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γδ T cells in physiology and pathology

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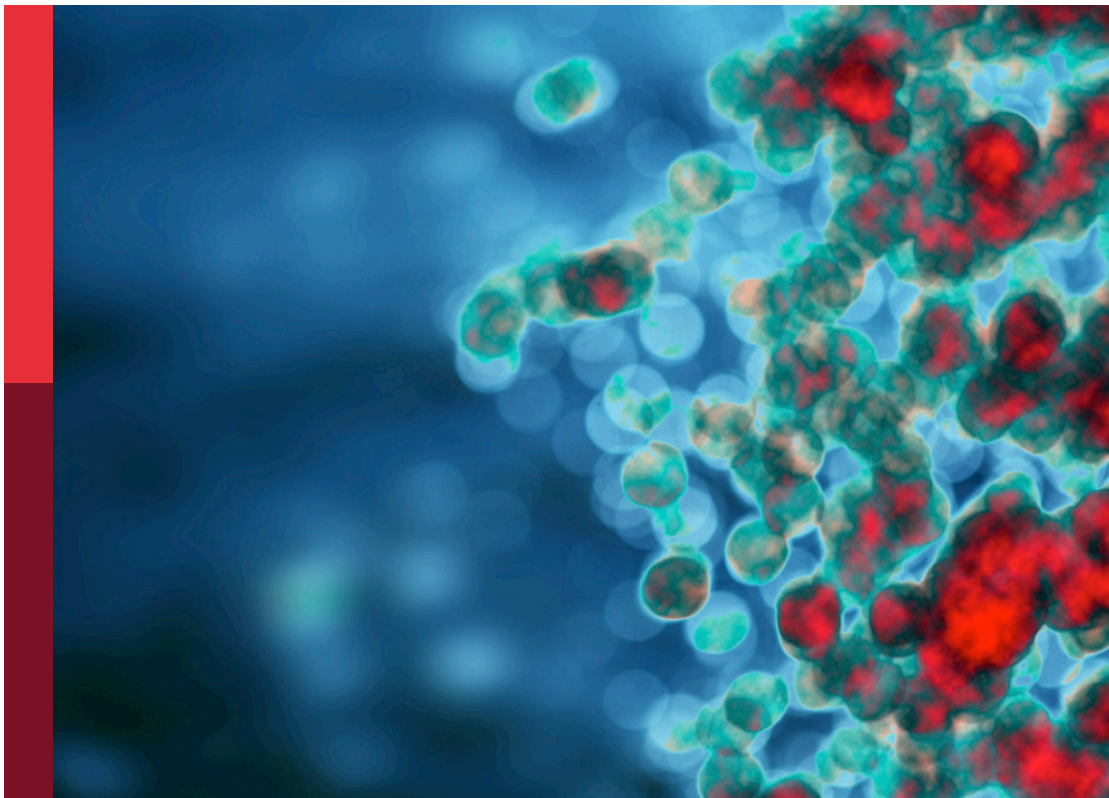

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γδ T cells in physiology and pathology

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PERSPECTIVE
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Cheap and Commonplace: Making the Case for BCG and γδ T Cells in COVID-19

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Antigen-specific vaccines developed for the COVID-19 pandemic demonstrate a remarkable achievement and are currently being used in high income countries with much success. However, new SARS-CoV-2 variants are threatening this success *via* mutations that lessen the efficacy of antigen-specific antibodies. One simple approach to assisting with this issue is focusing on strategies that build on the non-specific protection afforded by the innate immune response. The BCG vaccine has been shown to provide broad protection beyond tuberculosis disease, including against respiratory viruses, and ongoing studies are investigating its efficacy as a tool against SARS-CoV-2. Gamma delta (γδ) T cells, particularly the Vδ2 subtype, undergo rapid expansion after BCG vaccination due to MHC-independent mechanisms. Consequently, γδ T cells can produce diverse defenses against virally infected cells, including direct cytotoxicity, death receptor ligands, and pro-inflammatory cytokines. They can also assist in stimulating the adaptive immune system. BCG is affordable, commonplace and non-specific, and therefore could be a useful tool to initiate innate protection against new SARS-CoV-2 variants. However, considerations must also be made to BCG vaccine supply and the prioritization of countries where it is most needed to combat tuberculosis first and foremost.

Keywords: gamma delta T cell, Bacille Calmette-Guérin vaccine, trained immunity, non-specific immunity, COVID-19, innate immunity, vaccine, antiviral

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INTRODUCTION

In January 2020 the WHO declared Coronavirus disease 19 (COVID-19) a Public Health Emergency of International Concern (PHEIC), and a pandemic in March 2020. As of July 2021 this virus is responsible for nearly four million deaths worldwide (1). COVID-19 represents a broad spectrum of clinical syndromes, from asymptomatic disease, mild flu-like symptoms, to severe pneumonia and acute respiratory distress syndrome (ARDS). Safe and effective vaccines have now been developed to combat COVID-19 spread. However, the highly specific nature of these vaccines leaves them susceptible to escape mutations. This, along with additional concerns around supply, especially in low- and middle-income countries (LMICs), justifies the search for common, affordable and non-specific strategies to be used in combination with specific vaccines or as an

interim measure. Here we make the case for the Bacille Calmette-Guerin (BCG) vaccine and its role in stimulating gamma delta (γδ) T cells, particularly the Vδ2 subset.

The causative agent of COVID-19 is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 is a positive sense single stranded RNA virus, able to spread between humans in close contact, *via* respiratory droplets produced from coughs and sneezes, and probable fomites. The virus is able to enter the respiratory epithelial cells of the oropharynx and upper airway *via* its spike glycoprotein, which targets the angiotensin converting enzyme 2 (ACE2) receptor. Binding causes conformational changes in the spike protein, mediating the fusion of the viral and cell membranes and the release of the viral nucleocapsid into the cell (2). Part of the reason SARS-CoV-2 is more transmissible than SARS-CoV is because of structural differences on its surface proteins that allow stronger binding to the ACE2 receptor (3, 4).

SARS-CoV-2 variants are now being identified that have a multitude of further mutations that allow even stronger binding of the ACE2 receptor, and therefore are spread even more easily. An example of this is the N501Y mutation, present in the Alpha variant, which alters an amino acid within the six key residues in the receptor biding domain of the spike glycoprotein, which has arisen independently in various locations including the UK, South Africa and Australia (5). It has been shown that additional mutations may result in lessened antibody effectiveness (6), and there is growing concern around variants rendering existing vaccines less efficacious. The current principal variant of concern in the UK, the Delta variant, contains mutations in the spike protein, including E484K and L452R, that, in addition to strengthening ACE2 receptor binding, can reduce the ability of vaccine stimulated antibodies to attach to the altered spike protein (7, 8). In light of these concerns, vaccine strategies that are able to offer a broader base of protection, and therefore are more resistant to mutations than single target strategies, could prove an important additional tool in our arsenal against SARS-CoV-2 variants.

Vaccines against SARS-CoV-2 including those manufactured by Pfizer, Moderna and AstraZeneca, are currently being used in wealthy nations with great success. However, with production limited and demand greatly exceeding supply, it may be some years before LMICs are able to complete their own nationwide COVID-19 vaccination programs. This vaccine inequality only enhances opportunities for additional mutations to arise that further reduce vaccine protection. Continued research into additional strategies that could be used in conjunction with SARS-CoV-2 antigen specific vaccines to combat COVID-19 is needed.

BCG is the most widely used vaccine in the world, and in recent years has been used most extensively in LMICs. When it was first introduced to Europe in the 1920s it was observed that vaccination provided non-specific, otherwise known as off-target, protection against a range of diseases, particularly respiratory infections (9). Since the SARS-CoV-2 pandemic there have been many observational studies reporting a level of protection in BCG vaccinated adults and children (10–12). An ecological study found both cases and deaths in countries with national BCG vaccination programs were significantly lower in March 2020 than in countries without (10). Escobar et al., found that with every 10% increase in BCG index (an estimation of vaccination coverage) there was a corresponding 10.4% decrease in COVID-19 deaths (11). Additionally, in Japan, prefectures with higher BCG vaccine coverage had fewer COVID-19 infections (12). However, another study in Sweden looked at people born just before or just after 1975, when universal BCG vaccination ceased, and did not find any statistically significant difference in COVID-19 cases and hospitalizations (13). Twelve randomized control trials (RCTs) studying BCG and COVID-19 are presently underway in various countries, although results from most of these studies are still many months away. However, the findings from one randomized trial are now available in preprint; the ACTIVATE-2 study, which revaccinated elderly Greek patients with BCG, found a reduction in COVID-19 clinical and microbiological diagnoses compared to the placebo group (14).

Recent articles have outlined how BCG is able to reprogram the innate system, resulting in an altered innate immune response to subsequent infections (15). This so-called 'training' of innate immune cells, which includes epigenetic, transcriptional, and functional reprogramming, is thought to be largely responsible for much of the off-target beneficial impact of BCG on non-tuberculosis diseases, including viral diseases. The pathways impacted by trained immunity include those that may be important for the control of COVID-19 disease, as reviewed by others (16–22).

Much is now known about BCG and its 'training' of innate cells, but less is known about the role of γδ T cells in this non-specific action. γδ T cells, of which Vδ1 and Vδ2 cells are the main subtypes in humans, are unconventional T cells that bridge the innate and adaptive immune system. They have been shown to be a significant component of the early innate immune response to many viral infections. Importantly, Vδ2 T cells proliferate rapidly after BCG stimulation, as well as being one of the main producers of IFN-γ in this vaccination response. Studies have also shown they demonstrate recall responses. These long lasting, memory-like responses, which include rapid production of proinflammatory cytokines and cytotoxic granules essential for viral clearance (23), indicate γδ T cells might be a key player in BCG non-specific protection to viruses, including SARS-CoV-2.

THE HETEROGENOUS EFFECTS OF BCG VACCINATION

BCG is an attenuated form of *Mycobacterium bovis* which has been used in humans as a tuberculosis (TB) vaccine since the 1920s. BCG remains to this day a critical component of the strategy to combat TB, with the focus on vaccinating infants shortly after birth in endemic areas. Although there is a high efficacy against childhood TB (24), protection wanes with age,

and the efficacy of adult BCG vaccination varies widely in different studies from 0 – 80% (25). Revaccinating in adolescence has been proposed as one way to boost this protection, with Nemes et al. demonstrating revaccination reduced the rate of sustained QuantiFERON TB Gold InTube (QFT)-conversion, reflecting better bacterial control and clearance (26). The REVAX clinical trial is ongoing to assess whether revaccination of adolescence may be a useful tool for TB control.

After BCG was introduced in the 1920s, epidemiological studies reported that BCG vaccination greatly reduced infant mortality, beyond that which could be explained by a reduction in TB alone (9). These observations were confirmed by RCT studies, including one showing that giving BCG to low birth weight children could reduce mortality by 50% (27). The reduction in mortality was mostly from respiratory infections, which are for the most part viral, and sepsis. Another recent RCT study found BCG can protect the elderly against respiratory infections (14). Observational studies looking at BCG in humans have demonstrated protective roles for BCG in syncytial virus infection (28); respiratory tract infections and pneumonia in older individuals (29, 30); and yellow fever (31). This non-specific protective role in viral infections has also been demonstrated in vaccinated mice, where studies as early as the 1970s showed BCG vaccination reduced influenza virus titer (32), and provided a level of protection against herpes simplex virus (HSV) (33). Another study found that even the administration of just components of the mycobacterial cell wall was enough to provide some protection against vaccinia virus and herpes simplex virus 2 (HSV2) (34). Now studies are showing a similar effect with COVID-19, with one retrospective cohort study finding an association between BCG vaccine in the five years prior and a lower incidence of sickness and extreme fatigue during the COVID-19 pandemic (35). Where BCG can be used on its own to stimulate innate immunity, it has also successfully been used as an adjuvant in more specific vaccine strategies against SAS-CoV-2 infection (36).

The non-specific protection afforded by BCG is often referred to as 'trained immunity'. Although much is still uncertain regarding how this protection comes about, it is now known to involve long-lasting changes in cells of the innate immune system, including monocytes, macrophages, dendritic cells (DCs), mucosal associated invariant T (MAIT) cells, natural killer (NK) cells and γδ T cells. Most innate cells were previously believed to be static and unchanged after encountering stimuli (37), and therefore investigations into trained immunity have resulted in a shift of central immune system dogma. The changes that result in the non-specific protection BCG provides against many viral infections are likely a combination of epigenetic, transcriptional, and functional reprogramming, as well as the induction of memory-like cells (15, 38).

Epigenetic changes after BCG include the upregulation of innate cell transcripts in the bone marrow of hematopoietic cells, as well as inducing greater DNA-accessibility around genes associated with inflammation in existing innate cells (39). Chemical modifications (methylation and acetylation of histones) allow for greater accessibility of chromatin, and easier transcription of genes (40). This results in the rapid and sustained upregulation of antimicrobial responses in innate cells upon subsequent infection, of which monocytes and NK cells are the most characterized. Kleinnijenhuis et al., demonstrated that macrophages isolated from BCG vaccinated healthy adults showed enhanced production of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 when stimulated *ex vivo* with unrelated bacterial and fungal antigens (41). Similar findings have been seen in against viruses, with BCG vaccination inducing greater protection against attenuated yellow fever virus vaccine strain, which correlated with an increase in the upregulation of IL-1β (31). A further RCT in Ugandan infants found that just delaying BCG vaccination from birth to six weeks old, significantly increased infectious disease incidence. They found the protection afforded by BCG was related to histone trimethylation at the promoter region of pro-inflammatory cytokines, including TNF and IL6, indicating immune cells were primed for pro-inflammatory responses (42). Specifically, monocytes show a particular increase in H3K4me3 histone modification, involved in transcriptional activation of TLR4, TNFα, and IL6 genes (43, 44)

These responses are also longer lasting than initially thought possible by innate cells. BCG trained monocytes were identified in the blood three months after vaccination, when their normal half-life in circulation may only be up to one day (39). Both NK and γδ T cells have been shown to exhibit memory-like properties after BCG vaccination, that are sustained for several months (38). The memory phenotype of γδ T cells induced in response to BCG was observed by Hoft et al., in 1998, after PBMCs from BCG vaccinated humans were cultured with mycobacterial antigens. Seven days later the cell type that had undergone the greatest expansion in comparison to cells from unvaccinated control cultures was the γδ T cell (45). Primate studies demonstrated the occurrence of a recall expansion by γδ T cells after *Mycobacterial tuberculosis* (*M. tb*) infection, and the kinetics of the recall expansion was dissimilar to the *M. tb* primary expansion (46). This recall expansion coincided with protective immunity. Recently the expansion of Vδ2 T cells after BCG was confirmed in humans *in vivo* as well as the production of IFN-γ by Vδ2 T cells after vaccination (47). Interestingly, other donor unrestricted T (DURT) cells, such as MAIT and NK cells were not altered after BCG vaccination or revaccination in humans in this study.

