-γ which induces the expression of MHC class I molecules and triggers perifollicular $CD8^+$ T cell infiltration (101, 132, 133). The severity of alopecia areata is closely related to $CD8^+$ T cell gene expression, with a positive correlation between $CD8^+$ T cell-specific genes and alopecia areata severity (101). Autoreactive $CD8^+$ αβ T cells cause hair cycle arrest which then inhibits further hair growth (134).

Hallmark Th1 and Th2 cytokines are elevated in the blood of patients suffering from alopecia areata, while regulatory cytokines such as TGF-β are reduced (135). Patients with alopecia areata express a higher level of IL-23, IL-16, and IL-32 in the skin, which implicates the IL-17 inflammatory axis (136). Th17 cells are elevated in the scalp lesions of patients with alopecia areata, while $FOXP3^+$ T regulatory cells are reduced (137). Alopecia areata lesions also exhibit upregulated expression of genes, such as *CCL19*, *IL-2*, *IL-15/IL-15RA*, *IL-2RA/IL-2RB*, and *Janus kinase 3* (*JAK3*) responsible for T cell migration and activation compared to regions with normal hair growth in alopecia areata patients (**Table 2**) (136).

Genome-wide association studies (GWAS) were performed to further investigate how innate and adaptive immunity is involved in the pathogenesis of alopecia areata. Several susceptibility loci for alopecia areata were identified: *CTLA-4*, *IL-2*, *IL-2RA*, *HLA*, *ULBP*, and *Eos* (**Table 2**) (124). These genes are known to regulate T cell activation and proliferation. Both CTLA-4 and IL-2RA are critical regulators of regulatory T cells (124, 138). ULBP functions as a NKG2D ligand and is a stress signal, which activates γδ T cells, natural killer T cells, and $CD8^+$ T cells (124, 139).

TABLE 2 | Genes associated with T cell function and activation in patients with alopecia areata, psoriasis, diabetes mellitus type 2 or melanoma.

Region	Gene	Function	Involved in disease	Type of study	Reference
2q33.2	*CTLA-4*	Co-stimulatory family	Alopecia areata	Genome-wide association studies (GWAS)	(124)
4q27	*IL-21/IL-2*	T cell proliferation	Alopecia areata, psoriasis, diabetes mellitus type 2 (T2DM)	GWAS, Microarray	(124, 125)
9q31.1	*STX17*	Premature hair graying	Alopecia areata	GWAS	(124)
10p15.1	*IL-2RA*	T cell proliferation	Alopecia areata	GWAS	(124)
12q13	*ERBB3*	Epidermal growth factor receptor	Alopecia areata	GWAS	(124)
6p21.32	*MICA*	NKG2D activating ligand	Alopecia areata, psoriasis, T2DM	GWAS, microarray	(124, 125)
6p21.32	*HLA-DQA1*	Antigen presentation	Alopecia areata, psoriasis, melanoma	GWAS, microarray	(124, 126)
	HLA-DRA	Antigen presentation	Alopecia areata, melanoma	GWAS, microarray	(124, 126)
1p12	*NOTCH2*	T cell activation	T2DM	GWAS	(127)
8p21.3	*TNFRSF10A*	TNF receptor superfamily	T2DM	Microarray	(125)
	CTLA2A	Cytotoxic T lymphocyte-associated protein alpha	T2DM	Microarray	(125)
21q22.3	*ICOSLG*	T cell costimulator ligand	T2DM	Microarray	(125)
16Q12.1	*IL-4R*	T cell differentiation	Melanoma	Microarray	(126)

Murine studies further implicate $CD8^+NK2GD^+$ T cells in the induction of alopecia areata (140). Skin of alopecia-prone C3H/HeJ mice revealed an increased number of $CD8^+NK2GD^+$ T cells with smaller numbers of $CD4^+$ T cells, and mast cells (140–142). Similar to the GWAS studies, microarray analysis identified an upregulation of both IL-2 and IL-15 in skin from C3H/HeJ mice (140). IL-2 and IL-15 regulate the production of IFN-γ by $CD8^+$ effector T cells and NK cells which induces a positive loop, promoting a type I cellular immune response and inflammation in the hair follicle (140, 143, 144). More recent meta-analysis studies on alopecia areata have further identified candidates that regulate autophagy/apoptosis, T regulatory cells and the JAK/STAT pathway (145). Patients with alopecia areata have elevated JAK3 protein levels in the epidermis and phosphorylated JAK3 in the dermal infiltrate (146).

Together, these findings have led to the investigation of several treatments that help regrow hair in patients with alopecia areata (147). However, none of the treatments have been approved by the FDA due to severe study limitations and lack of pediatric treatment studies on alopecia areata (147). Corticosteroids may be administered orally, topically, or intralesionally but recurrence is likely. Clinical studies have focused on the delivery and timing of corticosteroid treatments to improve results for hair regrowth (148–150). Beyond corticosteroids, researchers have begun to study the effectiveness of JAK inhibitors. Ruxolitinib is a JAK1/2 inhibitor that blocks IFN-γ signaling, which is normally utilized by $CD8^+NKG2D^+$ lymphocytes (140). Ruxolitinib is currently approved by the FDA for the treatment of polycythemia vera and myelofibrosis; however, it is currently being studied in alopecia areata patients. In 2016, ruxolitinib was administered orally to 12 patients 2× daily with 20 mg for 3–6 month and 75% of the patients exhibited an average hair regrowth of 92% (151). Baricitinib, another immunomodulator that inhibits JAK1/2 is being studied in clinical trials for alopecia areata (152). A trial starting in 2012 enrolled one patient, and in 9 months that patient completely sustained hair regrowth (152). Tofacitinib is a small molecule JAK3 inhibitor. In an open-label pilot study, tofacitinib was administered to 12 patients, 8 patients exhibited more than 50% hair regrowth, 3 patients demonstrated less than 50% hair regrowth, while 1 patient did not demonstrate hair regrowth (153). Ongoing studies are further investigating how modulating cytokine signaling *via* the JAK/STAT pathway in T lymphocytes can effectively treat alopecia areata.

DIABETES MELLITUS TYPE II

Diabetes mellitus is a chronic condition of elevated blood glucose levels caused by insufficient insulin production and/or insulin resistance. Approximately 30.3 million people in the US have been diagnosed with diabetes, with 90–95% of these cases being diabetes mellitus type 2 (T2DM) (154). Obesity is a strong predictor for T2DM (155). Patients with T2DM exhibit a complex array of complications including a higher prevalence of chronic non-healing wounds, impaired leukocyte function, neuropathy, and vasculopathy (156, 157).

Chronic non-healing wounds in T2DM patients are caused by a disruption in one or more stages of the normal wound healing process (158, 159). The process of wound healing normally encompasses numerous overlapping stages. First there is clot formation from platelet aggregation followed by cytokine and chemokine secretion which elicits an inflammatory response. This leads to the proliferation of epithelial cells to restore the lost barrier. Finally, the tissue is remodeled to strengthen the new matrix. Skin-resident T cells participate in the early stages of wound healing through the production of growth factors, cytokines, and chemokines (7). Skin-infiltrating T cells arrive within a week to fight infection and secrete cytokines including IFN-γ (160). However, in T2DM the timing and level of immune cell function becomes altered.

Diabetic wounds become arrested in a chronic state of inflammation that is caused by pro-inflammatory cytokines, such as TNF-α, secreted by adipocytes and immune cells (161). In murine models of obesity and T2DM, epidermal γδ T cells become reduced in number as disease progresses (100). The remaining T cells in the epidermis exhibit reduced cytokine and growth factor production during wound repair (100). Growth factor production by the γδ T cells is partially restored upon blocking TNF-α with antibodies prior to injury (100). T cell dysfunction in

obese, T2DM mice results in a thinner epidermis with premature keratinocyte differentiation (85). In accordance, the blockade of TNF-α improves insulin resistance in animal models (162), but has not shown good efficacy in humans (163).

T cells isolated from patients with chronic non-healing wounds lack the ability to secrete growth factors such as IGF-1 upon stimulation (7). This suggests that skin T cells become refractory to stimulation as wound healing stalls. Obese subjects also exhibit a reduction in Vγ2Vδ2 T cells in the blood that negatively correlates with BMI (65). The remaining Vγ2Vδ2 T cells are less able to become activated and secrete reduced levels of IFN-γ in response to virus infected cells (65). The Vγ2Vδ2 T cells in obese subjects show an increase in differentiation from central memory to T effector memory T cells and T effector memory $CD45RA^+$ lymphocytes (65). Future studies are needed to further elucidate the contribution of T cell dysregulation in obese T2DM patients to chronic non-healing ulcers (164).

Conventionally, diabetic foot ulcers are treated with wound debridement followed by aseptic techniques that aim to keep the area clean and moist (165). Medications that reduce inflammation directly and/or indirectly, such as metformin, are being studied to determine whether they improve diabetic wound healing (166, 167). Upon activation of AMP-activated protein kinase, metformin suppresses the production of glucose by the liver (168). The activation of AMPK results in anti-inflammatory responses through the suppression of NF-κβ signaling (169). Metformin reduces Th17 differentiation and IL-22 secretion, decreasing chronic inflammation and improving immune responses (170, 171).

Systemic insulin therapy has shown some success in improving wound healing in rats and humans (156, 172). Insulin regulates glucose uptake, gene expression, and cell differentiation which all impact skin-resident T cells and wound repair. Other studies show that topical insulin injections accelerate the healing of diabetic foot ulcers by stimulating the AKT and ERK pathways (156). A large multicenter clinical trial is needed to determine the level of effectiveness and mechanisms used by insulin to promote proper T cell function and wound healing in diabetic patients.

MELANOMA

Melanoma only makes up approximately 1% of skin cancer cases, yet it is responsible for most skin cancer deaths with an estimated 9,730 deaths and 87,110 new cases in 2017 (173, 174). The majority of melanomas are caused by UV radiation from sun exposure (175). Malignant melanoma has a high metastatic rate, making it one of the most aggressive and dangerous cancers (176). Cytotoxic T cells recognize antigens associated with melanoma, including tyrosinase and tyrosinase-related proteins 1 and 2; however, the immune response is inhibited or repressed by the tumor environment (176–179).

There is a reduction in number and percentage of circulating Vγ2Vδ2 T cells in melanoma patients (180). Interestingly, 15–25% of tumor-infiltrating cells in patients are Vγ2Vδ2 T cells (180). γδ T cell cytotoxicity in melanoma patients is also significantly lower compared to healthy patients and this reduction in cytotoxicity is correlated with melanoma stages (180). In a study of 46 patients, 23 of the patients' melanoma-infiltrating γδ T cells into the skin were Vδ1+ T cells, 19 patients had predominantly Vδ2+, and 4 patients did not have a significant different in percentages of $Vδ1^+$ and $Vδ2^+$ T cells (60). Cytotoxic capability between $Vδ1^+$ and $Vδ2^+$ among these patients was substantially different. Most $Vδ1^+$ T cells performed cytotoxic activity against the melanoma cell line A375; however, only two out of eight $Vδ2^+$ T cells exhibited this capability (60). Thus, γδ T cell subsets play complex roles that contribute to immunosurveillance of melanoma in human skin.

Melanoma utilizes a wide variety of mechanisms to evade the host's immune system. The most recent mechanisms under extensive investigation are CTLA-4, programmed cell death protein 1 (PD1), and programmed death-ligand 1 (PDL1) immune checkpoints (**Figure 2**) (102). T cell expression of CTLA-4 and PD1 downregulates functions such as activation and antitumor activity by suppressing signals downstream of TCR stimulation (177, 178). CTLA-4 binds to CD80 and CD86 expressed by antigen-presenting cells. CTLA-4 disrupts CD80/CD86- CD28 binding, which suppresses co-stimulation and T cell activation (181). PD1 is a transmembrane protein that is expressed on activated T cells, DCs, B cells, and NK cells. It binds to the ligands PDL1 and PDL2 resulting in the suppression of T cell activation (182). Resting Vγ2Vδ2 T cells in the blood express PD1 at low levels but upon activation it is upregulated (183). PD1 regulated TCR-mediated activation thus maintains self-tolerance and prevents autoimmunity (184). Cancer cells express PDL1 and as a result bypass immune checkpoints and evade T cell recognition (181). Although anti-CTLA-4 and anti-PD1 treatments have shown promise, it is necessary to further investigate how these treatments impact $Vδ1^+$ T cells specifically.

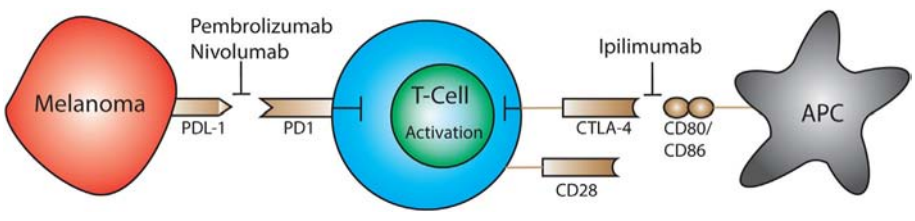

FIGURE 2 | Expression of PD-1 and CTLA-4 by T cells leads to downregulation of activation and antitumor cytotoxic activity by suppressing downstream TCR signals. Immunotherapeutics have been approved that block CTLA-4 (ipilimumab) or programmed cell death protein 1 (PD1) (pembrolizumab, nivolumab) which restores the ability of T cells to become activated and destroy tumor cells.

The FDA-approved drug, ipilimumab, targets CTLA-4 on T cells for the treatment of melanoma. This medication is also used to treat autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (185). Targeting CTLA-4 reduces Treg-mediated suppression and enables activation and proliferation of T cells (186–188). Beyond ipilimumab, the FDA has also approved immunotherapeutics that target the PD1 pathway: pembrolizumab and nivolumab. By blocking the PD1 receptor the therapeutic antibody prevents ligation with PDL1, which is expressed on tumor cells; this restores the ability of T cells to respond to melanoma antigens and initiate cytotoxic responses and cytokine production (189).

A phase III study of 945 patients with stage III and IV melanoma across 137 countries revealed that when combined, ipilimumab and nivolumab have synergistic effects against metastatic melanoma (185). Unfortunately, these patients also experienced side effects from the combined treatment including diarrhea, elevate liver enzymes, and colitis (185). This suggests that while a combination of CTLA-4 and PD1 targeted therapies can be more successful than a monotherapy, it is also likely to cause adverse effects by unregulated T cells.

CONCLUSION

αβ and γδ T cells are vital in the maintenance and homeostasis of the skin through the recognition of stressed or damaged cells and subsequent functions including the secretion of cytokines and growth factors. Current studies do not allow precise conclusions on the distinct roles of αβ and γδ in skin immunity to be drawn. However, both αβ and γδ T cells fight pathogens and cancer by directly destroying infected or transformed cells in the skin, while also maintaining immunological tolerance. γδ T cells recognize a wide variety of peptide and non-peptide antigens released by stressed, damaged, malignant, or infected cells in the skin while αβ T cells recognize peptides derived from pathogens or tumors presented by MHC. Through specific cytokine profiles $CD4^+$ αβ T cells aid in the recruitment and regulation of other immune cells, while $CD8^+$ αβ T cells exhibit cytotoxicity (190–192). The receptors responsible for T cell activation also differ between αβ and γδ T cells, suggesting unique roles in skin homeostasis and immunity. In addition, skin-resident versus recirculating T cells show distinct profiles indicating that the various roles and requirements for T cell function in the skin is complex and requires subsetting. Thus, the molecular mechanisms that regulate skin-specific αβ and γδ T cells are important to elucidate for the development and study of immunotherapies.

Cytokine and growth factor production by αβ and γδ T cells helps to maintain skin homeostasis, while factors produced upon activation contribute to wound repair and the eradication of tumors. Elevated and unregulated T cell activity can contribute to or cause chronic inflammatory related diseases including psoriasis, and alopecia areata. Conversely, defects in T cell function can increase susceptibility to melanoma. Current knowledge about skin αβ and γδ T cell activation and antitumor activity has advanced considerably, yet further studies are needed to identify specific molecular mechanisms that can be exploited for therapeutics that treat autoimmune diseases and cancer.

AUTHOR CONTRIBUTIONS

MC, AD, AR, and JJ wrote the manuscript, provided critical discussion in manuscript preparation, and revised the manuscript.

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REVIEW
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Gamma Delta T Cell Therapy for Cancer: It Is Good to be Local

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Human gamma delta T cells have extraordinary properties including the capacity for tumor cell killing. The major gamma delta T cell subset in human beings is designated Vγ9Vδ2 and is activated by intermediates of isoprenoid biosynthesis or aminobisphosphonate inhibitors of farnesyldiphosphate synthase. Activated cells are potent for killing a broad range of tumor cells and demonstrated the capacity for tumor reduction in murine xenotransplant tumor models. Translating these findings to the clinic produced promising initial results but greater potency is needed. Here, we review the literature on gamma delta T cells in cancer therapy with emphasis on the Vγ9Vδ2 T cell subset. Our goal was to examine obstacles preventing effective Vγ9Vδ2 T cell therapy and strategies for overcoming them. We focus on the potential for local activation of Vγ9Vδ2 T cells within the tumor environment to increase potency and achieve objective responses during cancer therapy. The gamma delta T cells and especially the Vγ9Vδ2 T cell subset, have the potential to overcome many problems in cancer therapy especially for tumors with no known treatment, lacking tumor-specific antigens for targeting by antibodies and CAR-T, or unresponsive to immune checkpoint inhibitors. Translation of amazing work from many laboratories studying gamma delta T cells is needed to fulfill the promise of effective and safe cancer immunotherapy.

Keywords: gamma delta, T cell, cancer, immuno-oncology, Vdelta2 gamma delta T cells, targeted immunotherapy

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INTRODUCTION

Human T cells expressing the Vγ9JγPVδ2 T cell receptor [also designated Vγ2Jγ1.2Vδ2 (1, 2)] comprise 1–5% of circulating lymphocytes in healthy adults. Treating peripheral blood mononuclear cells (PBMC) with phosphorylated intermediates from the isoprenoid biosynthesis pathway [isopentenyl-pyrophosphate (IPP) and (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP)] (3–5) or aminobisphosphonate inhibitors of farnesyl diphosphate synthase (FDPS) (6) stimulate proliferation, cytokine secretion, and cytotoxic effector function of Vγ9Vδ2 T cells. The Vγ9Vδ2 T cell response to phosphorylated compounds (phosphoantigens) or aminobisphosphonate is almost exclusive to Vδ2 cells with a JγP rearrangement and responding cells frequently use public Vγ9JγP sequences that are shared widely in the population [reviewed in Ref. (7)]. Because the Vγ9Vδ2 T cell response to phosphoantigen or aminobisphosphonate is ubiquitous in the healthy human population and the expanded cells have potent effector functions, many investigators are developing immunotherapies based on selective activation of the Vγ9Vδ2 T cell subset. Increasingly the focus of

γδ T cell research is on cancer immunotherapy. Here, we review oncology applications for this important component of natural tumor surveillance and discuss obstacles to clinical translation of our basic knowledge about γδ T cells.

Circulating Vγ9Vδ2 T cells are diverse due to length and sequence variation in the CDR3 regions of both γ and δ chains but their uniform activation by phosphoantigen or aminobisphosphonate gives the appearance of an innate response. Similarly, the circulating adult Vγ9Vδ2 T cell repertoire is shaped by constant positive selection due to the presence of ubiquitous phosphoantigens produced by host cells or resident microbes. Chronic positive selection increases the proportion of Vγ9Vδ2 T cells in blood and reduces population diversity due to amplification of T cell clones mostly expressing public Vγ9JγP chains. With a TCR repertoire altered by chronic positive selection, human and non-human primates along with a few other species (8) maintain a pool of Vγ9Vδ2 T cells that is dominated by central and effector memory phenotype, contains 1 of every 40 memory T cells in the body, and reacts to appropriate stimuli with the speed and uniformity of innate immunity. These features, particularly the uniform response to common stimuli without MHC restriction, make the Vγ9Vδ2 T cell subset especially attractive for a variety of therapeutic applications in man.

In earlier studies of human γδ T cells it was discovered that several tumor cell lines directly stimulated the Vγ9Vδ2 subset without exogeneous phosphoantigen or aminobisphosphonate addition. A good example is the Daudi B cell line, which is a selective activator of Vγ9Vδ2 T cell proliferation and effector function (9). The Daudi cell line is unusual because it does not express β_2-microglobulin, hence fails to MHC class I-related surface glycoproteins. However, other cell lines with MHC class I expression (10) are nonetheless capable of activating Vγ9Vδ2 T cells and of being targets for cytotoxicity. Using Daudi B cells to stimulate PBMC lead to expansion of multiple Vγ9Vδ2 T cell clones from healthy individuals and the pattern of responses was similar to the pattern of clonal expansion after IPP stimulation (11).

Multiple agents can be combined to achieve potent stimulation of Vγ9Vδ2 T cells. The best-known examples of phosphoantigen, aminobisphosphonate, or stimulatory cell lines were described above. In those examples, elevated levels of cellular IPP were critical to cell stimulation and IPP levels must be elevated in a cell that also expresses butyrophilin 3A1 on its surface (12–16). The butyrophilin 3A1 is only one part of a complex regulator of VγVδ2 T cell activation and is found within heteromeric complexes that control T cell activation (17). Evidence from physical studies supports a view that phosphoantigen binding to the cytoplasmic B30.2 domain of butyrophilin 3A1 induces a unique conformational change that propagates throughout the molecule (18–20). The idea that conformational change in butyrophilin 3A1 governs recognition by Vγ9Vδ2 T cells is consistent with positive selection of the Vγ9JγP rearrangement; JγP is the longest J segment and length seems to be crucial for TCR recognition (7, 11). The literature on butyrophilin and γδ T cell activation has been reviewed recently (18).

A requirement for the combination of IPP plus the human butyrophilin complex explains the lack of xenogeneic stimulation by non-human tumor cells despite their common production of IPP. Variations of this theme help to explain why cells infected with bacteria that are themselves capable of producing phosphoantigens similar to IPP (21–23) will stimulate Vγ9Vδ2 T cells. Combinations of T cell receptor cross-linking *via* antibody treatment plus cytokine or toll-like receptor agonists also stimulate Vγ9Vδ2 T cell proliferation and cytokine production (24).

The signals required to maximize cytotoxic effector activity are less clear, though C-type lectin receptors are known to be important. The NK receptor NKG2D is a potent activator of cytotoxic effector function and is expressed on the majority of stimulated Vγ9Vδ2 T cells (25). A smaller sub-population expresses the inhibitory receptor NKG2A (26, 27), and both subsets may contain activated Vγ9Vδ2 T cells expressing the CD16 low affinity Fc receptor, and are capable of being activated by IgG bound to target cells (28).

STRATEGIES FOR γδ T CELLS IN IMMUNO-ONCOLOGY (I/O)

The challenges to developing a cancer therapy based on activating γδ T cells are exemplified in the history of intravesical *Bacille Calmette–Guerin* (BCG), a strain of *Mycobacterium bovis* used for treating bladder cancer. Epidemiology studies in the early twentieth century linked tuberculosis with lower cancer incidence and lead to the introduction of BCG as a cancer vaccine in 1935 [reviewed in Ref. (29)]. By the 1970s BCG was becoming accepted for bladder cancer therapy and remains in use for this disease. It was reported that BCG is a potent stimulator for Vγ9Vδ2 T cells (30) and activated cells kill bladder cancer cells *in vitro* (31). These findings suggested a direct relationship between Vγ9Vδ2 T cell activation by locally administered BCG and subsequent destruction of tumors by direct cytotoxicity. Around 40 years later we know that Vγ9Vδ2 T cells are found at higher levels in urine from bladder cancer patients treated with BCG (32) and successful treatment is associated with increased levels of intratumoral CD19 B cells along with CD4, CD8, and γδ T cells (33). Today, bladder cancer treatment is evolving with the introduction of new immunotherapies despite our poor understanding of immune response triggered by BCG *in situ*, the extent to which γδ T cells are important for these responses, and the mechanisms of action for tumor reduction. Could we have a better therapy for non-invasive BCG based on a clearer understanding of the immune response? The answer is uncertain, but it is likely that such studies would produce more sophisticated biomarkers, better prognosis, and personalized treatment for bladder cancer. This review of γδ T cells and cancer therapy seeks to identify similar gaps in our current understanding of γδ T cell immunotherapy that may slow progress to the development of treatment strategies and clinical products.

Most studies on human γδ T cells have concentrated on the Vγ9Vδ2 T cell subset from peripheral blood. This reflects in part, the ease of obtaining primary cells for laboratory studies and the ability to grow large numbers of Vγ9Vδ2 T cells from human PBMC. More importantly, this choice reflects the biology of Vγ9Vδ2 T cells where the TCR repertoire is shaped by strong, positive selection pressure that maintains a circulating, innate-like

T cell population. Collective efforts in many laboratories created a detailed picture of the effector activities for Vγ9Vδ2 cells and outlined the road map for clinical applications.

Unique properties of circulating Vδ1 cells are less defined, although they have been studied for treatment of some neoplastic diseases (34, 35). The majority of Vδ1 cells comprise the intraepithelial lymphocyte population of mucosal epithelia where they react to signals of stress by producing abundant cytokines and chemokines that influence mononuclear cell infiltration into damaged, infected, or malignant epithelium (36–38). Recent progress in expanding Vδ1 cells from blood (39, 40) will undoubtedly increase our knowledge about this subset. The capacity for CD30+ Vδ1 T cells to produce IL-17A and create inflammatory microenvironment (41) suggests this T cell subset may promote or inhibit cancer progression depending on the cell type, location, state of disease, and other factors. Consequently, the role for Vδ1 cells remains unclear, especially in solid tumors, but these cells hold promise for treating a select group of malignancies including leukemia (35).

The Vδ3+ subset has also been considered for therapeutic use but less is known about stimulatory antigens or properties of these cells. The Vδ3+ subset has been implicated in the response to herpesvirus infections including cytomegalovirus and Epstein–Barr virus (42–44). Clinical studies correlated elevated baseline Vδ3 levels with fewer herpesvirus outbreaks after iatrogenic immune suppression such as that employed in the transplant setting (45). However, Vδ3 cells are relatively rare in blood and conditions for expanding these cells *ex vivo* are poorly defined. Cellular recognition of EBV- or CMV-infected cells has also been documented for Vδ1 or Vδ2 cells (42, 46) and in rare cases, the Vδ5+ subset also recognized herpesvirus-infected cells (44).

Our ability to define an I/O strategy based on the biology of γδ T cells is impacted by many factors including the limited information about how these cells participate in natural tumor surveillance. It is critical to decide whether a focus on the well-known Vγ9Vδ2 T cell subset offers more advantages compared to exploring tumor-infiltrating lymphocyte populations, and how can we balance the pro-tumor and anti-tumor roles for Vδ1 cells (47). Can we find unique properties of Vδ3 or other minor subsets that are compelling for cancer therapy? Finally, should we be looking for platform approaches to γδ T cell I/O or create unique approaches for each type of malignancy? Answers to these questions will help to define pathways for clinical development of γδ T cell immunotherapies.

IS THERE A ROLE FOR Vγ9Vδ2 T CELLS IN I/O?

There are compelling arguments for I/O strategies based on activating Vγ9Vδ2 T cells. This subset is abundant in blood and cells can be expanded *ex vivo* with simple protocols. Cytotoxic killing of many tumor types is well documented for Vγ9Vδ2 T cells and the range of targets is broad. Furthermore, activation of Vγ9Vδ2 T cells can be accomplished *ex vivo* or *in vivo* through stimulation with mammalian or microbial phosphoantigens, one of several widely used aminobisphosphonate drugs, TCR-cross linking monoclonal antibodies, butyrophilin cross-linking antibodies, or exposure to stimulatory tumor cells. This highly flexible system provides many opportunities for matching Vγ9Vδ2 T cell stimulation with a specific tumor target and allows for realistic consideration of both passive immunotherapy with *ex vivo* expanded cells, and *in vivo* therapy using direct activation of the Vγ9Vδ2 T cell subset.

By contrast, the list of tumor cell targets for Vδ1 or Vδ3 cells are narrow, but may be expanded in the future, and there is a concern regarding the pro-inflammatory nature of Vδ1 cells because of their propensity to express the cytokines IL-17 or IL-4. For an immediate, near-term I/O program we and others [reviewed in Ref. (48–51)] tend toward focusing on the natural tumor surveillance activities of Vγ9Vδ2 T cells.

Despite our enthusiasm for tumor immunotherapy involving Vγ9Vδ2 T cells, there is always doubt regarding their practical utility for clinical cancer care. We might (and should) ask: if natural tumor immunity is important and potentially useful for oncologic medicine, why does this surveillance system fail to catch and prevent malignant disease in the first place? In other words, do tumors escape immune surveillance or is the demonstrated tumor cytotoxicity of γδ, NK, NKT, MAIT, and other innate-like cells an *in vitro* property with little relevance to practical problems in cancer care? We will overcome these concerns by demonstrating consistent potency, clearly defined mechanisms of action, and objective clinical responses to γδ T cell immunotherapy.

TUMOR MECHANISMS FOR IMMUNE EVASION

Multiple mechanisms have emerged to explain tumor evasion of MHC-restricted responses. The conventional paradigm argues that innate immune activation precedes activation of antigen-specific T cells, especially CD8+ subsets, and in turn leads to recruitment of effector T cells capable of destroying the tumor [reviewed in Ref. (52)]. Multiple mechanisms at each step may result in tumor escape from T cell surveillance. Tumors may have an immunosuppressive microenvironment that fails to activate innate responses. Tumors may have reduced immunogenicity due to lack of tumor-specific antigens or downregulation of MHC molecules. Tumors may also inhibit the activation of T cell effector mechanisms through overexpression of ligands for immune inhibitory costimulatory molecules (checkpoints). The latter area has received much attention lately due to licensure of therapeutic monoclonal antibodies that prevent receptor:ligand engagement of inhibitory costimulatory receptors and their ligands resulting in activation of tumor-specific T cell responses.

Clinical studies with antibodies that block inhibitory costimulation documented a substantial impact on specific subsets of malignant diseases. For example, Merck's Keytruda product (monoclonal antibody against PD-1) for metastatic melanoma, non-small cell lung cancer, and tumors with high expression of PD-L1 has been highly successful in several clinical settings [reviewed in Ref. (53)]. Success with inhibiting PD-1/PD-L1 interactions by blocking PD-1 (Keytruda and Nivolumab) or PD-L1 (Atezolizumab, Avelumab, and Duvalumab), and blocking of CTLA-4 (Ipilimumab) has stimulated interest in blocking several other receptor/ligand interactions with similar mechanisms

of action. The continuing development of new checkpoint inhibitors is especially important because tumor escape from anti-PD-L1 has already been observed and involved upregulation of other inhibitory costimulation molecules (54). Clinical experiences will elucidate mechanisms for escape from checkpoint inhibitor antibody effects and drive the development of new monoclonal antibody drugs alone or in combination with other antibodies or alternate therapeutic modalities.

Whether PD-1 or other checkpoint inhibitors enable tumors to escape Vγ9Vδ2 T cell surveillance is an open question. Expression of PD-1 was increased after Vγ9Vδ2 T cell stimulation but pretreatment of PD-L1 Daudi tumor cells with zoledronic acid was sufficient to render them susceptible to Vγ9Vδ2 T cell killing irrespective of PD-L expression (55). Cytotoxicity was less potent when PD-L was present on tumor cells; some studies noted that tumor cell killing was reduced by up to fivefold in the presence of PD-L (56). Pennington's group (57) identified a CD24−/CD28−/CD16+ subset of Vγ9Vδ2 T cells, representing about 10% of total Vγ9Vδ2 T cells in blood from healthy donors, that expressed CD57 and had the highest proportion of PD-1 cells (14%) among all Vγ9Vδ2 T cell subsets. Both CD57 and PD-1 are presumed markers of inactivated or "senescent" T cells that have lost the potential for proliferation (58). When double-positive cells accumulated at the tumor site, it was usually taken as a sign of failed tumor immunity (59). However, recent literature suggests that effector function and capacity for proliferation may be differentiated on the basis of CD57 expression. In both CD4 T cells (60) and NK cells (61), CD57 expression identified cells that are potent for cytotoxicity but lack the capacity for expressing immune-suppressing cytokines including IL-10 or IL-21 and will not proliferate in response to stimulation (60). If Vγ9Vδ2 or other γδ T cells also become potently cytotoxic without producing regulatory cytokines, PD-1 and possibly CD57 may be markers for tumor effector activity and not signs of a failed immune response.

As noted above, expression of PD-1 increased after phosphoantigen or aminobisphosphonate stimulation of Vγ9Vδ2 T cells (55) and we might infer from studies of NK and CD4 cytotoxic T cells that PD-1 and CD57 identify non-proliferating but potently cytotoxic cells that do not express suppressive cytokines. It is important to define the conditions for generating such cells, to determine their life span in tumors and to understand whether they contribute meaningfully to tumor reduction. Such studies may also guide decisions about future clinical trials proposing combinations of Vγ9Vδ2-based treatment and checkpoint inhibitor antibodies.

Because the population of Vγ9Vδ2 T cells responds almost uniformly to phosphoantigen or aminobisphosphonate stimulation, there will be rapid proliferation of stimulated cells and continuing production of the PD-1+/CD57+ subset. The majority of Vγ9Vδ2 cells will have little or no expression of PD-1 resulting in tumor killing that is not abrogated by PD-L1 or PD-L2 ligands (56). While the impact of PD-1 on Vγ9Vδ2 T cells is still being evaluated, we can make a provisional conclusion that this immune checkpoint is not an obstacle to Vγ9Vδ2 T cell tumor therapy although it may impact potency. Whether other immune checkpoint molecules may be more important is still under study. At this time, it appears that Vγ9Vδ2 cell therapy may differ from αβ T cell effector mechanisms in the extent to which they are impacted by immune checkpoints. If this view holds true, it will become an important argument for the uniqueness of Vγ9Vδ2 T cell immunotherapy.

Beyond immune checkpoint regulation, there are complex interactions among T cells, NK cells, dendritic cells, mesenchymal cells, myeloid-derived suppressor cells, and even neutrophils that dictate the tumor microenvironment and benefit or inhibit the capacity for effective tumor immunity. Recent comprehensive reviews addressed many of the key mechanisms for immune suppression including the functions for regulatory γδ T cells and effects of their cytokines on tumor killing; we refer the reader to these excellent publications (62–66). In addition to the immunosuppressive cytokines, pro-inflammatory cytokines including IL-17A often promote tumor growth and may be produced at higher levels when intratumoral T cells are dominated by the Vδ1 subset (41, 67). Normally, Vδ2 T cells dominate peripheral blood in healthy individuals and are >2-fold more abundant than circulating Vδ1 cells. A study including more than 200 melanoma patients treated with the checkpoint inhibitor antibody ipilimumab (targeting CTLA-4) showed that individuals with an inverted ratio of blood γδ cell subsets (Vδ1 > Vδ2) had lower overall survival, and poorer outcomes were significantly associated with decreasing Vδ2 T cell levels during ipilimumab therapy (68). A similar relationship between Vδ2 and Vδ1 cells was noted for rectal carcinoma (67).

Inversion of the Vδ2 ÷ Vδ1 T cell ratio in blood was also observed in HIV disease [reviewed in Ref. (69)], where inversion is due to quantitative depletion of Vδ2 cells and expansion of the Vδ1 subset, similar to the melanoma case cited above. In HIV disease, Vδ2 T cell depletion is due to multiple factors including direct toxicity of the viral envelope glycoprotein (70) and inadequate levels of IL-18 (71). Expansion of the Vδ1 subset was linked to damage of the intestinal epithelium and translocation of stimulatory bacterial products into blood (72, 73). It is of interest that the melanoma study, which included ipilimumab therapy (68) and HIV disease are both characterized by rising Vδ1 cells plus falling Vδ2 T cell levels. Notably, cancer is an important comorbidity of HIV disease with rates greatly exceeding the general population along with increased susceptibility to a broad range of cancer types (74, 75). In future, we hope to apply knowledge from γδ T cell clinical cancer trials, to understanding and mitigating the increased cancer risk in HIV disease.

We do not yet understand why increased levels of Vδ1 T cells in blood is a risk factor for melanoma or HIV-associated cancer. This is due partly to the complex nature of the Vδ1 T cell subset. While Vδ1 from peripheral blood were cytotoxic for colon cancer cells (40), an IL-17A-producing subset of Vδ1 cells promoted tumor growth (76). Studies using peripheral blood may not reflect the properties of mucosal Vδ1 T cells. Overall, we will need to understand how to expand Vδ1 T cells *in vivo* or *ex vivo*, and to enrich beneficial cells while reducing the growth of regulatory or inflammatory subsets. These strategies must be refined for each tumor target because the tumor response to inflammation is not uniform across tumor types.

We know that many, but not all tumors have the capacity for activating Vγ9Vδ2 T cells. Perhaps the best-studied example

is the Daudi Burkitt's lymphoma cell. The Vγ9Vδ2 T cells are stimulated by contact with Daudi B cells (9) but not by the related Raji cells (10). Activated Vγ9Vδ2 T cells killed Daudi, Raji, and four other Burkitt's lines including HH514, DG75, Ramos, and Wilson, along with freshly isolated primary tumor cells (10). Subsequently, oligodendrocytes, fetal astrocytes, and glial cells were shown to induce Vγ9Vδ2 T cell activation and proliferation (77), and this activity was correlated with cell surface expression of heat shock proteins. An earlier report had noted the stimulatory properties of Daudi cells expressing a GROEL homolog of heat shock protein (9).

The list of tumors killed by Vγ9Vδ2 T cells is longer than the list of tumors capable of activating Vγ9Vδ2 T cells. Perhaps this is telling us that poor activation *in situ* is a mechanism for tumors to evade Vγ9Vδ2 T cell surveillance. Discrepancies in the literature, wherein *in vitro*-activated Vγ9Vδ2 cells kill tumors even if they express PD-1 ligands, may reveal that activation by direct exposure to tumor cells during normal immune surveillance is not strong enough to drive Vγ9Vδ2 T cell eradication of the tumor or to overcome inhibitory costimulation. When Vγ9Vδ2 T cells are activated by phosphoantigen or aminobisphosphonate and the culture medium includes IL-2, IL-15, or other cytokines, the resulting cells demonstrate potent tumor cell cytotoxicity. To date, we have only limited knowledge about Vγ9Vδ2 T cell activation *in vivo*, with or without stimulatory compounds and cytokines. We also know that some tumors may upregulate expression of FDPS, the enzyme responsible for converting IPP to farnesol (78). Such tumors may have smaller pools of IPP hence lesser capacity for directly activating Vγ9Vδ2 T cells. Tumors with upregulated FDPS are also insensitive to cytostatic effects of aminobisphosphonates because excess enzyme overcomes the drug's competitive inhibition of FDPS (79).

Importantly, tumors may not always be immunogenic in the conventional sense of activating naïve αβ T cells but may still be targets for Vγ9Vδ2 T cells. Downregulation of MHC is unlikely to affect Vγ9Vδ2 T cell recognition either for activation or as a target for cytolysis and might make tumors more sensitive to killing. Daudi, for example, is a potent activator of Vγ9Vδ2 T cells and a sensitive target despite having no detectable MHC class I expression due to a deletion in the beta2-microglobulin gene (9).

We also note the potential for modulating tumor immunity through specific costimulation of Vγ9Vδ2 T cells. We initially discovered the important role for costimulatory Vγ9Vδ2 T cells in activating tumor cytotoxicity by NK cells (80). Transient expression of 4-1BB (CD137) after phosphoantigen or aminobisphosphonate stimulation of blood Vγ9Vδ2 T cells upregulated this costimulatory molecule, increased NKG2D expression on NK cells, and increased NK tumor effector function. Subsequently, we discovered that Vγ9Vδ2 T cells also signal NK cells through an ICOS:ICOS-L interaction resulting in increased CD69 and 4-1BB expression on NK cells and increased levels of interferon-γ, TNF-α, MIP-1β, 1-309, RANTES, and soluble FasL in the culture. Perhaps most importantly, NK cells "educated" through the ICOS:ICOS-L pathway by Vγ9Vδ2 T cells gained the capacity to kill mature dendritic cells (81). Removing these dendritic cells would alter the tumor microenvironment by reducing inflammation. We also know that NK cells are normally responsible for "licensing" the dendritic cell population primarily by killing immature dendritic cells [reviewed in Ref. (82, 83)]. Both NK and Vγ9Vδ2 T cells interact reciprocally with dendritic cells (84–86) and examples cited earlier (81) showed that activated Vγ9Vδ2 T cells educate NK, which then destroy mature dendritic cells that would normally promote inflammation and tumor growth. Dendritic cells infected by *Brucella melitensis* are substantially impaired in their capacity for antigen presentation but the defect was corrected through a contact-dependent interaction with Vγ9Vδ2 T cells (87). This triangle of Vγ9Vδ2 T cells:NK cells:dendritic cells is part of a regulatory network affecting tumor cell cytotoxicity and regulating inflammation in the tumor microenvironment. We might imagine that mesenchymal stem cells, tissue macrophages, myeloid-derived suppressor cells, and potentially neutrophils or other inflammatory cells have similarly complex interactions that balance the requirement for activating protective immunity with a mechanism to limit destructive inflammation. Understanding these subtle interactions and finding ways to manipulate the regulatory networks may be one key to potent tumor immunotherapies focused on γδ T cells.

The Vγ9Vδ2 subset of γδ T cells is uniquely adapted for tumor immunity through: non-reliance on MHC expression, relative insensitivity to PD-1 inhibition, potent and broad tumor cytotoxicity, low contribution to IL-17A production, activation of NK cytotoxicity, and costimulation of NK for killing of mature (inflammatory) dendritic cells. Several of these mechanisms are unique to Vγ9Vδ2 T cells and fill critical gaps in tumor immunotherapy that are not approached through CAR-T cell therapy or use of checkpoint inhibitor antibodies. By careful selection of appropriate tumor types and understanding the critical markers signaling a healthy versus unhealthy balance of γδ T cell subsets, we can exploit the natural properties of γδ T cells and overcome several well-known mechanisms for tumor evasion of host immunity.

PRECLINICAL AND CLINICAL STUDIES OF Vγ9Vδ2 T CELL THERAPY

Activated Vγ9Vδ2 T cells kill a broad range of tumor cell lines, often with spectacular potency. Investigators have even observed potent killing with effector to target cell ratios below 1, meaning the effector cells recycle without being killed themselves or soluble death ligands are important contributors to cytotoxicity. Several preclinical and clinical studies have tested whether this level of potency translates to potent therapeutic effects *in vivo*.

The SCID mouse model was used to test the tumor surveillance capacity of Vγ9Vδ2 T cells. Mice were injected with Daudi cells followed by injecting PBMC from healthy adult donors. The Daudi cells were sufficient to stimulate Vγ9Vδ2 T cells, resulting in proliferation and transition to effector memory phenotype, along with suppression of tumor growth and survival of the mice (88). Subsequently, tumor killing by Vγ9Vδ2 T cells was demonstrated in several types of immune-deficient mice and with a variety of tumors including prostate cancer (89), melanoma (90), breast cancer (91, 92), ovarian cancer (93), and lymphoma (56, 94, 95) to name a few examples from this growing list.