GAMMA DELTA T CELLS

γδ T cells are important players in the early immune response to infections or malignant transformation, as well as being involved in the adaptive response. γδ T cells are powerful effector cells, despite only representing 0.5-5% of circulating T cells in homeostatic conditions (48). Their numbers rapidly expand in the circulation in response to stimuli due to the non-MHC restricted recognition of unprocessed antigens. γδ T cells also represent a much higher proportion of immune cells at barrier

surfaces such as mucosal and epithelial sites lending weight to their role as first-line effectors. Individual T cell receptor (TCR) variable region δ (Vδ) gene segments are associated with distinct ligand recognition and anatomical location. The positioning of these γδ T cells suggests a direct role of the TCR in each of these locations. The TCR may even be involved in retaining the cell at these locations (49). Thus, γδ T cells are usually categorized into two main types based on Vδ region: Vδ1 and Vδ2. In humans Vδ1 cells usually localize to tissues and are the main TCR type in the gut and skin. Some tissues contain highly specialized Vδ1 cells that are not found anywhere else in the body. For example, Vγ3Vδ1 skin dendritic epidermal T cells (DETC) arise exclusively in the epidermis, and Vγ5Vδ1 cells are only found in the intestinal epithelium. Vδ2 make up the largest population of γδ T cell family in the circulation of humans. The Vδ2 chain preferentially pairs with the Vγ9 (called Vγ2 in an alternative nomenclature) chain (50). These Vγ9Vδ2 cells comprise between 70 and 90% of the peripheral blood γδ T cell population. Although they make up less than 5% of total blood lymphocytes in healthy individuals, they can expand rapidly, up to 60% of peripheral blood lymphocytes, in certain infectious diseases due to their unique ligand recognition (51). This Vδ2 subtype is also responsible for the majority of the expansion in γδ T cells after BCG stimulation (45).

γδ T cells are involved in the first line of defense to a number of diseases, including cancer, bacterial infections, and viral infections. Studies have demonstrated their rapid activation and cytotoxicity to various viruses, including cytomegalovirus (CMV) (52, 53), influenza A virus (54–56), human immunodeficiency virus (HIV) (57–59), hepatitis B and C viruses (HBV and HCV) (60–62), Epstein Bar Virus (EBV) (63) and severe acute respiratory syndrome (SARS) virus (64), as reviewed by others (50, 51, 65–69). Additionally, γδ TCR knockout mice show an increase in viral titer or reduced survival when infected with West Nile virus or vaccinia virus (70, 71). After the 2003 SARS outbreak Poccia et al., evaluated lymphocytes in the circulation of survivors three months after initial infection. Interestingly, the number of αβ T cells did not differ from that of healthy uninfected subjects, but the numbers of Vδ2 T cells were substantially higher (64). This expansion was associated with higher anti-SARS-CoV immunoglobulin G (IgG). *In vitro* experiments showed that stimulated Vδ2 cells could kill cells infected with SARS-CoV, and that IFN-γ was involved in this response (64). Consequently, it is highly likely that γδ T cells could also be involved in the protective immune response to SARS-CoV-2.

Very few studies have investigated γδ T cells in SARS-CoV-2 infections, and the majority of information is in the context of severe disease. Laing et al., evaluated peripheral blood from hospitalized patients and showed lymphocytes were depleted in COVID-19 disease, the lymphocytes present were hyperactivated, whereas DC and monocyte functions were dampened. The drop in lymphocytes included γδ T cells, which were highly reduced in the circulation compared with healthy controls, especially the Vδ2 subset. This has also been reported by other studies (72–74). Lei et al., showed that the γδ T cells remaining in the blood had a CD25+ activated phenotype, although the very early activation marker CD69 did not increase compared to healthy controls, which the authors suggested may be because this marker was expressed earlier in infection (72). Notably, PD-1 expression did not change in these γδ T cells compared with controls, which suggests they were not exhausted. This contrasts with the finding that CD8+ cells showed heightened expression of both PD-1 and TIM3 related to disease severity, indicating a more exhausted phenotype in these cells as disease advances (75). Lastly, Lei et al., showed a dramatic increase in the proportion of γδ T cells co-expressing CD4, suggesting a role for this cell type, which is typically low in humans in homeostasis. Odak et al., showed a striking reduction in effector memory cells within the γδ T cell population, and an increase in naïve cells, and suggested that the effector memory γδ T cells may be recruited to the lungs. They also theorized that the reappearance of effector cells in the blood was associated with recovery from COVID-19 (74).

Many features of γδ T cells make them promising players in the SARS-CoV-2 response, including their key role in immunosurveillance of mucosal and epithelial barriers, their recognition of viral entry *via* a number of different pathways, and their functional responses that can act to kill virally infected cells as well as their ability to stimulate the adaptive immune system.

γδ T Cell Recognition of Viral Infection

The mechanisms behind γδ T cell recognition of viral infections like SARS-CoV-2 are not as clearly understood as other cell types. γδ T cells use many different pathways to recognize foreign antigens and stress signals, and it is likely that different combinations of these pathways work synergistically in distinct viral infections to initiate and amplify responses. The main pathways include toll like receptors (TLRs), the γδ TCRs, and natural killer-like receptors.

γδ T cells express a variety of TLRs which bind to pathogen associated molecular patterns (PAMPs). Of particular importance are TLR 2 and 4 expressed on their cell membrane, which can recognize viral glycoprotein and glycolipids, as well as TLR 3 and 7, expressed on endosomes, which recognize viral RNA (76, 77). The binding of TLRs to PAMPs induces transcription factor upregulation, leading to pro-inflammatory cytokine production. The synergistic effects of TCR and TLR stimulation has been demonstrated *in vitro* by Wesch et al., where IFN-γ production in response to direct TCR stimulation is dramatically increased when TLR 3 is also stimulated with a synthetic analogue of its natural PAMP (78). TLR recognition of SARS-CoV-2 glycolipids, glycoprotein and RNA is likely a vitally important step in this immune response.

The Vδ2 TCR can recognize small phosphoantigens in a way that is unique, and likely responsible for its prolific responses in cancer, mycobacterial infection, and BCG vaccination. The first small phosphoantigen found to stimulate Vδ2 cells was a pyrophosphate intermediate of the mevalonate isoprenoid synthesis pathway, isopentenylpyrophosphate (IPP) (79). This pathway exists in all mammalian cells, and during normal physiological conditions, IPP is at a low concentration inside the

cells and does not cause activation of Vδ2 cells. However, disruptions to the mevalonate pathway caused by a number of events, including dysregulated metabolism in tumors, pharmacological interference, or infections results in increases in intracellular IPP. Above a certain threshold IPP bound to the intracellular portion of butyrophilin-3A1 (BTN3A1) and BTN2A1 induces a conformational change that allows interaction of BTN2A1 with the Vγ chain of the TCR and likely also allow BTN3A1 to interact with the Vδ chain (80–82). Other small phosphoantigens that stimulate the BTN3A1 conformation change and subsequent Vδ2 TCR responses have now been identified. The most significant of these is produced by mycobacteria, including BCG, called (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). Microbial HMBPP, an intermediate of the MEP/DOXP pathway, has been found to activate Vδ2 cells with a potency 30000 times that of IPP (83).

Studies have suggested that the phosphoantigen/BTN mechanism of Vδ2 TCR activation may also have a role in viral infections, in addition to its importance in mycobacteria and cancer. Blocking the mevalonate pathway upstream with mevastatin, and therefore halting IPP synthesis, prevented the activation and proliferation of Vδ2 cells in an *in vitro* EBV infection (63). A similar outcome was seen when the mevalonate pathway was blocked in influenza A virus infection, where Vδ2 IFN-γ production was significantly reduced (54). It is currently unknown to what extent this pathway is active in SARS-CoV-2 infections. However, as it is likely responsible for much of the Vδ2 cell expansion after BCG, it is an important mechanism in the development of BCG primed anti-viral responses. Unlike Vδ2 cells, the Vδ1 TCR does not recognize phosphoantigen/BTN, and therefore Vδ1 cells proliferate less in response to BCG stimulation, although they are able to recognize BCG infected cells through the recognition of mycobacterial lipids on CD1. Recognition of CD1 in the context of viral infection is less understood, as there are no known virus specific lipids that exist in large enough quantities to be expressed on CD1. However, there is evidence that lipids derived from the host are presented on CD1 and can stimulate NK cells in viral contexts (84). The differentiation of CD1 displaying host lipid in homeostasis in comparison to viral infection, where substantial relocation of endosomal CD1 occurs has been hypothesized to mediate this stimulation (85). Some viruses, including Kaposi sarcoma associated herpesvirus (KCHV) and HIV actively induce the internalization of CD1, signifying CD1 presentation to NK cells or γδ T cells may contribute to protection (85).

In addition to TLRs and TCRs γδ T cells express other receptors, several of which are likely to be important in the recognition of viral infection, including NK type receptors (NKRs), DNAX Accessory Molecule 1 (DNAM1), and the Natural Cytotoxicity receptors (NCRs) NKp30, NK44 and NKp46. This review focuses on the NKR natural killer group 2-member D (NKG2D) only, as other NKRs have been recently reviewed by Caron et al. (69). NKG2D is an activating C-type lectin originally found on Natural Killer cells, but also highly expressed on both Vδ1 and Vδ2 T cells. It recognizes MHC class I polypeptide-related sequence A and B (MICA and MICB) and UL16 binding proteins (ULBPs). MICA, MICB and ULBPs can be expressed by the majority of cells, but are normally in very low abundance. Expression of these ligands is induced as part of the DNA damage response used by cells after stresses such as infection or malignant transformation (86). Once induced, their interaction with NKG2D on γδ T cells can assist activation and produce a powerful cytotoxic response. It is currently unknown the extent to which SARS-CoV-2 infection upregulates NKG2D ligands, however many ligands have been found to be upregulated on virally infected cells (69). For example, CMV infected cells have been shown to upregulate MICA and ULBP1-3 (87); EBV infected cells can upregulate MICA, MICB and ULBP4 (88, 89); and cells infected with either influenza A or Sendai virus can upregulate MICB (90). Blockade of NKG2D can also lead to a reduction in γδ T cell anti-viral responses (63).

γδ T Cell Responses to Viral Infection

γδ T cells can mediate the killing of virally infected cells through a number of mechanisms. These include directly killing infected cells *via* cytotoxic molecules, and expression of membrane bound TNF-family members FasL and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL), as well as indirectly *via* the production of pro-inflammatory cytokines, and assisting in DC maturation to stimulate the adaptive immune system. These responses are important in the defense against SARS-CoV-2 infection and COVID-19 disease progression (91).

γδ T cells can secrete cytotoxic granules containing granzymes, perforin, and granulysin. These molecules have various effects on target cells that promote cell death. Perforin is able to form pores in target cell membranes, disrupting the osmotic balance, leading to an influx of Ca+ ions present at the immune synapse and pro-apoptotic signaling. Perforin also allows entry of granzymes. Granzyme B directly cleaves proteins involved in the caspase pathway, resulting in caspase-mediated apoptosis. It can also initiate the mitochondrial cell death pathway by cleaving BH3 interacting-domain death agonist (BID) (92). Granulysin can cause cell death in similar ways to granzyme B, and can also interfere with the target cell's endoplasmic reticulum, which leads to pro-apoptotic signaling. Additionally, it has recently been shown that the 15kDa isoform of granulysin produced by γδ T cells, previously thought to be an inert precursor to the 9kDa isoform, can actually cause the migration and maturation of DCs (93). Other Granzymes that have been shown to kill virally infected cells in animal and *in vitro* models include granzyme M, H and K (94–96). Of interest, these lesser known granzymes may be able to inhibit viral replication by directly cleaving viral proteins, without necessarily killing the host cell, as exemplified by Granzyme M in a murine model of CMV infection (97).

γδ T cells can produce proinflammatory cytokines in response to viral recognition, including IFN-γ and TNF-α (98). These two cytokines trigger a multitude of pathways in target cells that can ultimately lead to the inhibition of viruses at all stages of their replication: viral entry, viral protein synthesis, viral assembly, and

viral release, as recently reviewed (69). Many γδ T cells produce multiple pro-inflammatory cytokines simultaneously, which have synergistic effects on virally infected cells, and are particularly effective for viruses that have evolved escape mechanisms from one or many of the cytokine-induced pathways.

BCG STIMULATION OF γδ T CELLS TO COMBAT NON-TUBERCULOSIS DISEASES

BCG stimulation of the immune system to target diseases other than TB is not a new concept, and has in fact been used for many decades before it was known how BCG could influence cells of the innate immune system, including γδ T cells. BCG has been used as a first line treatment for non-invasive bladder cancer since the 1970s, and can out-perform chemotherapeutic agents (99). BCG can also be used in the treatment of inoperable cutaneous melanoma (100–103). Studies have provided evidence that Vδ2 cells are contributing, at least in part, to BCG-induced regression of cancer cells, with BCG injections causing infiltration of Vδ2s into tumors and IFN-γ production (104). Other mycobacteria preparations are also in the process of commercialization, including IMM-101, an attenuated preparation of *Mycobacterium obuense*, which when used in combination with the first line treatment for inoperable pancreatic ductal adenocarcinoma (PDAC), the overall survival of patients improves (105).

BCG has many antigens that are potent stimulators of the immune system, and γδ T cells in particular. For example, BCG has a variety of cell wall lipids and proteins that are recognized by TLRs. Lipids from internalized BCG are also known to be displayed on CD1 molecules, that may be recognized by Vδ1 cells. As mentioned earlier, mycobacteria also produce the small phosphoantigen HMBPP, which potently stimulates Vδ2 TCRs. Therapeutics have been developed to specifically target this activation pathway using synthetic HMBPP and similar analogues, like Picostim (106, 107), as well as nitrogen-containing bisphosphonates (NBPs), which block the mevalonate pathway, leading to IPP accumulation (108, 109). Tu et al., expanded Vδ2 cells *in vitro* with the NBP pamidronate (PAM) and injected them into influenza infected humanized mice, demonstrating an improvement in disease severity and control of viral replication (110). Studies have also shown the NKG2D ligand MICA to be upregulated on epithelial and DCs after *M. tb* in humans (111), and mice NKG2D ligands Rae-2 and MULT1 are upregulated after BCG infection in the murine model (112).

Therefore, BCG can stimulate γδ T cell activation through a variety of pathways, many of which are still unknown, and these can have synergistic effects on transcription to amplify anti-viral responses. Anti-viral γδ T cells responses that may be induced by BCG include the production of cytotoxic molecules, including granzyme B, granulysin and perforin (113); inflammatory cytokines, including IFN-γ and TNF-α (23); and the upregulation of death receptor ligands (69). Activated γδ T cells can also enhance the maturation and migration of DCs and present antigens themselves, thereby stimulating the adaptive immune system. This is summarized in **Figure 1**. Although this review is focused on γδ T cells due to their potential in COVID-19, BCG also impacts biological pathways of other cells of the immune system, as already discussed, including macrophages, NK cells, and MAIT cells, inducing epigenetic modifications to genes such as IL1β, TNFα, TLR4 and IL6, marking these cytokines as important and allowing for their rapid upregulation (44). Taken together, BCG are able to activate γδ T cells in similar ways to viral infections, and induce the production of molecules that are critical to the anti-viral response. Therefore, it is likely that priming γδ T cells with BCG can actively contribute to SARS-CoV-2 control and moderate the severity of the COVID-19 disease.

CONCLUDING REMARKS

The BCG vaccine is affordable, commonplace, and non-specific. This makes it a rapid tool to implement in a pandemic such as COVID-19. Although we are only beginning to understand the innate mechanisms behind BCG's broad protection, its impact on non-tuberculosis morbidity and mortality has been noted for a century (9). BCG vaccination can expand and prime innate and effector cells, including γδ T cells. γδ T cells are of particular interest, as BCG vaccination can induce them to direct potent anti-viral responses against infected cells, as well as stimulate the adaptive immune system. They have also been shown to be activated and not exhausted after COVID-19 infection. However, we need to remain aware of the vital role BCG already has in protecting against TB, particularly in infants in LMICs. Neonatal BCG vaccination remains a crucial component of TB control, and any delay to vaccination, such as that observed by BCG shortages in the past years, can have significant impacts on TB meningitis rates (114) and would be a major setback to global TB strategies. Any approach using BCG as a tool against COVID-19 should first prioritize BCG vaccines where they are needed most in LMICs with a high incidence of TB.

Considerations should also be made to the target age group and impacts of boosting and revaccination. BCG vaccination in the elderly has been shown to help protect against respiratory diseases, like COVID-19, indicating that BCG can also impact the innate immune system later in life (14). However, the efficacy of using BCG vaccination in adults to control TB varies widely (25). Vaccinating adolescents could conceivably have dual effects reducing the transmission of SARS-CoV-2 and *M. tb* (26). Using this dual strategy could have the greatest impact on reducing morbidity. The efficacy of BCG vaccination also varies globally, thought to be due to a number of factors including strains used, genetic and socio-economic differences, as well as interference *via* prior mycobacterial exposures, called masking and blocking. These are all factors that need to be considered in any BCG strategy to combat COVID-19 as they may impact how long non-specific protection lasts and as well as the requirement for boosting vaccinations.

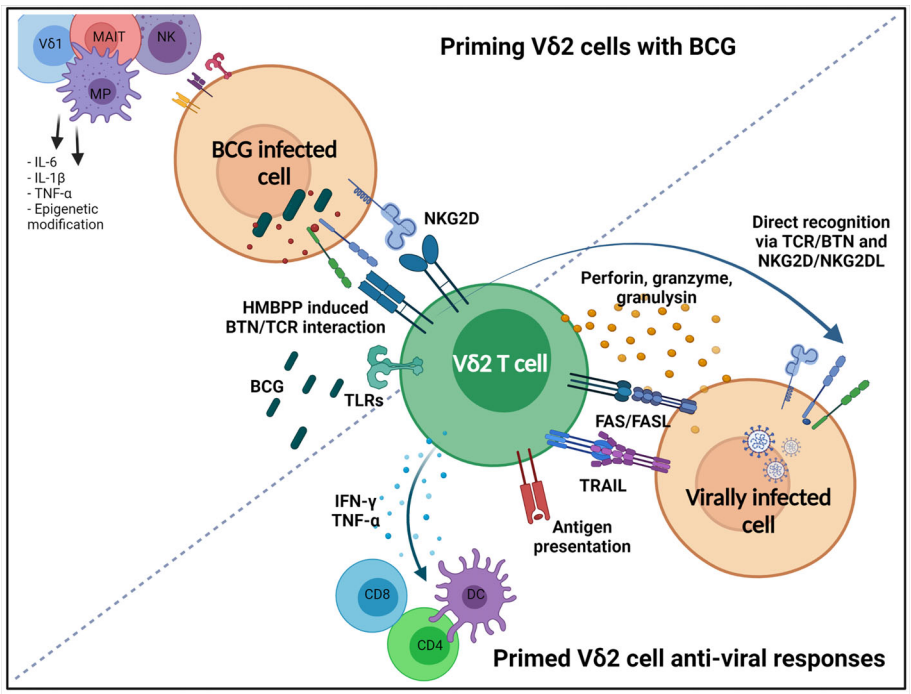

FIGURE 1 | Priming Vδ2 cells with BCG, and subsequent non-specific anti-viral responses. Vδ2 T cells are activated after BCG vaccination through a number of mechanisms. HMBPP produced by BCG infected host cell causes conformational changes on intercellular domains of butyrophilin (BTN) molecules, such as BTN3A1 and BTN2A1, which allows the extracellular domain to interact with the Vδ2 TCR. Mycobacteria have been shown to induce the expression of NKG2D ligands on cells which can activate Vδ2 cells through NKG2D. Vδ2 cells have many TLRs that can recognize BCG PAMPs. Non-specific responses induced that have anti-viral activity include directly killing infected cells through the secretion of cytotoxic granules containing perforin, granzymes and granulysin, or initiation of death-inducing pathways, FASL and TRAIL. They can also indirectly contribute to killing through the production of pro-inflammatory cytokines TNF-α and IFN-γ inducing the maturation and migration of DCs, leading to induction of the adaptive immune system. Vδ2 cells may also recognize virally infected cells directly *via* NKG2D and Vδ2 TCR. Infected cells can upregulate NKG2D ligands (e.g. MICA, MICB), and can have altered metabolisms, which induces conformation changes to BTN molecules. Created with BioRender.com.

Countries where TB rates are high often coincide with countries that have seen a delay in their antigen specific SARS-CoV-2 vaccine roll-out, and therefore are likely to be the countries where variants have full rein to develop. This last year has seen the rapid spread of variants across the world, and further mutations are expected to threaten the protection afforded by the current vaccines. BCG vaccination may provide a measure of protection independent of specific viral antigens, and therefore is unlikely to provide any selection pressure for new mutations, and is in fact likely to help control against new variants. If studies show BCG provides protection from COVID-19, a well-considered BCG strategy could contribute to the global effort against both COVID-19 and TB.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AM designed, wrote, and revised the manuscript. AW, SS, and MB-S revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Blocking of EphA2 on Endometrial Tumor Cells Reduces Susceptibility to Vδ1 Gamma-Delta T-Cell-Mediated Killing

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Background: Endometriosis is a common gynecological disease characterized by the presence of endometrial tissue outside the uterus causing chronic inflammation, severe pain, and infertility. However, the innate immunity of gamma-delta (γδ) T lymphocytes in endometriosis has not been characterized. Women with endometriosis present numerous endocrine and immune dysfunctions and elevated risk for endometrial, ovarian, and breast cancers. The tyrosine kinase EphA2 is often overexpressed in cancer including endometrial carcinoma.

Methods: We analyzed Vδ1 and Vδ2 γδ T cells in peripheral blood and paired peritoneal fluid samples in endometriosis patients (n = 19) and compared the counts with that of age- and sex-matched healthy donors (n = 33) using flow cytometry. Vδ1 and Vδ2 T cells isolated from healthy donors were used against KLE, RL-95, and Ishikawa endometrial tumor cells in 4 h flow cytometric cytotoxicity assays. The EphA2 blocking studies were performed using antibody, small-molecule inhibitor ALW-II-41-27, and the CRISPR/Cas9.

Results: We determined Vδ1 T cells substantially reduced in patients' peripheral blood ($p < 0.01$) and peritoneal fluid ($p < 0.001$). No differences were found for circulating Vδ2 T cells compared with peritoneal fluid samples. We observed inherent cytotoxic reactivity of Vδ1 and Vδ2 γδ T lymphocytes against endometrial tumor cells. Importantly, we found reduced specific lysis of EphA2-positive cell lines KLE and RL-95 by Vδ1 T cells in the EphA2 antibody blocking studies and by the EphA2 inhibitor. Furthermore, Vδ1 T-cell-mediated killing was significantly decreased in RL-95 cell EPHA2 knockout. Finally, potent cytolytic activity exerted by Vδ1 T cells was significantly reduced in EPHA2 knockouts in renal A-498 and colon HT-29 carcinoma cell lines.

Conclusions: We determined variable levels of Vδ1 and Vδ2 γδ T cells in endometriosis patients. We observed inherent cytotoxic reactivity of γδ T-cell subsets against endometrial cell lines. Specifically, we found that blocking of EphA2 expression resulted in significant inhibition of endometrial tumor killing mediated by Vδ1 γδ T cells.

These results suggest that EphA2 is involved in tumor cell lysis and contributes to susceptibility to Vδ1 γδ T cells cytotoxic reactivity.

Keywords: gamma-delta T cells, endometriosis, peritoneal fluid, tyrosine kinase EphA2, cytotoxicity, innate immunity

INTRODUCTION

Endometriosis is a hormone-dependent gynecological disease characterized by the presence of endometrial tissue outside the uterine cavity. The disease affects around 10% of reproductive-aged women (1). Retrograde menstruation is accepted for the pathogenesis when menstrual endometrial tissue fragments and viable cells escape apoptosis, evade normal immune surveillance, enter into peritoneal cavity where adhered, develop a blood supply, and grow into endometriosis lesions (1, 2). Hormonal treatments are believed to reduce proliferation of endometrial lesions by reducing estrogen activity. Increased concentrations of prostaglandins have been reported in peritoneal fluid of endometriosis patients and may be involved in the progression of the disease (3). It is well established that women with endometriosis exhibit numerous endocrine and immune dysfunctions. Specifically, they display aberrant numbers of immune cells and cytokines present in the plasma and peritoneal fluid (PF), which has been shown to contribute to chronic pain and infertility described by endometriosis women (4–7). The immune cells including macrophages, natural killer (NK) cells, cytotoxic T cells, and dendritic cells that lost the ability to effectively detect and destroy autologous endometrial menstrual tissue contribute significantly to the development of acute and chronic inflammation. In addition to decreased NK cell cytotoxicity (8–12) enhanced activation of monocytes and peritoneal macrophages (13, 14) have been well documented. It is still uncertain whether the aberrant activity of these immune cells causes endometriosis or whether they act as secondary enhancers of the disease. Recent evidence suggests that biology of endometriosis significantly overlaps those considered to be hallmarks of cancer and essential alterations in cell physiology including sustained proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, induction of angiogenesis, resistance to cell death, compromised immune detection, tumor promoting inflammation, and genome instability (15). It is understood that women with endometriosis present elevated risk for cancer by 90% for ovarian cancer, 40% for non-Hodgkin's lymphoma, and 30% for breast cancer. Many women with endometriosis are also diagnosed with polycystic ovary syndrome (PCOS).

Endometrial cancer (EC) is the most common malignancy of the female reproductive system (16). It tends to develop after menopause in women with a median age at onset of 63 years. Several risk factors have been identified, such as obesity (17), diabetes, PCOS, and infertility. Endometrial carcinoma arises from the lining of the uterus and can be broadly divided into two types: endometrioid carcinomas, affecting approximately 80% of patients, which can be graded according to the relative proportion of solid tumor and the nonendometrial carcinomas, which have a hormone-independent pathogenesis and unknown precursor lesion (16). An early stage EC patients' prognosis is generally favorable.

Human gamma-delta (γδ) T lymphocytes play critical roles in immune surveillance mediating potent inflammatory response and contributing to prominent tumor killing (18, 19). γδ T cells account for 1%–10% of T cells in the peripheral blood in adults and are often enriched as resident cells within the solid organs and mucosal tissues. They are considered the first line of innate immune defense, but they also have the possibility to create immunological memory and therefore also belong to adaptive immunity (20, 21). In contrast to conventional αβ T cells, γδ T cells display a non-MHC-restricted antigen recognition. Human γδ T cells can be divided according to their T-cell receptor (TCR) delta chain usage into two major populations, namely Vδ1 and Vδ2 T cells (22). Recent study highlighted the role of γδ T cells in cancer as the most significant favorable prognostic immune subset associated with overall survival outcomes across 39 malignancies (23). However, to our knowledge, γδ T cells in endometriosis patients have not been characterized.

The Eph receptors represent the largest family of receptor tyrosine kinases. Together with their respective ligands, they have been extensively studied for the roles they play during embryonic development, particularly within the central nervous system (24). As a unique feature, bidirectional signaling in Eph/ephrin ligands between cells is fundamentally involved in developmental processes, such as axonal guidance, remodeling of blood vessels or correct formation of crypt and villi in the intestinal epithelium (24, 25). Some Eph receptors, especially EphA2 is often overexpressed and functionally altered in many cancers including breast (26), ovarian (27), and endometrial (28, 29) carcinomas, which correlated with, e.g., increased invasiveness, increased metastatic potential, prominent vascularization, and consequently with poor patient outcome. Most recently, EphA2 has been identified as a stress antigen recognized by a Vδ1 TCR (30).

We conducted the present study to determine the numbers of γδ T-cell subsets in endometriosis patients. We demonstrate for the first time the prominent cytotoxicity of γδ T cells against endometrial tumor cell lines. Next, we show that the EphA2 receptor is highly important in tumor recognition and killing by Vδ1 γδ T cells.