Mouse xenograft studies demonstrated the potency of Vγ9Vδ2 therapy *in vivo* and the range of tumors that might be treated. In general, treatments were most successful when Vγ9Vδ2 cells were expanded *ex vivo* prior to injection, when cell treatments coincided with tumor cell implantation or occurred when tumors were first deemed "palpable" (meaning < 100 mm^3 volume) and required repeated administration of phosphoantigen or aminobisphosphonate drugs plus cytokine (usually IL-2). The mouse xenograft studies provided some assurance that Vγ9Vδ2 cell therapy might be successful, but solid proof-of-concept data will be difficult to obtain in this system and safety studies needed for regulatory approval will be challenging. Because human Vγ9Vδ2 T cells are exquisitely species-restricted, normal mouse tissues are not recognized and off-target effects may be obscured. The mouse studies mimic a treatment approach based on adoptive cell therapy but are less useful for studying primary Vγ9Vδ2 tumor responses. In this regard, it is important to note that most clinical studies were completed without serious adverse events.

Mouse model studies raised intriguing issues related to Vγ9Vδ2 T cell trafficking and tumor localization. Knowing that the circulating pool of Vγ9Vδ2 T cells contains both central and effector memory cells, it seems reasonable that at least the effector memory subset would be actively attacking tumors. Injecting Vγ9Vδ2 T cells without additional treatment failed to demonstrate tumor-infiltrating cells and failed to reduce tumor volume. Treating myeloma patients with zoledronic acid increased Vγ9Vδ2 T cell migration into the tumor and infiltration depended on IPP secretion (96). An earlier study on mouse Vγ9Vδ2 T cell migration into murine tumors used antibody blocking to show a requirement for T cell receptor in chemotaxis and tumor infiltration (91). By a mechanism that is not yet established, elevated IPP levels and T cell receptor-dependent mechanisms are associated with Vγ9Vδ2 T cell infiltration into tumors, which is exaggerated by aminobisphosphonate treatment and the resulting increases in IPP levels. Such observations are important, but mechanistic insight into tumor infiltration by phosphoantigen-specific Vγ9Vδ2 T cells is still lacking. It is very important to understand γδ T cell trafficking and mechanisms controlling tumor infiltration.

Human clinical trials have created the greatest promise for Vγ9Vδ2 T cell immunotherapy but also revealed important obstacles to success. The limited potency of γδ T cell immunotherapy is the most pressing problem. Potency is a critical parameter even early in clinical product development, because Vγ9Vδ2 T cell therapies will be compared to results from CAR-T treatments for lymphoma and myeloma. The spectacular cure rates for CAR-T in selected diseases have raised expectations among scientists, patients, advocates, and funders. Positive results from Vγ9Vδ2 therapy in Hodgkin's lymphoma or multiple myeloma clinical trials showed significant *in vivo* activation of Vγ9Vδ2 T cells among 55% of patients who were pre-screened for high *in vitro* responses to pamidronate/IL-2, along with objective clinical responses among 33% of the pre-screened patients (97). Viewed objectively, these outcomes do not compare favorably with high cure rates for CAR-T in similar diseases (98). Undoubtedly, γδ-centric immunotherapy is eventually less complex and probably safer than CAR-T because the T cells are not genetically modified, but differences in potency will impede both research and commercial development of γδ-centric therapeutics until more data are available.

Tumor immunotherapy with Vγ9Vδ2 T cells, including *in vivo* stimulation, adoptive transfer of expanded cells or combination protocols, may find better purchase in solid tumor treatments where CAR-T is less advanced (98). Immunotherapy based on Vγ9Vδ2 T cells is not limited to tumors with well-defined neoantigens and allogeneic cell products may be possible due to the MHC-unrestricted responses of Vγ9Vδ2 T cells. Treatments based on Vγ9Vδ2 T cells have been tested for head and neck cancer (99), renal carcinoma (100, 101), prostate cancer (102), neuroblastoma (103), mammary carcinoma (104), and lung cancer (105, 106) among others. In most cases, objective responses were noted but the proportion of complete remissions was low and long-term disease-free survival data are minimal. These clinical studies provided evidence for the clinical utility of therapies aimed at activating the tumor response of Vγ9Vδ2 T cells. Clinical and basic researchers in this field need to chart a course for improving these therapies in terms of potency and defining the mechanism of action. We need to understand conditions controlling tumor infiltration by γδ T cells, how cytotoxic and regulatory subsets are regulated, and to understand failures or examples of low potency. This is a complex field with many different approaches and emphases that cannot be covered here in sufficient detail. The reader is encouraged to access several excellent reviews of clinically relevant studies that provide additional examples and important insights into trial outcomes and future directions (48, 49, 51, 107, 108).

FINDING SOLID GROUND

Researchers in this field are searching for ways to achieve more impactful and curative γδ T cell immunotherapies. If we can realize the full potential of tumor surveillance by these cells, it will be possible to address malignant disease in a broader part of the population than can be reached by other I/O approaches. Several studies are already pointing to more potent strategies. When Vγ9Vδ2 T cells, aminobisphosphonate, and IL-2 were delivered intratumorally in a murine xenotransplant model for glioblastoma, potent tumor reduction was observed (109). Why was intratumoral delivery better than systemic delivery of aminobisphosphonate? Aminobisphosphonate drugs are tremendous for their intended purpose of treating osteoporosis but have unfavorable pharmacokinetics because they complex with calcium and precipitate in the bone matrix. Although the drug may remain in bone for 10 years or more, circulating aminobisphosphonate is eliminated rapidly by renal excretion (110). Rapid clearance of aminobisphosphonates from plasma impedes their use for tumor therapy except in special cases where accumulation in bone was related to the anti-myeloma activity of pamidronate (111). Direct intratumoral injection of aminobisphosphonate avoided the unfavorable pharmacology of this drug class. Poor systemic availability of aminobisphosphonate drugs accounts in part, for differences between extraordinary Vγ9Vδ2 tumor killing *in vitro* where there is no bone to trap the drug, and the lower potencies observed in clinical trials. One of the possible keys to exploiting Vγ9Vδ2 T cells for tumor therapy is to activate them locally and achieve higher potency. In many cases, it may be difficult to

continue repeated injections of cells, aminobisphosphonate, and cytokine as was done for glioblastoma in a murine model (109), but studies of this type are beginning to highlight the potential benefits for local activation of Vγ9Vδ2 T cells.

We also know that aminobisphosphonate drugs are competitive inhibitors of FDPS. The FDPS is upregulated in some cancers (112) resulting in resistance to aminobisphosphonate drugs (113). Drug resistance was reversed *in vitro* by transfecting small inhibitory RNA targeting the FDPS mRNA to lower the enzyme levels (113). Thus, we expect an inverse relationship between levels of the stimulatory phosphoantigen IPP and levels of FDPS; reducing FDPS through genetic manipulation is a strategy for increasing the levels of IPP and may be combined with aminobisphosphonate for even higher potency. Lentivirus vector delivery of shRNA targeting FDPS mRNA was tested *in vitro*. The B cell lymphoma line Raji, a poor stimulator of Vγ9Vδ2 cell proliferation or effector function, was transduced with lentivirus vector expressing shRNA against FDPS mRNA. The modified cells were cultured with primary Vγ9Vδ2 T cells to detect changes in phenotype or function. Transduction reduced FDPS levels and markedly activated Vγ9Vδ2 T cells cocultured with the modified Raji cells. Raji cells with decreased FDPS also had increased sensitivity to Vγ9Vδ2 cytotoxicity (114).

Locally administered cancer therapies are increasingly of interest for stimulating potent tumor immunity. Peritumoral, intratumoral, and intranodal therapies have already been tested for a variety of cancers. Viral vectors expressing cytokines, tumor targeting antibodies, and checkpoint inhibitor antibodies appear to be more potent when injected into the tumor microenvironment as opposed to systemic delivery. Intratumoral injection of checkpoint inhibitor antibodies was explored as a means for improving potency while reducing the toxicity encountered after systemic administration [reviewed in Ref. (115)]. Simultaneous intranodal administration of the tumor-targeting antibody Rituximab plus autologous dendritic cells plus granulocyte-macrophage colony stimulating factor caused objective clinical responses in 36% of patients with disseminated follicular lymphoma, an aggressive disease with no known cure (116). Intranodal injection of an adenovirus vector expressing CD40L (CD154) caused objective responses to chronic lymphocytic leukemia in 11 of 15 patients treated (117). Intranodal injection may be viewed as a way to localize therapy near a tumor mass, that also allows stimulation of the immune cells outside of the immunosuppressive tumor microenvironment. These and similar findings encourage us to consider whether potency of Vγ9Vδ2 T cell immunotherapy for cancer has been limited by efforts to stimulate cells through systemic administration of drugs and cytokines, especially when using aminobisphosphonate drugs with unfavorable pharmacokinetics? It seems possible, even likely, that treatment potency will increase dramatically once we achieve potent delivery of stimulating agents to the tumor itself, either by intratumoral or intranodal delivery. Local activation of Vγ9Vδ2 T cells that will infiltrate the tumor, may come closer to realizing the full potential of these innate-like T cells for attacking a broad range of cancers without genetic manipulation of lymphocytes themselves.

SUMMARY

The remarkable γδ T cells continue to be a focus for the development of new cancer immunotherapies because they are an important component of natural tumor surveillance. The Vγ9Vδ2 T cells are particularly attractive for tumor therapy because they comprise the largest group of memory T cells responding to a single antigen. These phosphoantigen-responsive cells represent about 2% of total T cell memory in the circulating population and respond with the speed and uniformity of innate immunity. Basic and clinical research on Vγ9Vδ2 T cells and other γδ T cell subsets is already demonstrating utility in cancer therapy, but the challenge is to increase potency and understand better the mechanisms of action. The improvements in patient outcomes will come through better definition of the balance between effector and regulatory subsets, the role for inhibitory costimulation pathways, factors governing tumor infiltration, and methods for increasing potency. The keys to potency and tumor elimination may be found in local administration of stimulating agents including chemicals, cytokines, and viral vectors. All of these obstacles pale in comparison to the value of an immunotherapy that attacks a broad range of tumor cell types, does not require identification of tumor-associated antigens, and does not require genetic modification of T cells. Local treatment delivered at the tumor site may be one way to increase Vγ9Vδ2 T cell potency. The promise is to achieve a near universal solution for malignant disease. The challenge is to translate the exquisite science of γδ T cell biology for the practical goal of cancer immunotherapy.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Functional Plasticity of Gamma Delta T Cells and Breast Tumor Targets in Hypoxia

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Interactions between immune and tumor cells in the tumor microenvironment (TME) often impact patient outcome, yet remain poorly understood. In addition, the effects of biophysical features such as hypoxia [low oxygen (O_2)] on cells within the TME may lead to tumor evasion. Gamma delta T cells (γδTcs) naturally kill transformed cells and are therefore under development as immunotherapy for various cancers. Clinical trials have proven the safety of γδTc immunotherapy and increased circulating γδTc levels correlate with improved patient outcome. Yet, the function of γδTc tumor infiltrating lymphocytes in human breast cancer remains controversial. Breast tumors can be highly hypoxic, thus therapy must be effective under low O_2 conditions. We have found increased infiltration of γδTc in areas of hypoxia in a small cohort of breast tumors; considering their inherent plasticity, it is important to understand how hypoxia influences γδTc function. *In vitro*, the cell density of expanded primary healthy donor blood-derived human γδTc decreased in response to hypoxia (2% O_2) compared to normoxia (20% O_2). However, the secretion of macrophage inflammatory protein 1α (MIP1α)/MIP1β, regulated on activation, normal T cell expressed and secreted (RANTES), and CD40L by γδTc were increased after 40 h in hypoxia compared to normoxia concomitant with the stabilization of hypoxia inducible factor 1-alpha protein. Mechanistically, we determined that natural killer group 2, member D (NKG2D) on γδTc and the NKG2D ligand MHC class I polypeptide-related sequence A (MICA)/B on MCF-7 and T47D breast cancer cell lines are important for γδTc cytotoxicity, but that MIP1α, RANTES, and CD40L do not play a direct role in cytotoxicity. Hypoxia appeared to enhance the cytotoxicity of γδTc such that exposure for 48 h increased cytotoxicity of γδTc against breast cancer cells that were maintained in normoxia; conversely, breast cancer lines incubated in hypoxia for 48 h prior to the assay were largely resistant to γδTc cytotoxicity. MICA/B surface expression on both MCF-7 and T47D remained unchanged upon exposure to hypoxia; however, ELISAs revealed increased MICA shedding by MCF-7 under hypoxia, potentially explaining resistance to γδTc cytotoxicity. Despite enhanced γδTc cytotoxicity upon pre-incubation in hypoxia, these cells were unable to overcome hypoxia-induced resistance of MCF-7. Thus, such resistance mechanisms employed by breast cancer targets must be overcome to develop more effective γδTc immunotherapies.

Keywords: gamma delta T cells, plasticity, hypoxia, breast cancer, tumor evasion, MHC class I polypeptide-related sequence A

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INTRODUCTION

Low oxygen (O_2) levels (hypoxia) characterize the microenvironment of many solid tumors, occurring as a consequence of structurally disorganized blood vessels and tumor growth that exceeds the rate of vascularization. Hypoxia is common within breast cancers, which have a median O_2 concentration of 1.4%, as compared to ~9.3% for normal breast tissue (1). In response to hypoxia, cells express genes that are essential for their survival. In tumor cells, this O_2-regulated gene expression leads to more aggressive phenotypes, including those that increase the ability of cells to resist therapy, recruit a vasculature and metastasize (2–4). Accordingly, there is a growing body of evidence correlating tumor hypoxia with poor clinical outcome for patients with a variety of cancers (5–7). O_2 availability has also been shown to regulate immune editing, allowing cancer cells to evade the immune system *via* a variety of mechanisms (8). For example, hypoxia upregulates hypoxia inducible factor 1-alpha (HIF1α)-dependent ADAM10 expression resulting in MHC class I polypeptide-related sequence A (MICA) shedding from the surface and decreased lysis of tumor cells (9). While many studies have focused on myeloid-derived suppressor cells or conventional CD8+ T cells (8), so far none have considered the impact of tumor hypoxia on gamma delta T cells (γδTcs).

While γδTc kill cancer cell lines, derived from both hematological and solid tumors alike [reviewed in Ref. (10)], it is unclear whether they are still active cancer killers when confronted with the harsh and immunosuppressive tumor microenvironment (TME) (10–13). We have focused on breast cancer, since there have been conflicting reports in the literature with respect to γδTc function in this disease. While *in vitro* studies clearly show that γδTc are able to kill breast cancer cell lines MDA-MB231, MCF-7, and T47D (14–16), it is unclear as to whether γδTc retain their cytotoxic properties once exposed to the breast tumor TME (11).

Here, we set out to determine how γδTc behave under low O_2, a TME factor likely encountered by γδTc in many malignancies. Carbonic anhydrase IX (CAIX) is a transmembrane protein that catalyzes the reversible hydration of carbon dioxide. It is expressed in response to hypoxia and is thus used as a surrogate marker for hypoxia (17). High CAIX expression indicates poor prognosis in many cancers, including breast cancer (18–20). Breast cancer cell lines express MICA, a ligand for the natural killer group 2, member D (NKG2D) receptor expressed by γδTc and implicated in γδTc cytotoxicity (21–25). Thus, we have further explored the integral role for NKG2D/MICA in γδTc cytotoxicity against breast cancer cell lines under hypoxia and normoxia.

Since γδTc are being developed for cancer immunotherapy (26–31), and have shown both safety and even some efficacy—despite advanced disease stage—in a Phase I trial for breast cancer (32), it is imperative that we learn how the TME impacts the function of γδTc infiltrating breast and other solid tumors.

Abbreviations: BP, band pass; CAIX, carbonic anhydrase IX; CalAM, Calcein AM; CD40L, CD40 ligand (or CD154); E:T, effector:target ratio; ER, estrogen receptor; FBS, fetal bovine serum; FMO, fluorescence minus one; γδTcs, gamma delta T cells; HIF1α, hypoxia inducible factor 1-alpha; HRP, horseradish peroxidase; IL, interleukin; LP, long pass; MICA, MHC class I polypeptide-related sequence A; MIP1α, macrophage inflammatory protein 1α [or CCL3 = chemokine (C–C motif) ligand 3]; MFI, median fluorescence intensity; NKG2D, natural killer group 2, member D; O_2, oxygen; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PIC, protease and phosphatase inhibitor cocktail; PR, progesterone receptor; RANTES, regulated on activation, normal T cell expressed and secreted (or CCL5); TBST, tris-buffered saline plus 0.05% Tween-20; TCR, T cell antigen receptor; TIL, tumor infiltrating lymphocytes; TME, tumor microenvironment; TNFα, tumor necrosis factor alpha; ZA, Zombie Aqua fixable viability dye.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the Research Ethics Guidelines, Health Research Ethics Board of Alberta—Cancer Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Health Research Ethics Board of Alberta—Cancer Committee.

Patients and Tissues

We assessed 17 surgically resected breast tumors from cancer patients diagnosed at the Cross Cancer Institute, Edmonton, AB, Canada from 1997 to 1998. Patient and tumor characteristics are listed in **Table 1**.

Immunohistochemistry

Anti-human T cell antigen receptor (TCR)δ staining was performed as reported (33). Briefly, 4.5 μm serial sections from formalin-fixed paraffin-embedded tumors were melted on a slide warmer at 60°C for a minimum of 10 min followed by de-paraffinization using a

TABLE 1 | Characteristics of breast cancer cohort.

n = 17	*n* (%)	Median (range)
Age at diagnosis		51 (40–69)
Histology		
Invasive ductal carcinoma	14 (82)	
Invasive non-ductal tubular	1 (6)	
Invasive non-ductal mucinous	1 (6)	
Non-invasive	1 (6)	
Tumor size (cm)		
<2	11 (65)	1.4 (0.2–5.5)
2–5	4 (24)	
>5	1 (6)	
Not specified	1 (6)	
Tumor grade		
1/3	4 (24)	
2/3	5 (29)	
3/3	8 (47)	
Nodal status		
Positive	9 (53)	
Negative	8 (47)	
Estrogen receptor		
Positive	12 (71)	
Negative	3 (18)	
Not available	2 (12)	
Progesterone receptor		
Positive	10 (59)	
Negative	5 (29)	
Not available	2 (12)	

fresh Xylenes (Thermo Fisher Scientific, Burlington, ON, Canada) bath. Sections were then hydrated with a series of graded ethanol (100, 95, 70, and 60%) followed by brief incubation in water, then tris-buffered saline plus 0.05% Tween-20 (TBST). Antigen retrieval was performed at 100°C for 20 min in target retrieval solution pH 9 (DAKO North America, Carpinteria, CA, USA). After cooling to room temperature, tissues were circled with an ImmEdge pen (Vector Laboratories, Burlingame, CA, USA) and blocked with Peroxidase Block (DAKO) for 5 min. Slides were washed in TBST for 5 min then blocked with Protein Block Serum Free (DAKO) for 10 min. Protein block was gently removed and replaced with 1:150 dilution of mouse monoclonal anti-human TCRδ antibody (clone H-41, Santa Cruz Biotechnology, Dallas, TX, USA) or 1:50 dilution of rabbit monoclonal anti-human CAIX [clone EPR4151(2), abcam, Cambridge, MA, USA] or corresponding isotype control diluted to the same antibody concentration; all dilutions were made in antibody diluent (DAKO). Known positive controls and isotype controls were included with each batch to ensure quality control of staining. Sections were incubated in a humidified chamber for 30 min at 25°C. Slides were then rinsed and washed five times in TBST for 5 min. Slides were then incubated with 100 µl secondary antibody, labeled polymer—horseradish peroxidase (HRP) anti-mouse or—HRP anti-rabbit (DAKO), for 60 min at room temperature in the humidified chamber. Washing was done as before, and then slides were treated with 75–100 µl 3,3′-diaminobenzidine chromogen solution (DAKO) for 8–10 min before the reaction was stopped by rinsing in water. Hematoxylin (DAKO) counterstaining was performed, slides were rinsed in water and then dehydrated using a series of graded ethanol (60, 70, 95, and 100%). Slides were then cleared with Xylenes, dried and coverslips mounted with VectaMount permanent mounting medium (Vector Laboratories).

Assessment of CAIX Expression and γδTc Infiltration

Light microscopy and semi-quantitative scoring for CAIX was performed by a single pathologist; scores were 0, no staining; 1, weak and/or very focal staining; 2+, strong but focal staining; and 3, strong and extensive staining. Serial sections stained for TCRγδ and CAIX were scanned. Areas of CAIX-positivity and negativity were defined, and images from slides superimposed to enable counting of γδTc in CAIX-positive and -negative areas. Five consecutive areas within each region were quantified for the frequency of γδTc infiltration.

Primary γδTc

We established and maintained primary human γδTc cultures as described (34). Briefly, healthy donor blood was diluted with phosphate buffered saline (PBS) and peripheral blood mononuclear cells (PBMCs) isolated using density gradient separation (Lymphoprep, Stem Cell Technologies, Vancouver, BC, Canada). PBMCs were cultured in a humidified atmosphere at 37°C with 5% CO_2 at 1×10^6 cells/ml in RPMI complete medium containing 1 µg/ml Concanavalin A (Sigma-Aldrich, Oakville, ON, Canada), 10% fetal bovine serum (FBS), 1× MEM NEAA, 10 mM HEPES, 1 mM Sodium Pyruvate (all Invitrogen, Burlington, ON, Canada), and 10 ng/ml recombinant human interleukin (IL)-2 and IL-4 (Miltenyi Biotec, Auburn, CA, USA). Cells were counted and viability assessed *via* Trypan Blue Exclusion Assay (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA); fresh medium and cytokines added to adjust density to 1×10^6 cells/ml every 3–4 days. After 1 week, αβ T cells were labeled with anti-TCRαβ PE antibodies (BioLegend, San Diego, CA, USA) and anti-PE microbeads (Miltenyi Biotec), and depleted after filtering (50 µm Cell Trics filter, Partec, Görlitz, Germany) and passing over an LD depletion column (Miltenyi Biotec). γδTcs, which did not bind to the column, were further cultured in complete medium plus cytokines (as above). For cytotoxicity and blocking experiments, γδTc cultures were used on days 19–21, as they were most cytotoxic then. Some hypoxia experiments were done at earlier time points. Donor cultures are identified as follows: donor number culture letter-culture day; thus, 7B-13 = the second culture derived from donor 7 on day 13. Culture purities and subset compositions are shown in Table S1 in Supplementary Material.

Breast Cancer Cell Lines

Human breast carcinoma cell lines, MCF-7 and T47D, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained as per ATCC guidelines. For surface marker staining of breast cancer cell lines, cells were harvested by washing with PBS followed by dissociation in Accutase (Sigma-Aldrich) for 20 min at 37°C.

Hypoxia Experiments

To examine the effects of hypoxia, cells were cultured in O_2 concentrations as indicated for 40–48 h using an X3 Xvivo Closed Incubation System (BioSpherix). After incubation under normoxic or hypoxic conditions, cell culture supernatants were collected, chilled on ice, and then frozen at −80°C until further analysis; harvested cells were used in cytotoxicity assays or stained for flow cytometric analysis. In some cases, cells were cold harvested, pellets frozen on dry ice, and stored at −80°C until lysis for Western blotting.

Flow Cytometry

Antibodies

For surface marker staining of γδTc, the following anti-human antibodies from BioLegend, unless otherwise indicated, were employed: TCRγδ PE (clone B1, 1:25); TCR Vδ1 FITC (Miltenyi, clone REA173, 1:10); TCR Vδ2 PerCP (clone B6, 1:25); NKG2D APC (BD Biosciences, Mississauga, ON, Canada, 1:25); CD56 FITC (clone MEM-188, 1:5); CD69 AF700 (clone FN50, 1:4); CD94 FITC (clone DX22, 1:5); CD95 APC (clone DX2, 1:100); HLA ABC PE (clone W6/32, 1:10); FasL PE (clone NOK-1, 1:5); and CD40L APC (clone 24–31, 1:5).

Anti-human MICA/B PE (BioLegend, clone 6D4, 0.1 µg) was used to stain breast cancer cell lines.

Surface Marker Staining

Gamma delta T cell and breast cancer cell lines were adjusted to 10×10^6 cells/1 ml, stained with 1 µl/10^6 cells Zombie Aqua fixable viability dye in PBS (ZA, BioLegend) for 15–30 min at

room temperature in the dark. γδTc were stained directly with fluorochrome-conjugated antibodies diluted in FACS buffer [PBS containing 1% FBS and 2 mM EDTA (Invitrogen)] as indicated above. Breast cancer cell lines at 10×10^6 cells/ml were blocked in FACS buffer containing 50 µl/ml Trustain FcX (BioLegend) and incubated on ice for 30 min prior to antibody incubation. After blocking, cells were centrifuged and supernatants removed, leaving 10 µl FACS buffer plus block/10^6 cells. Antibodies and FACS buffer were added to 20 µl, and cells incubated on ice 15–20 min followed by washing. All cells were fixed in FACS buffer containing 2% paraformaldehyde (Sigma-Aldrich), stored at 4°C and acquired within 1 week.

Flow Cytometer Specifications

Cells were analyzed using a FACS CANTO II (Becton Dickinson, Mississauga, ON, Canada) equipped with an air-cooled 405-nm solid state diode, 30 mW fiber power output violet laser, with 450/50 and 510/50 band pass (BP) [502 long pass (LP) detector]; a 488-nm solid state, 20-mW blue laser with 530/30 BP (502 LP), 585/42 BP (556 LP), 670 LP (655 LP), and 780/60 BP (735 LP) filters; and a 633-nm HeNe, 17-mW red laser with 660/20 BP and 780/60 BO (735 LP) filters. Calibration was performed with CS&T beads (Becton Dickenson, Mississauga, ON, Canada). Live singlets were gated based on forward and side-scatter properties. Fluorescence minus one (FMO) controls were used to set gates. Analysis was performed using FlowJo© software (Tree Star, Ashland, OR, USA, Version 10.0.8r1).

Cytokine Arrays

The Proteome Profiler Human Cytokine Array Kit, Panel A (R&D Systems, Minneapolis, MN, USA) was used to detect proteins secreted by γδTc cultured under normoxic or hypoxic conditions. Undiluted culture supernatants were used in these assays, which were carried out according to the manufacturer's instructions. Analysis of resulting films was done as follows: pixel intensities were measured using FIJI software (ImageJ Version 2.0.0-rc-15/1.49m) using a consistent circular region of interest; measured values from duplicate spots were subtracted from 255. The average intensity from the two negative spots was subtracted from all values to obtain net values. The intensities of the six reference spots (positive controls) were averaged and a multiplier was defined for each array (normalized to the array with the lowest pixel intensity). Values were adjusted accordingly and then values for the duplicates were averaged. Finally, ratios were calculated for each cytokine, normalized to normoxia.

ELISAs

1–2 ml aliquots of culture supernatants stored at −80°C were thawed on ice. Halt™ Protease and Phosphatase Inhibitor Cocktail (PIC, Thermo Fisher Scientific) was added to samples prior to use in ELISAs or further storage at 4°C. The following ELISA kits were used: ELISA MAX Deluxe regulated on activation, normal T cell expressed and secreted (RANTES/CCL5) (BioLegend), Human macrophage inflammatory protein 1α (MIP1α) and Human CD40L Quantikine ELISA kits (R&D Systems), and Human MICA ELISA Kit (abcam). For RANTES and CD40L ELISAs, culture supernatant samples were diluted up to 16-fold to obtain readings within range (1:2, 1:4, 1:8, 1:16). For MIP1α ELISAs, samples were diluted up to 1:20. For MICA ELISAs, culture supernatants stored at −80°C were thawed overnight in at 4°C, then 4 ml applied to Amicon Ultra-4 10 K spin columns (Merck-Millipore, Carrigtwohill, Ireland) that were subsequently centrifuged at 3,000 *g* for 2 h at 12°C. Concentrated media was then transferred into 1.5 ml Eppendorf tubes and diluted to 200 and 20 µl of a 1:10 dilution of PIC were added. For the ELISA, 100 µl per well were assayed in duplicate. All ELISAs were done according to the manufacturer's instructions. Absorbance at 450 and 550 nm was measured using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany) with Omega Software version 5.11. The difference linear regression fit of the standard curve was used for concentration calculations. ELISA data were normalized to γδTc cell numbers and culture volumes.

Immunoblotting

Cell lysates were prepared by mixing γδTc with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing PIC at 10 µl lysis buffer per million γδTc followed by incubation at room temperature for 10 min. Lysates were then centrifuged at 13,000 rpm for 15 min 4°C, after which supernatants were transferred to fresh tubes and 5× reducing sample buffer [0.0625 M Tris/HCl pH6.8, 2% SDS, 20% glycerol, 0.05% β-mercaptoethanol, 0.025% (w/v) Bromophenol Blue] added. Samples were boiled 5 min, cooled, and briefly centrifuged in a benchtop centrifuge prior to running on 10 or 12% SDS-PAGE gels. Proteins were transferred onto Immobilon-FL PVDF membranes (Millipore) using the Trans-Blot Turbo Transfer System (Bio-Rad, Mississauga, ON, Canada). The high molecular weight (MW) program was used when transferring proteins for HIF1α detection. Otherwise, the mixed MW program was used. Membranes were blocked 40 min in 3% milk in TBST, followed by overnight incubation in primary antibody baths at 4°C. After washing, membranes were incubated with the corresponding species-specific HRP-labeled secondary antibody for 1 h, followed by further washing and then detection using Clarity™ Western ECL Substrate (Bio-Rad). Primary antibodies were diluted in PBS containing 2% bovine serum albumin and 0.05% sodium azide at the following dilutions: 1:500 mouse anti-human HIF-1α (clone MOP1, BD Biosciences); 1:2,000 goat anti-human CCL3/MIP1α (R&D Systems); 1:1,000 mouse anti-human/primate CCL5/RANTES (Clone #21418, R&D Systems); 1:500 mouse anti-human CD40 ligand/TNFSF5 (Clone #40804, R&D Systems); 1:2,000 rabbit anti-human β-Actin (Cell Signaling Technologies, Danvers, MA, USA). Secondary antibodies were diluted in blocking buffer as follows: 1:10,000 goat anti-mouse IgG HRP (Bio-Rad); 1:20,000 goat anti-rabbit IgG HRP (Bio-Rad); and 1:1,000 donkey anti-goat IgG HRP (R&D Systems).

Quantification of Bands on Western Blots

Band intensities for CD40L, MIP1α, and RANTES were measured using FIJI software (ImageJ Version 2.0.0-rc-15/1.49m) on converted grayscale images using consistent rectangular regions of interest. Measured values for bands and background (region of same size beneath each band) were subtracted from 255, then background was subtracted from bands to obtain net values for

protein bands of interest and loading control bands (actin). The ratios of protein bands to loading control bands were then calculated. In the case of CD40L and RANTES, these values were multiplied by 10 to obtain values between 0.1 and 10. For calculation of induction, hypoxia values were divided by normoxia values, and average values for each protein were plotted. Calculations were done in Microsoft Excel version 15.3 (Microsoft, Redmond, WA, USA).

Cytotoxicity Assays

Target Cell Labeling With Calcein AM (CalAM)

As per the manufacturer's instructions, target cells were labeled with 5 μM CalAM (Invitrogen/Thermo Fisher Scientific). Cells were diluted to 30,000 cells/100 μl medium for cytotoxicity assays.

Blocking Antibodies

The following anti-human antibodies were used: LEAF purified anti-NKG2D (BioLegend, Clone 1D11); anti-human CCL3/MIP1α (R&D Systems); anti-human/primate CCL5/RANTES (Clone #21418, R&D Systems); and anti-human CD40 ligand/TNFSF5 (Clone #40804, R&D Systems). Mouse IgG (Sigma-Aldrich) was used as a control.

Blocking/Cytotoxicity Assay

For blocking and cytotoxicity assays, 6×10^6 cells/ml γδTc cells were re-suspended in complete medium: RPMI 1640 plus 10% heat-inactivated FBS; 10 mM HEPES; 1× MEM NEAA; 1 mM sodium pyruvate; 50 U/ml penicillin–streptomycin; and 2 mM L-glutamine, all purchased from Invitrogen. Blocking antibodies were added at 6 μg mAb per 600 μl cell suspension/test in Eppendorf tubes, then plated at 100 μl/well in a 96-well round-bottomed plate and incubated at 37°C for 30 min. Thereafter, 100 μl CalAM-labeled targets were added. For cytotoxicity assays, the effector:target (E:T) ratio is indicated; blocking assays were done at 20:1. Co-cultures were incubated at 37°C for 4 h, after which plates were centrifuged and supernatants transferred to black clear-bottom 96-well (flat) plates (Costar, VWR International, Edmonton, AB, Canada). CalAM fluorescence was then detected on a FLUOstar Omega, BMG labtech fluorimeter. Controls were untreated and IgG-treated cells (for blocking assays), CalAM-labeled target cells incubated alone (spontaneous release) as well as 0.05% Triton-X 100 (Thermo Fisher Scientific)-treated cells (maximum release). The calculation for percent lysis is: [(test-spontaneous release)/(maximum-spontaneous release)] × 100%.

Statistics

The following tests were used to determine significance: paired one-tailed Student's *t*-tests [**Figures 2A,B** only, Microsoft Excel version 15.3 (Microsoft, Redmond, WA, USA)]; paired two-tailed Student's *t*-tests [**Figures 2C–K**, Prism 7.0 for Mac OSX (GraphPad Software, San Diego, CA, USA)]; one-way ANOVA analysis (**Figure 4**, Prism); and Shapiro–Wilk normality tests followed by two-way ANOVA (**Figure 1E**, 3, 5, 6, Prism). Sidak's pairwise multiple comparison *post hoc* tests were performed alongside ANOVA analyses. The threshold for significance was set at $P < 0.05$; asterisks indicate degrees of significance as defined in the figure legends.

RESULTS

γδTc Can Be Found in Hypoxic Regions in Breast Cancer Cases

In order to determine whether γδTc are present in areas of hypoxia in breast tumors, we performed immunohistochemistry to detect the hypoxia marker CAIX and γδTc using single stains of serial sections from a panel of 17 breast tumors (**Table 1**). Examples from one case (case 14) are shown (**Figures 1A–D**), including images of a CAIX-positive region (**Figure 1A**), an area with no appreciable CAIX positivity (**Figure 1B**), and increased magnification of γδTc found in the same region depicted in **Figure 1A** (**Figure 1C**) and **Figure 1B** (**Figure 1D**). Of these 17 cases, 47% (8/17) stained positively for CAIX. In CAIX-negative cases, there was little γδTc infiltration; however, when γδTc were quantified in CAIX-positive versus CAIX-negative areas of breast tumors, γδTc frequency was greater in hypoxic regions, significantly so in three cases in particular (**Figure 1E**, cases 13, 14, and 17, $P < 0.0001$). Images for cases 13 and 17 are in Figure S1 in Supplementary Material. In our cohort, 71% (12/17) of tumors were estrogen receptor positive (ER+); most ER+ cases were CAIX-negative (**Figure 1E**, ER status indicated below case numbers).

Exposure to Hypoxia Reduces γδTc Density

Given the co-localization of γδTc and CAIX in breast tumors, we measured the effects of hypoxia on γδTc viability and density *in vitro*. We cultured γδTc for 12–19 days, then subjected them to 48 h in hypoxic (2% O_2) or normoxic (20% O_2) conditions. We found that exposure to hypoxia had variable effects on γδTc viability (**Figure 2A**, $P = 0.08$), and significantly decreased cell density (**Figure 2B**, $P = 5.7 \times 10^{-4}$). Immunophenotyping was performed using flow cytometric analyses of activation markers including γδTCR, NKG2D, CD56, CD69, CD95, CD40L, and HLA ABC as well as the inhibitory markers FasL and CD94. γδTc were stained with live/dead ZA prior to surface marker staining. Median fluorescence intensity values (MFIs) of hypoxia and normoxia samples were divided by the MFI of FMO controls to obtain fold-change values. Surface markers on γδTc cultures subjected to 48 h 20 or 2% O_2 were not significantly different (**Figures 2C–K**).

MIP1α, RANTES, and CD40L Are Secreted by γδTc in Hypoxia

Culture supernatants from three different donor γδTc cultures subject to 40 h of normoxia or hypoxia were analyzed by cytokine array. While IL-8 appears elevated in the cumulative results graph depicted here (**Figure 3A**), this cytokine was only greatly increased under hypoxia in one of three experiments (Figure S2B in Supplementary Material, $P < 0.0001$), was moderately increased in one experiment (Figure S2A in Supplementary Material, $P < 0.05$), and not significantly elevated in the third experiment (Figure S2C in Supplementary Material). Due to significant variation among

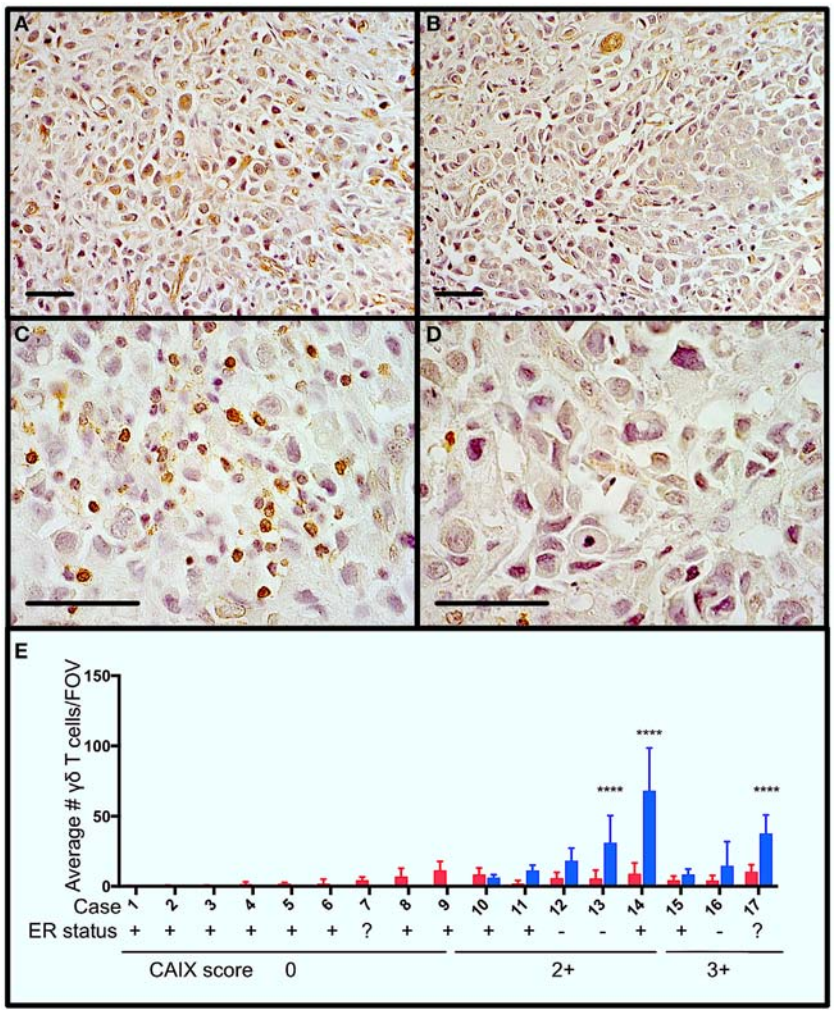

FIGURE 1 | Gamma delta T cells (γδTcs) are present in areas of hypoxia in estrogen receptor positive (ER+) breast tumors. Serial sections from ER+ breast tumors were stained for carbonic anhydrase IX (CAIX) and T cell antigen receptor δ. **(A)** Example of CAIX-positive staining at 400× magnification from case 14; **(B)** CAIX-negative field of view (FOV) from the same slide as in **(A)**; **(C)** γδTc in the same area as **(A)** at 1,000× magnification; and **(D)** γδTc in the same area as **(B)** at 1,000× magnification. Scale bars are 50 μm. Brown indicates positive staining. **(E)** Parallel staining for γδTc and CAIX suggests that γδTc infiltration increases in hypoxic regions. CAIX scoring is indicated below the case numbers: 0 = no staining; 1 = weak and/or very focal staining; 2+ = strong but focal staining; and 3 = strong and extensive staining. Quantification and statistical analysis of γδTc frequency in CAIX-positive versus -negative regions (blue and red bars, respectively) reveal significantly increased γδTc infiltration in hypoxic regions (two-way ANOVA, $^{****}P < 0.0001$).

donor cultures, cumulative results reveal significantly increased secretion of only CD40 ligand (CD40L or CD154) under hypoxia compared to normoxia (**Figure 3A**, $P = 0.0472$). However, in all three individual cytokine arrays, significantly increased secretion of MIP1α [or CCL3 = chemokine (C–C motif) ligand 3], RANTES (or CCL5), and CD40L under hypoxia compared to normoxia was observed (Figures S2A–C in Supplementary Material). Note that equal cell numbers were plated, and relative values at 2 and 1% O_2 were normalized to normoxia without taking harvested cell numbers into account. Considering the decrease in γδTc densities observed under hypoxia, this suggests an even greater effect would be observed if comparing the output of equal cell numbers.

ELISA validation for expression of RANTES, MIP1α, and CD40L was performed with culture supernatants from three different γδTc cultures (**Figures 3B–E**, hypoxia = 1 or 2% O_2 as indicated). For RANTES expression, an additional eight experiments were assayed, for secretion over 48 h at 20 or 2% O_2 (**Figure 3C**). In this case, and in contrast to the cytokine array data, ELISA values were normalized to cell numbers. Significantly increased secretion of these cytokines by γδTc was observed when cells were cultured in hypoxia compared to normoxia (asterisks indicate significance). A wide range of average secreted RANTES levels was observed, ranging from 93 to 521 pg/million γδTc in normoxia to 431 to 856 pg/million γδTc under hypoxia; the average ratio hypoxia:normoxia is indicated above the bars (**Figures 3B–E**). Likewise, secreted MIP1α and CD40L levels were quantified for three independent experiments using ELISA (**Figures 3D,E**). MIP1α levels ranged from 152 to 394 pg/million γδTc in normoxia to 1,406 to 2,509 pg/million γδTc under hypoxia,

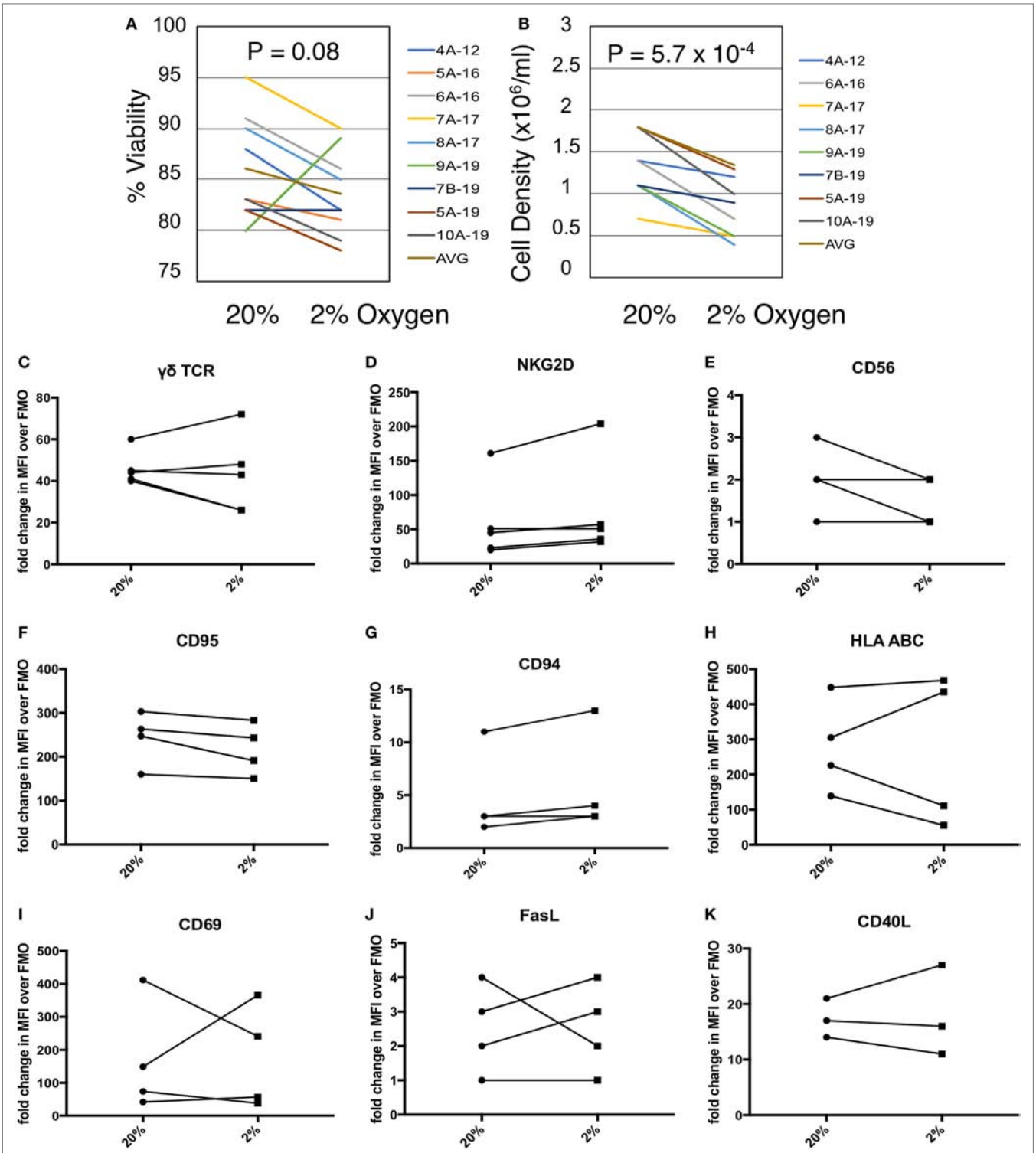

FIGURE 2 | Gamma delta T cell (γδTc) viability and proliferation under hypoxia/normoxia differ, but overall surface marker expression is not significantly impacted by oxygen levels. **(A)** Viability of γδTc cultured under 20% versus 2% O_2 for 48 h beginning on culture days 12–19, for eight cultures from seven different donors, assessed *via* Trypan Blue exclusion. Donor numbers are given with A and B indicating different cultures from the same donor; numbers after the hyphen are the culture days on which the experiment was begun. **(B)** Cell density assessment from experiment shown in **(A)**. **(C–K)** The indicated surface markers were assessed by flow cytometric analysis [**(C,D)** $n = 5$; **(E–J)** $n = 4$; **(K)** $n = 3$ different donor cultures].

with fold changes from 4.0 to 14.2 (**Figure 3D**). Similarly, CD40L secretion by γδTc increased significantly when cultured in low O_2, with 2% O_2 in one experiment yielding an average of 171 pg CD40L/million γδTc in hypoxia, a 4.9-fold increase over just 35 pg CD40L/million γδTc in normoxia (**Figure 3E**). Two experiments conducted with 1% O_2 yielded a wide range of CD40L secretion by γδTc in both conditions (**Figure 3E**, 120–395 and 536–653 pg CD40L/million γδTc in normoxia and hypoxia, respectively).

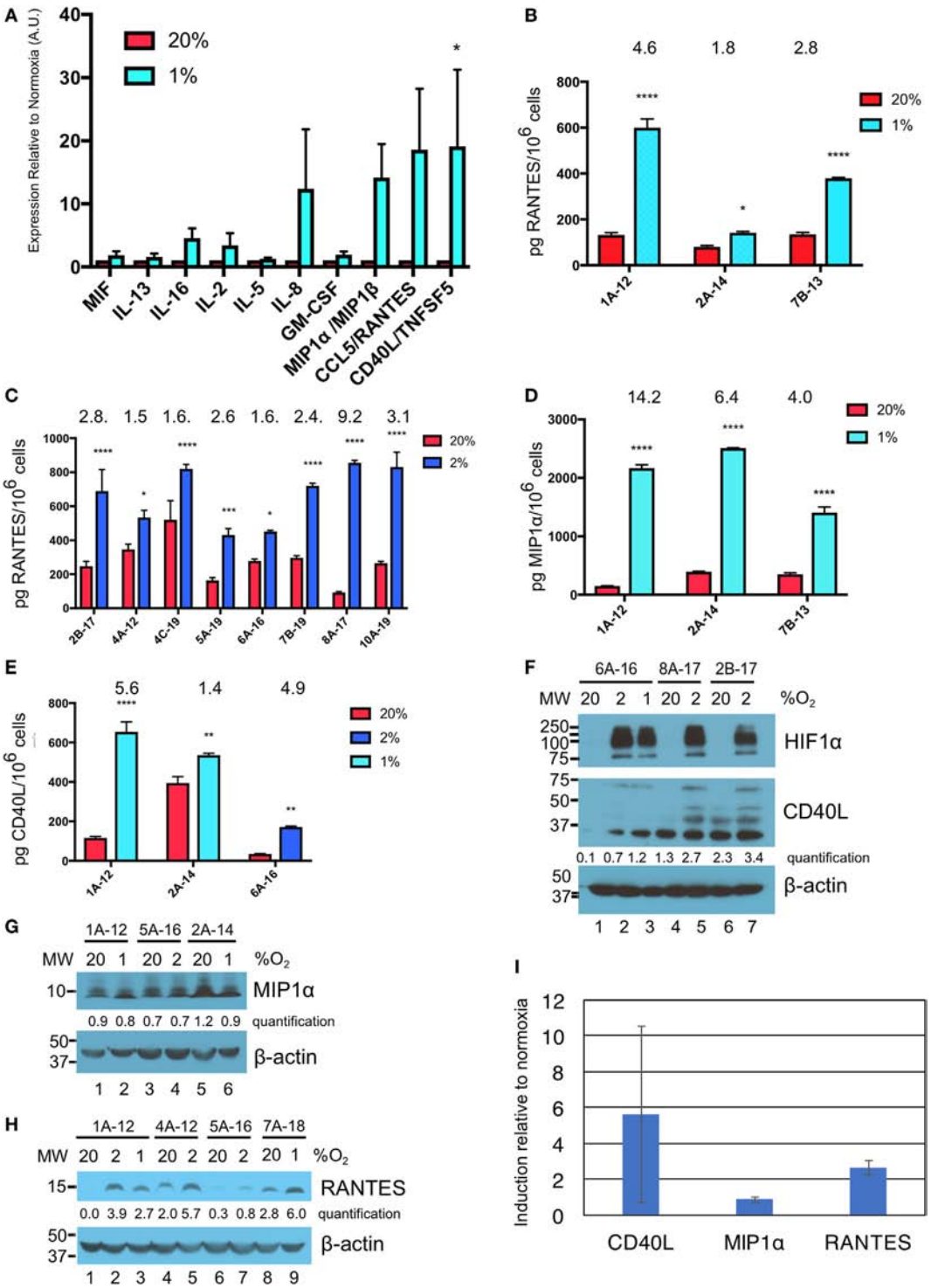

FIGURE 3 | Continued

FIGURE 3 | Hypoxia induces secretion of macrophage inflammatory protein 1α (MIP1α), CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), and CD40L/TNFSF5 by gamma delta T cells (γδTcs). **(A)** Culture supernatants from γδTc subjected to 40 h at 20 or 1% O_2 were analyzed by cytokine array. Cumulative results of three independent experiments for a panel of cytokines that were differentially secreted by γδTcs under hypoxia compared to normoxia are shown. Error bars are SEM; A.U. = arbitrary units; **(B)** ELISA validation of RANTES cytokine results shown in **(A)** for three independent experiments carried out at 20 and 1% O_2 for 40 h; **(C)** RANTES ELISA for eight hypoxia experiments carried out for 48 h at 20 and 2% O_2; **(D)** MIP1α ELISA for the same experiments shown in **(B)**; **(E)** CD40L ELISA for culture 6A-16 subject to 48 h 20 or 2% O_2, and two of the experiments shown in **(B,D)**. Statistical analyses for **(A–E)**: two-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; **(F–H)** Western blot analysis of lysates from γδTc cultures subject to 20, 2, and/or 1% O_2 for 48 h as indicated. γδTc culture identification is given above the blots and molecular weight (MW) markers are shown on the left; corresponding β-actin loading controls are shown in the bottom panels; relative band intensities were quantified and are indicated in arbitrary units; **(F)** three examples shown for detection of hypoxia inducible factor 1-alpha (HIF1α) ($n = 6$, 5 different donors) and CD40L ($n = 8$, 7 γδTc cultures from six donors); **(G)** MIP1α ($n = 7$, 6 γδTc cultures from five donors); **(H)** RANTES ($n = 7$); and **(I)** induction of proteins in **(F–H)** was determined by dividing protein band intensities from hypoxic samples by their corresponding normoxia control, and averaging these values. Error bars are SD.

Western blotting was done to verify induction of HIF1α in γδTc under hypoxia, and also to investigate whether intracellular levels of CD40L, MIP1α, and RANTES reflected those of secreted proteins (**Figures 3F,G**). HIF1α was clearly induced in γδTc at 2 and 1% O_2 in all cases; three examples from six independent experiments with five donor cultures are shown (**Figure 3F**, top panel, compare lane 1 versus 2 and 3, 4 versus 5, and 6 versus 7). CD40L appears visibly increased in hypoxia samples for γδTc culture 6A-16 (**Figure 3F**, middle panel, compare lane 1 versus 2 and 3), and quantification suggests this is also the case for the other two donor cultures shown (lane 4 versus 5 and lane 6 versus 7). Note that several forms of CD40L are evident here, which were included in the quantification of bands. Of eight experiments with seven γδTc cultures from six donors, intracellular CD40L was clearly visibly increased in three (38%). HIF1α and CD40L blots originated from the same gel, which was transferred and then cut at 75 kDa; thus, the β-actin loading control serves for both (**Figure 3F**, lower panel). MIP1α levels were not consistently higher in γδTc subject to hypoxia versus normoxia (**Figure 3G**, representative of seven experiments with six γδTc cultures from five donors), as demonstrated by very similar quantification values within each experiment. By contrast, RANTES was typically induced by hypoxia, with higher protein levels evident in cellular lysates from γδTc cultured in 1 or 2% O_2 compared to normoxia (**Figure 3H**, compare lane 1 versus 2 and 3, 4 versus 5, and 8 versus 9; n = seven independent experiments, seven donors, induction clear in six, unclear in one). Longer exposure of this blot also revealed RANTES induction in lane 7 versus 6 (Figure S3 in Supplementary Material). Full scans of Western blots can be found in Figure S4 in Supplementary Material. The average induction of CD40L, MIP1α, and RANTES in γδTc under hypoxia relative to normoxia was calculated using Western blot band intensity values, and confirmed elevated levels of intracellular CD40L and RANTES, but not MIP1α, under hypoxia (**Figure 3I**).

NKG2D Expressed on γδTc and MICA/B on Breast Cancer Targets Are Critical for γδTc Killing

MCF-7 and T47D are estrogen receptor (ER) positive luminal A breast carcinoma cell lines (35). Both of these cell lines express MICA/B on the surface as identified by flow cytometric analysis (**Figures 4A,B**). Blocking NKG2D on γδTc significantly decreased lysis of MCF-7 (**Figure 4C**, one-way ANOVA versus IgG control, $P < 0.0001$, representative of four independent experiments, $n = 4$) and T47D (**Figure 4D**, $P = 0.0002$, $n = 5$). Likewise, blocking the NKG2D ligand MICA/B on targets prevented MCF-7 and T47D cell lysis (**Figures 4C,D**, both $P < 0.0001$, $n = 2$ and 3, respectively). By contrast, no decrease in cell lysis of either line was observed when γδTc were pre-incubated with antibodies against MIP1α, RANTES, or CD40L (**Figures 4E,F**, $n = 3$ and 2, respectively). Since antibodies were not washed away prior to co-incubation with targets, blocking should have been effective against both membrane-bound and soluble proteins. Thus, it appears that MIP1α, RANTES, and CD40L are not directly involved in γδTc cytotoxicity against MCF-7 or T47D.

γδTc Cytotoxicity Against MCF-7 and T47D Targets Is Enhanced in Hypoxia

Cytotoxicity experiments were performed in which γδTc effectors and breast cancer cell lines were pre-incubated for 48 h under normoxia or hypoxia (2% O_2) and then co-cultured at 1:1, 10:1, and 20:1 E:T ratios in parallel under normoxia or hypoxia, as per target pre-incubation conditions, for 4 h. Pre-incubation in hypoxia enhanced γδTc cytotoxicity against MCF-7 targets cultured in normoxia (**Figures 5A,B**). In a representative example, significantly increased MCF-7 cell lysis was observed at 20:1 (**Figure 5A**, $P = 0.0005$); when data from all six experiments performed with day 21 γδTc from five different donors (six different cultures) were compiled and subject to statistical analysis, this result was confirmed (**Figure 5B**, $P = 0.007$). Likewise, γδTc cultured in hypoxia were better able to kill T47D cultured in normoxia (**Figures 5C–D**). In an example representative of five experiments with day 21 γδTc from four different donors, target cell lysis was significantly increased at all E:T ratios tested (**Figure 5C**, $P < 0.01$); analysis of compiled results from all five experiments revealed significantly increased lysis of targets by hypoxia-treated γδTc at 1:1 and 20:1 E:T (**Figure 5D**, $P < 0.05$).

Breast Cancer Targets in Hypoxia Are Resistant to γδTc Killing due to MICA Shedding

As outlined above, cytotoxicity experiments were performed in which breast cancer cell lines were pre-incubated for 48 h under normoxia or hypoxia (2% O_2) and then co-cultured with γδTc at 1:1, 10:1, and 20:1 E:T in parallel under normoxia or hypoxia for

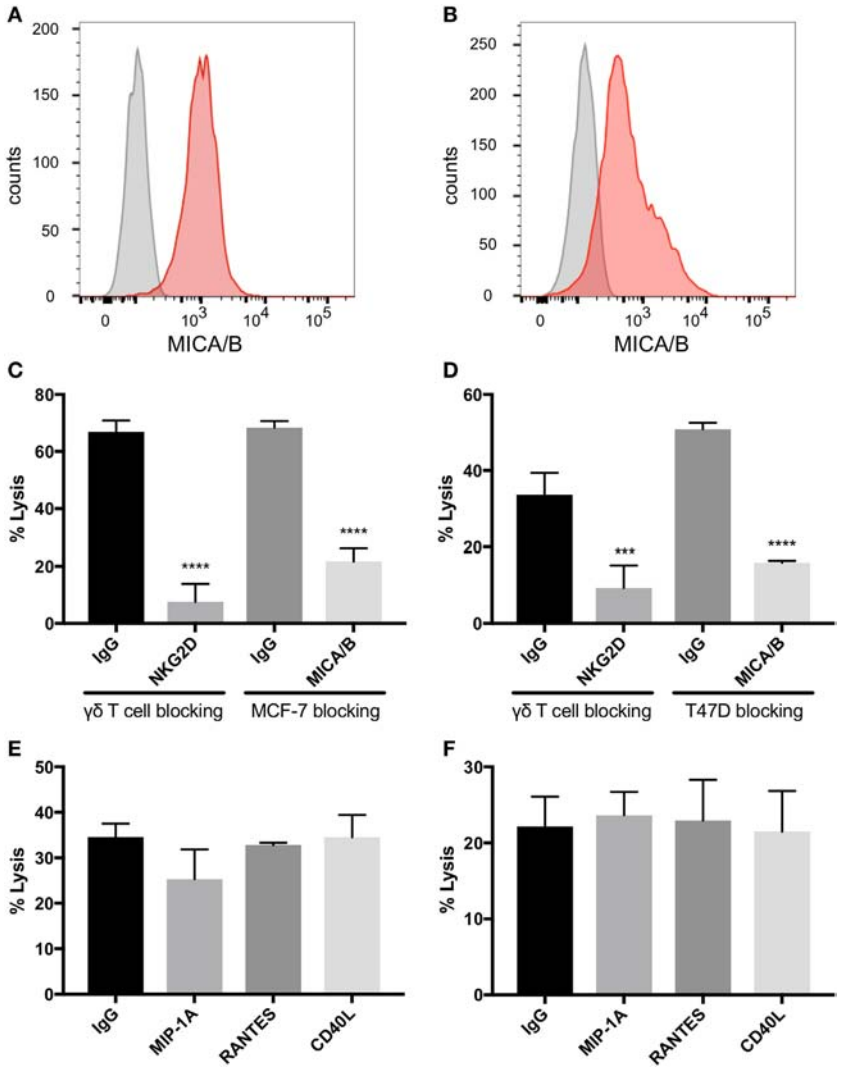

FIGURE 4 | Natural killer group 2, member D (NKG2D) on gamma delta T cells (γδTcs) and MHC class I polypeptide-related sequence A (MICA)/B on breast cancer cell lines mediate γδTc cytotoxicity. Flow cytometric analysis of **(A)** MCF-7 ($n = 4$) and **(B)** T47D ($n = 2$) confirms that both cell lines express MICA/B. **(C)** Cytotoxicity assays in which NKG2D on γδTcs or MICA/B on MCF-7 cells are blocked with antibodies confirm γδTc recognition of breast cancer targets *via* this receptor/ligand interaction ($n = 3$, representative of three independent experiments with three different donor cultures). **(D)** Blocking assays as in **(C)** using T47D targets ($n = 3$). **(E)** Blocking macrophage inflammatory protein 1α (MIP1α), CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), and CD40L/TNFSF5 does not decrease lysis of MCF-7 ($n = 3$ independent experiments with two different donor cultures) or **(F)** T47D ($n = 2$). Statistical analyses for **(C–F)**: one-way ANOVA, $^{***}P < 0.001$, $^{****}P < 0.0001$.

4 h. In most cases (4/6, 67%), pre-incubation in hypoxia induced MCF-7 resistance to γδTc cytotoxicity (**Figures 6A–C**). In a representative example from an experiment performed with γδTc culture 4B-21, significantly decreased MCF-7 cell lysis was observed at 10:1 (**Figure 6A**, $P = 0.0054$) and 20:1 (**Figure 6A**, $P = 0.0119$). By contrast, in two experiments with two different γδTc cultures from the same donor, no resistance was observed; one example is shown in which MCF-7 cultured under hypoxia appeared to be more susceptible to γδTc killing (**Figure 6B**, $P < 0.0001$ at 1:1 and 10:1). When data from five experiments performed with day 21 γδTc from five different donors were compiled and subject to statistical analysis, the overall effect of hypoxia inducing MCF-7 resistance was confirmed (**Figure 6C**, $P = 0.0011$). Likewise, T47D cultured in hypoxia were more resistant to γδTc killing at 20:1 than those cultured in normoxia (**Figure 6D**, $P = 0.0043$), although the 1:1 result is opposite ($P = 0.0076$); these compiled results were from four experiments conducted with four different γδTc donor cultures. Flow cytometric analysis of MICA/B surface expression on breast cancer lines subjected to 48 h normoxia or hypoxia revealed no significant change in MFI; representative examples are shown for MCF-7 (**Figure 6E**, $n = 4$) and T47D (**Figure 6F**, $n = 2$). Of note, Accutase was used for dissociation of these adherent cell lines, out of concern for potential trypsin sensitivity of surface MICA/B that might have confounded our results. Supernatants from MCF-7 and T47D subject to 48 h 20 or 2% O_2 were subject to MICA ELISA (**Figure 6G**). MICA could not be detected in supernatants directly, thus samples were concentrated and MICA ELISA was repeated. MICA in T47D

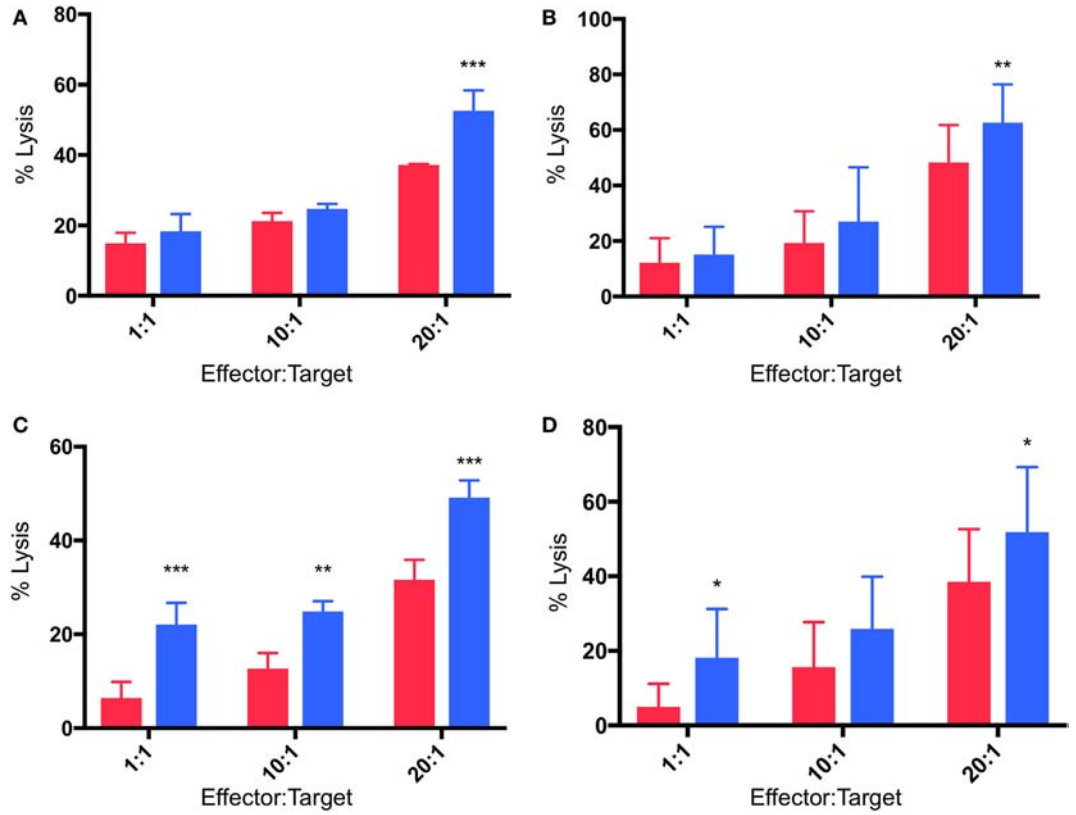

FIGURE 5 | Enhanced cytotoxicity of gamma delta T cells (γδTcs) cultured in hypoxia. Cytotoxicity assays comparing γδTc cultured in 20% (red bars) or 2% O_2 (blue bars) 48 h prior to co-culture with breast cancer target lines cultured at 20% O_2. **(A)** A representative example of γδTc targeting MCF-7 cells; **(B)** compiled results from six independent experiments with γδTc cultures from five different donors targeting MCF-7; **(C)** a representative example with T47D targets; **(D)** compiled results from five independent experiments with γδTc cultures from four different donors targeting T47D. Two-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

remained below the detection limit; however, after normalization to cell numbers, a significant increase in secreted MICA by MCF-7 cells under hypoxia was observed in 3/4 experiments (**Figure 6G**, $^{***}P = 0.0005$, $^{****}P < 0.0001$). These results match those observed in cytotoxicity experiments, with ELISA from MCF-7 targets used in cytotoxicity assays with 4B-21 showing increased MICA secretion under hypoxia that fits with the observed resistance to γδTc cytotoxicity in **Figure 6A**. Likewise, no difference in MICA secretion was observed in MCF-7 targets under 20 or 2% O_2 subject to cytotoxicity assays with γδTc culture 10B-21, which also showed no MCF-7 resistance to γδTc killing in **Figure 6B**. Thus, resistance to γδTc killing appears to be correlated with MICA secretion by breast cancer targets. Despite enhanced cytotoxicity of γδTc cultured under 2% compared to 20% O_2 against targets cultured under normoxia (**Figure 5**), they are unable to overcome resistance exhibited by MCF-7 under 2% O_2, as revealed by analysis of five compiled experiments comparing γδTc cultured under 20 or 2% O_2 against MCF-7 cells cultured in hypoxia (**Figure 6H**).

DISCUSSION

Gamma delta T cells are being developed as immunotherapeutic agents for a variety of cancer indications and clinical trials (Phase I/II) thus far have shown excellent safety profiles (36). Yet, they are known to embody remarkable functional plasticity, dependent on the environment in which they find themselves (24, 37–39). Thus, it is important to explore the function of γδTc infiltrating solid tumors, some of which may be hypoxic. In our small cohort of 17 breast cancer cases, 47% of tumors contained areas of CAIX positivity indicating hypoxia (**Figure 1**). The CAIX-negative cases were 89% ER+ (**Figure 1E**, cases 1–9, case 7 was of unknown ER status); of ER+ cases, 76% were CAIX negative. This confirms reports showing up to 80% CAIX negativity in studies assessing ER+ breast tumors; in these cases, CAIX negativity correlated with low histological grade (40). While our cohort was admittedly small, the very low levels of γδTc infiltrates in CAIX-negative tumors, correlated with low histological grade, confirm results showing that levels of γδTc infiltration correlate positively with higher histological grades (41). Unfortunately, our cohort size was too limited to determine whether γδTc infiltration correlated with patient outcome. We did, however, find γδTc in areas of hypoxia in some tumors. While we did not have the power in our study, or *in vivo* functional data, to claim that γδTc are preferentially attracted to hypoxic regions, our results at least provide an indication that γδTc can be found in hypoxic areas of tumors, and that studying

their function under low O_2 is worthwhile. As CAIX is associated more so with triple negative breast cancers (TNBC) (18, 42), future studies of γδTc and hypoxia should focus on a larger cohort of TNBC patients. Indeed, the groundwork for such studies has been laid by Hidalgo and colleagues, who recently reported on the pattern of distribution of γδTc in TNBC (43).

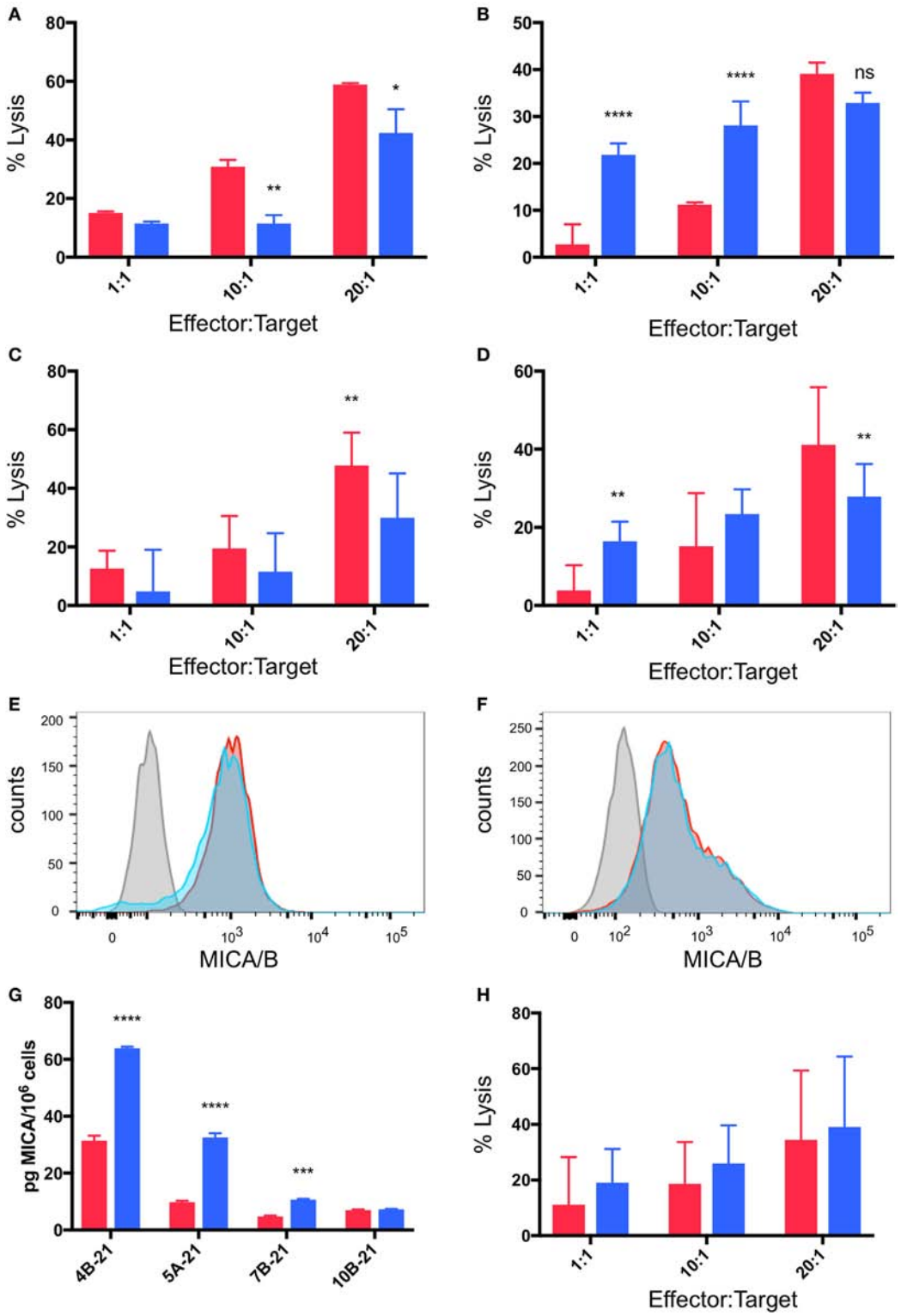

FIGURE 6 | Continued

FIGURE 6 | Breast cancer cell lines pre-incubated in hypoxia are resistant to gamma delta T cell (γδTc) killing. Cytotoxicity assays comparing the ability of γδTc cultured under normoxia to target breast cancer target lines cultured at 20% O_2 (red bars) or 2% O_2 (blue bars) for 48 h prior to co-culture under hypoxia; **(A)** a representative example in which MCF-7 cells were resistant to γδTc killing (4B-21); **(B)** an example in which MCF-7 cells cultured under 2% O_2 were susceptible to γδTc killing (10B-21); **(C)** compiled results from five independent experiments with γδTc cultures from five different donors targeting MCF-7; **(D)** compiled results from four experiments with four different donor-derived γδTc cultures targeting T47D; **(E)** surface expression of MHC class I polypeptide-related sequence A (MICA)/B on T47D remains unchanged under hypoxia versus normoxia; **(F)** surface expression of MICA/B on MCF-7 is not differentially impacted by hypoxia versus normoxia; **(G)** MICA ELISA on concentrated supernatants of MCF-7 from experiments in **(A)**; **(H)** compiled results from five independent experiments with γδTc cultures from five different donors cultured at 20% O_2 or 2% O_2 targeting MCF-7 cultured under hypoxia for 48 h prior to co-culture under hypoxia. Two-way ANOVA, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

It was unsurprising that γδTc cell density decreased under hypoxia (**Figure 2**), as terminally differentiated γδTc stop proliferating to become cytotoxic (44), and hypoxia enhanced γδTc cytotoxicity (**Figure 5**). Delayed cell-cycle progression was also noted in a study on PBMC in hypoxia (45). To our knowledge, the only study of γδTc in the context of hypoxia showed that circulating γδTc in patients with obstructive sleep apnea had elevated intracellular tumor necrosis factor alpha (TNFα) and IL-8 levels, increased TNFα and L-selectin-mediated adhesion properties, and enhanced cytotoxicity against endothelial cells compared to those isolated from healthy donors (46). While that study compared freshly isolated blood-derived γδTc from patients and healthy donors, we used healthy donor-derived *in vitro* expanded γδTc for our experiments, which potentially accounts for different results. TNFα secretion was not impacted by hypoxia in our study, as no differential effects were detected by cytokine array (data not shown). While we did observe strongly elevated hypoxia-induced IL-8 in the supernatant of one of the three γδTc cultures subject to cytokine array analysis (Figure S2 in Supplementary Material), this was not the case for the other two cultures.

More significant were cytokine array data pointing to increased secretion of RANTES, MIP1α, and CD40L by γδTc under low O_2 compared to normoxia that were confirmed by subsequent ELISAs (Figures S2A–C in Supplementary Material; **Figures 3B–E**). Intracellular protein levels induced by hypoxia matched ELISA results only in the case of RANTES (**Figure 3H**); the same could not be said for CD40L and MIP1α, where hypoxia treatment did not appear to increase intracellular levels (**Figures 3F,G**), and surface expression of CD40L was variable (**Figure 2K**). Since blocking these proteins appeared to have no impact on γδTc cytotoxicity against breast cancer target lines (**Figures 4E,F**), they must have an indirect function related to enhanced cytotoxicity of γδTc under hypoxia.

Human memory Vγ2Vδ2 cells were reported to store cytoplasmic RANTES that was secreted rapidly in response to TCR signaling, but little MIP1α protein was found in these cells (47). RANTES is a chemokine employed to recruit antigen presenting cells, such as dendritic cells (48, 49), and thus speaks to the anti-tumor function of γδTc in hypoxia, though breast tumors may use this to their own advantage to promote malignancy (50). RANTES and MIP1α expression were also reported to aid Vδ1 cell suppression of HIV replication (51). CD40 ligation is thought to enhance the immunogenicity of tumors (52), thus γδTc may secrete CD40L in order to better "see" tumor targets. CD40L may also inhibit growth of CD40-expressing tumors directly (52–55). Further investigation will be required to determine the functions served by these cytokines with respect to γδTc targeting solid tumors.

A study of the Vγ9Vδ2 γδTc subset in the context of breast cancer suggested that surface levels of MICA/B on breast cancer target cell lines were associated with γδTc cytotoxicity against these lines; however, direct blocking assays were not carried out (16). Both MCF-7 and T47D cells expressed surface MICA/B, in contrast to an earlier report suggesting a lack of MICA/B expression on MCF-7 (56). If trypsin was used to dissociate MCF-7 in that study, it might explain their inability to detect MICA/B; to avoid this issue, we used Accutase to dissociate our adherent cell lines, as detachment of cells is gentler and protects most surface epitopes. We have confirmed the involvement of NKG2D on γδTc and MICA/B on MCF-7 and T47D in cytotoxicity of γδTc against breast tumor targets (**Figure 4**), although differences in the ability of γδTc to kill targets pre-incubated in hypoxia or normoxia do not appear to be related to surface levels of MICA (**Figure 6**).

One mechanism of hypoxia-mediated tumor evasion is MICA shedding (57). MICA downregulation related to shedding under hypoxia, as well as downregulated expression of NKG2D on PBMCs incubated with culture supernatants of prostate cancer cells exposed to hypoxia—abrogated upon incubation with MICA blocking antibodies—has been reported (58). MICA shedding is not a universal evasion mechanism employed by all cancer cells, however, as glioblastoma cell lines did not shed MICA, although this study was only carried out under normoxia (59). While we assume that soluble MICA may bind NKG2D and block or downregulate this receptor to prevent γδTc recognition of breast cancer targets, a recent report suggests that, in mice, soluble NKG2D might activate NK cells and aid in tumor eradication, but this anti-tumor effect has yet to be shown in humans or with γδTc (60). By contrast, soluble MIC was shown to decrease γδTc cytotoxicity in pancreatic cancer (61) and has been implicated in evasion of human ovarian cancer cells from γδTc recognition (21). Thus, we were surprised that surface expression of MICA/B on MCF-7 and T47D breast cancer lines appeared unaffected by 48 h under hypoxia (**Figure 6**). However, MICA secretion did not correlate with MICA surface levels, as soluble MICA increased in the supernatants of MCF-7 cells cultured under hypoxia, while surface MICA levels remained unchanged (**Figure 6**). Thus, it appears that we would need to neutralize soluble MICA to improve γδTc cytotoxicity, since target surface expression did not appear to be affected by hypoxia. That said, we did not directly assess MICA expression during co-culture with γδTc, and it is possible that MICA was downregulated in the presence of γδTc, although the correlation between resistance to γδTc killing and soluble MICA levels in culture supernatants under hypoxia

speaks against this (**Figure 6**). One way to overcome MICA shedding may be to increase nitric oxide signaling (58), although its impact on γδTc would have to be assessed.

Although the γδTc tumor infiltrating lymphocytes (TIL) signature was deemed the most positive prognosticator across a range of cancers, including breast cancer (62), some reports suggest that γδTc may take on a regulatory phenotype within the breast TME (41, 56, 63, 64). In one study, γδTc TIL isolated from a breast tumor were expanded in high levels of IL-2 for several weeks prior to immunosuppression assays and proved to inhibit dendritic cell maturation and CD8+ T cell cytotoxicity (56); however, given the known functional plasticity of γδTc, such assays conducted on *ex vivo* expanded cells removed from the TME cannot inform the function of γδTc *in situ*. A positive correlation was observed between γδTc infiltration and breast cancer stage, leading the authors to suggest that γδTc may contribute to disease pathology; however, causality was not established (41). Although our cohort size was much smaller, we too observed a positive correlation between CAIX expression, indicating hypoxia—typically an indicator of cancer progression—and γδTc infiltration (**Figure 1**). This could just as easily indicate the greater need for γδTc attempting to eradicate disease. Our hypoxia experiments reveal enhanced cytotoxicity of γδTc exposed to 48 h of low O_2, suggesting that γδTc are indeed able to kill in this environment (**Figure 5**). Soluble MICA appears to inhibit γδTc cytotoxicity against breast tumor targets in hypoxia and, despite their increased killing capacity under low O_2, γδTc are unable to overcome resistance exhibited by MCF-7 under 2% O_2 (**Figure 6**), a condition under which γδTc must operate within at least some parts of a tumor. Further studies will be required to definitively identify γδTc function in breast tumors *in situ*.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Research Ethics Guidelines, Health Research Ethics Board of Alberta—Cancer Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Health Research Ethics Board of Alberta—Cancer Committee.

AUTHOR CONTRIBUTIONS

GS and L-MP contributed to research design. GS and ID conducted experiments; data analysis was carried out by GS, ID, and RL. GS wrote the manuscript; all authors provided feedback and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01367/full#supplementary-material.

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γδ T Cells and Tumor Microenvironment: From Immunosurveillance to Tumor Evasion

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γδ T cells possess cytotoxic antitumor activity mediated by production of proinflammatory cytokines, direct cytotoxic activity, and regulation of the biological functions of other cell types. Hence, these features have prompted the development of therapeutic strategies in which γδ T cells agonists or *ex vivo*-expanded γδ T cells are administered to tumor patients. Several studies have shown that γδ T cells are an important component of tumor-infiltrating lymphocytes in patients affected by different types of cancer and a recent analysis of ~18,000 transcriptomes from 39 human tumors identified tumor-infiltrating γδ T cells as the most significant favorable cancer-wide prognostic signature. However, the complex and intricate interactions between tumor cells, tumor microenvironment (TME), and tumor-infiltrating immune cells results in a balance between tumor-promoting and tumor-controlling effects, and γδ T cells functions are often diverted or impaired by immunosuppressive signals originating from the TME. This review focuses on the dangerous liason between γδ T cells and tumoral microenvironment and raises the possibility that strategies capable to reduce the immunosuppressive environment and increase the cytotoxic ability of γδ T cells may be the key factor to improve their utilization in tumor immunotherapy.

Keywords: γδ T cells, tumor microenvironment, immunotherapy, cyototxicity, immunosuppression

THE TUMOR MICROENVIRONMENT (TME)

Tumors develop in a composite and heterogeneous microenvironment consisting of endothelial cells, stromal cells, and immune cells; all of them act and cooperate either in direct or indirect way with tumor cells promoting tumor proliferation, invasion, and metastasis or actively interfering with its development.

It is well known that a large number of cells of both the innate and adaptive immune compartments are present at the tumor site since the early steps of cancer development, exerting immunosurveillance (1) and controlling spontaneous neoplastic diseases (2), even though the composition and extent of the immune infiltrates consistently varies among individuals (3, 4).

Tumors are able to escape from the host immune system and take advantage on the presence of infiltrating immune cells by modifying their functions and creating a TME favorable to tumor progression. In fact, tumor-infiltrating immune cells, together with stromal cells and extracellular matrix create an inflammatory milieu responsible for tumor expansion and dissemination and for

tumor evasion. Tumor escape from the host immune response is promoted by its ability to actively subvert antitumor immunity by interfering with cell development, differentiation, migration, and cytotoxicity or from host immunosuppression.

Cancer-associated fibroblasts (CAFs) (5–8), myeloid-derived suppressor cells (MDSC), regulatory T cells (Tregs), type-2 macrophages (M2 macrophages), tumor-associated neutrophils (9), inhibitory cytokines, and immune checkpoint receptors are components of the immune system acting together with cancer cells, responsible for the subversion of antitumor immunity (10, 11) (**Figure 1**). We will discuss any of these TME components in the following sections.

Cancer-Associated Fibroblasts

Cancer-associated fibroblasts are key players in the generation of an immunosuppressive TME and consequently in the promotion of tumor evasion from immune surveillance. It is well known that high presence of CAFs in the context of the tumor has been correlated with poor prognosis in several malignancies, including lung (12) and colorectal cancers (13). The mechanisms used by CAF to induce tumoral growth and immune escape are different: (i) the production and the release of a large amount of immunosuppressive cytokines and growth factors, such as interleukin (IL)-6 and IL-8, transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and insulin-like growth factor, that directly or indirectly influence the behavior of malignant cells (14) and (ii) the direct suppressor activity on T cell proliferation through regulatory molecules and immunosuppressive cytokines released by fibroblasts. In addition, the analysis of the expression of costimulatory molecules on CAF have showed that CD80 and CD86 are not expressed by CAFs or normal fibroblast while B7H1 and B7DC, that bind programmed death-1 (PD-1) on activating T cells trasducing a negative signal inside the cells, are expressed by CAFs, but not by normal fibroblasts. In non-small cell lung cancer (NSCLC) patients, Nazareth et al. have demonstrated that CAFs constitutively express the B7H1 and B7DC molecules (15) and their blocking completely restores activation of tumor-associated T cells.