MATERIALS AND METHODS

Patient Characteristics

Patients (n = 19) have been enrolled from the Department of Gynecology and Obstetrics, Faculty Hospital Brno. The study was approved by the local institutional ethics committee of the

Faculty of Medicine, Masaryk University. The study was performed in accordance with the Declaration of Helsinki. Written informed consents were obtained from all patients. Endometriosis was assessed according to the revised American Fertility Society (r-AFS) classification during laparoscopy. Patients received no hormonal therapy for a minimum of 3 months prior to laparoscopic surgery.

The patient characteristics are shown in **Table 1**.

Sample Collection and Preparation

Peripheral blood (PB) and peritoneal fluid (PF) samples were obtained from endometriosis patients and were processed within 2 h of collection. PF samples were taken during the planned surgery from lower pelvis cavity by fine needle suction from cavum Douglasi at the opening phase of diagnostic laparoscopy prior the surgical procedure as less invasive technique for more patients than tissue biopsy. At the same time, it allows to obtain sufficient volumes of biological material for subsequent analysis. Buffy coats from age- and sex-matched healthy volunteers (n = 33) were collected at the Transfusion and Tissue Bank, Faculty Hospital Brno. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation using Lymphoprep (Stem Cell Technologies) following the manufacturer's recommendations. Plasma samples were collected and stored at −80°C.

Cell Culture

Endometrial carcinoma cell lines KLE (ATCC® CRL1622™) and RL95-2 (ATCC® CRL1671™) were purchased from ATCC (American Type Culture Collection, VA, USA). Ishikawa cell line was purchased from Sigma-Aldrich (St. Louis, MO, USA). KLE and RL95-2 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (all Thermo Fisher Scientific); RL95-2 cells with the addition of 5ug/ml of insulin (Sigma-Aldrich, MO, USA). The Ishikawa cell line was maintained in MEM supplemented with 5% FBS and 2% penicillin/streptomycin.

TABLE 1 | Study subjects.

Parameters	Endometriosis patients	Controls
Number (*n*)	19	33
Age (years)		
Median	33	29
Range	24–48	18–48
Disease stage*		
I	3	
II	4	
III	5	
IV	7	
Menstrual cycle		
EPP	8	
LPP	3	
ESP	5	
LSP	3	

**Classification according to r-AFS.*
EPP, early proliferation phase; LPP, late proliferation phase; ESP, early secretory phase; LSP, late secretory phase.

In addition, human tumor cell lines including myeloma (U266, EJM) and chronic myeloid leukemia (LAMA-84, KYO-1) were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Germany). Renal (A-498), prostate (DU-145), breast adenocarcinoma (MCF-7) and histocytic lymphoma (U937), chronic myeloid leukemia (K562), acute monocytic leukemia (THP-1) and glioblastoma (U87 MG) cells were purchased from ATCC. Glioblastoma cells (U-373 MG and U251 MG) were purchased from The European Collection of Authenticated Cell Culture (ECACC). Myeloma cell lines (OPM-2, LP-1, KMS-11) were a kind gift from Dr. Krejci (Institute of Biology, Masaryk University Brno). Cells lines (U266, LAMA-84, KYO-1, K562, U-937, THP-1, EJM) were cultured in RPMI-1640 containing 10% FBS, 2mM L-glutamine, and 2% penicillin/streptomycin. Cell lines (A-498, DU-145, HT-29, U-87 MG, MCF-7) were cultured in modified Eagle's medium (MEM) (Sigma Aldrich) with 10% FBS, 2 mM L-glutamine, and 2% penicillin/streptomycin. The MCF-7 cells were supplemented with nonessential amino acids (NEAA, Sigma Aldrich). Cell lines (U251 MG, U-373 MG) were cultured in DMEM/F12 with 10% FBS. All cells were grown at 37°C in 5% CO_2 atmosphere up to 70%–80% confluence; adherent cells were harvested by using gentle dissociation solution TrypLE (Gibco, Thermo Fisher Scientific) and counted by using Trypan blue exclusion.

The EphA2 inhibitor ALW-II-41-27 was purchased from MedChem Express (Monmouth Junction, NJ, USA). It was dissolved in sterile DMSO at 10 mM stock concentration and solution stored in aliquots at −20°C.

Flow Cytometric Phenotyping

The cell phenotype was assessed by fluorescence-activated cell sorting (FACS) by using staining with the monoclonal antibodies MICA, MICB, CD112, CD155, B7-H6 (R&D Systems, clone 875001), and EphA2 (R&D Systems, clone 371805). The tumor cell lines were harvested, washed with cold phosphate-buffered saline (PBS, Sigma) containing 2% FBS, and incubated for 30 min on ice with fluorescently labelled monoclonal antibodies. Gamma-delta T cells were identified in freshly isolated PBMCs labelled with CD3 (Thermo Fisher Scientific, clone SK7), Vδ1 TCR (Thermo Fisher Scientific, clone TS8.2), Vδ2 TCR (BD Pharmingen, clone B6) or Vδ2 TCR (Sony, clone B6). CD27 (BD Pharmingen, clone M-T271), and CD45RA (Exbio, clone MEM-56) were used for immunophenotyping. Samples were washed and acquired using FACSCanto® (BD Biosciences) and data analyzed using FlowJo® software (FlowJo, Ashland, OR, USA). Forward and side scatter gating were used to discriminate live cells from dead cells and γδ T cells were derived from SSC *vs.* FSC-gated bulk PBMCs with doublet exclusion (FSC-A *vs*. FCS-H). To determine the placement of the gates, appropriate fluorescence minus one (FMO) and unstained controls were used.

Isolation of Polyclonal Vδ1 and Vδ2 γδ T Lymphocytes

Fresh γδ T-cell populations were sorted by positive selection using anti-TCR Vδ1 (Beckman Coulter, clone R9.12) or anti-TCR Vδ2 (BD Pharmingen, clone B6) monoclonal antibodies

and magnetic microbeads (Miltenyi Biotec, Germany) according to manufacturer's instruction. The cell purity was routinely greater at 97%.

Cytotoxicity Assay

Freshly sorted Vδ1 or Vδ2 γδ T lymphocytes were incubated with tumor target cells at indicated effector:target (E:T) 5:1 and 10:1 ratios in duplicates for 4 h co-culture at 37°C as described previously (31). Briefly, tumor target cells were washed in Hank's buffered saline solution (HBSS, Invitrogen Life Technologies) to remove FBS and culture media. Cells were resuspended in diluent C (Sigma) and labeled with PKH67 fluorescent dye (Sigma). To-Pro-3 iodide (1 μM in PBS) (Invitrogen Life Technologies) was added immediately prior to the acquisition on the flow cytometer. At least 10,000 target cells were acquired after gating out the green fluorescence of PKH67 dye and the proportion of To-Pro-3 iodide positive cells. Background target cell death was determined from the cells incubated in the absence of effector cells. In the blocking experiments, the EphA2 antibody (R&D Systems, clone 371805) and ALW-II-41-27 EphA2 inhibitor (10 μM, 1 μM) or DMSO as a control were added to tumor cultures prior the cytotoxicity assays.

RNA Extraction, cDNA Synthesis, Real-Time PCR

Total RNA has been extracted from tumor cell lines using RNeasy Mini kit (Qiagen) according to manufacturer's instruction. RNA was eluted in RNAse-free water and stored in −80°C. Complementary DNA (cDNA) has been synthesized using 20 ng/μl total RNA that has been reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene has been used as an internal control by quantitative real-time polymerase chain reaction (real-time qPCR). cDNAs were amplified using TaqMan® Gene Expression Assay (ID : Hs01072272_ml, Applied Biosystems). Samples were analyzed on StepOne™ Real-Time PCR Systems (Applied Biosystems).

Generation of EPHA2 Knockout by the CRISPR/Cas9 method

The EPHA2 gene knockout was performed with the EPHA2 CRISPR gRNA + Cas9 in Lenti-particles (supplied from antibodies-online GmbH) and used closely following manufacturer's instructions. Briefly, vector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro (product number ABIN5252263) was used to generate EPHA2 knockouts in human endometrial cancer line RL95-2, renal carcinoma cell line A-498, and colon carcinoma cell line HT-29. After infection, positive clones were selected by 3.5 μg/ml puromycin, and the single clones were transferred separately into 48-well plates and further passaged. The EPHA2 knockouts were confirmed by flow cytometry after antibody staining (anti-EPHA2, R&D Systems, clone 371805).

Statistical Analysis

Data analyses were performed using GraphPad Prism5 software (GraphPad Software Inc., La Jolla, CA). The Student's *t*-test was used to determine significant differences between groups. Differences between sample groups were evaluated with the nonparametric Mann-Whitney *U* test. $p < 0.05$ values were considered to be significant. Data are expressed as mean ± standard deviation (SD).

RESULTS

γδ T Cell Subsets in Peripheral Blood and Peritoneal Fluid Samples in Patients With Endometriosis

First, we aimed to determine the two major populations of γδ T cells (Vδ1 and Vδ2 subsets, respectively) in peripheral blood (PB) samples from patients with endometriosis ($n = 19$) and compared the frequencies with age- and sex-matched healthy donors (HD, $n = 33$). Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) where proportion of Vδ1 and Vδ2 γδ T cells among leukocyte gate followed by the percentage of CD3 lymphocytes is shown in **Figure 1A**. Immunophenotyping of Vδ1 (**Figure 1B**) and Vδ2 (**Figure 1C**) using the CD27 and CD45RA antibodies to determine the naïve/memory/effector memory and TEMRA phenotypes was analyzed, and representative flow plots are shown. We found significantly low percentages of Vδ1 T cells in PB ($p = 0.008$) (median 0.5%, range 0.1%–2.4%) in endometriosis patients compared with HD (median 0.9%, range 0.1%–3.8%), as shown in **Figure 2A**, whereas Vδ2 T cells showed no difference between the endometriosis patients (median 1.5%, range 0.2%–7.9%) and healthy controls (median 2.4%, range 0.3%–11.9%), as shown in **Figure 2B**. Next, the absolute counts of Vδ1 and Vδ2 γδ T cells in PB in patients compared with HD were determined in **Figures 2C, D**, respectively. We found dramatically reduced Vδ1 T-cell absolute counts in patients PB ($p = 0.0002$) (median 2.23 cells/μl, range 0.14–14.01) and HD controls (median 13.3 cells/μl, range 0.11–242.1). No differences in Vδ2 T-cell counts were observed between the patients PB (median 9.8 cells/μl, range 0.12–118.7) and HD controls (median 17.3 cells/μl, range 0.11–198).

Second, we analyzed γδ T-cell infiltration in patient' peritoneal fluid (PF) samples and compared the counts with paired PB. We found most patients with dramatically reduced Vδ1 T cells in PF (median 0.1%, range 0%–2.8%) compared with PB samples (median 0.5%, range 0.1–2.4%) ($p = 0.001$) in **Figure 2E**. Similarly, no significant differences were identified for circulating Vδ2 T cells (median 1.5%, range 0.2%–7.9%) compared with PF samples (median 2.0%, range 0%–11.5%), in **Figure 2F**. These results show for the first time the presence of Vδ1 and Vδ2 γδ T cells in peritoneal fluid in patients with endometriosis.

Third, we found most Vδ1 T cells of naïve (CD27+CD45RA+) and TEMRA (CD27-CD45RA+) phenotype in patients PB shown in **Figure 3A**. Peritoneal fluid samples showed majority of Vδ1 T cells at the memory stage of differentiation (CD27+CD45RA-) in **Figure 3B**. Vδ2 T cells in patients PB and PF samples were predominantly of memory phenotypes in **Figures 3C, D**.

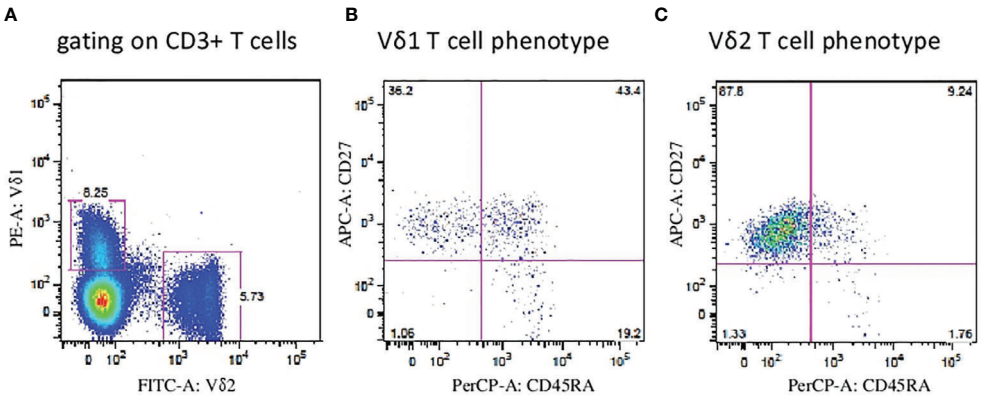

FIGURE 1 | Flow cytometric analysis of Vδ1 and Vδ2 γδ T cells. **(A)** Peripheral blood mononuclear cells (PBMCs) were analyzed where proportion of Vδ1 and Vδ2 γδ T cells among leukocyte gate followed by the percentage of CD3 lymphocytes. Immunophenotyping of Vδ1 **(B)** and Vδ2 **(C)** T cells using the CD27 and CD45RA antibodies to determine the naïve, memory, effector and TEMRA phenotypes was analyzed and representative flow plots are shown.

γδ T-Cell-Mediated Killing of Endometrial Tumor Targets

We analyzed the cytotoxic function of Vδ1 and Vδ2 γδ T cells freshly sorted from healthy donors against endometrial tumor cell lines including Ishikawa, KLE, and RL95-2. We determined the 4-h killing reactivity shown as percentages of specific lysis of Vδ1 and Vδ2 γδ T cells at 5:1 and 10:1 E:T ratio. All of the tested γδ T lymphocytes efficiently killed the tumor targets. First, the summary of Vδ1 T cell-mediated killing ($n = 4$) at 5:1 E:T against KLE (mean 29.8%, SD 4.2%), RL95-2 (mean 28.4%, SD 12.7%) and Ishikawa ($n = 3$, mean 25.1%, SD 3.8%) in **Figure 4** is shown. Importantly, significant antiendometrial reactivity of Vδ1 T cells was detected at 10:1 E:T against KLE (mean 34.8%, SD 1.3%), RL95-2 (35.9%, SD 14.2%), and Ishikawa (mean 37.8%, SD 4.3%).