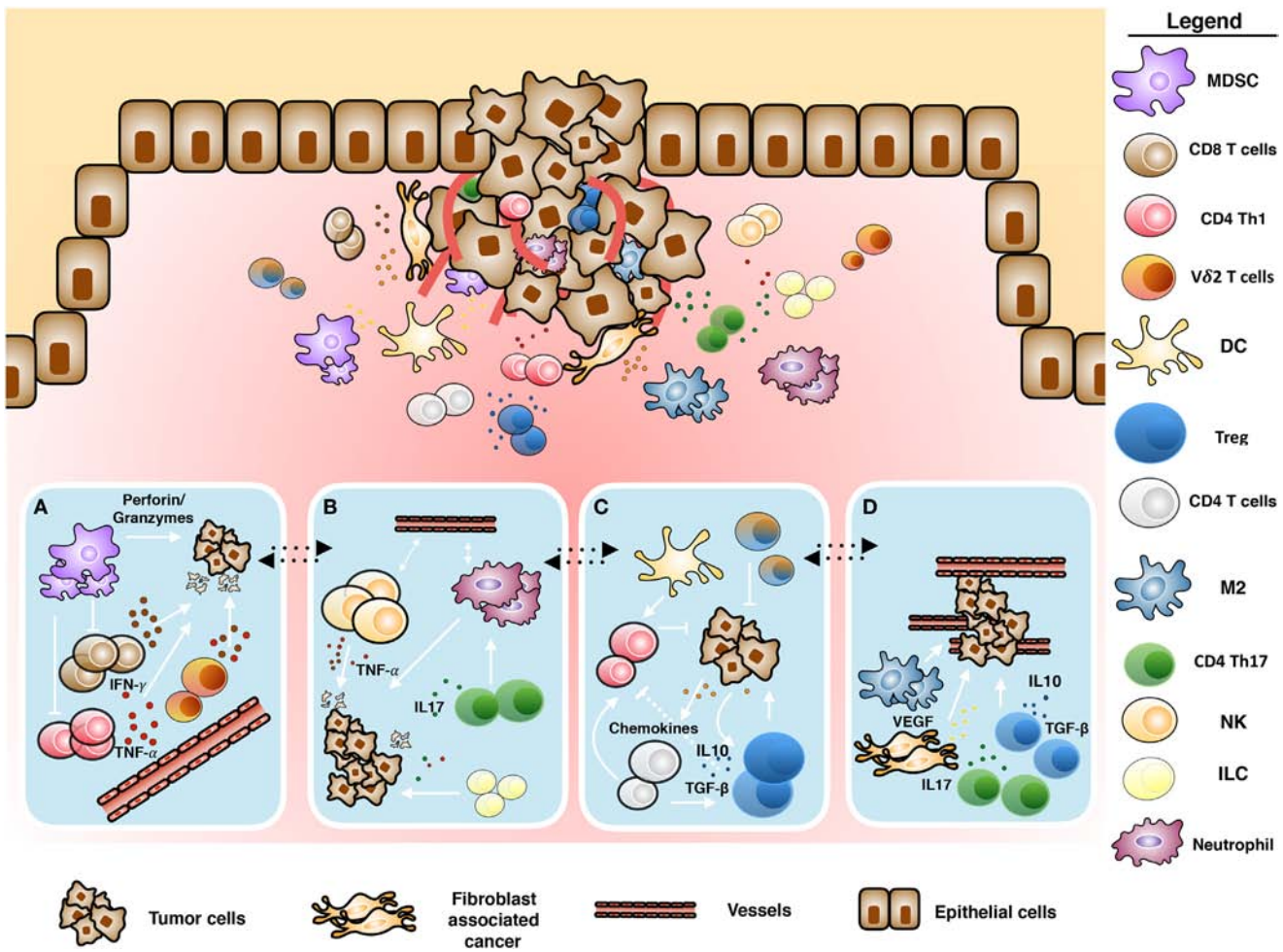

FIGURE 1 | Schematic representation of tumor microenvironment. Cross-talk between immune and tumoral cells: **(A)** antitumoral role of infiltrating γδ T cells mediated by proinflammatory cytokines and cytotoxic activity, **(B)** inflammation induced by tumor-infiltrating immune cells, **(C)** immunosuppressive polarization of immune cells, and **(D)** tumor promotion and neoangiogenesis induced by tumor-infiltrating immune cells.

Moreover, CAFs shows a direct function on the orchestration of TME, inducing preferentially T cell apoptosis and Tregs; in fact the production of IL-6, CXCL8 are responsible for tumor-associated macrophage (TAM) polarization toward M2 macrophage polarization and functions during the differentiation of circulating monocytes to TAMs (16–19) while TGF-β induce the recruitment of macrophages at the tumor site, promoting the effective tumor evasion of the host immune system (20, 21).

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells play several protumoral functions promoting tumor cell survival, angiogenesis, invasion of tumor cells, and initiation of metastasis formation (22). Moreover, they can directly and indirectly skew the immune response toward immune suppressive counterparts, using different strategies:

(1) MDSCs are able to inhibit T cell proliferation and activation by (i) the depletion of essential amino acids such as L-arginine, *via* arginase-1 (ARG)-dependent consumption, and L-cystein by sequestering, as demonstrated in renal adenocarcinoma (23, 24); (ii) the reduction of local tryptophan levels and production of cytotoxic metabolites by indoleamine 2,3 dioxygenase (IDO) (25); and (iii) the decrease of IL-2 production and inhibition of the IL-2 receptor signaling, by the reactive oxygen and nitrogen species NO, ROS, H_2O_2, and peroxynitrite, produced by arginase 1 (Arg-1), nitric oxide synthase (iNOS), and NADPH oxidase (NOX2) (26, 27).
(2) MDSCs induce T cell apoptosis by several mechanisms, such as decrease of Bcl-2 expression and upregulation of FAS (CD95 ligand) in T cells, expression of galectin 9, which binds the inhibitory surface molecule TIM3 (T-cell immunoglobulin domain and mucin domain) and by expressing inhibitory surface molecules that alter T cell viability and trafficking.
(3) MDSCs interfere with lymphocyte trafficking and viability through the downregulation of L-selectin (CD62L) on the surface of T cells, by expression of ADAM17 (disintegrin and metalloproteinase domain 17) and they also interrupt the migration of $CD8^+$ T cells to tumor sites by peroxynitrite modification of CCL2 (28, 29).
(4) MDSCs promote the differentiation of $CD4^+$ T cells into Tregs both by direct cell–cell interactions (including CD40–CD40L interactions) and the production of several cytokines (such as IL-10 and TGF-β) (30), and polarize TAMs toward the M2 phenotype (31).

Regulatory T Cells

In the TME, classic Tregs, as defined by expression of CD4, CD25, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4/CD152), the Forkhead Box P3 transcription factor (32, 33), and Helios (34), directly promote immune evasion and the formation of a pro-tumorigenic TME, and prompt the growth and metastasis of various malignant tumors such as lung, ovary, breast, and prostate (35). Tregs exert their immunosuppressive activity using different approaches: they release soluble inhibitory molecules as TGF-β, IL-10, adenosine, PGE_2, interfere with T effector cell activity and perforin/granzyme-mediated direct cytotoxicity by sequestration of IL-2 (36) and directly inhibit effector T cells by virtue of immune checkpoints and inhibitory receptors (CTLA-4, PD-1, and LAG-3) (37, 38).

M2 Macrophages

In the TME, macrophages typically differentiate to the M2 phenotype under the action of Th2 cytokines (such as IL-4 and IL-13) and glucocorticoids. M2 macrophages promote tumor growth by suppressing immune response, remodeling the extracellular matrix, and stimulating neoangiogenesis (39). The majority of macrophages that are recruited at the tumor site, called TAMs, acquire features closely similar to the M2 phenotype due to different stimuli present in the TME, such as IL-4 and TGF-β, accompanied by reduced antitumoral activity (40). TAMs play an important role for lymphangiogenesis through the release of VEGF-C and VEGF-D *via* VEGFR3, and neo angiogenesis by VEGF, TNF-α, CXCL8, PDGF-β, MMP2, MMP7, and MMP9, both of mechanism are critical steps for tumor growth, invasion, and metastasis (41).

EFFECTS OF THE TME ON γδ T CELLS

γδ T cells are considered as good candidates for effective antitumor immunotherapeutical approaches for their unique features as (i) the recognition of antigens shared by a variety of stressed and tumor cells (42) in the absence of major histocompatibility complex (MHC) restriction and co-stimulation, (ii) the production of cytokines with well-known antitumor effect as IFN-γ and TNF-α with cytotoxic activity against tumor cells directly and indirectly *via* stimulating macrophages and DCs (43–45), and (iii) the potent cytotoxic activity *in vitro* and in xenograft models *in vivo* mediated by several different effector mechanisms (46–48). Moreover, γδ T lymphocytes are recruited in several types of cancer (49) and analysis of expression signatures from a large number of human tumors identified them as the most significant favorable cancer-wide prognostic signature for outcome (50, 51). Moreover, data mining transcriptomes from a large cohort of colorectal cancer patients ($n = 585$) has revealed that the aboundance of tumor-infiltrating γδ T cells is related with the 5-year disease-free survival probability (51).

There are at least three major γδ T cell subsets in humans that exhibit different Vδ chain in the TCR: (1) the population expressing the Vδ2 gene paired with the Vγ9 chain (Vγ9Vδ2 T cells) represent the majority of circulating γδ T cell population; (2) the population expressing the Vδ1 gene and different Vγ chain, are confined to skin and mucosa; and (3) a third subset of Vδ3 cells are present in higher percentage in the liver (52).

Antigen recognition by γδ T cells is a field of intense research. Vδ1 T cells recognize MHC class I-related molecule A (MICA), MHC class I-related molecule B (MICB), and UL16-binding proteins, expressed on stressed and tumor cells (53–55), glycolipid presented by MHC-related class Ib molecules CD1c and CD1d (56, 57), and unidentified ligands that engage natural cytotoxicity receptors (such as NKp30 and NKp44) (58). It is known that Vδ3 T cells can be activated by a glycolipid bound to CD1d molecules, but the real activating ligand are not yet defined (59).

Finally, Vδ2 T cells recognize non-peptidic phosphorylated intermediates of the non-mevalonate and mevalonate pathways of isoprenoid biosynthesis called phosphoantigens (PAgs), in the absence of processing, presentation, and MHC restriction (60).

Thus, there is a substantial interest in γδ T cells in the context of immunotherapeutic strategies, considering the intracellular accumulation of isopentenylpyrophosphate leading to activation of Vδ2 T cells can be manipulated in the experimental assay and applied *in vitro* and *in vivo* cancer immunotherapy by two synthetic drugs, the synthetic PAg analog bromohydrin pyrophosphate and the aminobisphosphonate (n-BP) Zoledronate.

Nonetheless, recent flow cytometry or immunohistochemical studies of tumor-infiltrating γδ T cells have failed to provide clear-cut evidence that they correlate positively or not with tumor growth, or even fail to correlate with any prognostic feature in different types of cancer, as reviewed in Ref. (61).

The dual role of Vδ2 T cells against tumor cells, either antitumoral or protumoral, could be related to the plasticity of γδ T cells to differentiate into different functional subsets under precise polarizing conditions; thus, Vδ2 T cells may display Th1-, Th2- (62), Th9- (63), Th17- (64), or Treg-like (65) profiles and they can produce several immunosuppressive cytokines as TGF-β and IL-10. Recent papers indicate that IL-17 produced by Th17-like γδ T cells can directly promote the proliferation and dissemination of tumor cells in breast cancer (66–68) and in the TME IL-17 regulates other cell population, such as MDSCs and macrophages influencing indirectly the tumor immunosurveillance (69). Treg-like Vδ2 T cells participate in the immunosuppressive TME either by the release of soluble molecules and by cell-to-cell contact *via* CD86/CTLA-4 and PD-L1/PD-1 interactions (70, 71). Recently Hu et al. have identified a novel γδ Treg subset exhibiting CD39 expression that is polarized by TGF-β, with stronger immunosuppressive potential than $CD4^+$ Tregs and that suppresses the activity of human lymphocytes in an adenosine-dependent manner (72).

This plasticity of γδ T cells and the plausible idea that the TME drives their differentiation toward subsets equipped with immunosuppressive activities suggests the possibility that the TME can limit the effectiveness of the antitumor activity of γδ T cells (73).

How does then the TME induce the polarization of γδ T cells toward pro tumoral subsets?

Tumor cells and other cells of the TME produce inhibitory molecules which interfere with the proliferation and function of γδ T cells, such as TGF-β (74), prostaglandin-E2, adenosine (75, 76), and soluble NKG2D ligands (such as MICA/B) (77).

We have recently investigated the nature of the immunosuppressive soluble molecules present in secretomes from two different human cancer types. We first analyzed secretomes obtained from cancer stem cells (CSC) and CAF of non-melanoma skin cancer patients, and found that the secretome of SCC patients contains cytokines (IL-6, IL-1β, IL-23, and TGF-β) capable of polarizing the differentiation of γδ17 T cells (78, 79), confirming the transition from IFN-γ-producing to IL-17-producing γδ T cells in the TME, during tumor progression observed in these patients. Whether or not these cytokines alone are responsible for the polarization toward γδ17 T cells or additional cells/factors are required is currently under investigation: accordingly, we have recently found that activated plasmacytoid dendritic cells provide yet unknown signals which selectively induce γδ17 T cell polarization of Vγ9Vδ2 T cells (80), which was dominant over the PAg-induced IFN-γ response.

In a second study, we have also studied the immunosuppressive properties of secretomes of CAF and CSC obtained from CRC patients (51). Secretome from colon CSCs significantly inhibits proliferation and IFN-γ production by freshly γδ T cells and also by γδ, $CD4^+$, and $CD8^+$ αβ T cell lines and promotes production of IL-17. Conversely, secretome from CAF has limited suppressive ability and does not promote production of IL-17. Detailed analysis of CSC and CAF secretomes revealed only three cytokines differentially expressed by the inhibitory CSC secretome, but absent in the non-inhibitory CAF secretome, IL-8, IL-12, and VEGF. Because IL-12 does not have inhibitory activity on T cell proliferation and IFN-γ production, IL-8 and VEGF remain potential candidates of the immunosuppressive activities of the colon CSC secretome, which is probably not exerted directly on T cells but is rather mediated by other cell types like MDSCs, M2 macrophages, DCs, and Tregs (81, 82).

While the above findings indicate that soluble molecules present in the TME promote γδ T polarization to subvert antitumor immune response, it is likely that additional signals like prostaglandins (83), kynurenins (84), or potassium (85), are needed to achieve this effect.

Indeed, relating to the well-known ability of cancer cells to use inhibitory checkpoints to induce T cells apoptosis or anergy, Vδ2 T cells results not to be affected by this immunosuppressive mechanisms by the very low expression of PD-1 compared to conventional αβ CD8 and CD4 T cells; a recent paper have demonstrated that upon 4 days of *in vitro* stimulation by Zoledronate and IL-2, Vδ2 T cells increase the expression of PD-1 but very rapidly decrease nearly to baseline (86) as well as TIGIT that is another negative checkpoint receptor (Hayday, unpublished results). Moreover, several suppressive cells in the TME can inhibit the proliferation and cytotoxic effect of γδ T cells (87–90). For example, tumor cells promote γδ T cells polarization toward a Treg phenotype, that obstacle antitumor immunity (73), contributing to the immunosuppressive microenvironment that is characteristic of most tumor cells as breast cancer (91). Deficient γδ T cell functions have already been observed in various types of cancer, including hematological malignancies (92), liver, breast cancer (93), and HCC (94).

TARGETING TME FOR THERAPY

Cancer immunotherapy is a highly promising new cancer treatment, that enhances the host antitumor response, increasing the number of effector immune cells, reducing host immunosuppressive mechanisms, inducing tumor killing, and modulating immune checkpoints (93).

Better knowledge on how tumor cells escape immune response has been translated into innovative therapeutic strategies that redirect immune cells to tumors and restore their cytotoxic activity against tumor cells.

γδ T cell immunotherapy, either by the use of *in vivo* expanded T cells by administration of compounds that activate them or

by the adoptive transfer of *ex vivo*-activated γδ T cells, has been shown to be both feasible and safe (95).

However, there are technical and functional limitations to its use in cancer therapy; technical problems could be linked to the hyporesponsivness of γδ T cells in some patient or to the activation-induced γδ T cell anergy (lack of γδ T cell activation or expansion), while functional limitations could be related to the ability of *in vivo* or *in vitro*-expanded γδ T cells to reach and infiltrate tumors and to defeat the immunosuppressive environment (90, 96). To overcome this limitations, several immunotherapeutical approaches have been studied (**Figure 2**), we will discuss in the following sections.

Inhibitory Immunological Checkpoint

Combination of γδ T cell adoptive transfer with immune checkpoint inhibitors is a useful strategy to enhance the antitumor activity of these cells.

The cancer immunotherapy has obtained an important progress by the use of therapeutic antibodies to CTLA-4, PD-1, and PD-L1 that antagonize immune checkpoints even though not all cancers respond to these antibody therapies. The pharmaceutical industries are involved in the development of new monoclonal antibodies that target B7H1 (PD-L1) (97, 98), considering that the blockade of B7H1 seems to induce durable tumor regression and prolonged disease stabilization in patients with advanced cancers in a phase I trial (99). Vγ9Vδ2 T cells express very low levels PD-1 compared to CD8 and CD4 T cells, with a peak at 4 days of *in vitro* activation with Zoledronate and IL-2 but it is not durable considering their rapid decrease upon 7 days reaching the baseline level [(86) and our unpublished data]. Beyond PD-1, many other immune checkpoint inhibitors as CTLA-4, IDO, VISTA, galectin-9, LAG-3, and TIM-3 should be investigated to improve γδ T cell adoptive immunotherapy.

Chemotherapy

Some cytotoxic compounds, including doxorubicin and oxaliplatin, can trigger "immunogenic" cell death. These chemotherapeutic agents act on tumor cells in such a way that the host immune

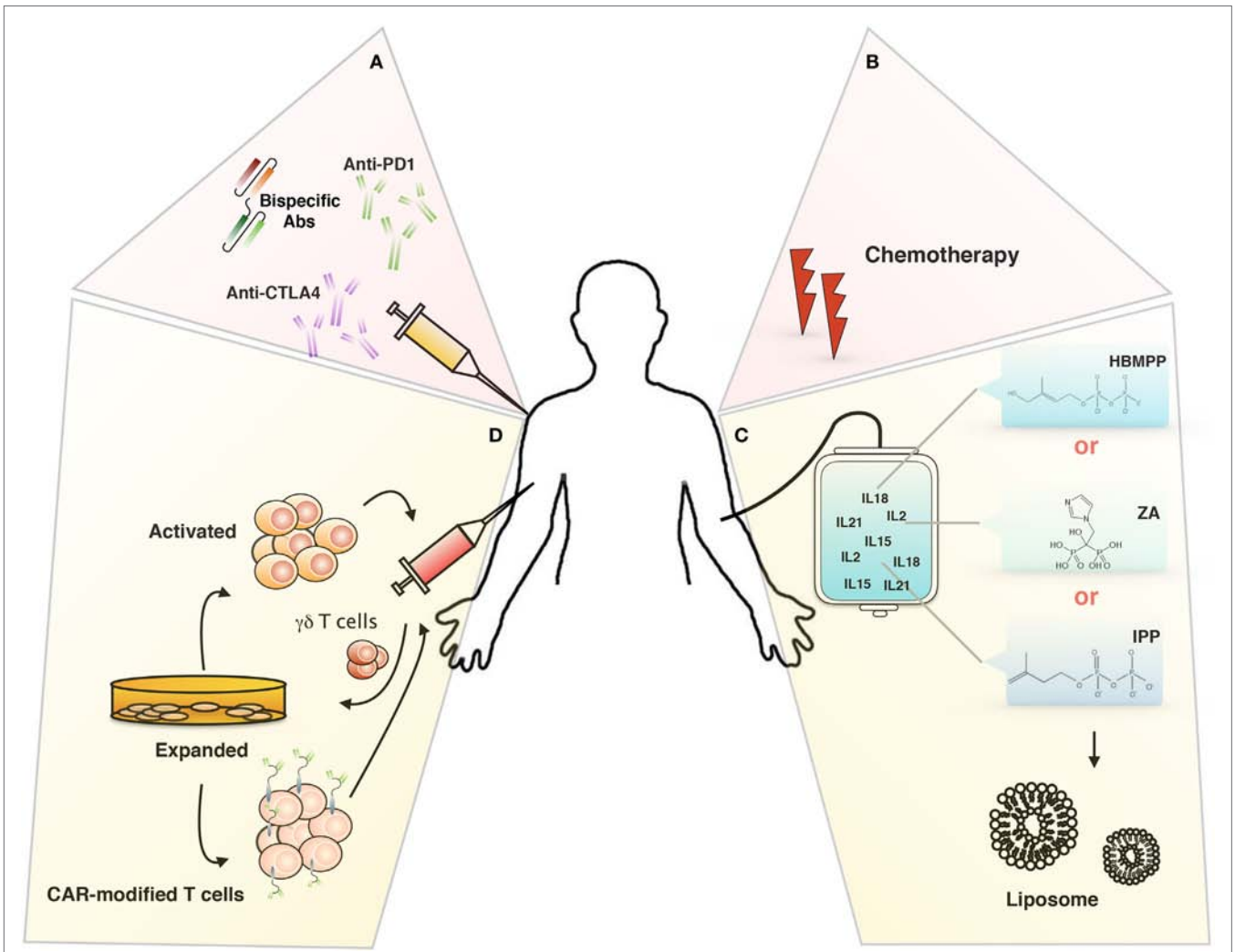

FIGURE 2 | Targeting tumor microenvironment for immunotherapy. Novel therapeutical approaches to improve γδ T cells-based immunotherapy: **(A)** inhibitory immunological checkpoint and bispecific antibodies, **(B)** chemotherapy, **(C)** liposomes, and **(D)** chimeric antigen receptor-T cells (CAR-T).

system recognizes the dying tumor cell. A tumor-specific immune response occurs during cell death, which results in an antitumor immune response leading to tumor eradication and prevention of relapse. This immunogenic cell death can prevent immune tolerance to tumor cells and is a crucial component of treatment efficacy (100). γδ T cells can be recruited to the tumor site after exposure to immunogenic chemotherapy and can contribute to its efficacy (67). *In vitro*, pre-treatment with low concentrations of chemotherapeutic agents (doxorubicin, cisplatin, etoposide, and vincristine) or even Zoledronate has been shown to sensitize tumor cells to killing by γδ T cells with additive or synergistic effects (101–104).

Therefore, such new effective immunotherapeutic approaches include the use of chemotherapeutic drugs that induce immunogenic cell death (100) or non-specific immune stimulation by cytokines such as IL-2 and IFN-α, monoclonal antibodies, and other biomolecules. Novel regimens that combine these drugs with PAgs or with γδ T cells are currently under investigation.

Liposomes

Aminobisphosphonates (N-BPs) have been shown to have anticancer activity both as a monotherapy and in combination with γδ T cells. Due to the biodistribution of N-BPs *in vivo*, encapsulation of N-BPs in a nanoformulation is a good technique for their use in the treatment of non-osseous tumors.

Liposomes, a closed bilayer phospholipid system, have been proposed as drug carriers in cancer therapy due to their ability to be preferentially taken up in tumors (105), to increase the therapeutic index of a drug and to reduce the side effects (106, 107). Moreover, nanoparticles such as liposomes pass very easily trough the blood vessel, expecially the neovessels inside the tumor that exhibit leaky endothelial lining. This effect is further reinforced by the lack of efficient lymphatic drainage of the tumor which causes liposomes to accumulate preferentially in the tumor area. This is known as enhanced permeation and retention effect (EPR) (108). Particles of 10–500 nm are thought to be able to extravasate into tumors as the pore sizes in the endothelial lining of leaky blood vessels in peripheral tumors are estimated to be 400–600 nm in diameter (109). However, particles with diameters <200 nm have been shown to be more effective at accumulating at tumor sites. This passive tumor targeting does not occur in all tumors and vessel leakiness may also be heterogeneous within a single tumor (110). Ligand-targeted or "active" targeting of liposomes may result in liposomes that are more selective to cancer cells, once passive targeting has taken place (107).

Toxic side effects have been observed *in vivo* when Zoledronate and Alendronate were encapsulated into liposomes even though liposome-encapsulated (L)-Alendronate was shown to be better tolerated than L-Zoledronate. Hodgins and colleagues have obtained promising results using *in vivo* L-Alendronate and γδ T cells for the treatment of experimental metastatic lung tumors in immunocompromised mice (110).

To increase the uptake of L-Alendronate by receptor-mediated endocytosis *in vivo*, Hodgins and colleagues have targeted L-Alendronate to the $\alpha_v\beta_6$ integrin receptor, which is overexpressed on cancer cells but absent on normal cells; combining the immunotherapy with γδ T cells, they achieved substantial sensitization of $\alpha_v\beta_6$ positive cancer cells to γδ T cells and a more efficient cell killing *in vitro*. Despite the promise of using targeted-L-Alendronate in a monotherapy regimen, no added advantage was observed in an experimental metastatic lung mice model by the combination of targeted-L-Alendronate and γδ T cells (111).

Bispecific Antibodies

The immunotherapeutical approaches using monoclonal antibody-based targeted therapy have obtained promising results, improved by the generation of bispecific antibody (bsAb) (112) capable of targeting multiple molecules as a single agent, even though the positive effects are not time durable because of their toxicity and cellular resistance mechanisms.

In order to be able to recruit and activate all T-cell subsets, most bsAbs target CD3, but as a consequence a wide range of T cells, including $CD4^+$, $CD8^+$, γδ T-cells, and also several immunoregulatory and immunosuppressive T-cell subsets are recruited.

Bispecific antibodies are very promising tools for γδ T cell-based immunotherapy with a lot advantages. There exist Vδ2γδT cell and γδ T cell (Vδ2 and Vδ1)-NK cell-specific bsAb which drastically enhance cytotoxic activity of these cells and did not recruit immunosuppressive γδ T cells (113–116).

Recently, de Bruin and colleagues produced a new bispecific nanobody that simultaneously targets Vγ9Vδ2 T cells and EGFR. This compound has shown a potent ability to activate Vγ9Vδ2 T cells and to induce their antitumoral activity *in vitro* and in mouse xenograft model *in vivo* independently on the mutational status of the tumor. Thanks to the conserved monomorphic nature of the Vγ9Vδ2 TCR that permits a more selective cell recruitment, this immunotherapeutic approach could be used in several different clinical settings and could be applied to a large group of cancer patients (117).

Chimeric Antigen Receptor-T Cells (CAR-T)

Chimeric antigen receptors (CARs) redirect T cell specificity to tumor-associated antigens (TAAs), such as CD19, independently on the genetic (MHC) restriction.

Given the natural recruitment of γδ T cells for the tumor site, their transduction with CARs might increase their cytotoxic activity without affecting their migratory capability toward the tumor and their polarization toward antigen-presenting cells phenotypes that prolong the intratumoral immune response (118).

γδ T cells directly recognizes unique TAAs, e.g., MICA/B, F1-ATPase, and PAgs, which are widely expressed by a variety of tumor cells (119) and thus, broad recognition of tumor cells and antitumor activities may be achieved by these T cells expressing a diverse γδ TCR repertoire.

The question concerning the optimization of the immunotherapeutical approaches using costimulatory molecules remains open and the synergy between TCR γδ and costimulatory molecules signals should be better explored for clinical expansion of Vδ2 T cells.

It is well known that γδ T cells express a series of costimulatory molecules such as CD27, CD28, and 4-1BB (CD137) that increase

their activation and effector function. Ribot et al. showed that CD28 is constitutively expressed on γδ T cells and play a role on the survival and proliferation *via* IL-2 production (120). deBarros and colleagues have shown the key role of CD70 molecule (CD27 ligand) on the *in vitro* expansion of Vγ9Vδ2 T cell by promoting the upregulation of Cyclin D2 and the anti-apoptotic gene regulator Bcl2a1, and on the effector function by the production of high levels of IFN-γ (121). Another costimolatory molecule investigated on Vγ9Vδ2 T cells was CD137L that is expressed at high levels when cells are activated and act as a ligand for CD137 on T and NK cells (122).

Capsomidis et al. have produced a new CAR by GD2-targeting, easily trasduced by both Vδ1 and Vδ2 subsets; the transduced cells have shown an increase cytotoxicity activity toward GD2-expressing cancer cell lines, a stable ability to migrate in tumor cells, take up tumor antigens and cross-present the processed peptide to responder αβ T lymphocytes (118).

Although these engineered immune cells have made remarkable success in the treatment of patients with hematologic malignancies, the therapeutic efficacy in solid tumors has been limited because of the complexity and the heterogeneity of TME.

CONCLUSION

Recent advances in cancer immunotherapy have revolutionized treatment for a number of cancers. By targeting checkpoint receptors, durable remissions have been achieved in patients with advanced metastatic melanoma, NSCLC, bladder cancer, and kidney cancer, that otherwise would have had little chance of survival with conventional chemotherapies or targeted therapies. Similarly, CAR-T, bearing receptors specific for CD19 have successfully treated patients with relapsing B cell acute lymphoblastic leukemia and diffuse large B cell lymphoma. However, both these treatments have limitations. Therefore, additional types of immunotherapy are needed to achieve the full potential of cancer immunotherapy. Harnessing γδ T cells toward tumor cells remains a fascinating immunotherapeutical approach, considering that their activation is not dependent on peptides presented by MHC proteins and is, therefore, MHC unrestricted. Finally, the efficacy of adoptive immunotherapy with Vδ2 T cells is independent of the mutational status of the tumor (123, 124), a limit for the efficacy of checkpoint blockade, and consequently could be also applied to patients with cancers that have low numbers of mutations, such as many of the pediatric cancers.

The well-known plasticity of γδ T cells upon interaction with TME limits the effectiveness of this therapy, even though the overall interactions of the cells in TME and their rapid modifications induced by the natural story of the tumor and of the host remain an enigmatic story. A better comprehension of these mechanisms will be useful to formulate really efficient and durable therapeutic strategies that combine different approaches and could restore antitumor immune responses, overcome tumor escape, and overcome tumor-induced immune deviation to enable the host immune system to more effectively control tumor growth.

AUTHOR CONTRIBUTIONS

SM and FD wrote the paper. ELP prepared the figures. ELP, GP, AMC, NC, and GS contributed to the discussion of the draft.

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Vγ9Vδ2 T Cells in the Bone Marrow of Myeloma Patients: A Paradigm of Microenvironment-Induced Immune Suppression

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Vγ9Vδ2 T cells are non-conventional T cells with a natural inclination to recognize and kill cancer cells. Malignant B cells, including myeloma cells, are privileged targets of Vγ9Vδ2 T cells *in vitro*. However, this inclination is often lost *in vivo* due to multiple mechanisms mediated by tumor cells and local microenvironment. Multiple myeloma (MM) is a paradigm disease in which antitumor immunity is selectively impaired at the tumor site. By interrogating the immune reactivity of bone marrow (BM) Vγ9Vδ2 T cells to phosphoantigens, we have revealed a very early and long-lasting impairment of Vγ9Vδ2 T-cell immune functions which is already detectable in monoclonal gammopathy of undetermined significance (MGUS) and not fully reverted even in clinical remission after autologous stem cell transplantation. Multiple cell subsets [MM cells, myeloid-derived suppressor cells, regulatory T cells, and BM-derived stromal cells (BMSC)] are involved in Vγ9Vδ2 T-cell inhibition *via* several immune suppressive mechanisms including the redundant expression of multiple immune checkpoints (ICPs). This review will address some aspects related to the dynamics of ICP expression in the BM of MM patients in relationship to the disease status (MGUS, diagnosis, remission, and relapse) and how this multifaceted ICP expression impairs Vγ9Vδ2 T-cell function. We will also provide some suggestions how to rescue Vγ9Vδ2 T cells from the immune suppression operated by ICP and to recover their antimyeloma immune effector functions at the tumor site.

Keywords: Vγ9Vδ2 T cells, immune checkpoints, multiple myeloma, immune suppression, bone marrow

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INTRODUCTION

Vγ9Vδ2 T cells have gained a solid reputation in cancer immunotherapy for their capacity to bridge innate and adaptive immunity and to participate to a multifaceted array of direct and indirect antitumor immune responses (1). Hematological malignancies, and especially B-cell malignancies, are privileged targets of Vγ9Vδ2 T-cell recognition and killing (2). This intrinsic susceptibility is due to the enhanced cell surface expression of stress-induced self-ligands and to the intense production of phosphorylated metabolites generated by the mevalonate (Mev) pathway. Isopentenyl pyrophosphate (IPP) is the prototypic Mev metabolite recognized by Vγ9Vδ2 T cells *via* TCR in association with the isoform A1 of the butyrophilin-3 (BTN3A1) protein family (3, 4). IPP is structurally related to the phosphoantigens generated by bacteria and stressed cells that are patrolled by Vγ9Vδ2 T cells as part of their duty to act as first-line defenders against infections and stressed cell at risk of malignant transformation (5).

One strategy commonly used *in vivo* and *in vitro* to activate Vγ9Vδ2 T cells is the stimulation of tumor cells, monocytes, and dendritic cells (DC) with aminobisphosphonates (NBP) like pamidronate and zoledronate (ZA) (6). These drugs inhibit farnesylpyrophosphate synthase in the Mev pathway (7) leading to intracellular IPP accumulation and extracellular IPP release which is sensed by Vγ9Vδ2 T cells *via* TCR and BTN3A1 (8). Wilhelm and colleagues (9) were the first to demonstrate that activation of Vγ9Vδ2 T cells with pamidronate and low-dose interleukin 2 (IL-2) could induce clinical responses in patients with B-cell lymphomas and multiple myeloma (MM). The ability of peripheral blood (PB) Vγ9Vδ2 T cells to proliferate *in vitro* after stimulation with pamidronate and IL-2 was a predictor of clinical response. A clinical trial of adoptively transferred *ex vivo* activated Vγ9Vδ2 T cells in combination with ZA and IL-2 was well tolerated, but showed very limited clinical efficacy (9). Additional studies in solid tumors have also fallen short of clinical expectations (10–13). Understanding why Vγ9Vδ2 T cells perform so poorly when intentionally recruited *in vivo* or *ex vivo* to kill tumor cells is mandatory to really exploit their antitumor properties. One possible explanation is that activated Vγ9Vδ2 T cells do not reach the tumor site or, if reached, they are overwhelmed by the immune suppressive contexture operated by tumor cells and neighboring cells in the tumor microenvironment (TME).

The TME is the protective niche which helps tumor cell to resist chemotherapy and escape immune surveillance (14). Although immune effector cells are often recruited in the TME by the tumor mutational load and the inflammatory milieu, their antitumor functions are blunted by direct or indirect inhibitory signals generated by tumor cells and neighboring cells in the TME (15). Vγ9Vδ2 T cells are not exempted from this immune suppressive contexture operated *via* soluble and cellular factors (16). Soluble factors include transforming growth factor-β, prostaglandins, and kynurenins (17–19). Cellular factors include regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSC), bone marrow-derived stromal cells (BMSC), and others. The discovery that immune checkpoints (ICPs) and their ligands (ICP-L) are abundantly expressed by tumor cells, immune effector cells, and immune suppressive cells have helped to understand the mechanisms promoting the immune suppressive cross talk in the TME and provided new opportunities of interventions.

In this review, we will discuss how the ICP/ICP-L circuitry undermines Vγ9Vδ2 T-cell function and how Vγ9Vδ2 T cells are very early and sensitive detectors of the TME immune suppressive contexture in MM patients. Lessons learned from Vγ9Vδ2 T cells in MM can be instrumental to improve Vγ9Vδ2 T-cell-based immunotherapy in cancer.

THE IMMUNE SUPPRESSIVE TME IN MYELOMA

Multiple myeloma is a prototypic disease where malignant myeloma cells actively remodel the bone marrow (BM) microenvironment to establish a protective niche to support their growth, immune evasion, and drug resistance. MM is invariably preceded by a precursor asymptomatic stage of monoclonal gammopathy of undetermined significance (MGUS) with an estimated risk of progression to symptomatic disease ranging from less than 1% to more than 3% per year. This range depends on risk factors traditionally ascribed to intrinsic features of myeloma cells. Genomic alterations determining clonal advantage are already detectable in MGUS indicating that the probability of progression is also dependent on extrinsic factors such as the composition of the surrounding TME (20). TME in MGUS and MM consists of a non-cellular component, the extracellular matrix, and of a heterogeneous cellular compartment that includes hematopoietic and non-hematopoietic cells. Both the non-cellular and cellular components are edited by myeloma cells to elude immune surveillance and insure their undisturbed survival and progressive expansion (14, 15, 21).

Immune escape in the BM of MGUS and MM patients is achieved by shifting the balance between immune effector and immune suppressor functions as in many other cancers. The immune suppressive mechanisms include the local recruitment and/or activation of immune suppressor cells like Tregs, MDSC, the protumoral polarization of tumor-associated macrophages and/or mesenchymal stem cells, and the differentiation and activation of Th17 cells (22). The wane of immune effector functions includes impaired phagocytosis, ineffective antigen presentation, and T-cell costimulation by DC, B-cell defects and humoral deficiencies, and NK and NKT cell dysfunctions (23). The protumoral immune shift in the TME is driven by soluble factors and cellular interactions, including the recently discovered ICP/ICP-L circuitry.

For many years, the conventional wisdom has been that the immune balance is tipped in favor of myeloma cell control in MGUS and early stages of MM, whereas the balance is tipped in favor of myeloma cell growth in advanced disease. The wisdom was based on the results obtained from experiments exploring the phenotype and function of T cells, NK cells, and NKT cells in MGUS and early MM stages compared to advanced MM stages (24–26). Nowadays, it is clear that the TME of MGUS subjects already harbor a number of immune dysfunctions. The functional interrogation of pAg reactivity of Vγ9Vδ2 T cells in MGUS and MM patients at different stages of the disease (diagnosis, remission, and relapse) has been particularly enlightening (**Figure 1**). We have previously shown that Vγ9Vδ2 T cells from approximately 50% of MM patients are anergic to ZA stimulation at diagnosis when this assay is performed in PB and monocytes are used to generate IPP (27) (**Figure 1B**). The anergy is reversible if ZA-treated DC, and not monocytes, are used to stimulate PB Vγ9Vδ2 T cells, one possible explanation being that the higher IPP production by DC after ZA stimulation (28, 29). The proportion of anergic MM patients increases to 80–90% if ZA stimulation is carried out in the BM using monocytes as IPP-presenting cells. Unlike PB, the strategy to use ZA-treated DC to recover Vγ9Vδ2 T-cell proliferation in the BM is ineffective (**Figure 1C**), and neither the removal nor the functional inhibition of suppressive cells like Tregs or MDSC are sufficient to recover BM Vγ9Vδ2 T-cell proliferation (30). Crossover experiments have clearly shown that the defective pAg reactivity is peculiar to BM Vγ9Vδ2 T cells, which do not proliferate no matter whether they

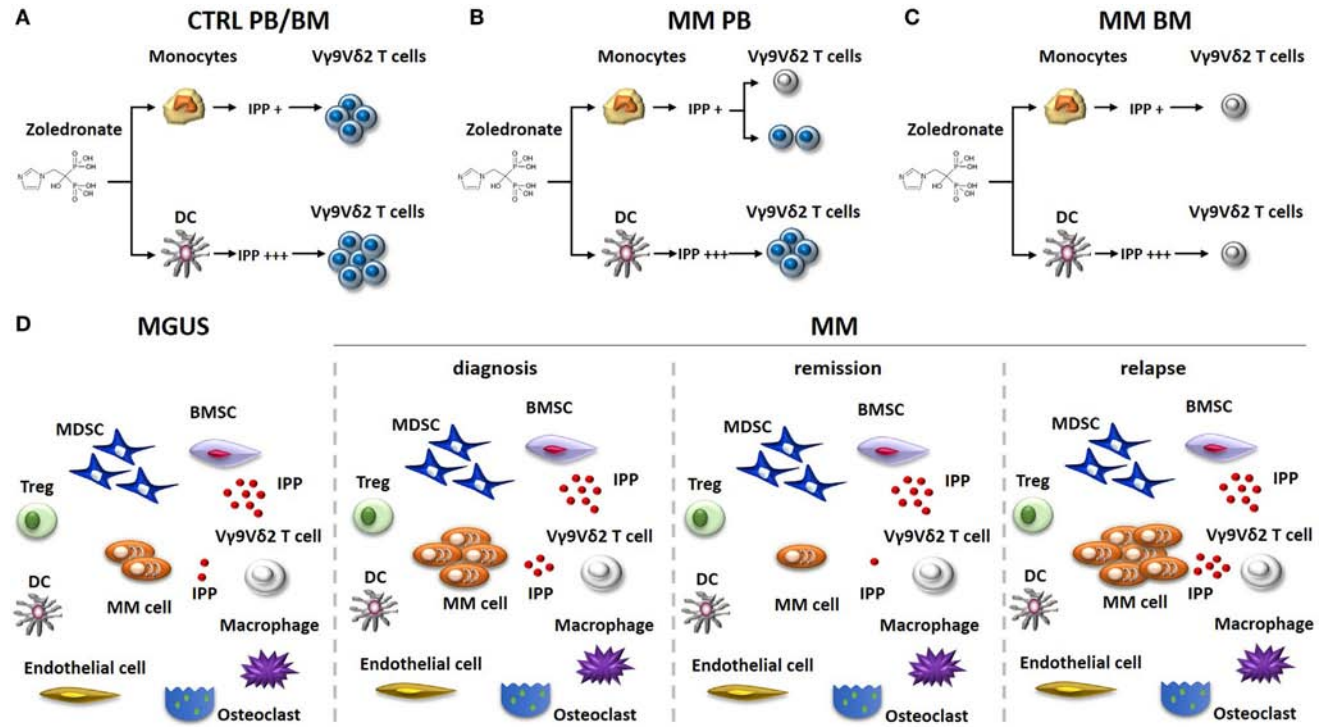

FIGURE 1 | Vγ9Vδ2 T-cell impairment in the immune suppressive TME of MM patients. The stimulation of monocytes or DC with zoledronate (ZA) and low dose of IL-2 is commonly used *in vitro* to induce IPP-dependent Vγ9Vδ2 T-cell activation. **(A)** This approach is uniformly effective in healthy donors (Ctrl), no matter whether Vγ9Vδ2 T cells are from the PB or the BM. ZA-treated DC are more effective than ZA-treated monocytes to induce Vγ9Vδ2 T-cell activation because they produce higher IPP amounts. **(B)** PB Vγ9Vδ2 T cells are refractory to ZA stimulation (gray colored) in about 50% of MM patients at diagnosis, when monocytes are used as stimulators. Anergy in these patients can be recovered if ZA-treated DC instead of monocytes are used as stimulators. **(C)** BM Vγ9Vδ2 T cells from MM patients are uniformly anergic to ZA stimulation and the anergy is not corrected by ZA-treated DC. **(D)** The immune suppressive TME of MGUS and MM patients includes suppressor cells like Tregs, MDSC, and BMSC (that produce high amounts of IPP). The proportion of these cells is very similar irrespective of the disease status. MDCS are PD-L1$^+$ in MGUS and MM irrespective of the disease status and release high amounts of IPP in the TME. Other cells playing a major role in the TME are macrophages, endothelial cells, and osteoclasts. The proportion of these cells is variable according to diagnosis and disease status. The defective pAg reactivity of Vγ9Vδ2 T-cell (gray colored) is present in MGUS, MM at diagnosis, MM in remission, and MM in relapse. Elimination of myeloma cells (that are PD-L1$^+$ and release IPP) is insufficient in the short/intermediate term to recover Vγ9Vδ2 T-cell functions. It is possible that the persistence of PD-L1$^+$ cells in the TME (i.e., MDSC), high levels of extracellular IPP, and PD-1 expression (and other ICP) on their cell surface continue to render functionally incompetent BM Vγ9Vδ2 T cells even in the remission phase. Abbreviations: DC, dendritic cells; ZA, zoledronate; IPP, isopentenyl pyrophosphate; PB, peripheral blood; BM, bone marrow; Ctrl, healthy subjects; MM, multiple myeloma; Tregs, regulatory T cells; MDSC, myeloid-derived suppressor cells; BMSC, bone marrow-derived stromal cells; MGUS, monoclonal gammopathy of undetermined significance; TME, tumor microenvironment; ICP, immune checkpoint; PD-1, programmed cell death protein 1.

are stimulated with BM- or PB-derived ZA-treated DC. Vγ9Vδ2 T-cell anergy is already detectable in the BM of MGUS individuals, largely anticipating the dysfunction of T and NKT cells. BM Vγ9Vδ2 T cells remain anergic to pAg stimulation also in MM patients who are in remission after autologous stem cell transplantation (30) (**Figure 1D**). Altogether, these data indicate that Vγ9Vδ2 T cells are unique among other immune effector cells in sensing the very early and persistent immune suppressive TME commitment in MGUS and MM.

PROGRAMMED CELL DEATH PROTEIN 1 (PD-1)/PD-L1 NETWORK IMPAIRS Vγ9Vδ2 T-CELL ACTIVATION IN THE TME

Immune checkpoints/ICP-L are expressed by a variety of immune cells to control the strength and duration of immune responses and maintain T-cell homeostasis and self-tolerance (31). Smartly, tumor cells have learned very quickly how to hijack the ICP/ICP-L circuitry to withstand immune recognition and onslaught. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and PD-1 are so far the ICP more often targeted for therapeutic purposes (32). PD-1 is expressed on the surface of dysfunctional T and B cells and inhibits T cell-mediated apoptosis after engagement by PD-L1 expressed by tumor cells (33–36). The devilish plot to protect tumor cells from immune recognition and killing begins in the tumor-draining lymph nodes where the PD-1/PD-L1 axis interferes with antigen presentation to blunt the activation of tumor-specific immune responses (37, 38). The inhibition of adaptive immune responses in secondary lymphoid organs is strategically implemented in the TME by ICP/ICP-L-dependent blockade of antitumor responses mediated by innate immunity (i.e., NK cells and Vγ9Vδ2 T cells) (30, 39). Based on these premises, ICP blockade (mainly pursued using anti-CTLA4

and/or anti-PD-1/PD-L1 mAbs) has been granted FDA approval in solid tumors and Hodgkin lymphoma (40, 41).

In the context of MM, increasing evidences suggest that the PD-1/PD-L1 pathway plays an active role in the generation of the immunosuppressive TME (42, 43). Myeloma cells offer high levels of PD-L1 to PD-1-expressing T and NK cells in the TME (43–45), and *in vitro* studies showed enhanced myeloma cell killing by T and NK cells after PD-1 and/or PD-L1 blockade (46). In a mouse model, Hallett and colleagues demonstrated that PD-L1 expression in myeloma cells decreases cytotoxic function, cytokine production, and proliferation of PD-1$^+$ T and NK cells leading to their functional exhaustion (47). Consistent with this observation, Paiva et al. reported a prolonged survival in disseminated myeloma-bearing mice after PD-1 blocking (42), corroborating the therapeutic exploration of PD-1 blockade in MM.

However, unsatisfactory results of single agent anti PD-1 mAb failed to meet the expectations in the clinical setting. Combination approaches with immunomodulatory drugs (lenalidomide or pomalidomide) and dexamethasone proved synergistic effects in phase I/II trials, nurturing hopes for therapeutic exploitation of PD-1 blockade in MM (48–50). Immunotherapy with daratumumab is currently under investigation as an alternative partner to improve efficacy of PD-1 blockade in a multiphase randomized clinical trial (NCT03357952) recruiting relapsed refractory myeloma patients. Only a deeper understanding of molecular mechanisms triggered by PD-1/PD-L1 signaling pathway may lead to rationally identify targeted strategies to overcome resistance to PD-1 blockade.

Although the function of PD-1 has been extensively studied in mouse and human conventional αβ T cells (51–53), little is known about the role of PD-1/PD-L1 signaling in human Vγ9Vδ2 T cells. Iwasaki et al. analyzed PD-1 expression in PB Vγ9Vδ2 T cells after pAg stimulation in healthy donors and breast cancer patient (54). They found that PD-1$^+$ Vγ9Vδ2 T cells in breast cancer patients produced less IFNγ had lower cytotoxic activity and CD107 degranulation than PD-1$^-$ cells after challenging with PD-L1$^+$ tumor target cells. Zumwalde et al. (55) have stressed the different kinetics of PD-1 expression in normal Vγ9Vδ2 T cells upon pAg stimulation compared with tumor-experienced Vγ9Vδ2 T cells. BM Vγ9Vδ2 T cells from myeloma patients represent a paradigmatic example of functionally impaired tumor-experienced Vγ9Vδ2 T cells. PD-1 expression in Vγ9Vδ2 T cells from normal donors peaks approximately 3–4 days after pAg stimulation afterward PD-1 expression returns to baseline values (55). This is very different compared with myeloma patients in which PD-1 expression increased in BM anergic Vγ9Vδ2 T cells after ZA stimulation, suggesting that these cells are intrinsically programmed to increase their threshold of refractoriness to pAg-induced TCR stimulation *via* PD-1 upregulation (30). Interestingly, PD-1 expression in myeloma BM Vγ9Vδ2 T cells is predominant in the central memory subset, which in normal conditions is the subset with the highest proliferative capacity to pAg stimulation (30).

One possible mechanism to explain PD-1 expression in BM Vγ9Vδ2 T cells, already detectable in MGUS when the myeloma cell infiltration is still low (<10% by definition), is the prolonged TCR engagement by pAg in the TME. Preliminary results from our lab indicate that myeloma cells are not the only IPP producers in the TME, and that BMSC in MGUS and MM also produce and release very high amounts of IPP in the extracellular microenvironment (8) (**Figure 1D**). Thus, it is possible that a chronic TCR engagement within an immune suppressive TME, characterized by inappropriate costimulatory signals and/or cytokines, leads to PD-1 expression and functional exhaustion of Vγ9Vδ2 T cells.

Our study has been the first to show that human MDSC are PD-L1$^+$ in the TME suggesting that this is an additional mechanism exploited by these cells to exert local immune suppression against PD-1$^+$ effector cells. Interestingly, the BM is highly hypoxic in MM (56) and experimental data in tumor-bearing mice have shown that the hypoxia-inducible factor-1α selectively upregulates PD-L1 in tumor-infiltrating MDSC, but not in MDSC from peripheral lymphoid organs (57). Hypoxia has been reported to increase the immune suppressive TME contexture *via* upregulation of a variety of ICP/ICP-L (58). Extracellular adenosine, which accumulates due to tissue hypoxia, also contributes to ICP/ICP-L upregulation (59), and adenosine levels are significantly higher in the BM of myeloma due to the highly coordinated expression of adenosinergic ecto-nucleotidases (CD39/CD73/CD38/CD203a) strategically located at the interface between myeloma cells and neighboring cells (60). Preliminary data from our lab indicate that BMSC, another major protumoral component in the BM niche of MGUS and MM patients, are PD-L1$^+$, further confirming that there is a redundancy of immune suppressor cells exploiting the ICP/ICP-L circuitry to hamper myeloma cell recognition and elimination by immune effector cells in the TME. The finding that BM Vγ9Vδ2 T cells are PD-1$^+$ in MGUS, MM at diagnosis, and even in remission, confirms the unique sensitivity of these cells to the immune suppressor imprinting operated by the TME which is not overcome even when myeloma cells have been cleared from the BM. One possible explanation is that the immune suppression is exerted by PD-L1$^+$ cells other than myeloma cells, like MDSC and BMSC, whose percentages and PD-L1 expression remain unchanged in the BM of MM in remission (30) (**Figure 1D**).

STRATEGIES TO RESCUE ANTITUMOR Vγ9Vδ2 T-CELL FUNCTION IN THE TME: LESSONS FROM MM

Clinical trials using anti-PD-1 mAbs as single agents in MM have failed to confirm the excellent premises of experimental data (61, 62). Interestingly, we have shown that single agent PD-1 blockade is insufficient to fully recover the antitumor activity of BM Vγ9Vδ2 T cells in MM (30). Thus, Vγ9Vδ2 T cells are excellent tools to decipher the mechanisms developed by Vγ9Vδ2 T cells and other immune effector cells to resist immune recovery triggered by ICP/ICP-L blockade in the TME (**Figure 2**). Understanding these mechanisms of resistance is important to improve the efficacy of immune interventions based on ICP/ICP-L blockade in MM and other cancers.

One mechanism could be the expression of alternative ICP under resting conditions or after pAg stimulation and/or PD-1 blockade (**Figure 2**, option A). Double PD-1 and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) expression in tumor-infiltrating lymphocytes from tumor-bearing mice identifies the most dysfunctional $CD8^+$ T cells in the TME of these mice, and concurrent PD-1 and TIM-3 blockade significantly improves the antitumor activity of these cells, much better than single inhibition (63). The expression of multiple ICP/ICP-L expression on individual immune cells and tumor cells has recently been proposed as a mechanism of acquired resistance to single PD-1 blockade also in human cancer (64, 65). TIM-3, lymphocyte-activation gene 3 (LAG-3), and TIGIT are examples of alternative ICP that could be expressed on the cell surface of BM Vγ9Vδ2 T cells and could restrain the efficacy of single PD-1 blockade. Targeting multiple ICP could be an attractive strategy to improve the recovery of antitumor Vγ9Vδ2 T-cell responses (**Figure 2**, option A).

Another mechanism could be the regulatory/suppressor polarization of BM Vγ9Vδ2 T cells driven by the TME (66, 67). This functional polarization cannot be reverted by single PD-1 blockade. Vγ9Vδ2 T cells with regulatory functions have initially been described by Casetti et al. (68), who reported the *in vitro* induction of $FOXP3^+$ regulatory Vγ9Vδ2 T cells after pAg stimulation in the presence of TGF-β1 and IL-15. Other groups have confirmed the emergence of regulatory/suppressor Vγ9Vδ2 T cells as a consequence of pAg activation in the presence of selected cytokines (69, 70). Ma et al. have reported an increased proportion of regulatory Vγ9Vδ2 T cells in the PB of MM patients which could suppress antimyeloma immune responses with the same efficiency of conventional Tregs (71).

Single PD-1 blockade may not be sufficient to revert the regulatory/suppressor polarization of BM Vγ9Vδ2 T cells. Preliminary data in our lab indicate that PD-1 blockade of BM Vγ9Vδ2 T cells in MM could even worsen this polarization by inducing the expression of additional inhibitory molecules (FOXP3, CD73, CD39, and CD38) and the release of suppressive factors like IL-10 (**Figure 2**, option B). In this case, PD-1 blockade should be integrated by strategies aimed at preventing the detrimental BM Vγ9Vδ2 T-cell polarization and/or the regulatory/suppressor functions exerted by polarized Vγ9Vδ2 T cells in the TME.

Another major hurdle preventing the full recovery of antitumor Vγ9Vδ2 T-cell functions by PD-1 blockade could be their immune senescence status (**Figure 2**, option C). Immune senescence is the hallmark of oligoclonal T cells which accumulate in the PB of MM patients with progressive and advanced disease (72–74). The immune competence of senescent cells is very hard to resurrect by single PD-1 blockade. The $CD57^+CD160^+KLRG\text{-}1^+CD28^-$ phenotype might portray a distinct population of senescent Vγ9Vδ2 T cells gathered in the BM of MM patients which require multiple approaches to overcome resistance to PD-1 blockade.

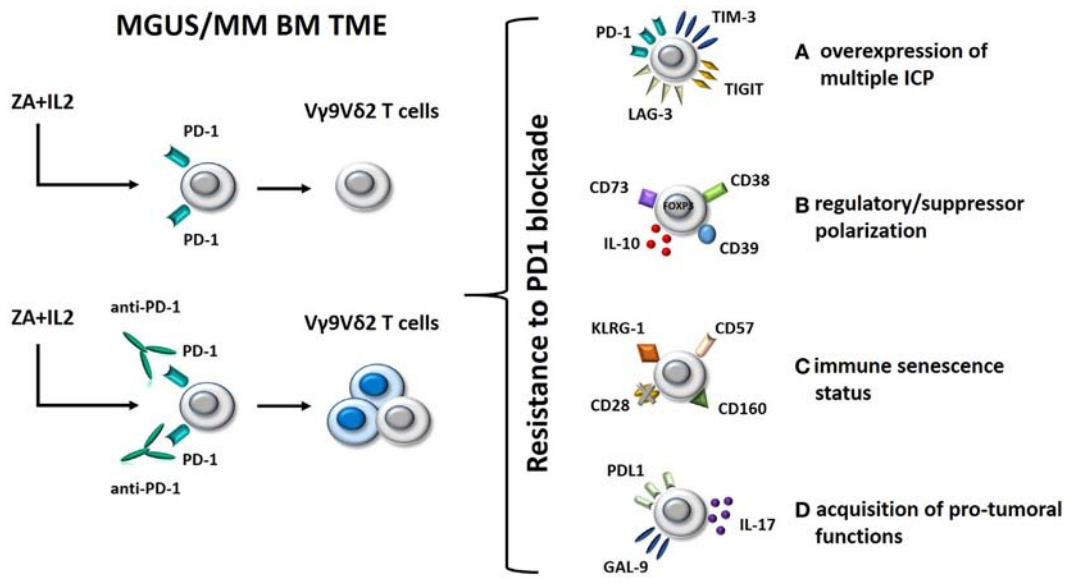

FIGURE 2 | Hypothetical mechanisms of resistance to PD-1 blockade in BM Vγ9Vδ2 T cells from MM patients. Left panel: BM Vγ9Vδ2 T cells in MGUS and MM are anergic to ZA + IL-2 stimulation; this anergy is only partially rescued by single agent PD-1 blockade. Right panel: possible mechanisms of resistance to immune recovery triggered by single agent PD-1 blockade. (A) Alternative inhibitory ICPs, such as TIM-3, TIGIT, and LAG-3, are expressed by BM Vγ9Vδ2 T cells to reinforce their anergy and resist to PD-1 blockade; (B) Vγ9Vδ2 T cells have been functionally polarized to express inhibitory molecules (FOXP3, CD73, CD39, and CD38) and release immune suppressive cytokines (IL-10) as a consequence of their long-term exposure to tumor cells in the BM; (C) BM Vγ9Vδ2 T cells have acquired a senescence status ($CD57^+CD160^+KLRG\text{-}1^+CD28^-$ phenotype) which is hard to reverse by single PD-1 blockade; (D) PD-1 engagement of anergic/senescent BM Vγ9Vδ2 T cells induces the expression of PD-L1 and galectin-9 (GAL-9), and IL-17 secretion leading to immune suppressive effects on other effector cells like $CD8^+$ cells. Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; BM, bone marrow; TME, tumor microenvironment; ZA, zoledronate; IL-2, interleukin 2; ICP, immune checkpoint; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; LAG-3, lymphocyte-activation gene 3; PD-1, programmed cell death protein 1.

Finally, the acquisition of protumoral functions by Vγ9Vδ2 T cells that are long-term resident in the TME could be another mechanism of resistance to single PD-1 blockade. The inappropriate expression of ICP-L such as PD-L1 and galectin-9 (GAL-9) in Vγ9Vδ2 T cells could affect *via* PD-1 and TIM-3 the antitumor responses of other immune effector cells in the TME (**Figure 2**, option D). Likewise, production of IL-17 by Vγ9Vδ2 T cells, as reported for selected γδ subsets in solid tumors (75), may contribute to reinforce the immune suppressive TME imprinting by recruiting MDSC (76) and polarizing neutrophils (77). This hypothetical scenario suggests that PD-L1/GAL-9 and IL-17 (or its receptor) could be novel targets to rescue antimyeloma Vγ9Vδ2 T-cell function.

Whether the mechanisms reported above are operative under baseline conditions or sharpened by pAg stimulation in the presence of PD-1 blockade is a matter of current investigation in our lab. Preliminary data suggest that the intracellular metabolic and signaling pathways evoked by PD-1 blockade could worsen the immune competence status of pAg-experienced Vγ9Vδ2 T cells.

CONCLUSION

Vγ9Vδ2 T cells are programmed by default to behave as very effective professional killers of malignant B cells, including myeloma cells. We propose that Vγ9Vδ2 T cells are very precociously neutralized by myeloma cells in cooperation with neighboring cells in the TME of MGUS and MM patients. Finalistically speaking, it makes sense that myeloma cells inactivate or co-opt in their favor those immune cells mostly well suited to threaten their survival. This is done very early at the stage of MGUS and the uncontrolled production of IPP by BMSC and myeloma cells is probably a relevant initiating event.

Functional interrogation of BM Vγ9Vδ2 T cells from MM patients in remission has revealed that clearance of myeloma cells does not automatically imply the recovery of a fully immune competent TME.

ICP/ICP-L abundantly expressed by tumor cells, immune effector cells, and immune suppressive cells are major promoters of immune suppressive cross talks in the TME at any stage and hamper the antimyeloma activity of BM Vγ9Vδ2 T cells. Single agent PD-1 blockade is insufficient to fully recover the antitumor activity of Vγ9Vδ2 T cells *in vitro*, especially in MM at diagnosis or in relapse. These data indicate that additional immune suppressive mechanisms are involved in the anergy of Vγ9Vδ2 T cells. A working knowledge of these mechanisms may yield insight into the development of more effective interventions to fully exploit the immune potency of Vγ9Vδ2 T cells in MM and other cancers. This knowledge could be profitably implemented by next generation sequencing studies investigating the genetic and epigenetic consequences of cell-to-cell interactions of Vγ9Vδ2 T cells and other cell subsets in the TME of MGUS and MM patients.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to text and figures and have approved the manuscript for submission.

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REVIEW
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Engineering Approaches in Human Gamma Delta T Cells for Cancer Immunotherapy

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Sharing both innate and adaptive immune properties, γδT cells are attractive candidates for cellular engineering. As the cancer immunotherapy field becomes increasingly busy, orthogonal approaches are required to drive advancement. Engineering of alternative effector cell types such as γδT cells represents one such approach. γδT cells can be modified using many of the techniques used in αβT cell engineering, with the added advantage of innate-like tumor recognition and killing. Progress has been made in T-cell receptor transfer to and from γδT cells as well as in a number of chimeric antigen receptor-based strategies. As the cancer immunotherapy field moves beyond repetitive iteration of established constructs to more creative solutions, γδT cells may offer an attractive chassis to drive anti-tumor responses that are not only broader, but also possess a more favorable safety profile.

Keywords: gamma delta, chimeric antigen receptor, adoptive transfer, alpha beta T cells, cancer immunotherapy

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INTRODUCTION

Cellular engineering has offered many options for redirecting immune responses against cancer. In some cases, the clinical responses have been remarkable (1–5) but there are still many challenges to overcome. Broadly speaking, redirection of T-cell responses against specific tumor-associated antigens (TAAs) has been achieved in two ways: T-cell receptor (TCR) gene transfer or chimeric antigen receptor (CAR) expression. TCR gene transfer involves expression of a TCR derived from a tumor antigen-reactive T-cell (6–8). The TCR is typically derived from a tumor infiltrating lymphocyte or from *in vitro* antigen-stimulated blood. Chimeric antigen receptors (CARs) are more synthetic in nature and comprise an ectodomain that directly binds a cell surface molecule specific for the tumor and endodomains, which provide T cell signaling. The ectodomain is most commonly a single-chain variable fragment derived from a monoclonal antibody, and the endodomains usually include CD3ζ in combination with one or more costimulatory domains derived from molecules such as CD28 or 4-1BB (9, 10).

The majority of cellular engineering approaches have been applied to αβT cells, which are easy to expand and purify from peripheral blood. Notable attention has been given to αβT cells engineered to express second- and third-generation CARs against targets such as CD19 (2, 11–14) and CAR-T cells targeting CD19 recently received FDA approval for sale in the United States for the treatment of diffuse large B-cell lymphoma and acute lymphoblastic leukemia (ALL).

Engineering approaches that redirect immune cells to target single antigens *via* a CAR or MHC-presented TAA epitopes have limitations. TCR transfer depends on the ability to isolate a HLA-matched TCR against a processed antigen presented by tumor cells (10), and is susceptible to tumor immune-evasion strategies such as downregulation of MHC (15) or loss of redundant neo-antigens (16). Transferred TCRs against TAAs can also lead to unexpected side-effects due to cross-reactivity with unrelated peptides. One study targeting MAGE-3A with a HLA-A*01 restricted TCR led to

fatal cardiotoxicity due to cross-reactivity with epitopes derived from the striated-muscle protein, titin (17), though a later study targeting the same molecule but using a different TCR construct did not generate this toxicity and led to objective partial responses in 9/17 patients (18). This difference may be explicable due to recognition of different epitopes, but highlights the potential for unexpected toxicity.

Chimeric antigen receptors remove the need for HLA-matching and antigen presentation on tumor MHC by bypassing the αβTCR entirely, but antigen selection presents a challenge. CAR-T cells target both healthy and tumor cells expressing their cognate antigen (10); for example, anti-CD19 CARs kill $CD19^+$ ALL as well as healthy $CD19^+$ B-cells (19). In the context of CD19, B-cell aplasia is considered an acceptable cost, but targeting of other antigens such as carbonic anhydrase IX or ErbB2 has led to unexpected and sometimes fatal toxicity (albeit only at very high T cell dose in the case of ErbB2) (20, 21). Furthermore, the specificity of CAR-targeting provides a prime opportunity for immune-evasion through antigen loss, which has proven to be a particular issue in anti-CD19 CAR-T therapy (22).

Use of alternative cell types in cancer immunotherapy is not a novel concept. Adoptively transferred allogeneic NK cells or cytokine-induced killer cells have shown clinical efficacy against metastatic melanoma (23), renal cell carcinoma, acute myeloid leukemia, and Hodgkins lymphoma (24). While engineering of these cell types has lagged behind that of conventional αβT cells, CAR transduced NK cell lines have been successfully directed against CD19 (25), CD20 (26), the disialoganglioside GD2 (27), ErbB2 (28), and other TAAs (29). NK cell specificity to tumors has been enhanced using exogenous constructs such as bispecific antibodies that enhance or manipulate the synapse between NK cell and target (30). NKT cells expressing CARs have also been developed (31). Such modified NKT cells targeting the ganglioside GD2 are about to enter phase I trials in patients with neuroblastoma (clinical trial ID NCT03294954). This range of approaches demonstrates the feasibility of using effector cells with an innate immune phenotype, possessing broader tumor recognition potential.

PROPERTIES OF γδT CELLS

In vitro and *in vivo*, γδT cells exhibit potent anti-tumor responses suggesting natural roles in tumor control and potential for therapeutic exploitation (32–35). Of particular interest, a recent correlation between the molecular profile of the tumor immune microenvironment and prognosis in over 5,000 tumor samples indicated that the presence of infiltrating γδT cells was the strongest predictor of positive outcome (36).

γδT cells comprise only 1–10% of circulating T-cells (37), diverging from αβT cells in the thymus, with lineage commitment completed by the DN3 stage of thymic development (38). The dynamics of the γδT cell repertoire during fetal development and later adult life are complex, and while initial evidence suggested that the Vγ9Vδ2 subset, being small at birth (39), expanded purely in response to environmental pathogens, Dimova et al. showed that effector $V\gamma9V\delta2^+$ cells make up the bulk of the γδT cell repertoire in second trimester fetuses. This population contracts and loses its dominance toward full gestation, when $V\gamma9^-V\delta1^+$ subsets predominate (40). These results were corroborated by Ravens et al. who used next-generation sequencing of the γδTCR repertoire in cord blood to reveal higher proportions of Vγ2–5 and Vδ1, 3 and 5 TCR chains compared to healthy adult circulation (41). Later in life, while adult human peripheral γδT cells expressing Vγ2–5, 8–9, and Vδ1–8 chains (42) can all be detected in peripheral blood of healthy donors and cancer patients (37), $V\gamma9V\delta2^+$ cells predominate in the circulation, and the age-related extrathymic increase in circulating $V\gamma9V\delta2^+$ proportions (39, 43) is well documented. Interestingly, this trend shows geographical variation; γδTCR repertoires of individuals from sub-Saharan Africa show greater enrichment of $V\delta1^+$ cells compared to that of Caucasians living in Europe or America. This difference is not linked to malaria exposure and raises the possibility that the circulating γδTCR repertoire is shaped by environmental factors such as the endemic microbiome (43, 44).

Human $V\gamma9V\delta2^+$ T cells have been subjected to closer analysis than other subsets. They respond to targets with a high phosphoantigen burden, associated with malignant transformation and disordered EGFR signaling (45, 46). Importantly, this recognition is not dependent on peptide epitopes bound to MHC, distinguishing Vγ9Vδ2 T cells from αβT cells (47). Isopentenyl-5-pyrophosphate (IPP), a phosphoantigen by-product of the mevalonate pathway of cholesterol biosynthesis is the prototypic phosphoantigen in the context of human Vγ9Vδ2 T cell–tumor interactions (48, 49), though other phosphoantigens such as bromohydrin pyrophosphate and the microbially derived (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate have much lower EC_{50} values for Vγ9Vδ2 T cell activation (50). Because IPP production can be enhanced using aminobisphosphonates, $V\gamma9V\delta2^+$ T cells can be easily expanded from the blood of healthy donors and cancer patients using inexpensive and well-validated compounds in combination with low-dose IL-2 (50). Aminobisphosphonates inhibit the mevalonate pathway enzyme farnesyl pyrophosphate synthase, which is downstream of IPP and leads to its accumulation (51, 52). The approach allows production of large numbers of highly purified $V\gamma9V\delta2^+$ T cells using a relatively simple protocol (37, 53).

The precise mechanism of Vγ9Vδ2 TCR stimulus is still being clarified. There is a high degree of CDR3 sequence homology between TCR chains from fresh $V\gamma9V\delta2^+$ γδT cells, those expanded using aminobisphosphonates and those which expand in response to co-culture with microbes such as *Escherichia coli* (54). There is also homology in Vδ chain CDR3 regions between cells from unrelated individuals following phosphoantigen exposure (37). These factors reinforce the evidence that the Vγ9Vδ2 TCR responds to a ligand held in-common across donors. While previous reports have implicated F1-ATPase as the ligand (55, 56), strong recent evidence points to butyrophilin 3A1 (BTN3A1) (57, 58), which is stabilized in the membrane and undergoes a conformational change when its intracellular 30.2 domain is bound by IPP.

γδT cells also receive inputs from multiple co-stimulatory receptors and receptors usually associated with NK cells (59, 60), such as NKG2D (61), DNAM-1 (62), and Fcγ receptors, such as FcγRIII (CD16) (34, 63). Consequently, $V\gamma9V\delta2^+$ T cells exhibit NK-cell like properties of potent antibody-dependent and

independent cytotoxicity (34, 53, 63). Less is known about the ligands of non-Vγ9Vδ2 γδTCRs, perhaps due to the diversity of targets and their MHC-independent activity. Numerous ligands have been identified but a clear pattern has not yet emerged, for example, Vδ1$^+$ γδT cells have been shown to have against cells expressing lipids, such as CD1c (64), CD1d-sulphatide (65), CD1d-α-GalCer (66), but also against the MHC-associated molecules MICA (67) and MICB (68).

TCR GENE TRANSFER IN THE CONTEXT OF γδTCR$^+$ CELLS

Transfer of specificity through the transfer of murine α and β TCR genes was first used to target the hapten molecule, fluorescein (69), an approach which has subsequently been used to redirect αβT cell immunity against antigens from viral (70) and tumor (71) targets, notably in highly immunogenic tumors, such as melanoma.

Transferring a new αβTCR gene construct into an αβT cells runs the risk of TCR chain mis-pairing unless the endogenous α and β chains are suppressed (72). Mis-pairing can lead to inefficient expression of the novel construct and may lead to the generation of self-reactive TCR clones leading to off-target toxicity (73). Using murine constant regions or altering arrangement of cysteines in the transferred TCRs prevents this (74). While there is a risk of the host mounting an anti-murine immune response with subsequent reduction in immunotherapeutic efficacy, this has not been seen in practice, and many leading groups favor murinized TCRs. One study in which 23% of patients developed anti-murine-TCR antibodies showed that these anti-murine responses had no effect on clinical outcome (75). Were an anti-murine response to be of concern, however, an alternative allowing use of entirely human TCRs is to use γδT cells as the substrate for gene transfer, as the γ and δ TCR chains do not mis-pair with transferred α or β chains (**Figure 1A**). Dorrie et al. (76) demonstrated that

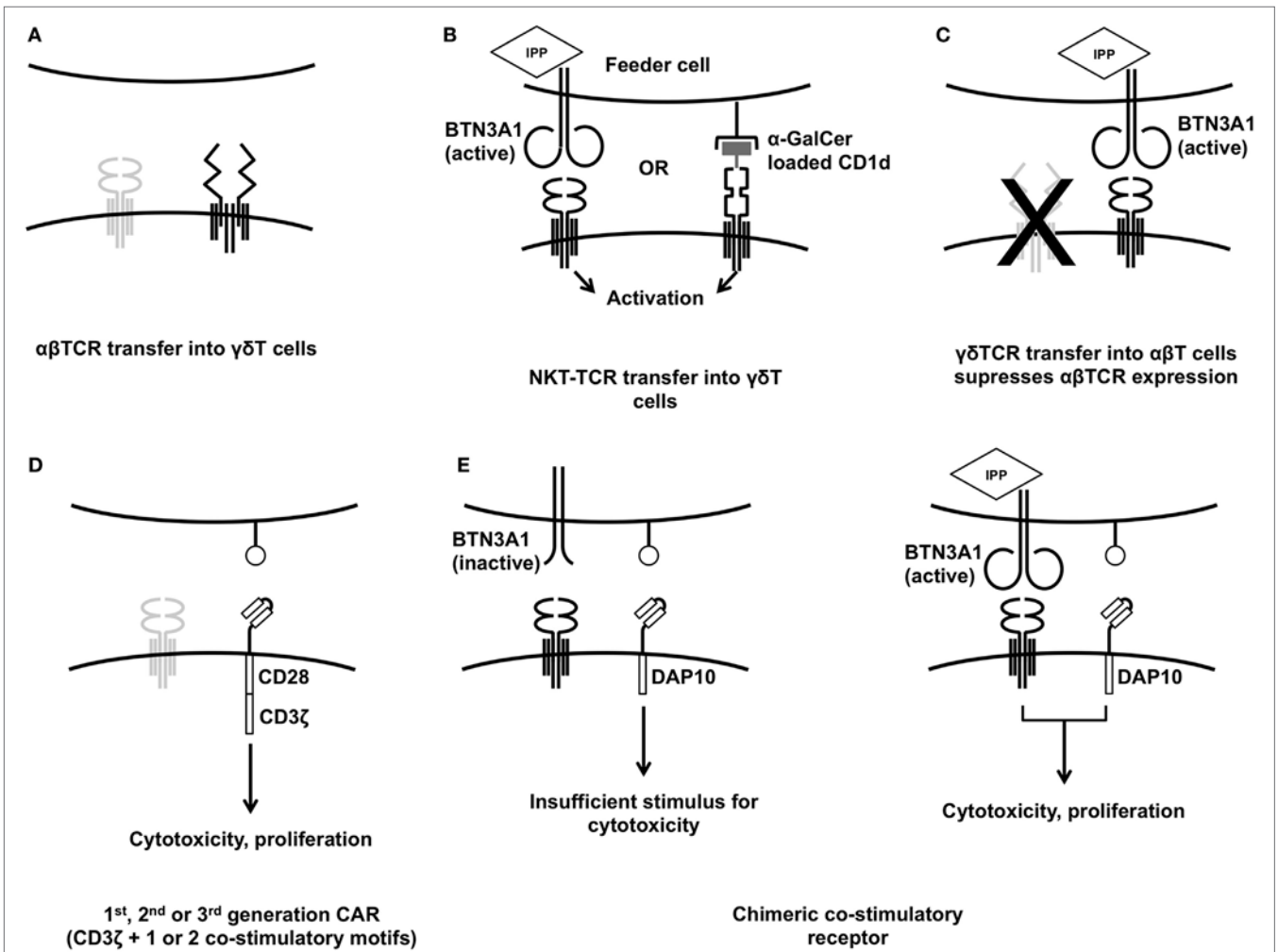

FIGURE 1 | Established strategies for engineering γδT cells. Transferring specific αβT-cell receptor (TCR) into γδT cells **(A)** gives greater control of the final cell product and is one strategy to avoid TCR mis-pairing. Transferring NKT-TCRs into γδT cells **(B)** allows them to be activated for cytotoxicity using feeder cells treated with αGalCer or zoledronic acid. Transfer of γδTCRs into αβT cells has been used to impart broader anti-tumor reactivity **(C)**. Anti-tumor cytotoxicity can be enhanced using conventional first-, second-, or third-generation chimeric antigen receptors **(D)** but the innate tumor recognition provided by the γδTCR can also be harnessed to deliver a more tumor-specific response **(E)**.

γδT cells could be induced to express a HLA-A*0101 restricted αβTCR targeting a peptide derived from an adenovirus hexon protein. Engineered γδT cells produced more IFNγ and TNFα than $CD8^+$ αβT cells expressing the same TCR and had equivalent cytotoxicity against autologous adenovirus-infected dendritic cells. Similar antigen-specific cytokine release was demonstrated by Harrer et al. when γδT cells expressing a gp100/HLA-A2 restricted αβTCR were exposed to $gp100^+$ melanoma cells (77).

Some researchers have highlighted the restrictions inherent in αβTCR gene transfer, in particular the restriction to particular HLA types and the possibility of antigen-negative escape variants (16). Transfer of TCRs derived from invariant natural killer T (iNKT) cells into γδT cells and transfer of γδTCRs into αβT cells (78) have both been used to overcome this. In humans, iNKT cells express the nearly invariant TCR encoded by Vα24Jα18 which responds to glycolipids presented on the HLA-class-I-like molecule CD1d. Like Vγ9Vδ2 TCR activation, this response is not MHC restricted (79). γδT cells expressing TCRs derived from iNKT cells can be stimulated by co-culture with either zoledronic acid treated HEK293T cells or HEK293T cells pulsed with the exogenous glycolipid α-galactosylceramide (α-GalCer, **Figure 1B**). Both stimulations led to enhanced cytotoxicity against the $CD1d^-$ leukemia cell line K562 (79). Continuing on the theme of using MHC-unrestricted TCRs, γδTCR gene transfer into αβT cells (**Figure 1C**) has yielded exciting results. The Vγ9Vδ2 TCR clone G115 (80) was expressed in αβT cells by Marcu-Malina et al. (78). They demonstrated that both the were required for either to be detected, indicating that mis-pairing with endogenous α or β chains was not occurring. The γδTCR-expressing αβT cells showed similar functional properties to "native" Vγ9Vδ2 cells including cytotoxicity against the Daudi cell line, release of TNFα and IFNγ, enhancement of cytotoxicity following target pre-treatment with aminobisphosphonates, and the ability to induce dendritic cell maturation. Vγ9Vδ2 transduced αβT cells showed a surprising lack of alloreactivity, linked to a downregulation of their endogenous αβTCRs (78), and were able to mount responses against a broad panel of tumor cell lines. This lack of γδT cell alloreactivity against non-transformed cells is corroborated by other *in vitro* data on both $Vδ1^+$ and $Vγ9Vδ2^+$ γδT cells (37).

Though the Vγ9Vδ2 TCRs derived from different T cell clones show varying anti-tumor responses, linked to small differences in the γ9 and δ2 CDR3 regions; no correlation was found between the expression of NKG2D, CD158a, NKAT-2, or NKB-1 and anti-tumor reactivity (81). This suggests that altering the functional avidity of interaction between BTN3A1 and the Vγ9Vδ2 TCR is a rich area for optimization. Using $CD4^+$ αβT cells as the recipient cells, Gründer et al. performed alanine scanning between positions $δ2\text{-}G115_{L109}$ and $δ2\text{-}G115_{T113}$ and between $γ9\text{-}G115_{E108}$ and $γ9\text{-}G115_{E111}$, to demonstrate that the length and sequences in these CDR3 regions were critical for ligand interaction, with particular importance being placed on $γ9\text{-}G115_{A109}$, in addition to the J-region residues $δ_{109}$ and $δ_{117}$ (81).

Such detailed knowledge of Vγ9Vδ2 avidity means that highly optimized γδTCRs can be expressed in more readily available αβT cells. γδTCR-engineered αβT cells prevented tumor growth in an immunodeficient (irradiated $Rag^{-/-}γc^{-/-}$) murine model of Burkitt lymphoma (Daudi) and multiple myeloma (OPM2) and also protected mice who had responded to initial treatment from re-challenge with OPM2 performed 120 days after the first tumor and T-cell injection (82). The downregulation of the αβTCR in the transduced cell population allows for facile selection of cells by αβTCR depletion, rather than positive selection of the transduced cells. This "untouched" cell product does not require co-expression of a marker gene and can be processed using pre-existing αβT cell depletion techniques currently used before some bone-marrow transplants, making it highly amenable to GMP-compliant manufacture.

γδT CELLS EXPRESSING CARs

While harnessing the innate potential of the γδTCR is a highly attractive option, manipulating cellular behavior in an antigen-specific manner using CARs (**Figure 1D**) remains one of the mainstays of modern immunotherapeutics. Compared to the substantial body of literature on αβT cells expressing CARs, there are relatively few reports of CAR-γδT cells. First described in 2004 (83), γδT cells expressing a first-generation CAR-targeting GD2 (14.G2aζ) which is expressed on the surface of neuroblastoma and Ewing sarcoma cells (84, 85) showed enhanced antigen-specific tumor reactivity. Following co-culture with the $GD2^+$ neuroblastoma cell line LAN-1, $14.G2aζ^+Vγ9^+$ cells showed greater production of the Th1 cytokine IFNγ compared to non-transduced zoledronate expanded $14.G2aζ^-Vγ9^+$ γδT cells. This effect was mirrored in the expression of the T-cell activation marker CD69, which also upregulated the presence of the tumor cells. In the absence of $GD2^+$ cells, $14.G2aζ^+Vγ9^+$ γδT cells showed only 1.5 ± 0.5% $IFNγ^+CD69^+$ but following co-culture with $GD2^+$ LAN-1 targets this rose to 33 ± 3%. Background production of IFNγ by non-transduced effectors exposed to LAN-1 was low (5.7 ± 1.2%). Similar results were seen when γδT cells expressing the CD19ζ CAR were co-cultured with $CD19^+$ cell lines Daudi, Raji, and Reh (83), with substantial increases in target-dependent IFNγ production by mixed populations of $CD19ζ^{+/-}$ γδT cells. While Daudi is known to engage the γδTCR and is highly susceptible to γδT cell-mediated killing in its own right, Raji is usually considered to be a γδT cell resistant cell line (86), and it was in this model that the highest IFNγ production was seen, suggesting that CAR expression could overcome some of the immune-escape mechanisms shown by the target cells.

Since the publication of the work of Rischer et al. (83), progress in immunotherapy using adoptively transferred γδT cells has focused on the expansion of un-engineered γδT cells (32, 35). Whereas earlier studies used aminobisphosphonates to generate a predominantly $Vδ2^+$ population (83, 87), a series of papers eventually demonstrated the possibility for expanding γδT cells with a broad range of γδTCR subsets using either plant-derived T-cell mitogens such as concanavalin A (88–90) or artificial antigen-presenting cells (aAPC) engineered to express co-stimulatory ligands and membrane-bound IL-15 (91). Two groups used a $CD19^+$ aAPC system to expand $Vδ2^-$ γδT cells, demonstrating that the repertoire of γδT cells produced could be influenced by the loading of anti-γδTCR antibodies to the CD64 expressed on the aAPC (37, 92). Furthermore, this approach can be used to

specifically propagate anti-CD19 CAR$^+$ γδT cells (93). Deniger and colleagues generated a CAR$^+$ γδTCR$^+$ population containing a broad range of Vγ and Vδ chain combinations using negative selection following CAR gene transfer to the whole peripheral blood mononuclear cell (PBMC) population. γδT cells were isolated on the day after electroporation and propagated on CD19$^+$CD64$^+$CD86$^+$CD137L$^+$IL-15$^+$ aAPCs in the presence of IL-2 and IL-21; the aAPCs were refreshed weekly. The resultant γδT cell population showed low expression of exhaustion markers such as CD57 and contained a heterogeneous mixture of memory phenotypes. This expansion technique has been shown to preserve the distribution of Vδ1$^+$, Vδ2$^+$, and Vδ1$^-$/Vδ2$^-$ γδT cell subsets within a donor PBMC sample (37, 92). Singh et al. had previously demonstrated that culture using this aAPC system produced a selection pressure for CAR$^+$ αβT cells (94) resulting in >90% CAR$^+$ αβT cells after 28 days of co-culture, but this effect was muted when CAR$^+$ γδT cells were expanded, presumably due to the inherent reactivity of non-transduced γδT cells against the aAPC leading to non-specific proliferation. aAPC based expansion may be particularly advantageous for γδT cells due to their expression of CD28 and CD137 which interact with CD86 and CD137L on the aAPC, and expression of CCR7 and CD62L by the CAR$^+$γδTCR$^+$ cells suggested that they had the capacity to home to the bone marrow and lymph nodes where CD19$^+$ leukemia is known to reside. The CAR$^+$ cells produced IFNγ, TNFα, MIP-1α, MIP1β, and RANTES following CAR activation through co-culture with a huCD19$^+$ murine cell line which does not engage the γδTCR due to inter-species differences (95, 96), and killed human CD19$^+$ cell lines with much greater efficacy than CAR$^-$γδTCR$^+$ cells (93). Immunodeficient mice xenografted with CD19$^+$ffLuc$^+$ NALM6 B-cell leukemia showed enhanced survival following CAR-γδT cell treatment compared to untreated, though a non-transduced or irrelevant CAR control was not included in the *in vivo* study so the *in vivo* activity is harder to dissect.