Second, summary of Vδ2 T-cell-mediated killing against KLE, RL95-2, and Ishikawa (**Figure 4**) is shown. At low E:T ratio of 5:1, the specific lysis was detected against KLE ($n = 5$, mean 44.4%, SD 10.3%), RL95-2 ($n = 4$, mean 26.0%, SD 8.6%), and Ishikawa ($n = 4$, mean 34.4%, SD 6.4%). Prominent ability of Vδ2 γδ T cells to recognize and kill endometrial tumor targets was observed at 10:1 E:T against KLE (mean 49.8%, SD 11.1%), RL95-2 (mean 33.2%, SD 8.8%) and Ishikawa (mean 40.7%, SD 10%). Altogether, the endometrial tumor killing was comparable for γδ T-cell subsets isolated from different donors and was reproducible between the assays for all cell lines.

The EphA2 Expressed on Endometrial Tumor Cells Is Involved in Vδ1 T-Cell-Mediated Killing

To elucidate possible mechanisms involved in γδ T cell cytotoxicity, we evaluated several molecules typically involved in γδ T-cell killing including the MICA and MICB as ligands for the NKG2D receptor; CD112 and CD155 as ligands for the DNAM-1 receptor; and ligand B7-H6 for the NKp30 receptor. We analyzed the surface expression of MICA, MICB, CD112, CD155, and B7-H6 on target endometrial tumor cell lines including Ishikawa, KLE and RL95-2 by flow cytometry. Variable expression of these markers is shown in **Figure 5A**. Furthermore, we analyzed the expression of the EphA2 receptor, which is known to be overexpressed in many human malignancies, including endometrial carcinoma. We showed activation and high expression of the EphA2 receptor on KLE and RL95-2 endometrial tumor cell lines but only weak expression on Ishikawa cells in **Figure 5A**. In addition, we determined the EphA2 RNA expression by the real-time qPCR in a panel of tumor cell lines as fold gene expression in **Figure 5B**. We found the highest EphA2 expression in solid tumors including prostate (DU-145), colon (HT-29), and renal (A-498) carcinoma cell lines in contrast to mostly negative hematological cell lines.

Next, we aimed to determine whether the EphA2 is involved in γδ T-cell killing. In the blocking experiments, we first preincubated the target cells with the EphA2 antibody prior to 4-h cytotoxicity assays and then analyzed the specific lysis of KLE (**Figure 6A**) and RL95-2 (**Figure 6B**) target cells in the presence/absence of the EphA2. Interestingly, all Vδ1 T cells isolated from healthy donors killed efficiently both tumor cell lines at 5:1 and 10:1 E:T ratios; however, the cytotoxicity was reduced when the EphA2 receptor was blocked. The inhibitory effect of anti-EphA2 on tumor cell killing was determined for KLE cells in the range 14%–40% (median 25%) and for RL95 cells in the range of 15%–40% (median 26%). Together, these results suggested that EphA2 was recognized by cytotoxic Vδ1 T cells in the tumor killing.

To validate these findings, we then tested the *in vitro* effects of the EPHA2 small-molecule inhibitor ALW-II-41-27 on KLE endometrial tumor cells. We incubated the KLE target cells with ALW-II-41-27 inhibitor at 1 and 10 μM concentrations and showed the specific lysis significantly reduced at both 5:1 and 10:1 E:T ratios in the range 50%–80% (median 71%) (**Figure 6C**). Importantly, the inhibition of cytotoxicity by Vδ1 T cells was

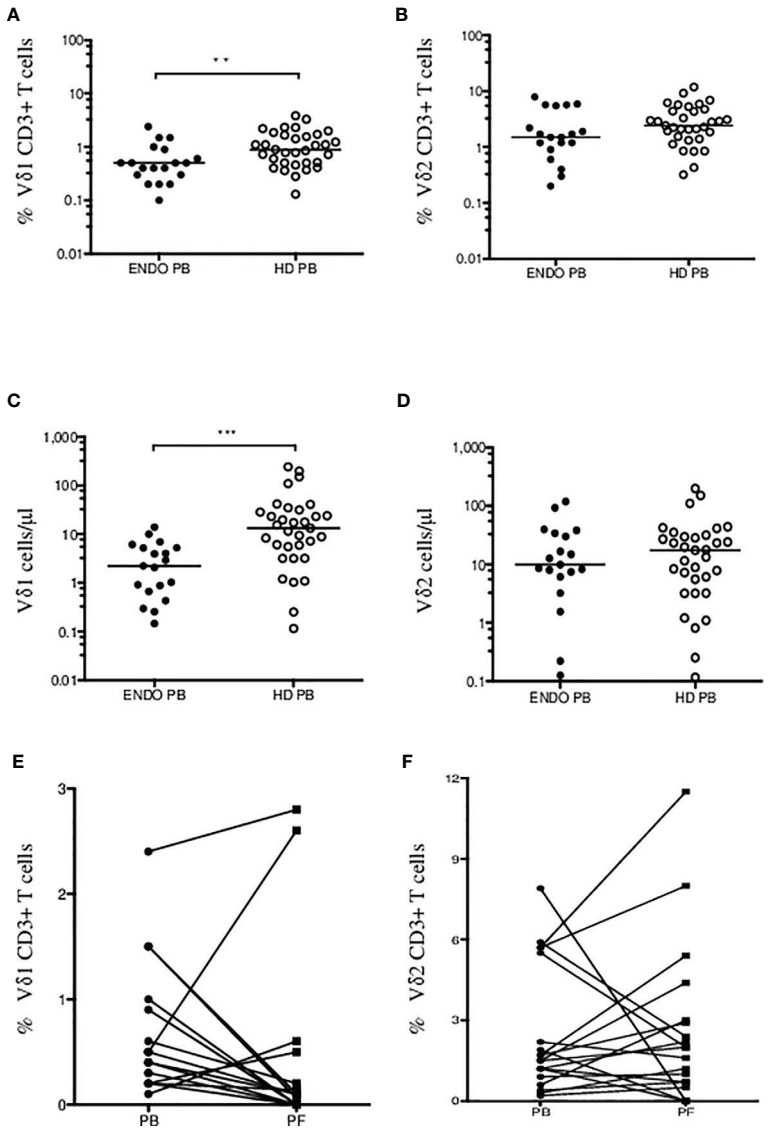

FIGURE 2 | Summary of Vδ1 and Vδ2 γδ T cells in endometriosis patients. Percentage of CD3+ Vδ1 **(A)** and Vδ2 **(B)** γδ T cells in endometriosis patients (ENDO, filled circles) and age- and sex-matched healthy donors (HD, empty circles) are shown. Absolute counts of Vδ1 **(C)** and Vδ2 **(D)** γδ T cells in PB in patients compared with HD were determined. Analysis of Vδ1 **(E)** and Vδ2 T cells **(F)** in patient's peripheral blood (PB) and paired peritoneal fluid (PF) samples. The median values are shown. Statistically significant differences are presented as $^{**}p = 0.008$; $^{***}p = 0.0002$.

shown as dose dependent. Next, we used the EphA2-negative Ishikawa cell line and determined the specific lysis at 10:1 E:T in the presence of ALW-II-41-27 (1 μM). No significant reduction of tumor killing was observed (**Figure 6D**). Of note, the pharmacological effect of inhibitor ALW-II-41-27 on cell viability in drug treatment sample relative to a DMSO control group was determined independently prior the killing assays and no increase of the spontaneous cell lysis was detected after 4h (data not shown). In addition, the inhibitor ALW-II-41-27 showed no change in the surface expression of EphA2, B7-H6 and stress ligands in 4-h cytotoxicity incubation (data not shown).

Finally, to confirm the inhibition of the EPHA2 resulting in significantly decreased *in vitro* tumor cell death, we generated EPHA2 knockouts (KO) in endometrial cell line RL95-2 by the CRISPR/Cas9 method. The loss of EPHA2 significantly reduced specific lysis by 35%–90% (median 45%) by Vδ1 T cells in knockout *versus* wild type (WT) at 5:1 E:T ratio is shown in **Figure 7A**. Next, we observed the Vδ1 T-cell cytotoxicity inhibition of RL95-2 WT *versus* KO cells and also in the addition of ALW-II-41-27 inhibitor (1 μM) at 10:1 E:T in **Figure 7B**.

To further verify these results, we generated EPHA2 knockouts in A-498 (renal) and HT-29 (colon) tumor cell

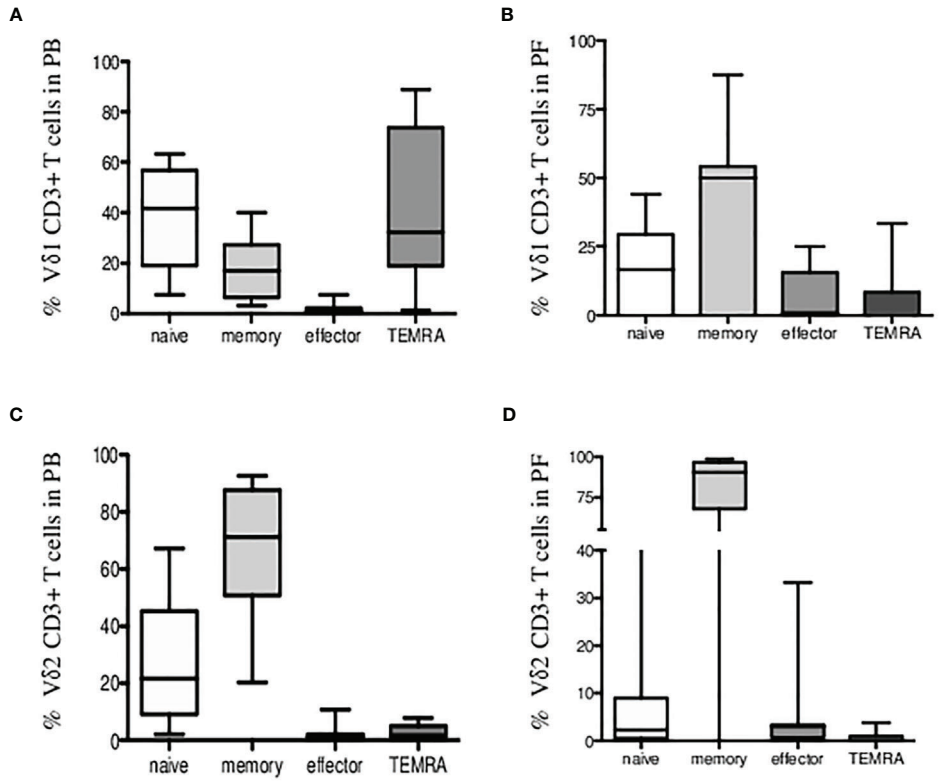

FIGURE 3 | Immunophenotyping of Vδ1 and Vδ2 γδ T cells in endometriosis patients. Percentage of CD3+ Vδ1 T cells **(A)** in peripheral blood and **(B)** in peritoneal fluid samples showing expression of CD27 and CD45RA markers for naïve/memory/effector and TEMRA phenotypes. **(C)** Percentage of CD3+ Vδ2 T cells in peripheral blood and **(D)** in peritoneal fluid samples is shown.

lines which had previously showed the highest EphA2 expression in **Figure 5B**. The summary of Vδ1 T cell cytotoxicity results is shown for A-498 in **Figure 7C** and HT-29 in **Figure 7D**. The A-498 KO cells showed significant protection from specific lysis mediated by Vδ1 T cells compared with WT cells at 10:1 ratio between 40% and 77% (median 61%). Similarly, HT-29 KO cells presented significant reduction of tumor killing than WT cells at 10:1 ratio between 42% and 75% (median 44%).

In summary, we evaluated Vδ1 γδ T-cell cytotoxicity against tumor cells and found consistently that EphA2 expressed on cancer cells show susceptibility to cell lysis by tumor-reactive Vδ1 T cells.

DISCUSSION

It is well accepted that women with endometriosis exhibit numerous immune dysfunctions and that the immune system plays a central role in its etiology, infertility, increased risk of ovarian carcinoma, or poor pregnancy outcomes (32). Pathogenesis of endometriosis is poorly understood, and the incomplete phenotyping of immune cells within the endometrium and peritoneal fluid of women with the disease warrants urgent research to identify biomarkers that could be used to predict or verify the disease.

In this study, we determined for the first time the numbers of Vδ1 and Vδ2 γδ T-cell subsets in peripheral blood and peritoneal fluids in patients with endometriosis. We observed dramatically reduced numbers of circulating Vδ1 T cells in endometriosis women compared with healthy donors; however, no differences were found for Vδ2 T cells between endometriosis patients and healthy controls. Interestingly, we described the presence of both Vδ1 and Vδ2 γδ T cell subsets in the peritoneal fluid.