Engineering strategies which harness the innate properties of Vγ9Vδ2 T cells would seem to be the best justification for using them as an alternative "chassis" for CAR-T cell therapy. CARs were initially developed to bypass the αβTCR, limited as it is by MHC restriction and a requirement for specific TAA epitopes to be presented. The Vγ9Vδ2 TCR is not subject to these limitations; through its MHC-unrestricted detection of moieties associated with cellular stress. As such, there is an opportunity to "tune" the CAR-T cell response by modulating the level of stimulus delivered by the CAR. So far, this has been demonstrated in the context of neuroblastoma, against which Vγ9Vδ2$^+$ T cells have minimal innate cytotoxicity (37), in part due to the tumor shedding soluble NKG2D ligands which block NKG2D activation (97, 98). If further stimulus is provided to the γδT cell, this cytotoxicity can be restored, either *via* a conventional second-generation CAR (90, 99) (**Figure 1D**), opsonization of the target cell (34, 37) or, as was recently shown, by restoring the NKG2D signal using a chimeric costimulatory receptor (CCR), that lacks CD3ζ, but contains the endodomain motif from the NKG2D adaptor, DAP10 (GD2-DAP10, **Figure 1E**). This approach enhanced killing of GD2$^+$ neuroblastoma cells but did not induce cytotoxicity against GD2$^+$ cells that did not engage the Vγ9Vδ2 TCR. Cytokine release was also controllable using this "AND gate" system; IL-2, IFNγ, and TNFα were only released from GD2-DAP10$^+$Vδ2$^+$ cells when they received both CD3 and CCR stimulus, whereas in GD2-28ζ$^+$Vδ2$^+$ cells, only CAR stimulus was required (99).

Chimeric costimulatory receptors have also been used in the context of αβT cells. They can deliver an isolated costimulatory signal to support antigen-specific proliferation (100), enhance tumor specificity by dividing CAR and CCR stimuli such that two antigens are required for activation (101), or reverse the suppressive effects of tumor PD-L1 through a PD1-CD28 chimeric receptor (102). In two of these studies, a separate CD3 signal was provided, either using OKT3 anti-CD3 (102), or a separate CD3ζ containing CAR (101). The earlier work by Krause et al. was particularly innovative; a chimeric anti-GD2 receptor with a CD28 endodomain supported antigen-specific proliferation of αβT cells in a TCR or CD3-dependent manner. When tested in the context of GD2$^{+/-}$ tumor cells, they also confirmed that signal 1 could be provided by the TCR (100) and that their CCR would function under these conditions. The promiscuous, MHC-independent reactivity of γδT cells to danger-associated molecular patterns rather than MHC-restricted peptide epitopes could offer an opportunity to broaden this approach.

In addition to the possibility of avoiding on-target off-tumor toxicity, CAR expressing γδT cells retain the ability to antigen to cross-present (53, 90, 103–105). A recent study indicated that γδT cells transduced with second-generation anti-GD2 CARs (GD2-28ζ) retain the ability to cross-present TAAs leading to a clonal expansion of αβT cells. Using a 25 amino acid fragment of the melanoma antigen MART-1 which encompasses a 10 amino acid epitope but is too long to be MHC-presented in its un-processed form, Capsomidis et al. demonstrated that HLA-A201$^+$V2δ$^+$GD2-28ζ$^+$ cells pulsed with the long peptide were able to elicit secondary expansions in αβT cells expressing a HLA-A201-restricted MART-1 αβ TCR (90). Vδ2$^+$GD2-28ζ$^+$ cells also retained the ability to migrate toward tumor cell lines; GD2-28ζ expression had no effect on migration in either Vδ1$^+$ or Vδ2$^+$ subsets in an *in vitro* trans-well assay. The next step in these investigations would be to show that γδT cells can cross-present antigens derived from cells that they have themselves killed. If this were successful it would raise the possibility that the broad anti-tumor reactivity of γδT cells could be used to prime a diverse population of autologous αβT cells against many tumor-derived antigens simultaneously. Demonstration of enhanced anti-tumor activity by "γδT-cell primed" αβT cells would further validate this approach.

TRANSDUCTION STRATEGIES FOR γδT CELLS

When engineering γδT cells it is important to select appropriate tools. In general, long-lasting transduction strategies which work well for αβT cells work well for γδT cells also. The predicted shorter lifespan of infused γδT cells offer the opportunity to use more transient engineering approaches as well, as the infused cells may not persist in the host long-term.

Many groups continue to use gammaretroviral vectors for transducing γδT cells. High transduction efficiencies are achievable using a Maloney murine leukemia virus-based vector, SFG (87), pseudotyped with the envelope of the feline endogenous retrovirus RD114 (99), or gibbon-ape leukemia virus envelopes. Gammaretroviral transduction has the advantage of allowing preparation of large, high titer batches of virus because of the availability of packaging cell lines which can be stably transduced to produce virus containing the construct of interest (106). Gammaretroviruses, lacking the machinery to penetrate the nucleus, require the cells to be actively cycling in order to achieve transduction as viral nucleic acids can enter through nuclear pores (107). This is not a restriction in the engineering of αβT cells, and the specific and rapid expansion of Vγ9Vδ2$^+$ T cells in response to aminobisphosphonates allows for similar strategies to be applied. Transduction of other γδT cell subsets using gammaretroviruses following concanvaulin A driven expansion is less predictable, however, with variable yield (90). There has been some concern regarding the potential for insertion-site-mediated mutagenesis following gammaretroviral gene transfer (108), which has prompted some in the field to favor lentiviral vectors which have a safer insertional profile (107). There is little published data to compare lentiviral transduction techniques for γδT cells, though one group did find that the use of a vesicular stomatitis Indiana virus G-protein containing envelope in combination with a simian immunodeficiency virus transfer vector consistently provided higher transduction efficiency than a human immunodeficiency virus-based vector with the same envelope (transduction efficiency 65% vs 42%, $p = 0.04$) (109). New genome editing technologies, such as CRISPR-CAS, allow targeting integration of viral vectors with several potential advantages including avoidance of integration into oncogenic loci, and integration into loci that optimize CAR or TCR expression (11, 110).

Non-viral methods of transduction have provided particular advantages when engineering γδT cells. The Sleeping Beauty Transposon system (111, 112) uses enzymes originally derived from fish to insert new genetic material into host cells. It has not yet been established that the SB-transposon system is more efficacious than lentiviral transduction, and there is an unknown potential for insertional mutagenesis (113). Cells must be electroporated in order for gene transfer to occur, but do not require a specific proliferative stimulus. As described above, Deniger et al. used this to good effect to express an anti-CD19 CAR (CD19RCD28) in a polyclonal repertoire of γδT cells which were subsequently expanded using CD19$^+$ aAPCs (91, 93). Unlike proliferation-driven transduction techniques, there was no "skewing" of the γδT cell population toward a particular Vγ/Vδ subset. This may be of particular interest if engineered γδT cells were to be directed against epithelial tumors, as non-Vδ2$^+$ γδT cells are enriched in epithelial surfaces, a tropism which could be harnessed.

Viral or transposon-based gene transfer generates stable construct expression over time by integrating into the transduced cell genome. This has been considered important in the CAR-T cell field as it allows for persistence of CAR-T cells for weeks or months. More transient CAR expression strategies have been suggested as a means of reducing toxicity following CAR-T cell infusion. mRNA transfection using electroporation was used to generate γδT cells expressing NKT-cell derived TCRs (79) and, more recently, HLA-A2/gp100-specific TCR or CARs targeting melanoma-associated-chondroitin-sulfate-proteoglycan (MCSP). MCSP is a tumor-associated antigen expressed on melanoma (114), glioma (115), triple-negative breast cancer (116), and sarcomas (117). Expression peaked at around 24 h after transfection, returning to baseline by around 72 h (77). Similar techniques have been tested clinically in the context of CAR$^+$ αβT cells, where they were used to transfect cells with a construct targeting mesothelin (118), though repeated infusions of CAR-T cells were required, presumably because of the need to "top-up" the reservoir of circulating CAR-T cells as expression was lost.

CONCLUDING COMMENTS

While there is promising data to suggest that gene-modified γδT cells may be an attractive candidate for clinical studies, the bulk of enthusiasm in the cellular immunotherapy field focuses on αβT cells. Undoubtedly, more data are available on αβT cell engineering, increasing the likelihood of introducing novel constructs to the clinic. γδT cells appear to cause less graft-versus-host disease than αβT cells while retaining graft-versus leukemia activity in the hematopoietic stem-cell transplant setting (119). *In vivo* data on the function of CCR expressing γδT cells and/or the antigen cross-presentation capacity of γδT cells are not yet available. Reduced toxicity and the potential for antigen cross-presentation are compelling arguments for the potential benefits of γδT cells over αβT cells as a substrate for CAR expression. The difficulty of modeling the more subtle aspects human γδT cell activity in a murine system makes this data particularly hard to generate. However, within the increasingly crowded field of cancer immunotherapy, orthogonal approaches to cellular engineering are required to move the field forward. The challenges of off-tumor toxicity, poor penetration of solid tumors and tumor immune evasion need to be addressed. It is no surprise that CD19 CAR-T therapies have been more successful than others, depletion of healthy CD19$^+$ B-cells is considered an acceptable toxicity and the disease resides in the hematological compartment. In other cases, on-target off-tumor toxicity has been severe or fatal, experiences which have shaped the way that target antigens are chosen (20, 21). Investigating the potential of alternative CAR "chassis" to harness the innate characteristics of particular cell types factorially increases the number of options available. As cells sharing adaptive properties of conventional αβT-cells and innate properties of NK-cells, γδT cells are a highly attractive and potentially efficient candidate for this process of optimization.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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New Insights Into the Regulation of γδ T Cells by BTN3A and Other BTN/BTNL in Tumor Immunity

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Recent findings in the immunology field have pointed out the emergent role of butyrophilins/butyrophilin-like molecules (BTN/BTNL in human, Btn/Btnl in mouse) in the modulation of γδ T cells. As long as the field develops exponentially, new relationships between certain γδ T cell subsets, on one hand, and their BTN/BTNL counterparts mainly present on epithelial and tumor cells, on the other, are described in the scientific literature. Btnl1/Btnl6 in mice and BTNL3/BTNL8 in humans regulate the homing and maturation of Vγ7+ and Vγ4+ T cells to the gut epithelium. Similarly, Skint-1 has shown to shape the dendritic epidermal T cells repertoire and their activation levels in mice. We and others have identified BTN3A proteins are the key mediators of phosphoantigen sensing by human Vγ9Vδ2 T cells. Here, we first synthesize the modulation of specific γδ T cell subsets by related BTN/BTNL molecules, in human and mice. Then, we focus on the role of BTN3A in the activation of Vγ9Vδ2 T cells, and we highlight the recent advances in the understanding of the expression, regulation, and function of BTN3A in tumor immunity. Hence, recent studies demonstrated that several signals induced by cancer cells or their microenvironment can regulate the expression of BTN3A. Moreover, antibodies targeting BTN3A have shown *in vitro* and *in vivo* efficacy in human tumors such as acute myeloid leukemia or pancreatic cancer. We thus finally discuss how these findings could help develop novel γδ T cell-based immunotherapeutical approaches.

Keywords: γδ T cells, butyrophilins, BTN3A, tumor immunity, immunotherapy

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γδ T CELL SUBSETS AND RELATED BTN/BTNL PROTEINS

γδ T Cell Subsets

Arising from the same common multipotent double negative precursor than the αβ T cells, and differentiated earlier in the thymus, γδ T cells comprise a heterogeneous group of cells that are considered to be a link between innate and adaptive immunity.

The main characteristic that defines the γδ T cells is the expression of its distinctive TCR composed by a γ-chain and a δ-chain, that is called γδ TCR. In humans, 0.5–16% of all CD3+ cells in adult peripheral blood and organized lymphoid tissues (thymus, spleen, tonsil, and lymph nodes) are γδ T cells, they usually represent less than 5% in tongue and reproductive tract and 10–30% in intestine (1, 2). In adult mice, 1–4% of the entire T cell compartment in thymus, secondary lymphoid organs and lung are γδ T cells. Higher numbers of γδ T cells are found in other mucosal sites, reaching until 10–20% of all T cells in female reproductive organs (3), 20–40% of the intestinal intraepithelial T cells (4), and 50–70% of skin dermal T cells (2, 5, 6).

Like B cells and αβ T cells, γδ T cells have an RAG-mediated rearranged antigen receptor by the combination of V (variable), D (diversity), and J (joining) gene segments. In human, there are only a few Vγ (Vγ 2,3,4,5,8,9) and Vδ (Vδ 1,2,3,4,6,7,8) germline genes that can be used to rearrange (7). Nonetheless, the CDR3 loop of Vδ-chain shows a high degree of diversity thanks to multiple D gene segments. In addition, the insertion or loss of N-nucleotides during the junctional diversification process further enriches this diversity (8). Available Vγ and Vδ genes are not randomly used. Thus, certain Vδ chains tend to pair almost exclusively with another Vγ chain (like Vγ9 and Vδ2 in humans), and some γδ T cells expressing certain Vγ and Vδ genes preferentially reside in well-defined tissue locations (**Table 1**).

In contrast to conventional αβ T cells, the antigen recognition mechanism in γδ T cells is not MHC-restricted. Indeed, γδ T cells seem to be implicated in the recognition of different antigens than αβ T cells. While αβ T cells recognize non-self-peptide fragments restricted by MHC molecules, γδ T cells can recognize unconventional antigens as stress molecules (MICA and MICB), non-peptidic metabolites of isoprenoid biosynthesis, heat-shock proteins, and so on (12).

As cytotoxic CD8+ T cells do, γδ T cells can elicit a broad cytotoxic activity against infected and transformed cells. This cytotoxic activity is based on death receptor/ligand (Fas/FasL) signals and perforin/granzyme to destabilize cellular integrity (12). γδ T cells also secrete various cytokines (16) and chemokines including proinflammatory Th1-like cytokines such as IFN-γ and TNF-α in order to activate several immune mediators (dendritic cells and Th1), arrest proliferation, and kill target cells (17). Thanks to these cytotoxic properties, γδ T cells highlight among other immune mediators, along with CD8+ T and NK cells, as a powerful tool for cancer immunotherapy.

The BTN/BTNL Protein Families

The butyrophilins (BTN) and butyrophilin-like (BTNL) genes are part of the immunoglobulin superfamily. They are structurally related to the B7 proteins, which comprise co-stimulatory (B7-1, ICOS, etc.) and co-inhibitory (PD-L1, PD-L2, B7-H3, etc.) molecules involved on T lymphocytes regulation. The seven human BTN genes are clustered in the MHC class I region of chromosome 6 (18, 19) into three phylogenetically related subfamilies: BTN1, BTN2, and BTN3, in humans. The BTN1 subfamily consists only in the BTN1A1 gene, whereas the BTN2 and BTN3 subfamilies have three genes: BTN2A1, BTN2A2, and BTN2A3 pseudogene, and BTN3A1, BTN3A2, and BTN3A3. The BTN proteins show high structural homology, with BTN1, BTN2, and BTN3 subfamilies sharing the 50% of their amino acid identity. The homology between the BTN2 and BTN3 is even closer, reaching on average 80% identity (20). The high homology rates observed suggest that the BTN genes have undergone tandem duplication. Although further, the BTNL family still shares considerable homology (on average 40% identity) to the BTN-family members. Five identified BTNL genes are encoded in the human genome. BTNL2, the best-known member of this family, is clustered with the BTN genes in the MHC class I region of chromosome 6, while the chromosome 5 harbors the coding sequences of BTNL3, BTNL8, and BTNL9. The genomic sequence of SKINTL pseudogene is found in the chromosome 1.

In the mouse genome, however, only two Btn proteins are found: Btn1a1, who plays a role in the regulation of milk fat globule secretion (21), and Btn2a2. Both genes are clustered on chromosome 13, and are orthologs of human BTN1A1 and BTN2A2, respectively. Eight different murine Btnl genes have been described so far (Btnl1, 2, 4, 5, 6, 7, 9 and skint-1). Two of them are predicted to be pseudogenes (Btnl5 and Btnl7). Six of them are located in the same genomic region, at the MHC class II locus (chromosome 17), while Btnl9 is the only Btnl gene found on chromosome 11, whereas the Skint-1 (**Figure 1**) gene is encountered on chromosome 4. Given their high extracellular identity, Btnl9 and Btnl2 are thought to be orthologs of human BTNL molecules. Recent advances in the field have pointed out the role of BTN3, Skint-1, Bntl1/Btnl6, and BTNL3/BTNL8 as key immune regulators of γδ T cells in humans and mice (**Figure 1**) (22–26).

REGULATION OF SPECIFIC γδ T CELL SUBSETS BY BTN/BTNL PROTEINS

Skint-1 and Dendritic Epidermal T Cells (DETCs)

The study of the T cell repertoire in mice with a Skint-1−/− genetic background pointed out the relevance of the interaction

TABLE 1 | γδT cell subsets and tissue locations in human and mouse.

Species	V gene segment pairing	V(D)J diversity	Tissue location	References
Human	Vδ1-T cells	High	Thymus, spleen, dermis, liver (with Vδ3), and gut epithelia (with Vδ3)	(9, 10)
	Vγ9Vδ2-T cells	Intermediate	Main peripheral blood γδ T population (paired with Vγ9)	(9, 11)
	Vδ3-T cells	High	Liver, higher numbers in chronic viral infections and leukemia, gut epithelia	(9, 10, 12)
	Vδ4, Vδ6, Vδ7, Vδ8-T cells		Peripheral blood of lymphoma patients	(13)
Mouse	Vγ1/Vγ4 γδ-T cells	High	Predominant in spleen	(14)
	Vγ7-T cells	Intermediate	Gut epithelia (paired with Vδ4, Vδ5, and Vδ6)	(14)
	Vγ4/Vγ6-T cells	Intermediate	Lungs	(14)
	Vγ6Vδ1-T cells	Invariant	Main population in reproductive organs	(14)
	Vγ5Vδ1-T cells (dendritic epidermal T cells)	Invariant	Major subset in mice skin	(15)
	Diverse	High	Adult thymus	(10)
	Diverse	High	Lymph node	(10)
	Vγ1Vδ6/3, Vγ4 and Vγ6-T cells	Intermediate	Liver	(10)

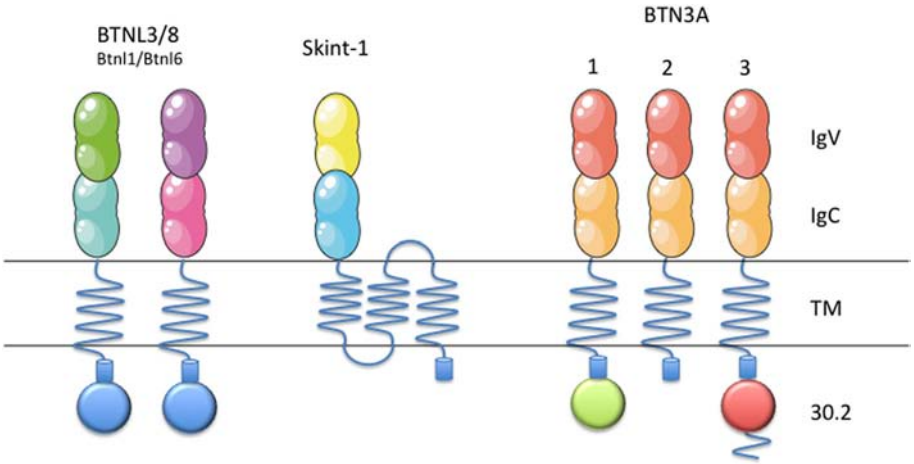

FIGURE 1 | Schematic representation of BTN3, Btnl1, Btnl6, BTNL3, BTNL8, and Skint-1. Schematic representation of BTN/BTNL molecules in mice (Btnl1, Btnl6, and Skint-1) and humans (BTN3, BTNL3, and BTNL8), that have been shown to regulate specific γδ T cell subsets. Each subfamily member contains an extracellular, N-terminal IgV, and a membrane-proximal IgC domain connected to a single-pass transmembrane domain. BTN3A1 and BTN3A3 contain intracellular B30.2 domains, as well as BTNL3 and BTNL8, which is missing in BTN3A2. This figure was created in part using graphics from Servier Medical Art (https://smart.servier.com/) with permission.

between Btn/Btnl proteins and γδ T cells, as these mice do not develop canonical Vγ5Vδ1+ DETCs (27, 28). Skint-1 is a transmembrane protein without any known ortholog in humans (**Figure 1**), but it shows a high degree of homology with a subfamily of BTNL molecules, which are conserved in humans (25, 29, 30). Skint-1 is expressed at detectable levels exclusively by keratinocytes and thymic epithelial cells, where it promotes the IFN-γ production and TCR hyporesponsiveness of DETC progenitors (**Figure 2**) (31, 32). Mature DETCs exist in a semiactivated state under homeostatic conditions. This activation is explained, at least in part, by a constitutive TCR engagement through ligand recognition on surrounding keratinocytes within this tissue *in vivo* (33). Nevertheless, while a DETC TCR ligand was expressed on the surface of keratinocytes at the wound edge in FVB-Tac mice (a substrain of FVB mice, harboring a mutation in Skint-1, specifically deficient for Vγ5Vδ1 DETCs), Skint-1 was not able to directly bind the DETC TCR, neither detected on the surface of keratinocytes (19, 25, 31). Thus, although Skint-1 expression is fundamental for the development of canonical Vγ5Vδ1+ DETCs, the underlying mechanisms by which Skint-1 promotes the maturation of these cells remain poorly understood. However, DETC tetramers did inhibit wound closure *in vivo* (34) suggesting that Skint-1 might not be the ligand, or at least the only ligand, of the DETC TCR in keratinocytes. It has to be noted that direct binding between γδ TCRs and any Btn/Btnl has not been described so far in the scientific literature.

Intriguingly, Skint-1 was not readily expressed at the cell surface of HEK293 cells transfected with WT Skint-1 (28). This finding suggests that an accessory protein might help Skint-1 to properly localize at the cell membrane compartment. Unfortunately, this accessory protein has not been identified so far.

Btnl1/Btnl6 and Vγ7+ IELs in Mice, BTNL3/BTNL8 and Vγ7+ IELs in Humans

In mice, several Btnl proteins are only expressed at protein level in the intestinal epithelium, concretely on enterocytes of the small-intestinal villus epithelial cells (25, 26). In this way, the expression of Btnl1 by small-intestinal villi at an early time point in life was recently found to critically and selectively promote Vγ7+ intraepithelial lymphocytes (IELs) maturation and expansion within the tissue (**Figure 2**) (26). The first evidence came from the study of IEL populations on four different strains of Btnl1−/− mice, where Vγ7+ IEL numbers were depleted by ~90%, with Vγ7+ Vδ4+ cells almost ablated. The specificity of the interaction between Vγ7+ IELs and Btnl1 was emphasized by the fact that Btnl4−/− mice displayed no overt defects in any major IEL subset.

In 2016, Lebrero-Fernandez et al. reported an enhancement on cell surface expression of Btnl1 on Btnl1-transfected MODE-K cells when these cells were concomitantly transfected with Btnl4 and Btnl6 (35). In the same way, Btnl1 greatly enhanced the expression of Btnl6 on the cell surface *in vitro* (26). Conversely, co-transfecting Btnl1 or Btnl6 did not augment the cell surface expression of Btnl4. These results match with the fact that Btnl4−/− mice displayed no overt defects in any major IEL subset.

Vγ7+ IELs co-cultured with MODE-K stably expressing Btnl1+ Btnl6 cells overexpress the T cell activation marker CD25, downregulate the TCR and CD122 expression levels and show higher levels of granulocyte–macrophage colony-stimulating factor, CCL4, and IFN-γ *in vitro* (26). Likewise, it was observed that human gut epithelial cells as well express BTNL3 and BTNL8, and that concomitant expression of BTNL3 + BTNL8 induces selective TCR-dependent responses of human colonic Vγ4+

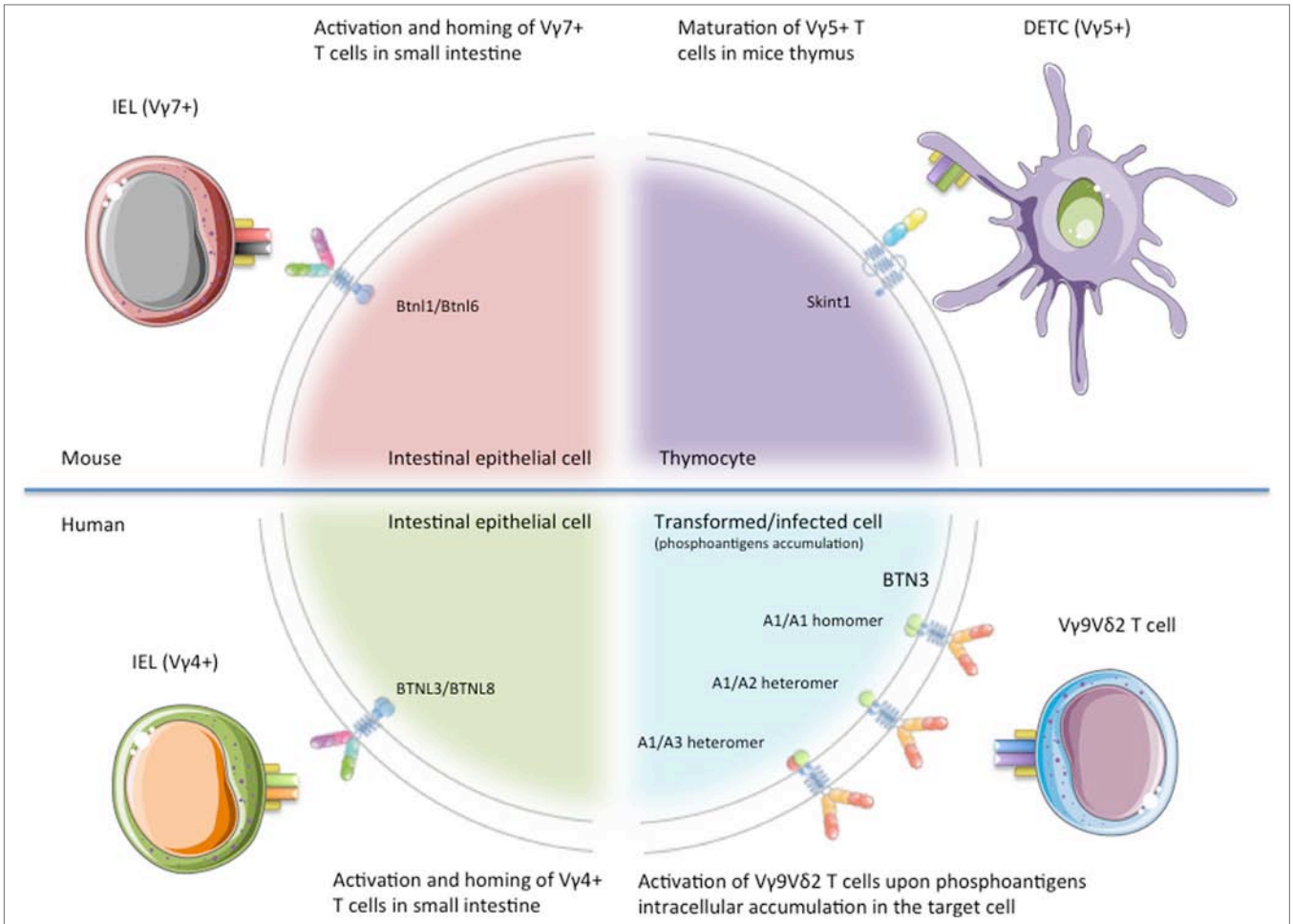

FIGURE 2 | Schematic representation of the different γδ T cell subsets regulated by Btn/Btnl proteins. Representation of the published functions of BTN/BTNL proteins on specific γδ T cell subsets. Skint-1 has been shown as critical for the maturation of Vγ5Vδ1+ dendritic epidermal T cells (DETCs) in mice thymus. Btnl1/Btnl6 promote Vγ7+ intraepithelial lymphocytes (IELs) maturation and expansion within mice small intestine, whereas BTNL3/BTNL8 regulate the activation of Vγ4+ IEL in human gut epithelium. Finally, BTN3A molecules play a mandatory role in the recognition of tumor or stressed cells by human Vγ9Vδ2 T cells. This figure was created in part using graphics from Servier Medical Art (https://smart.servier.com/) with permission.

cells (**Figure 2**) (26). When HEK293 cells were transfected with BTNL3, BTNL8, or BTNL3 + BTNL8, only Vδ2− cells co-cultured with HEK293 cells co-expressing BTNL3+ BTNL8 undergo a marked TCR downregulation (26). Among all the γδ T cell subsets included on the Vδ2− population, only those expressing Vγ4 effectively downregulated TCRs in co-cultures with L3 + L8 cells. Similar to Skint-1, investigations have failed to report direct γδ IEL-TCR–BTNL molecule interactions up to date. As for BTNL1 and BTNL6 described above, neither BTNL3 nor BTNL8 protein was efficiently expressed on cells transfected with their respective genes, unless both were co-expressed (36).

Vγ9Vδ2 T Cells and BTN3A Proteins

Due to the pairing restrictions observed in the formation of the γδ TCR, the Vδ2 chain can be combined almost exclusively with Vγ9 forming the Vγ9Vδ2-TCR. Vγ9Vδ2 T cells represent the major γδ T cell subset in the human peripheral blood, with values ranging from 50 to 95% of γδ T cells. These immune mediators, which have been only found in humans and non-human primates and are evolutionarily conserved in selected species like alpaca (*Vicugna pacos*) (37), stand out as having the capacity to "sense" several infected and malignant cells. This Vγ9Vδ2 T cell reactivity has been associated with intracellular accumulation of organic pyrophosphate-containing molecules, also called phosphoantigens (pAgs) (38). These molecules can be produced by microbes, such as hydroxy-methyl-butyl-pyrophosphate (HDMAPP, also known as HMBPP), a microbial intermediate of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway or can be synthetized endogenously, such as isopentenyl pyrophosphate (IPP), an intermediate of the mevalonate pathway in mammal cells that can accumulate in transformed cells during tumorigenesis (39).

After these findings, nitrogen-containing bisphosphonates (N-BPs) like Zoledronic acid have emerged as drugs that, indeed, can raise up the intracellular levels of IPP by the inhibition of farnesyl diphosphate synthase (FPPS) (40, 41), thus facilitating

the activation of human Vγ9Vδ2 T cells against tumor cells. In the same way, several synthetic analogs of HMBPP-like bromohydrin pyrophosphate (BrHPP) have been synthetized up to date, showing all of them potent stimulation of Vγ9Vδ2 T cells (42).

Although the Vγ9Vδ2-TCR of Vγ9Vδ2 T cells is sufficient for pAg recognition (38), the cell–cell contact is mandatory for the proper Vγ9Vδ2 T cell activation against cancer cells [although some pAgs, like BrHPP can activate Vγ9Vδ2 T cells without necessity of cell–cell contact (43, 44)], suggesting the presence of a "pAg presenter molecule" in target cells. In addition, none non-primate cell can stimulate the Vγ9Vδ2 T cells (45), indicating that this antigen-presenting molecule (APM) is characteristic of primates (like Vγ9Vδ2 T cells). On the other side, Vγ9Vδ2 T cell activation relies on the expression of the Vγ9Vδ2-TCR as Jurkat cells became reactive against pAgs intracellular accumulation in their targets when they were transfected with Vγ9Vδ2-TCR (46). Finally, Harly and coworkers demonstrated the role of the butyrophilin-3A (BTN3A, also called CD277) subfamily of proteins as key mediators of pAg signaling (47).

The second part of this review will be focused on the peculiar antigenic activation process of human Vγ9Vδ2 T cells through BTN3A proteins, and consequently on the mechanism of tumor cell recognition by Vγ9Vδ2 T cells. Recent publications have shed new light on the mode of action of BTN3A.

FOCUS ON BTN3A ROLE ON THE ACTIVATION PROCESS OF HUMAN Vγ9Vδ2 T CELLS

BTN3A1/A2/A3 Isoforms

Due to the recent discovery of these proteins, most concepts about these molecules remain still obscure. BTN3A subfamily of molecules belongs to the B7 co-stimulatory family of molecules. BTN3A subfamily is composed by three members in humans: BTN3A1, BTN3A2, and BTN3A3 (**Figure 1**) (18). They exhibit 95% identity between them in their extracellular part, thus forming a monophylogenetic group along with the BT-related members (48). Each subfamily member contains an extracellular, N-terminal IgV, and a membrane-proximal IgC domain connected to a single-pass transmembrane domain. BTN3A1 and BTN3A3 both contain an intracellular B30.2 domain, which is not found in BTN3A2. After its B30.2 domain, BTN3A3 has a unique cytoplasmic tail of 70 amino acids.

All the three isoforms, BTN3A1, BTN3A2, and BTN3A3, after treatment with the 20.1 agonist monoclonal antibody (mAb) can stimulate Vγ9Vδ2 T cells, thus activating them through mechanisms involving decreased mobility (47) and a multimerization of BTN3A molecules (49). These findings suggest the involvement of their extracellular domains in the activation process. Nonetheless, only the BTN3A1 isoform, and to a much lesser extent BTN3A3, can trigger the activation of Vγ9Vδ2 T cells upon phosphoantigen burst (47).

The way BTN3A1 presents pAgs to Vγ9Vδ2 T cells has remained controversial over the last years. The receptor for BTN3A, similar to the receptor for other BTNs or B7-H4, remains to be identified. The very first model of pAgs presentation hypothesized that BTN3A might present pAgs directly to Vγ9Vδ2 T cells *via* its extracellular IgV domain (50). This model fails to explain why BTN3A2 is unable to activate Vγ9Vδ2 T cells, taking into account that the IgV domains of BTN3A1 and BTN3A2 share the 95% of their amino acid sequence. However, the binding of pAgs to the BTN3A IgV domain was not confirmed by other groups. Harly and coworkers pointed out the importance of the B30.2 intracellular domain of BTN3A1 in the activation process of Vγ9Vδ2 T cells by swapping the B30.2 intracellular domains of BTN3A1 and BTN3A3 (47). Thus, the chimeric BTN3A3 protein carrying the BTN3A1 B30.2 intracellular domain became stimulatory. In fact, the activation levels reached by Vγ9Vδ2 T cells when co-cultured with cells expressing the BTN3A3 chimeric protein were higher than those obtained when Vγ9Vδ2 T cells were cultured with cells expressing BTN3A1 WT. Conversely, cells transfected with chimeric BTN3A1 protein carrying the BTN3A3 B30.2 were no longer able to activate Vγ9Vδ2 T cells. Based on these results, a second model for pAgs presentation to Vγ9Vδ2 T cells was proposed. In this model, supported by several articles, BTN3A1 might act as an indirect antigen presentation molecule. Thus, Vγ9Vδ2 T cells would not recognize directly the pAg binding to the IgV domain of BTN3A, but they might recognize the conformational changes triggered in BTN3A1 by the binding of pAgs to its B30.2 domain. Even more recent data focusing on the BTN3A1 B30.2 domain show that only the B30.2 intracellular domain of BTN3A1 can directly bind pAg through a positively charged surface pocket under physiological conditions. Several basic residues along this binding pocket including histidines (His351 and His378), arginines (Arg412, Arg418, and Arg469), and a lysine (Lys393) were probed essential to allow pAgs binding as they provide a highly positive charged environment complementary to the negative charge of the pyrophosphate moiety of pAgs. By contrast, in the B30.2 domain of BTN3A3, a single amino acid change in the position 351 from histidine (in BTN3A1) to arginine (in BTN3A3) prevents the binding of pAgs to the surface pocket and, thus, impedes the subsequent conformational changes that, ultimately, will achieve the effective activation of Vγ9Vδ2 T cells (51).

As we have seen above, the BTN3A2 isoform lacks the B30.2 intracellular domain, reason why this receptor might be unable to activate Vγ9Vδ2-T cells upon pAgs intracellular accumulation (**Figure 1**). Surprisingly, several reports highlighted the importance of BTN3A2 and BTN3A3 expression in the activation of Vγ9Vδ2 T cells against their targets. Indeed, BTN3A1, A2, and A3 KD/KO cell lines were unable to activate Vγ9Vδ2-T cells as much as their WT counterparts upon pAgs intracellular accumulation (36, 52, 53). Recently, Vantourout et al. showed that HEK cells KO for BTN3A1 alone, failed to activate Vγ9Vδ2 T cells, even in the presence of BTN3A2 and/or BTN3A3, confirming that BTN3A1 is strictly required for Vγ9Vδ2 T cell activation. However, HEK cells expressing BTN3A1 but neither BTN3A2 nor BTN3A3 did not activate Vγ9Vδ2 T cells. This fact suggests that BTN3A2 and BTN3A3 are somehow involved in the activation mechanism of Vγ9Vδ2 T cells. In 2014, Riano et al. pointed out through transductant experiments that gene(s) on Chr6 in

addition to BTN3A1 are mandatory for PAg-mediated activation of Vγ9Vδ2 T cells (54).

BTN3A Homo- and Heteromers

Last published evidences shed some light to this controversy. Adams et al. showed that BTN3A1 tends to form heterodimers with BTN3A2, as well as BTN3A1 homodimers in a lesser extent, in a native cellular environment. During their experiments, the stability of BTN3A1/A2 heterodimers was higher than those of BTN3A1 homodimers. This observation suggests that BTN3A1 tends to pair with BTN3A2 in a physiological context (55). Further confirmation of BTN3A1–A2 interaction arrived in 2018. In their work, Vantourout and coworkers showed that BTN3A2 is able to complex with BTN3A1 in an IgC-dependent manner. They also achieved to co-immunoprecipitate BTN3A3 along with BTN3A1 (**Figure 2**). Moreover, they demonstrated that BTN3A1 was intrinsically inefficient at cell surface localization when transfected alone in a BTN3 triple KO background. Strikingly, surface expression of BTN3A1 was greatly enhanced by co-transfecting BTN3A2 or BTN3A3 with BTN3A1. Thus, BTN3A1 transfected alone largely colocalized with the endoplasmic reticulum (ER). Nevertheless, BTN3A1 showed reduced ER colocalization in cells cotransfected with BTN3A2. These findings demonstrate that BTN3A2 helps BTN3A1 to properly localize at the cell membrane, where it exerts its action (36). It should also be noted that in their functional assays, double BTN3A2/A3 KO cells were unable to activate Vγ9Vδ2 T cells, suggesting than BTN3A1 alone is unable to mediate Vγ9Vδ2 T cell activation without A2 and/or A3. Reconstitution with either BTN3A2 or BTN3A3 in combination with BTN3A1 in BTN3 KO cells recovered the ability of cells to activate Vγ9Vδ2 T cells, which means that BTN3A2 and BTN3A3 might have a redundant role in the activation process of Vγ9Vδ2 T cells.

Once again, similarly to the behavior of Btnl1 and Btnl6 in mice, as well as BTNL3 and BTNL8 described above, BTN3A1 was not efficiently expressed unless co-expressed with BTN3A2 or BTN3A3. As BTN3A2 and BTN3A1 did, Btnl1/Btnl6 coexpression reduced their colocalization with the ER. BTNL3 and BTNL8 behave similarly in order to be co-expressed at the cell membrane compartment. Strikingly, this cellular mechanism, which allows the localization of one protein to the cell membrane only when its partner protein is also expressed, seems to be conserved among several members on the BTN/BTNL families and between rodents and humans (26), even though these proteins are not predicted to be orthologs.

Other BTN3A1 Interactors

From 2015, Periplakin was also identified as an important interactor of BTN3A1 in the activation process of Vγ9Vδ2 T cells (52). Rhodes and coworkers demonstrated that the cytoskeletal adaptor protein Periplakin interacted with a di-leucine motif, C-terminus to the B30.2 domain of BTN3A1. However, Periplakin did not interact with BTN3A2 or BTN3A3, which do not contain the di-leucine motif. Interestingly, Periplakin depleted cells did not activate Vγ9Vδ2 T cells upon N-BPs treatment, suggesting a key role for Periplakin binding in the activation process of Vγ9Vδ2 T cells.

Last insights into the complex relationships governing the interactions of the actin cytoskeleton with BTN3A proteins came out in 2016, when Sebestyen et al. highlighted the critical role of RhoB small GTPase in the stabilization of BTN3A1 in the membrane, as well as its attachment to the actin cytoskeleton (56). Thus, they show that RhoB localizes mainly to the nucleus in cells resistant to Vγ9Vδ2 T cells-mediated lysis and, by contrast, it preferentially localizes to the cell membrane in cells that are sensitive to Vγ9Vδ2-T cells lysis. Besides that, it is also shown that the inhibition of RhoB GTPase activity on target cells with C3 transferase dramatically reduced the activation levels of Vγ9Vδ2TCR+ T cells by LCL48 cells. In addition, RhoB was selectively excluded from nuclear areas in cells that are able to activate Vγ9Vδ2 TCR+ T cells, like leukemic blast of acute myeloid leukemia. Conversely, healthy stem cells from the same donor, which express RhoB mainly in the nucleus, did not activate Vγ9Vδ2 TCR+ T cells. Thus, exclusion of RhoB from the nucleus might be a cellular event which confers sensitivity to lysis by Vγ9Vδ2 TCR+ T cells. Taken together, these results suggest a critical role of Periplakin and RhoB in the activation mechanism of Vγ9Vδ2 T cells mediated by BTN3A.

BTN3A Functions in Other Immune Cells

Activating γδ T cells may not be the only function of BTN3A molecules. Some studies have highlighted the role of BTN3A in the regulation of TCR-mediated αβ T cells responses. In fact we demonstrated in 2004 using the BTN3A mAb 20.1 that the molecule was largely expressed on most immune cells (48). A first study showed that the engagement of BTN3A1 expressed in co-stimulatory artificial APC, coated with CD3 mAb, through its counter-receptor expressed on CD4 T cells resulted in a decrease of CD3/CD28-dependent proliferation of CD4 T cells (57). The effect of BTN3 engagement itself on αβ T cells has been confirmed by two studies. Anti-BTN3A 232.5 mAb cross linking on CD4 and CD8 T cells induced BTN3A3 phosphorylation and inhibited CD3- and IL-2-induced T cell activation (58). Artificial APC consisting of CD3/CD28/BTN3A 20.1 mAb coated beads enhanced co-stimulation-induced CD4 T cell proliferation and cytokine production through enhanced TCR signaling (59).

In contrast with CD4 T cells, BTN3A2 is the most abundant transcript found in NK cells compared to BTN3A1 and BTN3A3 isoform, and the only one able to inhibit NK cells-induced cytokine production. As BTN3A2 is devoid of B30.2 domain, one could postulate that BTN3A2 could be a decoy receptor. This hypothesis was further supported by the decrease of NKp30-induced cytokine production following the specific engagement of BTN3A2 but not BTN3A1 triggering on KHYG-1 transfected cells (59).

When engaged on monocytes and immature dendritic cells (iDCs) with plate-coated anti-BTN3A 19.5 or 20.1 mAbs, BTN3A protected cells from apoptosis and induced the expression of co-stimulatory and APMs. BTN3A stimulation with mAb synergized with toll-like receptor stimulation to increase chemokine and proinflammatory cytokine production (60). These data suggested that BTN3A subfamily could enhance and amplify inflammatory signals that are initiated by other receptors.

On THP-1 cells [human monocytic cell line derived from acute myeloid leukemia (AML) patient], depletion of BTN3A1 inhibited the cytoplasmic nucleic acid- or virus-triggered activation of IFN-β production, suggesting that BTN3A1 may be a novel regulator of type I IFN responses (61).

To note, cross linking of BTN3A mAbs is needed to obtain a biological effect on monocytes, iDCs, and αβT cells and this effect is the consequence of direct engagement of BTN3A expressed by responder immune cells. In marked contrast, soluble BTN3A mAbs can, in the absence of any other stimuli, sensitize the tumor cell to mediate Vγ9Vδ2 T cell activation.

CURRENT ADVANCES ON BTN3A EXPRESSION AND REGULATION IN TUMORS

BTN3A Expression in Tumors

BTN3A members are widely expressed in various tumors of hematological origin such as acute myeloid leukemia (48, 62) and solid tumors such as breast, colon (52, 63), ovarian (64), and more recently in gastric cancer (65) and pancreatic ductal adenocarcinoma (PDAC) (53) (**Table 2**). Immunohistochemical analysis of PDAC tissue microarray confirmed BTN3A expression in all the tested tumor samples, whereas BTN3A expression was either absent or barely detectable in control pancreatic tissue. Regarding the isoforms, BTN3A2 was the most expressed isoform in primary AML blasts (62). In addition, BTN3A overexpression and a dominant expression of the BTN3A2 isoform were strongly associated with a poor prognosis, in gastric cancer and PDAC (62, 65). This overexpression of BTN3A2 gene was associated with an increased proliferation and invasion of gastric cancer cells. Given that BTN3A2 lacks the B30.2 intracellular domain and could potentially be considered as a decoy receptor, its predominant expression in AML blasts or other tumors could constitute an immune escape mechanism to Vδ9Vδ2 T cell recognition. Another study showed that epithelial BTN3A expression evaluated by immunohistochemistry was significantly associated with better prognosis in high grade serous epithelial ovarian cancer patients, and correlated with higher density of infiltrating T cells (64). The role of BTN3A isoforms may be complex and BTN3A function in tumors could be regulated by the combinations of isoforms.

BTN3A Regulation by the Tumor Microenvironment

BTN3A is upregulated under T_H1 stimulation on the surface of normal tissue namely human vein endothelial cells (48). Other studies have demonstrated that certain factors of the tumor microenvironment are able to regulate BTN3A expression (**Table 3**): inflammatory cytokines and hypoxia-associated mediators such as VEGF, CCL3, and IL-10 upregulate BTN3A expression in ovarian cancer (57). In a recent study, we have shown that hypoxic and metabolic stress increase BTN3A2 isoform transcript in pancreatic cell lines and patient-derived xenograft-cell lines. In addition, soluble BTN3A isoforms including soluble BTN3A1 are found in the supernatants of pancreatic cell lines and in the plasma of PDAC patients (53). The soluble isoform results from BTN3A shedding that is in part MMP-dependent, similar to that previously described for the NKG2D ligands MICA/B (66). Soluble BTN3A and sBTN3A1 were associated with a decreased overall survival in PDAC patients, and consequently could represent new attractive prognosis biomarkers. The mechanism underlying the pro-tumoral effect of sBTN3A has to be clarified. One hypothesis could be

TABLE 2 | BTN3A expression in tumors.

Cancer	Method	Observations/correlation with prognosis	Reference
Cancer cell lines	• FC	Cell surface BTN3A expression on: • T, B or monocytic leukemia cell lines • Solid tumor (breast, pancreas, and ovary) cell lines	(48)
Ovarian cancer	• IHC	• BTN3A2 observed on epithelial cells, some tissue cores, and stroma • Correlated with CD3+ immune infiltrate • High BTN3A2 associated with increased OS and disease free progression	(64)
Breast cancer	• IHC	Increased BTN3A1/BTN3A3 staining in tumors sections compared to normal epithelium	(52)
AML blasts	• FC • qRT-PCR • WB	• BTN3A cell surface expression • BTN3A2 most abundant isoform both at transcriptional and protein level	(62)
Colorectal cancer	• qRT-PCR • WB • IHC	• Three isoforms detected by WB • BTN3A1 detected on epithelial cells and TAFs	(63)
Gastric cancer	Exome array analysis	BTN3A2 associated with poor prognosis, increased proliferation and invasion of gastric cancer cell lines	(65)
PDAC	• IHC • FC • qRT-PCR • WB	• BTN3A epithelial expression in PDAC, associated with invasiveness • BTN3A2 most abundant isoform both at transcriptional and protein level • High BTN3A2 transcript associated with reduced OS	(53)

AML, acute myeloid leukemia; FC, flow cytometry; IHC, immunohistochemistry; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, real-time quantitative reverse transcription PCR; TAF, tumor-associated fibroblast; WB, western blot.

TABLE 3 | Factors driving BTN3A regulation in the tumor microenvironment.

	Observations	Reference
Inflammatory cytokines: TNFα, IFNγ, CCL3, and IL-6	BTN3A upregulation on DCs and HUVECs	(48, 57)
Hypoxia-associated mediators such as VEGF, PlGF, and IL-10	BTN3A upregulation in ovarian cancer BTN3A2 transcript upregulation in PDAC	(53, 57)
Nutrient deprivation	BTN3A2 transcript upregulation in PDAC	(53)
Soluble BTN3A isoforms	Plasma sBTN3A associated with decreased OS	(53)

DCs, dendritic cells; HUVECs, human vein endothelial cells; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma.

that sBTN3A may prevent Vδ9Vδ2 T cells from exerting their cytotoxic activity on tumor cells. This feature of soluble form is also shared with other B7 family members such as CTLA-4 and PD-L1 and has been linked to patient prognosis (67, 68). The soluble forms of proteins are usually generated by proteolytic cleavage of the membrane-bound form, as in the case of soluble tumor necrosis factor receptor (69) and sB7-H3 (70), or by translation of alternative spliced mRNA, as in the case of sB7-2 (71, 72), as well as sCTLA-4 (67, 68) and sB7-H3 (73). Increasing evidences suggest that sPD-L1 is a prognostic biomarker associated with aggressive disease in malignant tumor, such as multiple myeloma (74), diffuse large B-cell lymphoma (68), renal cell carcinoma (75), and ovarian cancer (76). In the same way, sBTN3A may provide new biomarkers easily detectable in clinical practice.

Interactions Between γδ T Cells and BTN/BTNL Proteins: Therapeutic Opportunities

γδ T cells, both Vδ1+ and Vδ2+ subsets, have been shown to infiltrate a wide range of solid tumors, as well as blood cancers such as follicular lymphoma, AML Burkitt's lymphoma. Strikingly, intratumoral γδ T cells have emerged as the most favorable prognostic immune population among many cancer types (77). Their capacities to secrete effector cytokines and to kill tumor cells make them attractive as new immunotherapeutic targets. Lawand et al. have recently reviewed the main features of γδ T cell subsets in cancer (78). However, multiple signals within the tumor microenvironment can influence the functional outcome (79). The major challenge remains thus to determine how to specifically boost the anti-tumor effects of γδ T cells. Two synthetic drugs, the pAg BrHPP and N-BP Zoledronate activate γδ T cells *in vitro* and in clinical trials *in vivo*. However, their utility in clinics has been dampened by their bad pharmacokinetics and pharmacodynamics properties which favor their local accumulation at the bone surface and rapid renal clearance (80). New approaches have to be considered to optimize the use of γδ T cells in therapeutics. As reviewed here, BTN and BTNL have demonstrated a potent immunomodulatory role for certain γδ T cell subsets. The T-cell-stimulatory and inhibitory activity of some BTN/BTNL proteins suggests that they might be taken into account as novel targets for checkpoint inhibition (like CTLA-4, PD1, PD-L1, etc.) to potentially target γδ T cells (81).

BTN3A and Vγ9Vδ2 T Cells

BTN3A has been shown as crucial for mediating Vγ9Vδ2 cytolytic functions against tumor cells. We have developed anti-BTN3A antibodies which selectively promote an active (mAb 20.1) or inactive (mAb 103.2, 108.5) BTN3A conformation on the cell surface, and might broadly open the Vγ9Vδ2-T cells immunotherapy field for cancer and autoimmune diseases. Notably, the 20.1 mAb was shown to sensitize primary AML blasts and to circumvent their resistance to allogeneic Vγ9Vδ2 T cells lysis. This effect induced the clearance of primary leukemic blasts from the bone marrow *in vivo* in an NSG human AML-xenografted mouse model (62). The 20.1 mAb was also shown to boost BTN3A-mediated Vγ9Vδ2 T cells cytolytic functions against PDAC even under hypoxic conditions, overcoming this stress-related characteristic of the PDAC microenvironment (53). Harly et al. have also shown that 20.1 mAb induced the activation of Vγ9Vδ2 T cells against a wide range of tumor cell lines (47). A recent study highlighted that 20.1 mAb differentially activates Vγ9Vδ2-TCR clonotypes, and that the responsiveness strongly depends on CDR3 sequences of the TCR (82).

Vγ9Vδ2 T cells were also shown to be able to kill colorectal cancer cell lines exposed to Zoledronate, this effect being partly related to BTN3A1 expression and its cellular re-distribution, in the membrane and cytoskeleton-associated fraction (63). Cross linking of BTN3A on glioblastoma-derived cell lines increased IFN-γ secretion by Vγ9Vδ2 T cells (83).

As discussed above, BTN3A2 was the most expressed paralog along the BTN3A proteins in several AML (62), PDAC cell lines and primary tumors (53), and gastric cancer (65). Interestingly, the 20.1 antibody stands out for its capacity to stimulate Vγ9Vδ2 T cells independently on the predominant BTN3A paralog expressed at the cell membrane. Hence, the dominant expression of BTN3A2 isoform in tumors do not preclude the triggering of BTN3A molecules by the agonist 20.1 mAb and the effective activation of Vγ9Vδ2 T cells. This effect of priming and sensitization of tumor cells to killing by Vγ9Vδ2 T cells would be a first therapeutic approach. We have also shown that soluble BTN3A are increased in the plasma of PDAC patients and could potentially interfere with the tumor killing activity of effector cells. Development of antibodies to mop-up soluble BTN3A could thus be another strategy.

Therefore, agonistic anti-BTN3A antibodies might represent a novel therapeutic opportunity to treat cancer. Conversely, humanized inhibitory anti-BTN3A antibodies might be taken into account as immunosuppressive drugs for the treatment of autoimmune diseases genetically associated with BTN3A, like rheumatoid arthritis or schizophrenia (84).

Oppositely, patients with ulcerative colitis (UC) showed downregulation of BTN1A1, BTN2A2, BTN3A2, and BTN3A3 levels compared to healthy controls (85). This result might be explained by the fact that BTN3A proteins trigger opposite cell responses in Vγ9Vδ2 T cells (co-activation) and conventional T lymphocytes (co-inhibition) (86, 87). For example, BTN3A

expression levels, upregulated by tumor microenvironment signals in ovarian cancer, have been suggested to contribute to immune evasion by dampening the activity of infiltrating T cells (57). However, BTN3A2 has been shown to be associated with a good prognosis in patients with ovarian cancer, associated with an increased infiltrate of CD4+ T cells (64). Unfortunately, Vγ9Vδ2 T cell infiltrate was not assessed in this study. We can postulate that BTN3A2 would mediate other effects in other type of immune cells. As an example, a co-stimulatory role has been demonstrated for BTN3A in αβ T cells that could explain different functions depending on the cellular type engaged and the tumor context. BTN3A2 promoter and ORF were related also to gastric cancer (65) and type I diabetes (88), respectively. Besides that, Gene-Based Association Analysis revealed BTN3A3 associations with rheumatoid arthritis, lower risk of relapse in ovarian cancer (89), and so on. Aberrant BTN3A3 methylation patterns were also observed on bipolar disorder and schizophrenia (90). Meta-analysis of GWAS Data also related BTN3A2 and BTN3A1 with schizophrenia susceptibility (91).

Other BTN/BTNL Proteins

The recently described role of BTNL3 and BTNL8 in the homing and maintenance of a semi activated state on Vγ4+ γδ T cells in the human gut might be relevant for the onset of gut autoimmune diseases like UC and inflammatory bowel disease (85). Conversely, it was observed a downregulation of BTNL2, BTNL3, BTNL8, and BTNL9 mRNA in colon tumors, suggesting a co-stimulatory role for BTNL3/BTNL8 (85). Thus, the modulation of the activation signal coming from BTNL3/BTNL8 to Vγ4+ γδ T cells might be a useful approach to treat both diseases in the future.

CONCLUDING REMARKS

Recent studies have highlighted the expression of BTN3A in tumors and its correlation with patient prognosis. Notably, *in vitro* as well as *in vivo* experiments have opened new perspectives in Vγ9Vδ2 T cell-based immunotherapies and shown the potential of agonists BTN3A mAbs toward enhancing Vγ9Vδ2 T cell anti-tumor functions. In addition, sBTN3A may play significant role in tumor pathogenesis, immune responses, and prediction. The modulation of other BTN/BTNL molecules such as BTNL3/BTNL8 seems also of interest in other pathologies such as colon tumors or autoimmune diseases. Targeting BTN/BTNL could thus represent an attractive strategy, alone or in combination with current therapies (i.e., pAgs and monoclonal antibodies targeting immune-checkpoint).

AUTHOR CONTRIBUTIONS

JL-B, AB, and CP wrote/revised the manuscript. DO supervised/revised the manuscript. JL-B, AB, and CP contributed equally to this work.

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Human Vγ9Vδ2 T Lymphocytes in the Immune Response to *P. falciparum* Infection

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Malaria is an infectious disease caused by the protozoan parasite *Plasmodium sp*, the most lethal being *Plasmodium falciparum*. Clinical malaria is associated with the asexual replication cycle of *Plasmodium* parasites inside the red blood cells (RBCs) and a dysregulated immune response. Although the mechanisms of immune responses to blood—or liver-stage parasites have been extensively studied, this has not led to satisfactory leads for vaccine design. Among innate immune cells responding to infection are the non-conventional gamma-delta T-cells. The Vγ9Vδ2 T-cell subset, found only in primates, is activated in response to non-peptidic phosphoantigens produced by stressed mammalian cells or by microorganisms such as Mycobacteria, *E.coli,* and *Plasmodium*. The potential protective role of Vγ9Vδ2 T-cells against infections and cancer progression is of current research interest. Vγ9Vδ2 T-cells have been shown to play a role in the early control of *P. falciparum* parasitemia and to influence malaria adaptive immunity *via* cytokine release and antigen presentation. They are activated and expanded during a primary *P. falciparum* infection in response to malaria phosphoantigens and their activity is modulated upon subsequent infections. Here, we review the wide range of functions by which Vγ9Vδ2 T-cells could both contribute to and protect from malaria pathology, with a particular focus on their ability to induce both innate and adaptive responses. We discuss how the multifunctional roles of these T-cells could open new perspectives on gamma-delta T-cell-based interventions to prevent or cure malaria.

Keywords: gamma-delta T cells, malaria, falciparum, immunity to malaria, antigen presenting cell (APC), cytotoxicity

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INTRODUCTION

Over the last decades, the importance of a specific subset of γδ T-cells in malaria infection is becoming increasingly apparent, namely Vγ9Vδ2 T-cells. Restricted to human and non-human primates, Vγ9Vδ2 T-cells constitute a non-conventional T-cell subset activated in a non-MHC dependent manner, by phosphorylated intermediates of isoprenoid biosynthesis pathways of mammalian cells and microorganisms, known as phospho-antigens (Ph-Ag) (1). The known most potent of these, HMBPP [(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate] is produced by the DOX-P pathway used by several microorganisms (2) including the parasite responsible for malaria, *Plasmodium spp* [reviewed (3)]. Once

activated, Vγ9Vδ2 T-cells expand, produce cytokines, exert cytotoxic functions, and stimulate cells such as monocytes, resulting in improved monocyte antigen presentation capabilities (4).

Despite major global effort, malaria remains a major public health concern. Nearly half of the world's population live in malaria endemic regions, the majority in sub-saharan Africa, and it is responsible for ∼216 million cases and 445,000 deaths each year (5). Efforts to create an effective vaccine are hampered by lack of understanding of the parasites interactions with our immune system.

There are five species of *Plasmodium* that infect humans: *P. falciparum*, *P. vivax*, *P. ovale, P. malariae,* and *P. knowelsi*. *P. falciparum* is the most prevalent and deadly. *P. falciparum*, similar to other *Plasmodium,* is transmitted through the bite of a female *Anopheles* mosquito. The extracellular, liver-invasive form, the sporozoite, is injected into the skin, where it enters the blood flow and travels to the liver. Here the parasite eventually invades hepatocytes, wherein it differentiates and divides to form the extracellular form called merozoites. Merozoites are released into the blood stream and invade red blood cells (RBCs) where they progress through a 48 h life cycle before RBC rupture and merozoite release. Clinical disease manifests during this blood stage and is characterized by cyclical episodes of fever paroxysms. Severe malaria can be fatal and presents an array of severe symptoms including severe anemia, respiratory distress caused by severe metabolic acidosis, cerebral-malaria, multi-organ failure, and in pregnant women, placental malaria (6).

For over 100 years, it has been observed that partial immunity to malaria in endemic areas is only acquired after multiple disease episodes (7–9). In endemic settings, immunity is developed first to severe malaria (usually before 5 years old) then to clinical malaria (by 10–15 years old) (8, 10–12). Acquired immunity appears to be strain- and variant-specific and in endemic areas people are frequently re-infected by novel variants with novel antigen combinations. This complicates the assessment of protective immunity, however it is commonly accepted that sterile immunity is rarely reached and low parasitemia with no clinical symptoms is instead maintained (13, 14). Malaria infection causes dysregulation of immune responses, including inhibition of DC maturation and antigen presenting capacity (15–17) and expansion of atypical memory B cells, the functionality of which is not yet understood (18–20). The role of the innate immune responses, and the cellular and humoral branches of the adaptive immune response has been excellently reviewed elsewhere (11, 21–25).

Concerning γδ T-cells, much of the early *in vivo* work on Vγ9Vδ2 T-cell responses to *P. falciparum* infection was done in primary infected adult patients, usually Caucasians living in non-endemic regions, where Vγ9Vδ2 T-cells are the dominant subset of γδ T-cells. However, it has been shown that in malaria endemic regions, where the populations are exposed to numerous malaria infections and possibly chronically infected, Vδ1 T-cells are the major subset (26, 27). It is not yet known if this is a genetic peculiarity, or different microbiota and pathogen exposure early in life that drives expansion and contraction of these subsets. An in-depth discussion on the reasons for these geographical differences, and the role played by non Vγ9Vδ2 T-cells in malaria infection is beyond the scope of this review, which focuses on Vγ9Vδ2 T-cells. Vγ9Vδ2 T-cells have features associated with both innate and adaptive T-cells, and increasing evidence suggests they act as a bridge between the innate and adaptive immune systems [reviewed (28–30)]. Vγ9Vδ2 T-cells have a wide range of effector functions [reviewed (30, 31)], and it is becoming increasingly clear that during *P. falciparum* infection they contribute to both protection and pathology. In this review, we discuss their role as cytotoxic killer cells and their ability to initiate both innate and adaptive immune responses against *P. falciparum* malaria infection via cytokine release and direct antigen presentation to CD4 and CD8 T-cells.

Vγ9Vδ2 T-CELLS ARE ACTIVATED DURING MALARIA INFECTION

γδ T-cells have long been observed to expand *in vivo* in the peripheral blood of primary infected *P. falciparum* malaria patients, with the major subset being Vγ9Vδ2 T-cells (32, 33). Interestingly, expansion in the peripheral blood is greatest during recovery, after acute infection has passed (34), indicating either a delay in response, or homing to tissues during acute infection. Vγ9Vδ2 T-cells were found to be increased in human spleens during infection (35, 36), a phenomenon that was confirmed in monkey models (36). The rapid expansion of Vγ9Vδ2 T-cells during infection and their homing to sites of known importance in parasite clearance indicated that Vγ9Vδ2 T-cells could play a role in the response to infection.

Our previous work has demonstrated that the bioactive molecule released by infected red blood cells (iRBC) is a Ph-Ag of the DOX-P pathway, which is released concomitantly with iRBC rupture. We also showed that presentation of parasite Ph-Ag to Vγ9Vδ2 T-cells involves BNT3A1 on non-erythrocyte bystander cells, as RBCs and iRBCs are devoid of BNT3A1 (37). In addition to HMBPP various other signals, including IL-2, IL-15 (38, 39), CD4 T-cell interaction activation (40) and CD28 co-stimulation (41), are needed for effective Vγ9Vδ2 T-cell activation, and stimulation of Vγ9Vδ2 T-cells in different cytokine milieus emphasizes different functional behaviors (42).

CYTOTOXIC Vγ9Vδ2 T-CELLS DIRECTLY TARGET BLOOD STAGE *P. falciparum*

In vitro studies have built a picture of how Vγ9Vδ2 T-cells directly inhibit the erythrocyte stage life-cycle. The first studies showed that Vγ9Vδ2 T-cells targeted the iRBCs in a contact dependent manner, and suggested that merozoites were the target, as inhibition of parasite life-cycle was not seen until after parasite reinvasion (43–45). Active granulysin release by the Vγ9Vδ2 T-cells was implied in mediating parasite growth inhibition, as granulysin production correlated with life-cycle inhibition (44). Experiments with granulysin and perforin deficient Vγ9Vδ2 T-cell lines confirmed that Vγ9Vδ2 T-cell inhibition of parasites was indeed granulysin-mediated but not perforin-dependent (46). Finally, in an experiment where

Vγ9Vδ2 T-cells were co-cultured with late stage iRBC and removed before rupture there was no impact on the parasite reinvasion. This showed definitively that merozoites are the target, as schizonts are not affected by granulysin release (46).

CYTOKINE RELEASING Vγ9Vδ2 T-CELLS ACT AS A TRIGGER FOR BOTH INNATE AND ADAPTIVE IMMUNE RESPONSES

Vγ9Vδ2 T-cells are highly interactive, and much of their impact on the course of an immune response stems from their modulation of other innate and adaptive immune cells by cytokine release and direct cell-cell interaction (30). Existing evidence indicates that Vγ9Vδ2 T-cells are implicated in impacting the scale and nature of both innate and adaptive immune responses to *P. falciparum* infection. A large feature of the immune response to *P. falciparum* infection is the production of inflammatory cytokines. *In vitro* studies of schizont-activated PBMCs from naïve donors, Vγ9Vδ2 T-cells have been found to produce TNFα and be the major source of IFNγ, more than NK cells or macrophages (47–50). They have also been shown to express TNFα, TGF-β, and IL-8, and occasionally IL-10, IL-2, and IL-5 (48). In *ex vivo* analysis of cord blood from mothers in an endemic setting who had experienced malaria during pregnancy, the Vγ9Vδ2 T-cells produced significantly more IFNγ and TNFα than those from healthy mothers, as did the peripheral Vγ9Vδ2 T-cells from the mother (51). This inflammatory cytokine production by Vγ9Vδ2 T-cells has been associated with both protection and pathogenesis.

Vaccination studies have been performed where healthy, malaria naïve, volunteers are exposed to three doses of *P. falciparum* (via the bite of 12–15 infected mosquitos) with the accompaniment of chloroquine treatment. This permits the parasite to mature to blood stage, when it is then swiftly killed before disease symptoms can develop. After challenge by the bites of five infected mosquitos, the inoculated volunteers remained parasite-free, indicating that they had developed a sterilizing immunity (52, 53). Vaccinated (protected) volunteers showed increased IFNγ, TNFα, and IL-2 production compared to non-vaccinated (non-protected) when PBMCs, taken pre-challenge and 1 day post-challenge, were stimulated by iRBC *in vitro* (52, 53). IFNγ levels were also increased in PBMCs from vaccinated volunteers taken days 9, 35, 140, and 400 post-challenge when stimulated by both iRBC and sporozoites (53). γδ T-cells were found to be the major IFNγ contributors, with αβ T-cells the next largest. The majority of responding cells were effector memory, indicating recall responses, and IFNγ-producing γδ T-cells were demonstrated to be a major contributor to parasite-specific recall responses (53). Thus, in these vaccines, IFNγ production by lymphocytes including γδ T-cells, correlated with acquired immunity to *P. falciparum* infection. It should be noted that Vγ9Vδ2 T-cells were not specifically measured in this study. However, as Vγ9Vδ2T-cells are the predominant subset in the periphery of malaria naïve individuals from non-malaria endemic regions, it is reasonable to assume they were the major responding γδ T-cell subset in this study.

In longitudinal studies of semi-immune children from Papua New Guinea, the *in vitro* response of PMBCs to iRBC was measured, and subsequent malaria incidence recorded. Increased IFNγ production by PBMCs correlated with reduced risk of future moderate and high-density *P. falciparum* infection. Further, though there was much donor heterogeneity, γδ T-cells were the predominant IFNγ producing cell population (54). However, a different longitudinal study of children from Papua New Guinea suggests that γδ T-cell cytokine production is involved in severe malaria. *Ex vivo* stimulation of PBMCs from children with either severe or uncomplicated malaria or healthy controls showed that γδ T-cells and monocytes were responsible for inflammatory cytokines associated with 'high odds' of severe malaria (55). Several studies together have shown that Vγ9Vδ2 T-cell cytokine production is abrogated with repeat malaria exposure, and this contributes to decreasing clinical symptoms in subsequent infections.

Decreased peripheral activity of Vγ9Vδ2 T-cells has been found during the acute stage of infection in primary *P. falciparum* infected adults. Vγ9Vδ2 T-cells taken from the peripheral blood during paroxysms were found to expand less and produce less TNFα in response to IPP stimulation than Vγ9Vδ2 T-cells taken during recovery, post-treatment (though still expanded compared to uninfected controls). It was also found that there are less Vγ9Vδ2 T-cells [particularly Vγ2Jγ1.2γδ T-cells (US nomenclature), the TCR subset that is particularly reactive to Ph-Ags] in circulation during *P. falciparum* paroxysm than during recovery (34).

In a longitudinal study of Ugandan children, the percentage of Vγ9Vδ2 T-cells in peripheral blood was found to be inversely correlated with prior incidence of malaria infections. *Ex vivo,* Vγ9Vδ2 T-cell proliferation, TNFα, and IFNγ production and immune-modulatory gene expression was also negatively associated with prior malaria episodes—indicating decreased peripheral blood Vγ9Vδ2 T-cell activity with increasing exposure to the parasite. Lower *in vitro* Vγ9Vδ2 T-cell responsiveness to iRBC correlated with lower subsequent incidences of symptomatic infection, but to increased probability of higher parasitemia (56). This Vγ9Vδ2 T-cell dysfunction was shown to occur because of frequent malaria episodes in childhood, an effect that was abrogated by chemoprevention in early childhood (57). The mechanism of Vγ9Vδ2 T-cell regulation is as yet unknown. Vγ9Vδ2 T-cells are very susceptible to activation-induced cell death by Fas-Fas-L interaction as demonstrated for *M. tuberculosis* (58), though active regulation cannot be ruled out.

Together, these studies indicate that while Vγ9Vδ2 T-cell inflammatory cytokine responses can control parasitemia, excessive stimulation of these cells may also result in pathology suggesting that clinical immunity to malaria may be associated with reduced Vγ9Vδ2 responses.

Several accumulated data in mice, where the equivalent of human Vγ9Vδ2 T-cell subset is not yet certain, also show the importance of the cytokine secretion activity of murine γδ T-cells (59, 60). A recent study (61) showed that clonal expansion of a subset of γδ T-cells producing macrophage colony stimulating factor (M-CSF), prevents parasitemic recurrence. While it is

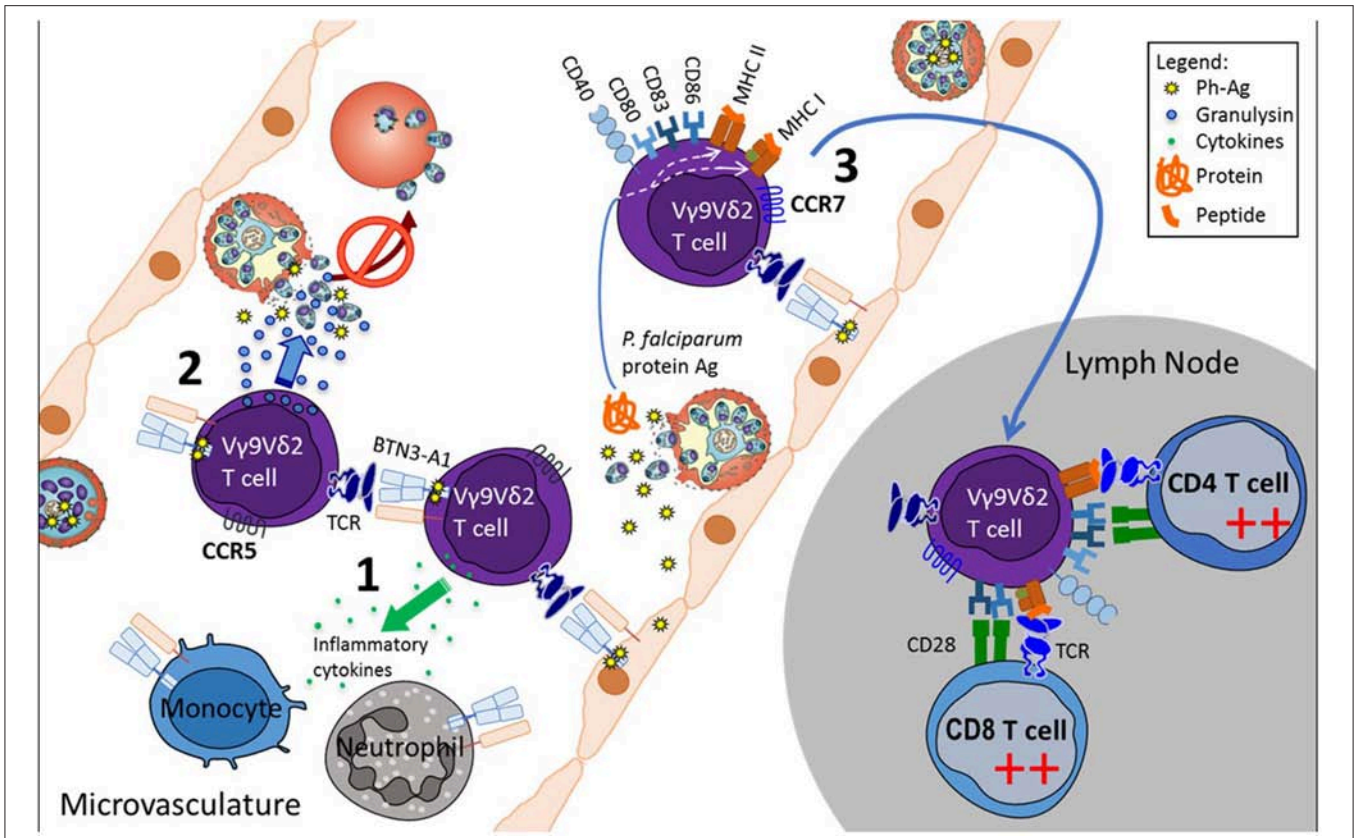

FIGURE 1 | Proposed model of Vγ9Vδ2 T-cell functions in the microvasculature during *P. falciparum* infection. Plasmodium-infected red blood cells sequester to the endothelium in the microvasculature, where they release phosphoantigens concomitantly with the red blood cell rupture. Phosphoantigens stimulate Vγ9Vδ2 T-cells via BTN3A1 available on neighboring cells, including Vγ9Vδ2 T-cells, the endothelial cells and innate immune cells. Activated Vγ9Vδ2 T-cells (1) modulate innate cells by cytokine secretion, (2) inhibit free parasite reinvasion of red blood cells, by releasing the cytotoxic granulysin, and (3) acquire APC phenotype and the capability to migrate to lymph nodes where they initiate an adaptive immune response.

perhaps a stretch to expect a direct murine equivalent of Vγ9Vδ2 T-cells, certainly one is not yet identified, it is likely that one or more murine γδ T-cell subsets have evolved which perform the same protective and/or pathologic functions in malaria infection as human Vγ9Vδ2 T-cells. "TγδM" cells are a good candidate for one such functional equivalent of Vγ9Vδ2 T-cells.

ANTIGEN PRESENTING Vγ9Vδ2 T-CELLS STIMULATE ADAPTIVE IMMUNE RESPONSES

Another way in which Vγ9Vδ2 T-cells influence the course of an immune response is by antigen presentation to αβ T-cells. Over the last 12 years it has been demonstrated that Vγ9Vδ2 T-cells can take up, process and present exogenous Ag, both via the classical pathway to CD4 T-cells and the cross-presentation pathway to CD8 T-cells. They even have shown the ability to act as professional antigen presenting cells (APCs) and stimulate naive CD4 and CD8 T-cells (62–70). γδ T-APC resembling cells are present in malaria infected individuals, and *in vitro* iRBC stimulated Vγ9Vδ2 T-cells not only take on an APC phenotype but also can cross-present Ag to a memory cell line and activate naïve CD4 and CD8 T-cells in a mixed-lymphocyte reaction (71). Where this might be occurring *in vivo*, or what the implication of repeated malaria infection could be is worth investigated. However, interesting work from liver stage malaria vaccines could shed some light on this (see below).

Overall, the data allow us to propose a global model of how peripheral Vγ9Vδ2 T-cells could control parasitemia and initiate both innate and adaptive responses (**Figure 1**). Whether the same cells are responsible for these functions or whether different subsets of Vγ9Vδ2 T-cell are concerned is still to be worked out.

Vγ9Vδ2 T-CELLS: CORRELATES OF PROTECTION FOR WHOLE ORGANISM MALARIA VACCINE?

Vγ9Vδ2 T-cells have been implicated in protection against liver stage immunity after vaccination with whole sporozoites.

In a mouse model of irradiated sporozoite vaccinations, it was clear that the γδ T-cells were required for induction of protective CD8 T-cell responses, but not antibodies, and were not

acting as effectors in controlling liver stage parasite replication (72). An as yet undefined subset of mouse γδ T-cells are able to function by inducing downstream αβ T-cell responses. Further studies are required to establish which mouse γδ T-cell subsets mirror the various activities of Vγ9Vδ2 γδ T-cells and explore the effect of irradiated sporozoite vaccination dose on these cells. In humans, in the first field trial of the Sanaria® PfSPZ vaccine in Mali, it was demonstrated that the Vγ9Vδ2 T-cells were highest in vaccines that remained uninfected throughout an intense malaria transmission season, compared to infected vaccines or the placebo group (72). These findings were comparable to those observed in malaria naïve individuals vaccinated with either the PfSPZ vaccine or a chemoprophylaxis vaccination, who also had a remarkable increase in Vγ9Vδ2 T-cells (73). Overall, these findings are intriguing in that they suggest that liver-stages growth of *P. falciparum* can stimulate Vγ9Vδ2 T-cell activation. This activation could have several explanations: first, locally in an infected liver, hepatocytes displaying BNT3A1 or other presentation molecules could activate Vγ9Vδ2 T-cells *in situ*. Second, Vγ9Vδ2 T-cells could be activated in the draining lymph nodes of the site of infection where a substantial fraction of the sporozoites migrate, as shown by Amino et al. in mouse model (74). Third, HMBPP produced by liver stages of *Plasmodium* could be sensed in the periphery by exquisitely sensitive Vγ9Vδ2 T-cells, as seen during blood stage *P. falciparum* infections (37). Finally, the activation of Vγ9Vδ2 T-cells could be due to recognition of other antigens or metabolites.

It should be noted that in subsequent trials which used a higher dose of the PfSPZ vaccine, Vγ9Vδ2 T-cell expansion did not distinguish protected vs. unprotected vaccines (75, 76). Interestingly, liver stage induced Vγ9Vδ2 T-cell expansion has not been observed in volunteers undergoing controlled human malaria infections (77). The reasons behind this are not yet understood, but given the plasticity of Vγ9Vδ2 T-cells, it may be that varying antigen loads modulate the phenotype and function of these cells.

CONCLUDING REMARKS

In conclusion, the Vγ9Vδ2 T-cell is an enigmatic cell, with a wide range of functions that can both contribute to and protect from malaria pathology. It is important to better consider this subset of γδ T-cells, especially their role in malaria vaccine protection. Given their sensitivity to Ph-Ag's such as HMBPP and apparent functional plasticity under different cytokines and stimuli dose, a cocktail of Ph-Ag and cytokines could be envisioned as an adjuvant to boost efficacy of both liver and blood stage malaria vaccines.

AUTHOR CONTRIBUTIONS

JH, IZ, and MM-M wrote the manuscript. SL and OM-P contributed to the manuscript. JD-M secured fundings.

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frontiers in Immunology

MINI REVIEW
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Human Vδ1$^+$ T Cells in the Immune Response to *Plasmodium falciparum* Infection

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Naturally acquired protective immunity to *Plasmodium falciparum* malaria is mainly antibody-mediated. However, other cells of the innate and adaptive immune system also play important roles. These include so-called unconventional T cells, which express a γδ T-cell receptor (TCR) rather than the αβ TCR expressed by the majority of T cells—the conventional T cells. The γδ T-cell compartment can be divided into distinct subsets. One expresses a TCR involving Vγ9 and Vδ2, while another major subset uses instead a TCR composed of Vδ1 paired with one of several types of γ chains. The former of these subsets uses a largely semi-invariant TCR repertoire and responds in an innate-like fashion to pyrophosphate antigens generated by various stressed host cells and infectious pathogens, including *P. falciparum*. In this short review, we focus instead on the Vδ1 subset, which appears to have a more adaptive immunobiology, but which has been much less studied in general and in malaria in particular. We discuss the evidence that Vδ1$^+$ cells do indeed play a role in malaria and speculate on the function and specificity of this cell type, which is increasingly attracting the attention of immunologists.

Keywords: gamma-delta (γ/δ) T lymphocytes, Vdelta1 gamma delta T cells, malaria, *Plasmodium falciparum*, innate immunity, acquired immunity, immune regulation

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INTRODUCTION

The most serious form of malaria is caused by the hemoprotozoan parasite *Plasmodium falciparum*. The disease is a major humanitarian and economic burden on societies affected by it, mainly in sub-Saharan Africa, and it leads to the death of about half a million children every year (1, 2). Immunity to the disease is gradually acquired after years of exposure and many disease episodes, and is mainly mediated by IgG antibodies targeting the asexual blood stages of the infection, which are responsible for all the clinical symptoms and complications (3–5). T cells are nevertheless also of obvious importance in acquisition of immunity, not least to enable B-cell class switching and affinity maturation.

Most circulating T cells express αβ type T-cell receptors (TCR-αβ), but a minority of T cells instead expresses the alternative γδ TCR heterodimer (TCR-γδ). The pivotal role of αβ T cells in immunity to *P. falciparum* malaria is well-established. The αβ T cells function both directly as cytotoxic effector cells against infected hepatocytes, and indirectly as CD4$^+$ helper cells for a variety

of innate and adaptive immune responses to all stages of the parasite life cycle in the human host. Much less is known about the function and significance of γδ T cells in this immunity.

The αβ and γδ T-cell compartments share several features. In both, the TCR constitutes the antigen recognition element of the multi-molecular TCR complex, which also includes several signal transduction components, such as CD3. TCR diversity is generated by somatic recombination events during T-cell maturation in the thymus. As for αβ T cells, the TCRs of γδ T cells are clonally distributed, such that each T-cell clone expresses a single, rearranged TCR variant, which determines the antigen specificity of the clone—at least in the case of αβ T cells.

The two compartments also exhibit important differences. Thus, αβ T cells respond predominantly to protein antigens that are processed by antigen-presenting cells (APCs) and subsequently displayed as short peptides bound to major histocompatibility complex (MHC) molecules on the APC surface. In contrast to αβ T cells, which typically express either CD4 or CD8, γδ T cells often express neither, in particular in the Vγ9$^+$Vδ2$^+$ subset. In keeping with this lack of MHC restriction elements, recognition of antigen by "double-negative" γδ T cells is not MHC-restricted. Furthermore, Vγ9$^+$Vδ2$^+$ T cells universally respond to non-peptide prenyl pyrophosphate metabolites (termed phospho-antigens, or P-Ag) (6). These antigens, which are produced by a variety of stressed cells (isopentenyl pyrophosphate, IPP, produced via the host mevalonate pathway) and by infectious pathogens, including *P. falciparum* [(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate, HMB-PP, produced via the microbial non-mevalonate pathway] are structurally related. Accordingly, the Vγ9 chains expressed by these cells are relatively invariant (7, 8) due to convergent and recurrent recombinations (9). In addition, the Vγ9$^+$Vδ2$^+$ TCR repertoire is already restricted from birth, and contains a high proportion of Vγ9 clonotypes that are shared by many clones in a given individual, and conserved between many individuals (i.e., "public" repertoires). Furthermore, the repertoire of these cells does not exhibit dramatic clonotypic focusing in adults relative to neonates (9, 10). The Vγ9$^+$Vδ2$^+$ T-cell subset, which is usually the dominant γδ T-cell subset in the peripheral blood of healthy individuals without exposure to *P. falciparum*, can thus be described as an "innate-like" T-cell subset.

To date, the Vγ9$^+$Vδ2$^+$ cells are the γδ T cells that have attracted by far the most attention in relation to malaria (11, 12). However, we focus here instead on a largely complementary subset that is characterized by a TCR composed of Vδ1 paired with a variety of γ-chains, and that appears to adopt a distinct immunobiology relative to the innate-like Vγ9$^+$Vδ2$^+$ subset (13). Unlike Vγ9$^+$Vδ2$^+$ T cells, Vδ1$^+$ T cells typically constitute a minority (≤20%) of adult peripheral blood γδ T cells. However, the subset is enriched relative to the Vγ9$^+$Vδ2$^+$ T-cell subset in tissues, where they have been reported to recognize a variety of host and microbial antigens (14–16). Also in marked contrast, the TCR repertoire of Vδ1$^+$ T cells—and of Vγ9^{neg}Vδ2$^+$ T cells (17)—is highly diverse at birth, and largely non-overlapping between individuals (i.e., "private" repertoires). Furthermore, the TCR repertoire of this γδ T-cell subset becomes increasingly focused over time as a result of selective expansion of specific clonotypes, most likely following antigenic stimulation (9, 18–20). The Vδ1 subset therefore appears to be much more "adaptive-like" than the Vγ9$^+$Vδ2$^+$ subset (21), and it bears substantial similarities to conventional αβ T cells. Nevertheless, there is certainly evidence that Vδ1$^+$ T cells play a distinct role from αβ T cells in the immune response to several infections—including *P. falciparum* malaria.

Increased Proportions and Numbers of Vδ1$^+$ T Cells in Malaria Patients and Healthy Residents From Malaria-Endemic Areas

Within a few years of the discovery of the γδ TCR, several groups reported modest but protracted expansions of γδ T cells in adult *P. falciparum* and *P. vivax* patients with little or no previous malaria parasite exposure (22–24). A later study of malarious children from a highly malaria-endemic area and employing a pan-γδ TCR-specific antibody reported similar findings, and did not find significant differences in peripheral blood γδ T-cell frequencies between children with uncomplicated and severe malaria, respectively (25). The authors also reported significantly decreased absolute numbers of γδ T cells at the time of admission to hospital with malaria (regardless of severity), followed by a transient increase to numbers above normal during convalescence. This was also observed among the few adult first-time malaria patients included in the study (25). Overall, the γδ T cell-specific findings appeared similar in patients with or without prior exposure to malaria, and also resembled earlier reports regarding the αβ T-cell response to malaria, namely an inflammation-induced withdrawal of these cells from the peripheral circulation, followed by their release back into the peripheral blood after successful chemotherapy [reviewed in Hviid (26)].