Next, we demonstrated for the first time the cytotoxicity of γδ T-cell subsets against endometrial tumor cell lines including Ishikawa, KLE, and RL95-2. Both Vδ1 and Vδ2 γδ T cells were able to lyse tumor cell lines at low 5:1 E:T ratios with specific lysis ranging between 20% and 68% in the 4-h killing assays. We have shown earlier similar cytotoxicity of γδ T cells against solid tumor cell lines including DU145 (prostate), MCF7 (breast), and A498 (renal) carcinomas (31). Together, our results show frequencies of γδ T-cell subsets in endometriosis patients and their cytotoxicity function against endometrial tumor cell lines. Recent studies have highlighted the correlation of tumor-infiltrating γδ T lymphocytes with patient disease outcome that further confirms the role of γδ T cells in cancer immune surveillance (33, 34). Importantly, γδ T lymphocytes are being

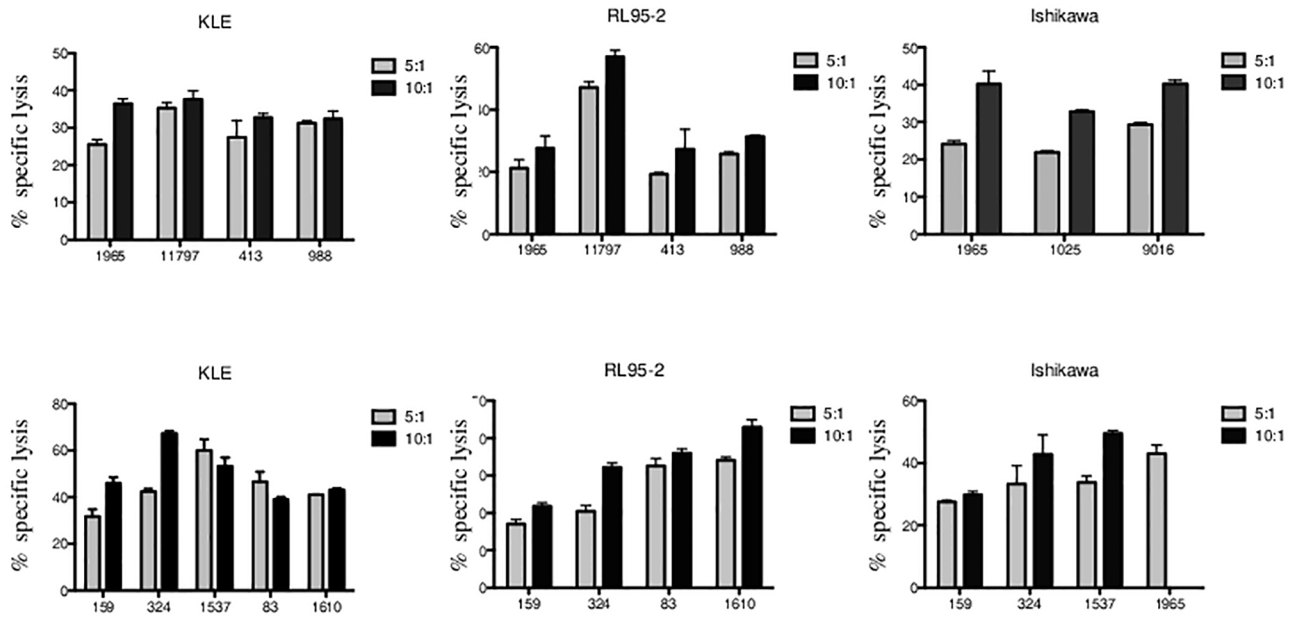

FIGURE 4 | Vδ1 and Vδ2 γδ T-cell-mediated killing of endometrial tumor cell lines KLE, RL95-2, and Ishikawa. Freshly sorted γδ T cells from three to five healthy donors (numbered anonymously) were co-cultured with tumor targets for 4 h, and specific lysis was determined at 5:1 and 10:1 E:T ratio. The results from independent experiments of Vδ1 γδ T-cell cytotoxic reactivity against KLE, RL-95, and Ishikawa is shown as the mean ± SD of sample duplicates. Summary data of specific lysis and prominent cytotoxicity of Vδ2 γδ T cells against KLE, RL-95, and Ishikawa is shown as the mean ± SD of independent experiments performed in duplicates.

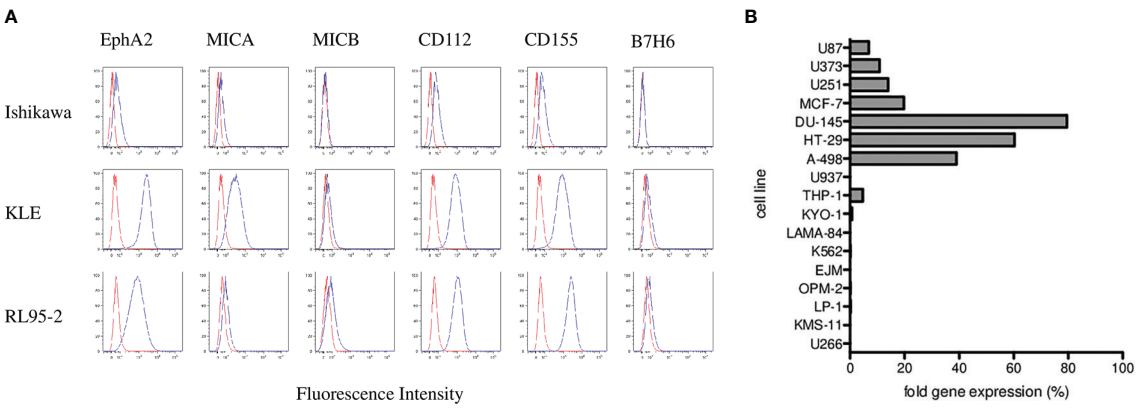

FIGURE 5 | Phenotyping and surface expression of EphA2, MICA, MICB, CD112, CD155, and B7-H6 on target endometrial tumor cell lines including Ishikawa, KLE, and RL95-2 by flow cytometry. **(A)** Representative plots are shown as histograms of the unstained controls (red) and histograms representing the stained samples (blue). Data are expressed as mean fluorescence intensity (MFI, *x*-axis) *versus* number of cells (*y*-axis). **(B)** Summary of the EphA2 expression analyzed by the real-time qPCR in a panel of tumor cell lines presented as fold gene expression.

intensively investigated towards better clinical applications and new immunotherapeutic interventions (35–37).

In order to elucidate possible mechanisms involved in γδ T-cell cytotoxicity, we chose the EphA2 as it is often overexpressed in many cancers including endometrial carcinomas (28, 29) and also ranked 25th of cancer antigens prioritized for translational research (38). We showed high expression of the EphA2 receptor on KLE and RL-95 endometrial tumor cell lines, and these were used as targets in the EphA2 blocking studies. First, we showed reduced cytotoxicity of Vδ1 T cells after we preincubated KLE and RL-95 target cells with the EphA2 antibody prior to 4-h cytotoxicity assays. Second, we used the EPHA2 small-molecule inhibitor ALW-II-41-27 on KLE endometrial tumor cells and also showed specific lysis significantly reduced at both 5:1 and 10:1 E:T ratios. Third, to

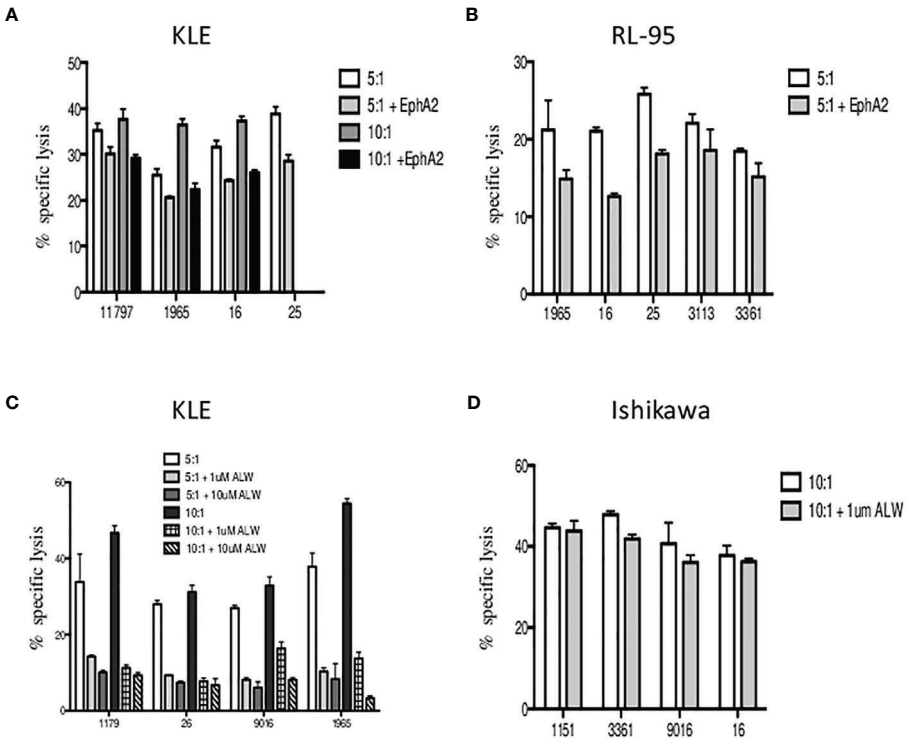

FIGURE 6 | The inhibition of Vδ1 T-cell-mediated killing by blocking of EphA2 expression on endometrial tumor cells. The target cells were preincubated with the EphA2 antibody prior to 4 h cytotoxicity assays and the specific lysis of KLE **(A)** and RL95-2 **(B)** cells was determined at 5:1 and 10:1 E:T ratios. Summary data of specific lysis and cytotoxicity reduction in the presence of EphA2 antibody is shown as the mean ± SD of independent experiments performed in duplicates (HD numbered anonymously). **(C)** Analysis of the *in vitro* effects of the small-molecule inhibitor ALW-II-41-27 at 1 and 10 μM on KLE endometrial tumor cells. Dose-dependent inhibition of cytotoxicity by Vδ1 T cells is shown at 5:1 (pale grey bars) and 10:1 (dark grey bars) E:T ratios. **(D)** Vδ1 T-cell-mediated killing of EphA2-negative Ishikawa endometrial cell line was analyzed at 10:1 E:T with/without the presence of small-molecule inhibitor ALW-II-41-27 (1 μM) and is shown as the mean ± SD of independent experiments performed in duplicates.

confirm the effect of EphA2 inhibition, we generated EPHA2 knockout in endometrial cell line RL95-2 by the CRISPR/Cas9 method and showed significantly reduced specific lysis by Vδ1 T cells in knockout *versus* wild type at 5:1 and 10:1 E:T ratios. Fourth, to further validate the inhibition of cell lysis by tumor-reactive Vδ1 T cells, we used EPHA2 knockouts in renal and colon carcinoma cell lines. Both A-498 KO and HT-29 KO cells showed significant protection from specific lysis mediated by Vδ1 T cells compared with WT cells at 10:1 ratio.

In recent years, studies have been accumulating on differential expression of Eph receptors and their ligands. In particular, the EphA2 triggers cellular events that are deregulated and implicated in carcinogenesis (39). In normal adult tissue, EphA2 expression is absent or present at low levels whereas in malignant cells is overexpressed and functions as a powerful oncoprotein. Targeting Eph receptors with antibodies, peptides and small molecule inhibitors have been widely explored (40–42). Targeting EphA2 is especially attractive in ovarian cancer, in which overexpression is present in over 75% of cases. It was shown in multiple preclinical models of ovarian, breast, and pancreatic cancers that inducing EphA2 downregulation by antibody-mediated inhibition of signaling, antibody-mediated downregulation of total EphA2 expression, and siRNA-mediated inhibition of expression the tumor growth is decreased, further prolongs survival and inhibits angiogenesis (43). Similarly, the pharmacologic inhibition of EPHA2 by the small molecule inhibitor ALW-II-41-27 reduced the viability of resistant tumor cells and inhibited tumor growth *in vivo* in lung cancer models (44). Moreover, high expression of EphA2 was found in endometrial carcinoma and was significantly associated with adverse patient outcome (45).

In summary, we showed for the first time the infiltration of Vδ1 and Vδ2 in peritoneal fluid samples in patients with endometriosis. We determined inherent γδ T-cell cytotoxic reactivity of both subsets from healthy donors against endometrial tumor targets. Importantly, we found that blocking of EphA2 expression significantly inhibits cytotoxicity of tumor reactive Vδ1 γδ T cells. Modifications of EphA2 expression may alter the susceptibility to Vδ1 γδ T-cell-mediated tumor recognition and killing that might be highly relevant in therapies targeting EphA2 in solid tumors and EphA2-positive leukemia (46). Most recent study has identified EphA2 as an antigen recognized by a Vδ1 TCR (30). Our functional data of blocking EphA2 on three different solid tumor cell lines by CRISPR/Cas9 had significantly modified Vδ1 γδ T-cell-mediated tumor lysis.

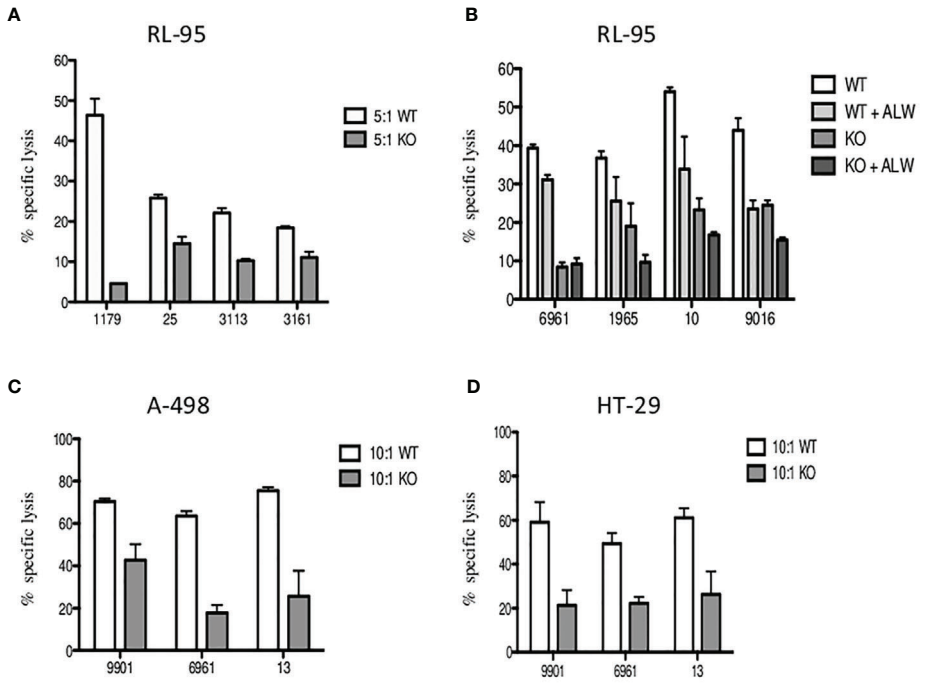

FIGURE 7 | The inhibition of specific lysis of EphA2-positive RL-95 endometrial cell line. **(A)** Freshly sorted Vδ1 T cells were co-cultured with tumor targets for 4 h, and cytotoxicity was determined at 5:1 E:T ratio for the wild type (WT, white bars) and the EPHA2 knockout (KO, grey bars) shown as the mean ± SD of independent experiments performed in duplicates (HD numbered anonymously). **(B)** Similarly, inhibition of specific lysis by Vδ1 T cells was determined at 10:1 E:T ratio for the wild type (WT, white bars) and the EPHA2 knockout (KO, dark grey bars) with/without the presence of small-molecule inhibitor ALW-II-41-27 (1uM) and is shown as the mean ± SD of independent experiments performed in duplicates. **(C)** The EPHA2 knockouts of renal tumor cell line A-498 (grey bars) and colon adenocarcinoma tumor cell line HT-29 **(D)** were generated and specific lysis was compared with WT cells (white bars) at 10:1 ratio. Significant inhibition of cell lysis mediated by tumor-reactive Vδ1 T cells was shown as the mean ± SD of independent experiments performed in duplicates.

Further expression and functional studies are warranted to demonstrate the therapeutic values of inhibiting the EphA2 in different malignancies, which may however compromise the antitumor Vδ1 γδ T-cell cytotoxicity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Faculty of Medicine, Masaryk University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AK designed the study, performed the experiments, analyzed and interpreted data, and wrote the manuscript. MP and BK contributed to research and collected and analyzed the data. IS and RH are in charge of patient accrual and collected the clinical data. RH and MP critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Advanced Imaging Techniques for Differentiating Pseudoprogression and Tumor Recurrence After Immunotherapy for Glioblastoma

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Glioblastoma (GBM) is the most common malignant tumor of the central nervous system with poor prognosis. Although the field of immunotherapy in glioma is developing rapidly, glioblastoma is still prone to recurrence under strong immune intervention. The major challenges in the process of immunotherapy are evaluating the curative effect, accurately distinguishing between treatment-related reactions and tumor recurrence, and providing guidance for clinical decision-making. Since the conventional magnetic resonance imaging (MRI) is usually difficult to distinguish between pseudoprogression and the true tumor progression, many studies have used various advanced imaging techniques to evaluate treatment-related responses. Meanwhile, criteria for efficacy evaluation of immunotherapy are constantly updated and improved. A standard imaging scheme to evaluate immunotherapeutic response will benefit patients finally. This review mainly summarizes the application status and future trend of several advanced imaging techniques in evaluating the efficacy of GBM immunotherapy.

Keywords: glioblastoma, immunotherapy, treatment response, pseudoprogression, tumor recurrence, advanced imaging

INTRODUCTION

Glioblastoma is the most common malignant brain tumor in adult and is extremely aggressive. The current standard treatment involves maximal safe resection, followed by radiotherapy and adjuvant chemotherapy (1). Despite this active treatment, the prognosis remains poor, with a median survival of less than 2 years (2). The main reason is that glioblastoma is strongly aggressive and grows rapidly, specifically, tumor cells are prone to infiltrate the normal brain parenchyma aside the lesion (3, 4). Thus, there is a risk of tumor recurrence once tumor stem cells remain after the resection and follow-up treatment. Many other treatments have been studied, such as immunotherapy, aiming to stimulate or mobilize the immune system and enhance the antitumor immunity in the tumor microenvironment, so as to control and kill tumor cells (5). This treatment concept has derived a

variety of treatment strategies, and remarkable progress of those methods has been made in the treatment of patients with intractable solid tumors such as melanoma and nonsmall cell lung cancer (6, 7). There are also many immunotherapy studies of glioma not only basic but also clinical. Due to the existence of blood-brain barrier (BBB) in the central nervous system (8), obvious loss of lymphatic reflux system (9), and the strong heterogeneity of GBM (10), the effectiveness of immunotherapy for brain tumors might be limited. Fortunately, it has been found that immunotherapy has the potential to induce immune changes in brain tumors (11, 12).

One of the challenges in the treatment of GBM is how to assess the treatment response accurately in order to make more informed clinical decisions. It is important to evaluate the treatment response to immunotherapy in an early stage by using noninvasive imaging, which can reduce unnecessary clinical complications. However, conventional imaging techniques are usually difficult to distinguish between pseudoprogression and tumor recurrence. Immune response is usually accompanied with inflammatory reaction characterized by the enlargement of enhanced foci, which is easily confused with the behavior of tumor relapse. Effective immunotherapy may be mistakenly terminated if being misdiagnosed, thus causing a negative impact on the prognosis. To solve this problem, researchers have carried out a lot of researches on advanced imaging techniques. This review describes the definition and clinical significance of pseudoprogression, generalizes the response evaluation criteria of GBM, summarizes the status and future development direction of advanced imaging techniques relevant to immunotherapy in GBM, and discusses the strengths and deficiencies of artificial intelligence (AI) in monitoring therapeutic response in GBM.

PSEUDOPROGRESSION OF GBM

About 30% of GBM patients who received radiotherapy and adjuvant temozolomide-based chemotherapy had pseudoprogression, which mainly occurred within 3 months after treatment (13). According to the Response Assessment in Neuro-Oncology (RANO) criteria, pseudoprogression was defined as the appearance of new lesion or an increase in contrast-enhancing areas, but these changes gradually faded or stabilized without changing the treatment (14). At present, it is believed that the enlargement of enhanced foci may be caused by the infiltration of inflammatory factors after radiotherapy and chemotherapy, but the real cause of pseudoprogression remains to be further studied. In addition, the methylation status of the O6-methylguanine-DNA methyltransferase (MGMT) promoter was associated with pseudoprogression, and about 2/3 of GBM patients with MGMT methylation exhibited pseudoprogression (15).

Patients with pseudoprogression usually have no clinical symptoms and only show new or enlarged enhanced lesions on images. Such patients usually only need symptomatic treatment and do not need to change the treatment project, while patients with tumor recurrence probably need to resect the lesion again or find another cure. If there is no accurate distinction between them, the effectiveness of treatment may be reduced. Therefore, correct identification of pseudoprogression and tumor recurrence is of great significance to guide clinical decision-making.

RESPONSE EVALUATION CRITERIA OF GBM

Noninvasive imaging for GBM can help define widely applicable treatment response criteria to assess disease progression and make clinical decisions. In order to address imaging challenges such as pseudoprogression, multidisciplinary experts developed RANO criteria (14), which suggested that the original treatment regimen can be maintained for patients with no clear clinical symptoms and only tumor progression on imaging. These patients only need regular follow-up. At present, the RANO criteria have been widely accepted in the field of neuro-oncology and applied in clinical and scientific researches. However, evaluating the therapeutic response to immunotherapy only by RANO criteria may not be sufficient. For example, the mechanism of pseudoprogression caused by immunotherapy may be different from that of standard therapy, which may be due to the infiltration of immune cells and inflammatory cells. It is necessary to establish corresponding imaging response criteria for immunotherapy in GBM.

Based on the important factors above, experts developed immunotherapy Response Assessment in Neuro-Oncology (iRANO) criteria for patients with GBM receiving immunotherapy to provide guidance for imaging changes in the early stage of progression (16). According to the iRANO criteria, the time window for pseudoprogression after immunotherapy is 6 months. Hence, the criteria recommend that patients with no significant clinical symptoms and evidence of early imaging progress within 6 months after immunotherapy should continue to receive immunotherapy before follow-up imaging confirms the tumor progression. In other words, patients with evidence of imaging progress outside the time window after immunotherapy will have a higher probability of potential true tumor progression, and these patients should be advised to discontinue ongoing immunotherapy.

APPLICATION OF ADVANCED IMAGING IN IMMUNOTHERAPY OF GBM

At present, the researches of glioma immunotherapy strategy mainly include the following: (1) specific peptide vaccine; (2) immunotoxin therapy; (3) immune checkpoint inhibitors (ICIs) therapy; (4) dendritic cell (DC) therapy; and (5) chimeric antigen receptor T-cell (CAR-T) Immunotherapy (17–21). The feasibility and safety of DC vaccine in the treatment of glioma have been proved, and it could induce immune response (20). It is worth noting that a new type of gamma delta T (γδ T)-cell therapy is becoming a rising star of cancer immunotherapy (22). Unlike the alpha beta T (αβ T) cells involved in most T-cell

researches and clinical applications, γδ T cells recognize their target cells independently of major histocompatibility complex (MHC) and do not cause graft-*versus*-host disease. γδ T cells infiltrate in a variety of tissues, which can quickly respond to the target cells and release effector cytokines. Furthermore, the recognition and killing of tumor by γδ T cells do not depend on the expression of single antigen (23). Based on the advantages of γδ T cells, a new CAR-T therapy can be developed to break through the limited application of αβ T-cell-based CAR-T-cell therapy in solid tumors (including gliomas) (24, 25). Currently, γδ T-cell therapy has been studied in the treatment and prevention of recurrence of solid tumors including head and neck cancer, breast cancer, and lung cancer (26–28). The therapeutic effect in glioma still needs to be verified in a large number of clinical trials.

Advanced imaging techniques based on physiological or metabolic characteristics may reflect the state of tumor more accurately, so various advanced imaging techniques are being studied to correctly identify immunotherapy-related changes and tumor progression and provide a credible basis for the treatment of patients. The advanced imaging techniques used in GBM currently include perfusion-weighted imaging (PWI), diffusion imaging, amide proton transfer (APT), magnetic resonance spectroscopy (MRS), positron emission tomography (PET) (i.e., **Table 1**). Some of these imaging techniques have been used to evaluate the immunotherapy efficacy of glioma. The following will introduce the basic concepts of these imaging techniques and describe the latest research progress and future application prospects that support them in the evaluation of therapeutic response to immunotherapy.

Perfusion-Weighted Imaging

PWI can reflect tissue perfusion by quantitatively calculating perfusion parameters including relative cerebral blood volume (rCBV), relative cerebral blood flow (rCBF), mean transit time (MTT), and time to peak (TTP). When the tumor progresses, neovascularization and increased perfusion could be observed in the lesion area. As pseudoprogression is usually caused by inflammation, there is no neovascularization and the perfusion is relatively low. As a consequence, these perfusion parameters can be used to distinguish between pseudoprogression and tumor recurrence in GBM patients receiving standard treatment or immunotherapy (30, 40).

DSC-MRI is the most commonly used perfusion technique in clinic. Evidence has shown that adding perfusion imaging to conventional MRI in patients with gliomas is helpful for clinical decision-making (41, 42). A recent meta-analysis including 35 studies on the role of various advanced imaging techniques in evaluating the therapeutic response of high-grade gliomas indicated that the diagnostic accuracy of perfusion imaging was only second to MR spectroscopy (MRS). The sensitivity and specificity of DSC were 87% and 86%, respectively, while the sensitivity and specificity of DCE were 92% and 85%, respectively (43). In addition, a retrospective study comparing the value of DSC-MRI and DCE-MRI combined with T1WI enhancement and DWI imaging in predicting the recurrence of GBM revealed that both the two perfusion imaging could significantly improve the diagnostic accuracy, and there was no significant difference in diagnostic performance (42). Similarly, some studies have compared the diagnostic accuracy of DSC-MRI with three-dimensional pseudocontinuous arterial spin labeling (3D-pcASL) and suggested that the ability of 3D-pcASL perfusion imaging in distinguishing between pseudoprogression and tumor recurrence in GBM patients is almost the same as that of DSC, but 3D-pcASL is superior to DSC when the lesions are disturbed by magnetic susceptibility artifacts (44, 45). The reason is that the fast spin echo (FSE) technology used in GE 3D-ASL can effectively overcome the disadvantages of DSC being vulnerable

TABLE 1 | Studies of applying advanced imaging techniques to assess immunotherapeutic responses in GBM.

References	Advanced imaging	Evaluation parameters	Tumor type	Immunotherapy category	Evaluation criteria
(29)	DSC-MRI DWI-MRI	ΔrCBVmax rADC	GBM	DC vaccination	RANO
(30)	DSC-MRI DWI-MRI	Maximum lesional rCBV ratios Minimum ADC	Recurrent GBM	DC vaccination	Macdonald
(31)	DSC-MRI	rCBV	GBM	Immunogene-treated	NA
(32)	DCE-MRI	Ve	GBM (rats)	mAb9.2.27+NK	NA
(33)	DSC-MRI DWI-MRI	Interval change in rADC	Recurrent GBM	ICIs	mRANO
(34)	DWI-MRI	Serial parametric response mapping of ADC	Pediatric diffuse intrinsic pontine glioma	Peptide-based vaccine	NA
(35)	DWI-MRI	IADC VOI	Recurrent GBM	ICIs	RANO Pathological
(36)	DWI-MRI	RSI	GBM	ICIs	Pathological
(37)	MRS	Cho, NAA, Crea, Lac	GBM	IL-4 toxin	Pathological
(38)	Amino acid PET	18F-FET PET/CT	GBM	DC vaccination	RANO
(39)	dck PET	[18F]-CFA PET/CT [18F]-FAC PET/CT	GBM (human) Orthotopic malignant gliomas (mice)	DC vaccination and/or PD-1 mAb blockade	NA

Ve, extravascular extracellular space volume fraction; IADC, intermediate ADC; VOI, volumes of interest; mAb9.2.27, a monoclonal antibody-targeting NG2; NK, natural killer cells; IL-4, interleukin 4; mRANO, modified RANO.

to susceptibility artifacts. The artifacts can attenuate the imaging signal, usually when the focus is on the skull base, paranasal sinuses or large surgical resection cavity with blood residue. Another deficiency of DSC imaging is that the contrast medium may leak into the space where the BBB is destroyed. When it happens, the values of rCBV parameters cannot reflect the real perfusion level (46). Also, there is no unified standard between different imaging parameters and postprocessing methods. These factors will affect the diagnostic accuracy of DSC perfusion imaging to varying degrees.

So far, there are still few researches about the application of PWI on assessing the immunotherapeutic response of GBM. In a study of advanced MRI assessing dendritic cell immunotherapy against GBM, it was found that the difference of relative cerebral blood volume ($\triangle$rCBVmax) could effectively differentiate tumor recurrence from pseudoprogression, with a sensitivity of 67% and specificity of 75% ($p = 0.004$), suggesting that the value of $\triangle$rCBV might be more helpful to distinguish them than the absolute value of rCBV during follow-up (29) (**Figure 1**). Research by Vrabec et al. showed that the maximal rCBV ratios in the contrast-enhancing area were potential radiological indicators to distinguish between inflammatory response induced by immunotherapy and tumor recurrence (30). Another follow-up study on immunogene-treated glioblastoma multiforme with DSC perfusion imaging combined with contrast-enhanced MR imaging also supported this view (31).