Substantial γδ T-cell subset heterogeneity was also reported (27–30). These early papers indicated that the γδ T-cell response to *P. falciparum* malaria extends beyond Vγ9$^+$Vδ2$^+$ cells, although that subset remained the dominant one among the non-immune patients that were studied. However, it was reported shortly after that in semi-immune African children and adults with acute *P. falciparum* malaria, the γδ T cells responding *in vivo* are completely dominated by cells expressing Vδ1, with little contribution from Vγ9$^+$Vδ2$^+$ T cells (31, 32). A study of children and adults from *P. falciparum*-endemic Lao People's Democratic Republic very recently reported similar findings (33). The expanded Vδ1$^+$ subset had an activated phenotype, produced pro-inflammatory cytokines, used a diversity of Vγ chains, and showed spectratyping evidence of clonal focusing (31–33). In fact, the Vδ1$^+$ subset appeared to dominate even among healthy *P. falciparum*-exposed individuals living in areas with stable transmission of these parasites (20, 34). In the absence of acute malaria, these cells were CD45RA$^+$, resting (CD69^{neg} and HLA-DR^{neg}), and about half of them were CD8$^+$ (in contrast to the majority of Vγ9$^+$Vδ2$^+$ cells, which are double-negative). They were clonally restricted in most adults, but less so in children (20). They thus appear phenotypically similar to the Vδ1$^+$ cells found in epithelia (35). While Vγ9$^+$Vδ2$^+$ cells from such individuals could respond when stimulated *in vitro* by *P. falciparum* pyrophosphate antigens (34)—similar to

Vγ9$^+$Vδ2$^+$ cells from donors without previous malaria exposure [reviewed in Howard et al. (11)]—this response did not appear very prominent *in vivo*.

Vδ1$^+$ T Cells in Malaria: What Do They See and What Do They Do?

Essentially nothing is known about the function or antigen specificity/specificities of the dominant Vδ1$^+$ γδ T-cell subset in *P. falciparum*-exposed individuals (12). A few studies have indicated that these cells might recognize, respond to, and have a direct effector function against infected erythrocytes in a manner resembling Vγ9$^+$Vδ2$^+$ cells (11, 33, 36). However, already early on May Ho and colleagues speculated that the expansion of Vδ1$^+$ T cells in *P. falciparum* malaria might instead involve "unidentified host factors" (29). Their prediction is supported by the findings that Vδ1$^+$ cells from parasite-exposed individuals do not respond markedly to *P. falciparum* antigens *in vitro* (34), including the parasite-derived pyrophosphate antigens recognized by Vγ9$^+$Vδ2$^+$ cells (37, 38).

Although it is not known what drives the expansion and differentiation of the adaptive-like Vδ1$^+$ subset in malaria, Vδ1$^+$ T-cell expansion has been observed in several other pathological conditions (16). Examples include infections with human immunodeficiency virus (HIV) (39–42), cytomegalovirus (CMV) and other herpes viruses (43–45), *Onchocerca volvulus* parasites (46), as well as autoimmune diseases such as Takayasu arteritis (47), inflammatory bowel disease and Crohn's disease (48, 49). The possibility that the Vδ1$^+$ T-cell response in these diseases involves recognition of host-encoded components is supported by studies of CMV. In that infection, Vδ2^{neg} T cells display shared reactivity against both CMV-infected target cells and uninfected epithelial cells, consistent with recognition of host-encoded antigens (50). Moreover, endothelial protein C receptor (EPCR) has been identified as an antigenic target for a Vδ2^{neg} γδ TCR expressed by a clonotype heavily expanded after infection with CMV (51), which is known to infect endothelial tissues. T-cell activation was dependent on integration of TCR/EPCR-mediated signals with a TCR-extrinsic "multi-molecular stress signature" induced upon infection of target cells that included CMV-mediated increases in ICAM-1 and LFA-1 expression. Conceivably, this may represent one route for Vδ2^{neg} γδ T-cell recognition of "stressed self." It may be of interest in the context of malaria that EPCR has been identified as a clinically important receptor for *P. falciparum*-infected erythrocytes (52, 53).

Dysregulation of the B-cell compartment might constitute another pathogen-induced change that could be sensed by "adaptive-like" γδ T cells. Of relevance, *P. falciparum* malaria, and indeed a number of other diseases associated with Vδ1$^+$ T-cell expansions, is characterized by massive B-cell activation, both of B cells that are specific for the infection causing the disease and B cells that are not (54, 55). This often leads to reactivation of latent EBV (and CMV) infection, and further B-cell proliferation (56–58). From this perspective, it is tempting to speculate that the selective expansion of Vδ1$^+$ T cells observed in individuals living in areas with stable transmission of *P. falciparum* occurs in response to antigens expressed by activated B cells, perhaps serving as part of an auto-regulatory response to curb excessive B-cell activation and proliferation. In addition, Vδ1$^+$ cells can recognize EBV-transformed B-cell lines (59, 60), and EBV infection can result in expansion of clonally restricted Vδ1$^+$ cell populations after stem cell transplantation (61, 62). Conceivably, CD1c/TCT.1/Blast-1 might be an antigen recognized by these cells. Thus, CD8$^+$ Vδ1$^+$ cells heavily expanded *in vitro* have been shown to recognize this antigen (63, 64), which is expressed/upregulated on some activated and transformed B cells (65, 66). This is not least the case in the spleen, where Vδ1$^+$ cells are also abundant (67), and further increase in numbers in response to *P. falciparum* malaria (68). In addition, Vδ1$^+$ T-cell reactivity to CD1c tetramers has been demonstrated (69), although to date only involving a low percentage of the Vδ1 T-cell repertoire. It therefore remains unclear whether CD1c-specific cells overlap with *in vivo* expanded clonotypes (21). In summary, while other possibilities cannot be discounted, responses to "stressed self" via recognition of host antigens may contribute to Vδ1-mediated adaptive surveillance in the context of malaria, which could be linked to immune, stress-linked, or EBV/CMV-related sequelae of parasite infection. Such adaptive surveillance of stressed self has strong relevance for the proposed role of Vδ1$^+$ T cells in cancer (16, 70–72).

CONCLUDING REMARKS

There is an increasing interest in the role of γδ T cells and other similar cells, such as NK cells, in the immune response to malaria (11, 73, 74). However, the Vδ1$^+$ subset has attracted only limited attention so far. Based on the ideas and studies highlighted in this review, we believe that there is a strong case for extending the focus of γδ T-cell studies in malaria beyond the innate-like Vγ9$^+$Vδ2$^+$ subset, to include adaptive-like γδ T cells. Although, we have focused here on Vδ1$^+$ T cells, it is worth noting that clonal expansion of γδ T cells that express Vδ2 chains paired with γ-chains other than Vγ9 has been described in a variety of conditions. Those cell populations also appear to display an "adaptive-like" immunobiology, positioning them functionally much closer to Vδ1$^+$ cells than to the innate-like Vγ9$^+$Vδ2$^+$ cell subset [reviewed in Davey et al. (17)]. Moreover, recent data further suggest that γδ T cells that express neither Vδ1 nor Vδ2 (e.g., Vδ3$^+$ cells) exhibit features of such adaptive immune subsets (13, 75). In light of this emerging adaptive immunobiological human γδ T-cell paradigm, examining the contributions of γδ T-cell subsets other than Vγ9$^+$Vδ2$^+$ in the immune response to malaria is an underexplored and important avenue for investigation.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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OPINION
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Bordeaux 2018: Wine, Cheese, and γδ T Cells

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INTRODUCTION

The 8th "International conference" was held at the University of Bordeaux, 7–10 June 2018. The lead organizer was Julie Déchanet-Merville who together with the other members of the organizing committee; Maria Mamani Matsuda, Matthias Eberl, Myriam Capone, Lionel Couzi, Hannah Kaminski, Layal Massara, Pierre Merville, Sonia Netzer, Angela Pappalardo, Charlotte Domblides, and Vincent Pitard, provided an interesting and exciting programme covering recent progress and developments in all aspects of γδ T cell research. The conference had a very good attendance with 294 delegates from 28 countries on the five continents (**Figure 1**). In total, 160 abstracts were submitted; resulting in 57 oral presentations and 103 poster presentations. In addition, eight "special lectures" were given after invitation. A "best poster" and "oral presentations" competition was held, covering all sessions, resulting in 10 and 5 awards, respectively (funded by AAI). Thirty-six travel awards were provided for students and post-docs by generous donations from NIH, European Federation of Immunological Societies and Gamma Delta Therapeutics Inc.

The conference presentations were divided into "basic" and "applied/clinical" sessions. Below we provide a snapshot of the progress we mainly heard about from the oral presentations during these 4 days.

γδ TCR REPERTOIRES AND SPECIFICITIES

Alongside the need for new γδ TCR ligands to be identified, the requirement to align ligand specificities of individual γδ TCRs with properties of the overall γδ TCR repertoire was discussed in this session.

Erin Adams (University of Chicago, USA) highlighted some of the distinct and unconventional features of current structurally defined γδ TCR/ligand interactions (1–3) relative to αβ TCRs, including a dominance of the genetically diverse TCRδ CDR3 region in binding to the T10/T22 ligand.

Sarina Ravens (Prinz laboratory, Hannover Medical School, Germany) presented an analysis of the TRD repertoire in children from Europe or Africa, and highlighted relatively slow changes in diversity over the first years of life, including over time periods where relevant challenges such as CMV infection (4) likely took place, or after measles vaccination. A discussion point was whether these kinetics reflected differential regulation of such responses in early childhood.

Ben Willcox (University of Birmingham, UK) summarized an analysis of the human liver γδ T cell compartment (5) which highlighted high levels of clonal focussing consistent with an

effector cell status, and also revealed a functionally specialized subset of hepatic γδ T cells that are liver-resident and clonotypically distinct from those in blood.

Juan Carlos Zúñiga-Pflücker (University of Toronto, Canada) exploited the *in vitro* OP9DL system to generate from haematopoietic stem cells γδ T cells that were specific for the melanoma-associated antigens MART-1 and gp100. Although relevant γδ TCRs exhibited MHC-restricted recognition, a point of discussion was whether such specificities would be induced *in vivo* during melanoma (6).

Martin Davey (Willcox laboratory, University of Birmingham, UK) presented an analysis of human $V\delta2^+$ T cells (7) highlighting an unrecognized adaptive subset comprising $V\gamma9^-V\delta2^+$ T cells. This population is not phosphoantigen reactive, and instead features a TCR-diverse naive repertoire that becomes greatly focussed after CMV infection, alongside differentiation to effector status, similar to $V\delta1^+$ T cells (8).

Alina Fichtner (Herrmann laboratory, University of Wurzburg, Germany) presented results on γδ T cells from Alpaca, the first non-primate species shown to possess phosphoantigen- reactive T cells. While other placental mammals share some elements of the phosphoantigen- sensing pathway, alpacas have VγJγP and Vδ2 gene segments, and a single BTN3 molecule that includes a B30.2 domain capable of interacting with IPP/HMBPP. This system may prove useful to help define the minimal requirements for BTN3-mediated phosphoantigen recognition.

γδ T CELL REGULATION BY BUTYROPHILINS

Recently, the butyrophilin (Btnl/BTN) family of genes has been shown to regulate the differentiation and activation of γδ T cells (9, 10); however, the underlying mechanisms by which these molecules are activated and influence TCR signaling remains unclear.

Anna Vyborova from Zsolt Sebestyén's laboratory (Utrecht, The Netherlands) generated Vγ9Vδ2 multimers and showed that a Vγ9Vδ2 TCR induces T cell activation through a multistep activation model. The TCR first exhibits a scanning mode that can be enhanced by pamidronate, followed by a recognition mode dependent on CDR3-mediated affinity and the detection of microclusters of BTN3A1 on the cell membrane through Vγ9Vδ2 TCR.

In support of the inside-out model of butyrophilin signaling in response to phosphoantigen, Lola Boutin from Emmanuel Scotet's laboratory (Nantes, France) showed that mutation of the juxtamembrane domain of BTN3A1 can modulate Vγ9Vδ2 T cell activity. This suggests that additional domains outside of the B30.2 region are critical for molecular interactions. Moreover, the formation of BTN3A1/3 heterodimers depends on the context of antigen activation, and that treatment with statins could abrogate these interactions.

A series of reports from Adrian Hayday's laboratory (London, UK) addressed the role of TCR/butyrophilin signaling in epithelial γδ T cell populations.

First, Duncan Mckenzie showed that the expression of Skint1 in keratinocytes focuses TCR expression to the tips of Vγ5Vδ1 dendrites. Loss of Skint1 expression in response to epithelial stress coincides with the dissociation of T cell-keratinocyte contacts. Although Vγ5Vδ1 TCR signal strength was increased following epithelial stress, the loss of cellular interaction alone was not sufficient to activate the γδ T cells, suggesting that a tonic signal maintains TCR activation and a secondary signal is required for a stress-induced response.

Daisy Melandri reported that the ability of intestinal intraepithelial lymphocytes to respond to Btnl1 and Btnl6 is a property of the Vγ7 chain. Further, the interaction with butyrophilins was mediated by the HV4 region of the TCR, and not the CDRs, suggesting that an innate non-clonal region of the Vγ chain regulates responsiveness to these molecules (11).

Pierre Vantourout showed that similar specificity exists among human colonic γδ IELs in which Vγ4, but not Vγ2 IELs were responsive to BTNL3 and BTNL8. *In silico* modeling showed that Vγ4Vδ1 TCR likely directly interacts with BTNL3, and that this binding interaction is distinct from that observed between TCR and CD1d. Together, these reports presented a model in which innate-like TCR reactivity results in the generation of a tonic signal that is required for IEL tissue residence.

Robin Dart presented clinical applications of these findings showing that co-culture of BTNL3 and BTNL8-expressing HEK293T cells with colonic γδ T cells isolated from healthy patients induced TCR downregulation. However, BTNL-dependent TCR downregulation was severely attenuated in Vγ2/3/4 cells isolated from patients with inflammatory bowel disease (IBD), which could be recapitulated following culture of Vγ2/3/4 from non-IBD controls with pro-inflammatory cytokines. Identification of a BTNL3/8 polymorphism that fails to regulate Vγ2/3/4 cells revealed that patients homozygous for this mutation exhibited a higher incidence of ileocecal disease, suggesting that BTNL-γδ dysregulation may predispose individuals to develop IBD.

γδ T CELL ACTIVATION, REGULATION AND FUNCTION

This third session was introduced by David Vermijlen (Brussels, Belgium) who provided a complete overview about the diversity of γδ T cell repertoire in human, as previously presented by Martin Davey (7) and Sarina Ravens (4). Beyond the periphery, David presented provocative new data from RNA sequencing of fetal vs. post-natal thymic γδ T cells, showing that innate γδ T cells are functionally programmed in the fetal—but not post-natal—thymus.

Dmitry Ushakov from Adrian Hayday's laboratory (London, UK) presented a 3i Immunophenotyping analysis of single cell confocal images of T cells and Langerhans cells in the epidermis. In data gathered from over 3,000 individual mice from >550 knockouts, 24 knockouts showed a γδ T-cell-specific phenotype, 20 of these were specific to the skin, and 8 of those were specific to the epidermis.

Weili Xu from Anis Larbi's laboratory (Singapore) showed that in response to aging and cytomegalovirus history, human Vδ2$^+$ T cells are more protected from cellular senescence compared to all other αβ and γδ T cells.

Benjamin Gully from Jamie Rossjohn's group (Melbourne, Australia) identified the N-terminal scavenger receptor cysteine rich (SRCR) domain structure of the cell surface co-receptor on bovine γδ T cells, Workshop cluster-1 (WC-1), which allowed insight into potential antigen binding surfaces.

Mathilde Raverdeau from Lydia Lynch's laboratory (Dublin, Ireland) reported that CD27$^-$ and CD27$^+$ γδ T cells exhibit fundamental differences in their metabolic profiles at steady state. Whereas, IL-17$^+$ γδ T cells primarily generate energy through oxidative phosphorylation, those producing IFNγ$^+$ use a glycolytic pathway.

Jonathan Boyson (Burlington, VT) showed that heterogeneous expression of Slam receptors mark functional γδ T cell subsets, with SLAMf1 and SLAMf6 expression being associated with IL-17$^+$ and IFNγ$^+$ γδ T cell populations, respectively. These Slam receptor profiles were established during thymic development, and global deletion of the SLAM adapter protein (SAP) resulted in a loss of thymic RORγt$^+$ γδ T cells thus reducing the SLAMf1 IL-17$^+$ γδ T cell population.

Tiago Amado from Bruno Silva-Santos's group (Lisbon, Portugal) reported that microR146a was upregulated in CD27$^-$ γδ T cells producing IL-17$^+$ and functioned to restrict IFNγ production by targeting Nod1 mRNA (12).

γδ T CELL HOMING IN TISSUES

Increasing evidence over the last several years has revealed the importance of γδ T cells in promoting tissue homeostasis. Identification of crosstalk between γδ T cells and other cells (epithelial, stromal and myeloid) within the tissue microenvironment has shed light on new functional roles for specific γδ T cell subsets under steady-state conditions and expanded our view of how these cells are primed to respond to local infection.

Providing novel insight into the functional role of γδ IELs in the intestine, Karen Edelblum (Newark, USA) showed that Vγ7 IELs were required for shedding of apoptotic intestinal epithelial cells at the villus tip under pathological conditions such as systemic LPS exposure. Further, intravital microscopy showed that this subset of IELs directly interacts nearly half of shedding enterocytes immediately prior to their expulsion into the lumen.

Multiple reports highlighted novel molecular regulators of Vγ6Vδ1 T cell plasticity across a broad range of tissues. Darshana Kadekar from Vasileios Bekiaris's laboratory (Copenhagen, Denmark) reported the identification of a novel intestine-specific RORγT$^+$ Tbet$^+$ γδ T cell population. Initially, RORγT$^+$ γδ T cells populate the neonatal intestine and then upregulate Tbet expression during the first week of life through a STAT5-dependent mechanism. Within the ileal and colonic lamina propria, these double-positive cells co-produce IL-17, IL-22, and IFN-γ.

Claire McIntyre from Vicky Morrison's laboratory (Glasgow, UK) showed that loss of β2 integrin (CD18) expression resulted in a 10-fold expansion of γδ T cells in the lung, spleen, blood, and uterus under steady-state conditions. This substantial increase in γδ T cell number was specific to IL-17-producing Vγ6Vδ1 T cells due to an absence of CD11a ($\alpha_L\beta_2$), indicating that β2 integrin expression negatively regulates this subset of γδ T cells.

Lydia Lynch (Dublin, Ireland) showed that PLZF$^+$ Vγ6Vδ1 T cells produce both TNF and IL-17A in adipose tissue (13). Crosstalk between these innate PLZF$^+$ γδ T cells and adipose stromal cells regulates endogenous IL-33 production to maintain core body temperature. Mice deficient in γδ T cells fail to appropriately thermoregulate in response to cold challenge, which may be due to an inability to induce IL-17-mediated brown fat activation necessary for thermogenesis.

Besides fat, Vγ6Vδ1 T cells were also found in the female reproductive tract. Leticia Monin from Adrian Hayday's laboratory (London, UK) reported that uterine γδ T cell compartment is more abundant in young mice <5 weeks of age. These Vγ6Vδ1 T cells secrete IL-17A, IFNγ- or both cytokines within the uterine stroma. Further, uterine γδ T cells confer protection against Candida infection through the recruitment of neutrophils, which is lost in γδ T-cell-deficient mice.

Vγ6$^+$ IL-17-producing T cells were also reported to infiltrate the stromal tissue of the testes by Julie Ribot (Lisbon, Portugal). During puberty, expansion of this γδ17 population was mediated by androgen-driven changes in the gut microbiome and myeloid cell IL-23 and IL-1 expression downstream of TLR4. Intra-testicular infection with *Listeria monocytogenes* was more severe and resulted in increased lethality of γδ T cell- or IL-17-deficient mice, indicating that testicular IL-17 producing γδ T cells are critical for limiting local bacterial infection.

γδ T CELL EVOLUTION AND DEVELOPMENT

The session opened with an overview of murine γδ T cell development from Daniel Pennington (London, UK). This introduced a consensus view of the stages of γδ T cell development and focused on the factors that affect thymic commitment to subsequent effector fates (i.e., to become γδ17 or γδIFN cells). The idea that γδ17 cells may not be generated from a common αβ/γδ progenitor in the thymus was discussed, and consistent with peripheral data presented earlier in the day by Mathilde Raverdeau from Lydia Lynch's laboratory (Dublin, Ireland), IL-17-, and IFN-γ- producing γδ T cells were revealed to adopt profoundly different metabolic programs at the very earliest stages of their development.

The session continued with two interesting comparative immunology presentations that emphasized how a sole focus on human and rodent biology may provide a distorted perspective. Breanna Breaux (Texas, USA) first described the TCRγ and TCRδ loci in the Florida manatee, which belongs to the afrotherians, a clade of eutherian mammals that includes elephants. For the gamma locus, initial data suggest there are many multiclusters with repeated Vγ and Jγ segments (i.e., high segmental diversity

FIGURE 1 | Group picture of the participants during the 8th International Gamma-Delta T Cell Conference in the Agora, Domaine du Haut-Carré, Bordeaux, France.

compared with human and mouse). By contrast, the delta locus has restricted combinatorial diversity as only one Dδ and Jδ segment was identified. Nonetheless the CDR3δ region was of a comparable length to those seen in other species. A VHδ segment, that has been observed in frogs, birds, and monotremes, was also identified (a first in a eutherian species).

In the next presentation, Rob Miller (Albuquerque, USA) continued the comparative biology theme by discussing the fifth TCR chain (TCRμ) in non-eutherian mammals (marsupials and monotremes). TCRμ pairs with TCRγ and contains a second Vμ domain (i.e., a third extracellular domain) similar to the VNAR domain that is observed in cartilaginous fish. "γμ cells" are transcriptionally distinct from both αβ T cells and γδ T cells in the opossum and represent ∼10% of the T cells in the spleen. The presence of a TCRμ locus and VHδ regions provides an interesting evolutionary perspective to the origin of the TCR loci.

After the brief journey into comparative immunology, Apostol Apostolov (Lyon, France) returned to the session's general theme of γδ T cell development, describing a CD4 fate mapping approach that identified a $CD4^+$ bone marrow precursor that could give rise to various subsets of murine γδ T cells.

Juliette Roels (Ghent, Belgium) from Tom Taghon's laboratory followed this by describing an RNA deep sequencing approach on ten human thymocyte subsets that represent various stages of αβ and γδ T cell development. Interesting observations on human vs. mouse T cell development were highlighted; proliferation was largely conserved yet regulation of preTCR components appeared different. γδ T cell biased genes were enriched in NK and CD8 T cell-associated signatures. Moreover, expression of RORγt, c-Maf, and Sox13 were all evident, despite the relative lack of γδ17 T cell development in human.

The next presentation by Sagar (Freiburg, Germany) returned to murine γδ T cell development, this time using a single-cell RNA sequencing approach. The study identified a TCR signal strength-related propensity to develop as either γδ17 or γδIFN cells in the $CD25^+$ progenitor subset. It also identified c-Maf as a key regulator of the γδ17 program with Sox-13/c-Maf/RORγt sequential gene expression. Indeed, c-Maf KO mice lacked γδ17 cells and γδ progenitors in these animals appeared to have an increased signal strength gene profile. Finally, c-Maf KO mice were unsurprisingly protected from γδ17-driven immunopathology.

The c-Maf theme was continued by Maria Ciofani (Durham, USA). Deletion (via IL-7-Cre) of c-Maf in all lymphoid progenitors completely abrogated γδ17 cell development, and expression of genes associated with a γδ17 program (e.g., RORγt and Blk) was lost. Deletion of c-Maf in γδ17 cells (via RORγt-Cre) also demonstrated a requirement for c-Maf to maintain the γδ17 cell program. c-Maf expression levels were inversely proportional to signal strength from various transduced γδTCRs (e.g., KN6) and appears to antagonize Tcf-1 function that is a negative regulator of γδ17 cell development (14).

The session ended with Paola Tieppo (Brussels, Belgium) as we again switched to human γδ T cell development. Notably, human fetal γδ T cells are enriched for $V\delta2^+$ cells (unlike post-natal γδ T cell populations) that use invariant, public sequences. The generation of these early invariant γδ T cells appears to be dependent on a specific fetal precursor. Interestingly, the

transfer of an unidentified RNA binding protein to post-natal hematopoietic progenitors converts them to a fetal mode in which they increase generation of invariant Vδ2$^+$ cells.

NEW CONCEPTS IN IMMUNOLOGY

The evening session was an entertaining departure from the "conventional" session format. The first speaker Thomas Pradeu (Bordeaux, France) adopted an intentionally philosophical approach to understanding the key concepts that underpin immune responses. He introduced the idea of a "discontinuity" theory in which the immune system has evolved to recognize changes from a "normal" state, mainly reacting to changes rather than status quo presence of antigens. Thomas suggested that this theory had advantages over models that suggested danger as a key initiator of immune responses, as it explained scenarios in which danger had not really been present.

This was followed by an equally provocative talk from Adrian Hayday (London, UK). Adrian used the opportunity to discuss (γδ T cell-driven) tissue immunosurveillance in the context of a "validation" theory. Adrian argued that for conventional αβ T cell responses the sensor (i.e., the TCR) crucially requires a validation signal (e.g., B7-mediated) before cells are activated. γδ T cells lack CD28, so what replaces the B7/CD28 validation axis? Skint1 and the BTNL family of genes are related to B7 and have recently been shown to be key TCR-binding regulators of specific tissue resident γδ T cell subsets. Interestingly, there is now evidence that these B7-like molecules may interact with the TCRγ chain in a non-CDR-mediated fashion. Adrian left the audience to consider whether tissue resident T cells required that sensor input via conventional γδTCR-CDR/ligand interactions must be validated by tissue stress surveillance (i.e., is the tissue "normal"?), also through the TCR, but by this intriguing family of B7-like molecules.

γδ T CELL FUNCTION IN INFECTION AND INFLAMMATION

Besides recent studies on the mechanisms required for steady state γδ T cell homing and homeostasis in tissues where they can contribute to local physiology, a series of presentations has focused on their functions in a pathogenic context. While their protective role against infection has been widely recognized in different mouse models and human diseases, γδ T cells can also be associated with deleterious outcomes, by exacerbating the inflammatory response.

The session was introduced by Willi Born, who gave a brilliant overview of the research program he has been leading for the past 30 years, together with his colleague Rebecca O'Brien. He mainly focused on the atypical regulatory role of IL-4 producing Vγ1$^+$ γδ T cells (15) and its impact on B cell development, activation and IgE production (16, 17). On behalf on the γδ T cell community, we would like to warmly acknowledge Willi and Rebecca for their important contribution to the field and wish them a great and well-deserved retirement.

Christophe Paget (Tours, France) reported a key protective cross talk in the lung between IL-1β neutrophils and IL-17-producing Vγ6 T cells in a model of invasive pneumococcal infection. This process was mediated by the neutrophilic NLRP3 inflammasome activated by macrophage-derived TNF-α bacterial pneumolysin (18).

Johnny Guo from Paul Thomas' laboratory (Memphis, USA) demonstrated a critical role for IL-17 producing γδ T cells upon neonatal influenza infection that enhanced the production of IL-33 in lung epithelial cells. This important cross talk was shown to promote a protective type 2 response, namely by inducing the secretion of amphiregulin by Tregs and ILC2s. Importantly, this mechanism may also exist in human, as suggested by the positive correlation observed between IL-17 and IL-33 in the nasal fluid of infected children.

Besides IL-17, Murad Mamedov from Mark Davis' laboratory (Stanford, USA) identified Macrophage-Colony-Stimulating-Factor (M-CSF) as a novel protective cytokine produced by an oligoclonal Vγ1Vδ6.3+ γδ T cell subset in a mouse model of malaria infection. Interestingly, γδ T cells expanded rapidly after resolution of acute parasitemia, in contrast to αβ T cells that expanded at the acute stage and then declined. This dynamic was also observed in *Plasmodium falciparum* infected subjects, suggestive of a key mechanism that could be targeted for the prevention of malarial recurrence in humans (19).

By analyzing cord blood samples from neonates of women infected with placental malaria, Cristiana Cairo (Baltimore, USA) showed that phosphoantigens released by *Plasmodium falciparum* during placental sequestration primed fetal Vδ2$^+$ γδ T cells, altering their phenotype (increased PD-1 expression) and function (reduced cytotoxic potential).

Further advances in our understanding of the anti-infectious function of γδ T cells have been made by the team of Zheng Chen (Chicago, USA) through studies in a macaque model of tuberculosis (20). In this congress, he presented a vaccination strategy based on phosphoantigen HMBPP-producing attenuated Listeria vector, which targets specifically the Vγ9Vδ2 T cell subset, therefore mounting efficient memory-like responses that reduce pulmonary bacterial burden after challenge.

Allen Cheung (Hong Kong) reported the presence of a novel population of Vδ2$^+$ γδ T cells that accumulate in the gut of HIV patients upon acute infection. These cells were characterized by the constitutive expression of Δ42PD1 (a PD-1 isoform) and tissue homing receptors such as CCR9 and CD103. Further *in vivo* experiments in humanized mice showed that Δ42PD1 interacted with TLR4 to promote innate immune activation and intestinal pathogenesis, thus highlighting a novel mechanism of mucosal inflammation.

Anne Hahn from Thomas Winkler's group (Erlanger, Germany) monitored the TCR repertoire of γδ T cells in different tissues along the timecourse of murine Cytomegalovirus (mCMV) infection. Using a Nur77-GFP reporter assay for TCR activation, she screened for γδ T cell clones that recognize mCMV infected target cells. This work may shed light on the nature of specific Ag for γδ T cells by identifying novel TCR ligands in mice, following up on the work in human previously

reported by the groups of Ben Willcox and Julie Déchanet-Merville (21, 22).

Hannah Kaminski from Julie Déchanet-Merville's laboratory (Bordeaux, France) described a paradoxical effect of the mTOR pathway on effector T cell functions. While it is used as an immunosuppressive drug in transplantation, mTOR inhibitors (mTORi) were associated with less CMV infections in transplanted patients. She showed that mTORi increases Vδ2- T cell *in vitro* expansion and IFN-γ production, as confirmed by proteomic analysis of purified Vδ2- γδ T cells from mTORi-treated patients. Mechanistically, she suggested that mTORi could inhibit mTORC1 while inducing a negative feedback increase of AKT phosphorylation, leading to phosphorylation of S6, and expression of T bet. This study parallels previous data from the group of David Pauza on the Vδ2$^+$ γδ T cell counterpart (23).

Simone Cuff from Matthias Eberl's laboratory (Cardiff, UK) presented a novel diagnosis strategy based on algorithms that rely on the analysis of local immune fingerprints from patients with acute peritonitis. She showed that incorporating the Vγ9Vδ2 T cell response into machine learning models is key to the production of an accurate immune profile of peritonitis and in particular, to immune profiles associated with specific pathogens.

In addition to their relevance against infections, the three following oral communications focused on the role of γδ T cells in the pathogenesis of autoimmune and inflammatory diseases.

Inga Sandrock from Immo Prinz's laboratory (Hannover, Germany) presented a new knock-in mouse line (Tcrd-GFP-DTR Luciferase mice), in which γδ T cells can be conditionally depleted with diphtheria toxin (24). She showed that acute depletion of γδ T cells in these mice results in protection from IMQ-induced psoriasis and from spondyloarthritis-resembling inflammation induced by *in vivo* overexpression of IL-23. This mouse model revealed compensatory mechanisms for IL-17 production normally mediated by αβ T cells and ILC3 in the constitutive γδ T-cell-deficient mice. Of note, ILC3 compensated for IL-17 production 9 weeks after γδ T cell depletion induced upon diphtheria toxin injection.

Julie Jameson (San Marcos, USA) showed that obesity impaired γδ T cell persistence in the gut, by downregulating adhesion molecules and chemokine receptors (CD103, CCR9). The remaining intestinal γδ T cell functions were dysregulated, as obese mice were more susceptible to DSS-induced severe colitis. Importantly, the process was reversible upon a 7-week administration of a diet inducing weight loss.

Following up on his previous investigation (25), Jun Yan (Louisville, USA) reported a critical role of the IL-1-IL-1R signaling in psoriasis pathogenesis (26). IL-1β induces dermal γδ T cell proliferation and IL-17 production via the IL-1R-MyD88-mTOR signaling pathway. IL-1β also stimulated keratinocytes to secrete chemokines such as CCL20, which chemoattract peripheral CCR6$^+$ IL-17$^+$ γδ T cells. Interestingly, endogenous IL-1β secretion was regulated by the skin microbiota to maintain dermal IL-17$^+$ γδ T homeostasis. The transfer of Corynebacterium isolated from human psoriatic skin on naïve mouse skin significantly stimulated IL-1β production, leading to dermal IL-17$^+$ γδ T cell expansion and psoriatic lesions.

γδ T CELL FUNCTION IN CANCER

In addition to their role in infection and inflammation, γδ T cells are widely recognize to display key anti-tumor activities through their IFN-γ production and potent cytotoxicity. Notwithstanding, recent data have now highlighted unexpected pro-tumoral functions linked to IL-17-producing γδ T cells. Thus, further studies are required for a better understanding of the potential of γδ T cell modulation in cancer immunotherapy.

Sofia Mensurado from Bruno Silva-Santos' group (Lisbon, Portugal) identified suppressive tumor-associated neutrophils that specifically inhibited the proliferation of pro-tumoral IL-17 producing γδ T cells in mouse. By expressing low levels of the antioxidant glutathione, this subset was shown to be particularly sensitive to neutrophils-derived-reactive oxygen species. Interestingly, she suggested that these findings could be applied to human, as Vδ1$^+$ γδ T cells, which contain most IL-17 producing γδ T cells found in cancer patients, also displayed low glutathione levels (27).

José Villacorta Hidalgo from Paul Fisch's laboratory (Freiburg, Germany) analyzed the distribution of γδ T cells infiltrating sentinel lymph nodes in human triple negative breast cancer and suggested that high endothelial venules may be critical to regulate γδ cell entry from the blood into the tumor.

Daniela Wesch from Dieter Kabelitz (Kiel, Germany) demonstrated a critical immunosuppressive role for β-galactoside-binding protein galectin-3 in pancreatic ductal adenocarcinoma and ovarian cancer. Galectin 3 is released by tumor cells and interacts with α3β1 integrin expressed by Vδ2$^+$ γδ T cells. While it did not impact on cell-cytotoxicity or survival, galectin 3 clearly impaired Vδ2$^+$ γδ T cell-proliferation.

Following up previous work on the identification of NK receptors expression by anti-tumoural Vδ1$^+$ γδ T cells (28), Elena Bruni from Domenico Marvilio's laboratory (Milan, Italy) showed that the expression of NKp46 was restricted to Vδ1$^+$ (but not Vδ2$^+$) γδ IELs and associated with an increased production of granzyme B and IFN-γ. NKp46$^+$ phenotype is a feature of IL2/IL15-induced human infant thymic γδ T precursors (29) and correlates with significantly lower tumor progression in CRC patients.

Finally, Jean Jacques Fournié (Toulouse, France) presented recent computational methods that perform cell type-specific quantifications from the transcriptomic analysis of tissue samples. Using CIBERSORT, an algorithm that allows the deconvolution of bulk tumor transcriptomes to find tumor infiltrating lymphocytes (TILs), previous studies have identified γδ TILs as the most significant favorable cancer prognostic cell population (30). By implementing machine learning from purified γδ T cell microarray data, Jean Jacques Fournié reported an updated improved version of CIBERSORT that enumerate and characterize Vγ9Vδ2 γδ TILs in 10,000 cancer biopsies from 50 types of hematological and solid malignancies (31). Jean-Jacques also presented new data on γδ T cell single cell RNA sequencing that define a specific γδ T cell signature enabling characterization of these cells in complex tissues RNA sequencing analyses. This new tool will help pave the way for critical findings that will be highly relevant for immunotherapy.

γδ T CELLS IN IMMUNOTHERAPY

γδ T cells are now fully appreciated as being functionally profoundly different from their αβ T cell counterparts. The increased understanding of their basic biology, as evidenced in other sessions in this conference, has meant that increasing numbers of research groups as well as clinical centers are now considering their use in cancer immunotherapy. In addition, the pharmaceutical and biotech industry are now actively entering this new area of immunotherapy. A number of encouraging oral as well as poster reports during this conference highlighted the considerable progress made in this area since the last γδ T cell conference. They all helped to form the strong impression that γδ T cells will become a very important addition to current immunotherapy strategies, at a minimum, and quite possibly a profound improvement at best—some might go as far as to say, a revolution of future immunotherapy strategies directed at (and possibly beyond) cancer.

Marta Barisa (from UCL Institute of Child Health, London, UK) reported on a new generation of Chimeric Antigen Receptor (CAR) constructs designed to suit γδ T cells. Whilst CARs expressed in αβ T cells provide "signal 1" for activation through the use of endodomains for that purpose, this γδ T cell-specific strategy makes use of the ability of the γδ TCR to provide "signal 1" through "stress" recognition of tumor cell targets. The γδ T cell CAR is instead engineered to provide a co-stimulatory "signal 2" following recognition of a molecular target on a cancer cell. This strategy arguably provides two distinct advantages: firstly, the well-known cancer cell ability to down-regulate molecules that are able to provide natural "signal 2" stimulation (such as MICA/B) is replaced by a "therapeutic signal 2," and secondly, it avoids on-target, off-tumor activation which is a well-recognized problem in current αβ T cell-based CAR treatment protocols.

Trudy Straetemans presented further results from the Jürgen Kuball group (Utrecht, Netherlands) on their strategy to confer the ability of γδ T cells to recognize "stressed" tumor cells on αβ T cells by expressing a defined γδ TCR in addition to their endogenous αβ TCR (TEGs). They selected a particular γ9δ2 TCR (TEG001) for testing of transduced αβ T cells in a 3D model consisting of multiple myeloma cells within the context of a humanized bone marrow stromal niche. The data presented showed that the tumor cells, but not the stromal cells, were specifically targeted in association with cytokine production. The findings demonstrate the potential clinical utility of this strategy.

B-cell malignancies are able to inhibit Vγ9Vδ2 γδ T cell anti-tumor activities. In her presentation, Barbara Castella (Massimo Massaia, Turin, Italy) showed that several functional impairments contribute to this inhibition of Vγ9Vδ2 γδ T cell reactivities. This includes multifaceted immune check-point expression in the tumor microenvironment. By combining PD-1 and TIM-3 blockade the ability of Vγ9Vδ2 γδ T cells to proliferate was restored. This type of work can be predicted to improve the efficacy of γδ T cell therapies against multiple myeloma as well as other malignancies.

Zhinan Yin (Guangzhou, China) presented some intriguing and hopeful results from *in vitro* expansions and clinical trials using allogeneic Vγ9Vδ2 γδ T cells. *Ex vivo* expansions followed by adoptive transfer of autologous Vγ9Vδ2 γδ T cells can be problematic as these are often impaired in various pathologies. Following an improved *ex vivo* expansion protocol, the allogeneic Vγ9Vδ2 γδ T cells were adoptively transferred to 80 breast cancer patients. Preliminary results indicated that this allogeneic cell transfer was safe, cancer progression slowed down, survival increased and immune function was improved in a majority of the patients, highlighting that the use of allogeneic γδ T cells in cancer immunotherapy constitutes a viable and very welcome alternative to autologous therapies.

Anne-Charlotte Le Floch (Daniel Olive, Marseille, France) reported on further use of their agonist antibody against the Vγ9Vδ2 TCR candidate ligand BTN3A (20.1). They have found that its use increases the cytotoxicity of Vγ9Vδ2 T cells against acute myeloid leukemia cells. They showed that the main mechanism appears to be increased degranulation and expression of DNAM-1 by/on Vγ9Vδ2 T cells.

Biagio Di Lorenzo (Bruno Silva-Santos, Lisbon, Portugal) provided new data on their "Delta One T" (DOT) expanded $V\delta1^+$ γδ T cells. The DOT cells were shown to be effective in killing human acute myeloid leukemia cells, including against clones of AML which were chemoresistant. These positive results were also translated into efficient cytotoxicity in a xenogeneic *in vivo* mouse model of AML. These results thus provide a very welcome novel means to potentially improve on the otherwise poor outcomes for AML patients.

Craig Morita (Iowa City, USA) showed data on a combination treatment of human prostate tumors in a xenogeneic mouse model by using Vγ9Vδ2 T cells in combination with PD-1 mAb blockade. The combination treatment reduced tumor burden to nearly to zero after 5 weeks—promising another means by which previously disappointing Vγ9Vδ2 T cell treatments of human tumors can be substantially improved.

In another combination treatment approach, Lawrence Lamb (University of Alabama, Birmingham, USA) demonstrated that using Temozolomide (TMZ) to induce upregulation of NKG2D ligands on human glioma tumors, in a xenogeneic glioma tumor mouse model, drastically increased the efficacy of adoptively transferred GMP-grade γδ T cells efficacy. This study suggests that this combination treatment could now be used in human clinical trials.

Noemie Joalland (Emmanuel Scotet group, Nantes, France) presented striking results from a xenogeneic human/mouse model showing that by combining existing chemotherapeutic and surgical strategies with immunotherapeutic transfer of allogeneic Vγ9Vδ2 γδ T cells and GMP grade aminobiphosphonates can significantly improve the survival of epithelial ovarian carcinoma-carrying animals. The results showed that chemotherapy treatment may not harm the beneficial effects of adoptively transferred γδ T cells.

Hans-Heinrich Oberg (Daniela Wesch, Kiel, Germany) showed data on the use of a tribody directed against the HER2 antigen on cancers of epidermal origin and the CD16 antigen on γδ T cells and NK cells [(HER2)xCD16]. The results from clinical trials in breast cancer, ovarian tumor, and pancreatic cancer

patients showed better results than the use of mAb trastuzumab and the main reason for the promising results was shown to be an increased degranulation of immune cells, presumably γδ T cells and NK cells.

CONCLUDING REMARKS

Bruno Silva-Santos (Lisbon, Portugal) closed the congress leaving us with the meeting's main highlights and future perspectives for γδ T cell research. Within the most significant advances made since the previous γδ T cell conference, the growing evidence that some γδ T cell subsets adopt an adaptive biology and clonally expand in response to pathogen infection was highlighted (4, 5, 7, 8). Also within such advances, Bruno mentioned butyrophilins as molecular mediators of tissue surveillance, namely their role in sensing stress, as well as new determinants of effector γδ T cell differentiation including new transcription factors (14) and the contribution from metabolic pathways. Bruno also underlined novel roles of γδ T cells impacting on tissue physiology at steady state, namely regulating thermogenesis (13) and neuroplasticity (32).

The development of γδ T cell based-therapies are part of the exciting future directions that were mentioned. Besides efforts being pursued against infection and autoimmunity, new clinical trials in cancer immunotherapy will be launched in the coming years. We look forward to hearing about the next discoveries in the field: the 9th γδ T cell conference is scheduled for 2020 in Beijing (China).

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KE and JR wrote the manuscript with the help of KG, DP, and BW.

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