It is worth noting that both DCE-MRI and ASL techniques have not been widely explored in GBM patients treated with immunotherapy, which may be due to the lack of standardized acquisition parameters of DCE-MRI and the poor image signal of ASL perfusion imaging. However, these two perfusion techniques still have their own advantages. For instance, DCE-MRI can measure vascular permeability by pharmacokinetic parameters to quantify the movement of contrast media through BBB (47, 48). Compared with DSC-MRI, the ability of DCE-MRI of quantifying the permeability can make the calculation of cerebral blood volume more precise. 3D-pcASL can avoid the influence of magnetic susceptibility artifacts. If we could combine the advantages of various perfusion imaging to make up for the shortcomings, we would have a powerful supplementary tool to evaluate immunotherapeutic response in GBM.

Diffusion Imaging

Diffusion-Weighted Imaging (DWI) reflects the diffusion of water molecules in the tissue of interest. The most widely used quantitative parameter is the apparent diffusion coefficient (ADC), which is inversely proportional to the cell density (49, 50). Based on this characteristic, it has been used in tumor identification, grading, and therapeutic response monitoring (51–54). In patients with recurrent gliomas, the diffusion of water molecules within the tumor was limited and the ADC values decreased, while treatment-related response, such as pseudoprogression, had higher ADC values than recurrent gliomas. This point of view was confirmed by a meta-analysis of diffusion magnetic resonance imaging combined with ADC measurements for distinguishing between glioma recurrence and pseudoprogression. Six cohort studies were included in the meta-analysis, and different ADC values were analyzed, including mean ADC values, relative ADC (rADC), and 5th percentile values. The results proved that the ADC values of pseudoprogression was higher than that of tumor recurrence, which provided a reliable foundation for the differentiation of the two (55). To date, some researches have applied this technique to the assessment of glioma immunotherapy and studied the evaluation effect of different ADC values. Song et al. conducted a retrospective study of 19 patients with recurrent GBM to evaluate whether the early changes in the quantitative parameters of diffusion and perfusion MRI before and after immunotherapy can determine the treatment-related changes. They calculated the rADC values and several perfusion parameters of the lesions before and after treatment and found that only the change of rADC could be used as an early marker to evaluate the response within 6 months after treatment (33). Another study also proved that rADC could help predict the immuno-therapeutic response and survival rate in patients with GBM (29) (**Figure 2**). Moreover, serial parametric response mapping of ADC performed at multiple time points of therapy may help identify pseudoprogression as an imaging biomarker in vaccine therapy for pediatric diffuse intrinsic pontine glioma (34). However, some studies believe that the application of the mean ADC values on differentiating pseudoprogression from tumor recurrence has some limitations, because the ADC values of cystic and necrotic areas are higher than that of solid tumors, which will affect the accuracy of the final results. It is considered that the 5th percentile values are better for the distinction (56, 57). Although ADC has good diagnostic value as a whole, the practicability of these different ADC parameters needs to be further studied. In addition, these results need to be verified in multicenter and larger cohorts.

Another kind of imaging technique commonly used in clinic is diffusion tensor imaging (DTI), which uses the diffusion anisotropy of water molecules for imaging. The fractional anisotropy (FA) images can show the structure and anisotropy of white matter fibers in the brain, and the change of FA can evaluate the therapeutic effect. Wang et al. combined DTI and DSC-MRI and found that the best models to distinguish between true progression and none-true progression (pseudoprogression and mixed progression) included FA, linear anisotropy coefficient (CL), and rCBVmax. It is suggested that the combination of DTI and DSC perfusion parameters could help evaluate the therapeutic response of gliomas (58). Although DTI has not been applied to the evaluation of immunotherapy in GBM, a recent study on the association of T cell density and diffusion tensor MRI changes in brain metastases revealed that FA in the peritumoral region was closely related to the density of CD^{3+} T-cell infiltration, indicating that FA could reflect the tumor immune microenvironment. This finding supports future researches and can be used to detect the sensitivity of neurological tumors to immunotherapy (59).

Furthermore, researchers also explored the role of some advanced diffusion models in assessing therapeutic response of brain tumors. These techniques are mainly used in scientific

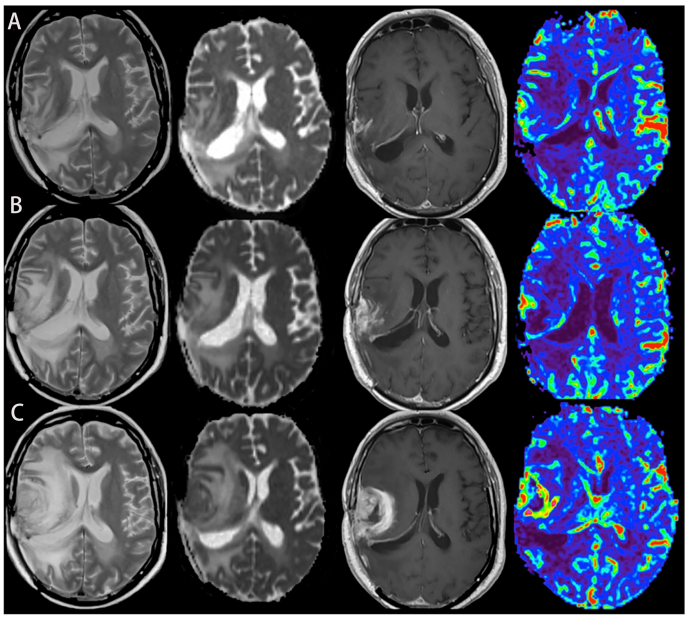

FIGURE 1 | A case of glioblastoma relapsed during immunotherapy, T2, ADC map, T1-enhanced, and CBV map from left to right. **(A–C)** MRI was performed in the 2nd, 6th, and 8th months of immunotherapy, respectively, showing that the edema degree of the lesion was gradually aggravated, the enhancement was more obvious, and the perfusion was higher.

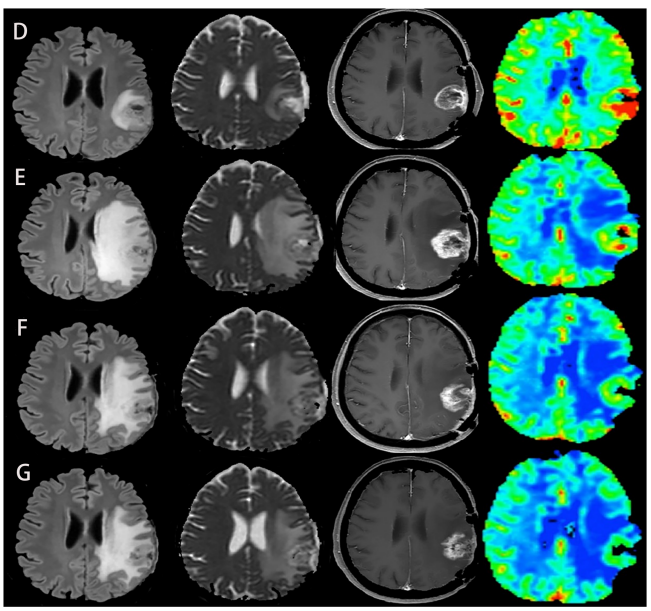

FIGURE 2 | Another case of glioblastoma developed pseudoprogression during immunotherapy, with FLAIR, ADC map, T1-enhanced, and CBV map from left to right. **(D–G)** MRI was performed before immunotherapy and 2, 4, and 6 months after immunotherapy, respectively. Although tumor recurrence was suspected at the second month, the subsequent two MRI showed that the lesion became smaller, the degree of edema and imitation of diffusion alleviated, and the perfusion decreased. These two cases demonstrate that the combination of conventional MRI and advanced MRI imaging can accurately identify pseudoprogression and tumor recurrence of glioblastoma after immunotherapy. The above two figures were reproduced with the permission of (29) (Copyright at Multidisciplinary Digital Publishing Institute).

researches, such as intravoxel incoherent motion (IVIM) MRI, restriction spectrum imaging (RSI) MRI, etc. Based on the double exponential model, IVIM can simultaneously obtain diffusion and perfusion parameters reflecting tumor cellularity and vascularity. Fast diffusion coefficient (D*) mainly reflects perfusion information, slow diffusion coefficient (D) represents real diffusion information, and perfusion fraction (f) reflects blood flow (60). At present, it has been successfully used in gliomas for grading and distinguishing between treatment-related changes and tumor progression (61–63). However, this technique has not been used to monitor the response to immunotherapy yet. RSI-MRI is an advanced DWI technique, which provides a direct method for measuring tumor cellularity *in vivo* (64). Compared with the traditional DWI model, RSI can improve the conspicuity and delineation of high-grade tumors (65), better distinguish between true and pseudoresponse in antiangiogenic therapy (66), and better display white matter tracts in peritumoral edema areas (67). These advantages indicate that RSI-MRI have a good application prospect in the immunotherapy of neurological tumors. In a case report of immunotherapy for GBM, authors demonstrated that RSI could differentiate between pseudoprogression and tumor relapse while conventional DWI imaging could not provide more information (36). Despite that these advanced DWI techniques can provide better tissue structural characteristics than traditional DWI, the potential pathophysiological mechanism of tumor is still unknown.

Amide Proton Transfer

Currently, APT is a relatively popular MR molecular imaging technique that can quantify free proteins with noninvasion and nonradiation. It reflects the changes of concentration and environment by detecting amide proton (NH) in endogenous low-concentration proteins or peptides. This technique displays its application value in a variety of central nervous system diseases (68–73) and shows great potential in glioma grading and curative effect evaluation (74, 75). Ma et al. used three-dimensional APT imaging technique combined with several conventional MRI sequences to evaluate the imaging features of tumor recurrence and pseudoprogression in 32 patients with gliomas who received standard treatment. It was found that the two kinds of progression had similar performance on conventional MRI. On the contrary, patients with tumor recurrence exhibited high signal intensity (relative to contralateral normal brain tissue) on APT-weighted (APTw) images, while patients with pseudoprogression showed equal to mild hyperintensity on APT-weighted images. Quantitative results demonstrated that compared with conventional MRI sequences, APTw could greatly improve the ability of MRI to distinguish between pseudoprogression and tumor recurrence (76). Additionally, as the therapeutic benefit and prognosis of glioma are related to its molecular subtypes and the expression of some proteins, APT imaging can detect the expression of MGMT protein before operation and provide relevant information for the possible drug resistance during treatment and the corresponding targeted therapy (77).

APT also has some shortcomings. In APTw images, red represents higher protein content, but not all red areas represent lesions or high-grade gliomas. Some tissues present high signal intensity on APTw images as well, like fat, cysts, and blood vessels. In addition to gliomas, there are other lesions that may also show high signal intensity, such as meningiomas, lymphomas, and some metastases. Most APTw images remove skull information because of the high signal of skull, which may hide potential lesions near the cerebral cortex. For this reason, using APT imaging alone to judge the nature of lesions may not be accurate enough, and it is best to combine multiple sequences to make a comprehensive diagnosis. Up to now, no research has reported the use of APT in the evaluation of immunotherapeutic response in GBM, but previous studies have shown that APT imaging is of great help to improve the diagnostic accuracy. If it is to become a powerful tool to assess immunotherapeutic response, it is necessary to continue exploiting and developing this technique and carrying out more clinical and scientific researches.

Magnetic Resonance Spectroscopy

MRS uses the phenomenon of magnetic resonance chemical shift to determine the molecular composition of substances. It can simultaneously measure the concentrations of several metabolites in brain tissue and tumors and can be used to diagnose, grade, and evaluate the curative effect of brain tumors (78). The metabolism of brain tumor is exuberant, while that of chronic inflammation is lower. From the metabolism degree of lesion, we can decide its composition and distinguish between benign and malignant tissues (79, 80). The typical proton magnetic resonance spectroscopy (1H-MRS) manifestation of glioma exhibits obvious inversion of Cho/NAA ratio, while inflammatory lesions are characterized by increased Cho/Cr ratio and normal or decreased NAA/Cr ratio (81, 82). Thus, the response induced by immunotherapy and tumor progression in glioma patients can be distinguished by the concentration of metabolites. Floeth et al. found that the metabolic data of MRS may help to distinguish between tumor recurrence and pseudoprogression after local immunotherapy of GBM and contribute to further decision-making (37). In addition, a recent meta-analysis suggested that among the advanced MRI techniques, MRS had the highest diagnostic accuracy in distinguishing between treatment-related changes and tumor recurrence, with a sensitivity and specificity of 91% and 95%, respectively, which showed the good diagnostic performance of MRS (43).

MRS has some limitations in detecting small lesions compared with other MR imaging techniques due to its low spatial resolution, and it needs to be collected in high quantities because of the low concentration of metabolites in tumor tissues, which needs more time. The determination of metabolite concentration may also be affected by MR equipment, pulse sequence and data postprocessing methods. Lastly, MRS requires experienced operators to define exactly areas of interest, which is faced with technical challenges in clinical practice (78).

Positron Emission Tomography

Positron emission tomography-computed tomography (PET-CT) is a metabolic functional imaging technique, which is applied to diagnose and analyze lesions by imaging radioactive

markers. It is commonly used in clinical tumor staging, curative effect evaluation, and therapy. The most widely used PET tracer is 18F-fluorodeoxyglucose (18F-FDG) based on glycolysis, whose tracer concentration occurs in hypermetabolic lesions. Every technology has some deficiencies, and FDG-PET is no exception. First of all, the resolution of PET is relatively low, and normal brain tissue also shows high metabolism. If the lesion is close to the cerebral cortex, the measured FDG uptake value cannot reflect the true condition of the lesion. Furthermore, treatment-related necrotic reactions can also be characterized by increased glucose metabolism, resulting in increased FDG uptake (83). Although FDG-PET is widely used in clinic, it may be for some reasons above that make the accuracy of differential diagnosis of tumor recurrence and pseudoprogression not high (84, 85). Therefore, radioactive tracers with higher tumor-background uptake ratio have been studied.

Due to the increased proliferative activity and amino acid transport of malignant brain tumors, and the relatively low level of amino acid uptake in normal brain tissue, the use of amino acid-based radioactive tracers can improve the tumor-background ratio to some extent and identify tumors better (86). Till now, some radioactive tracers based on amino acids have been developed, such as ^{11}C-methyl-L-methionine (^{11}C-MET) and O-(2-[18F] fluoroethyl)-L-tyrosine (^{18}F-FET). Studies have indicated that these two tracers have good accuracy in making a distinction between treatment-related response and tumor recurrence, and their manifestations are similar (87). However, ^{11}C-MET is difficult to be commonly used in clinic owing to its short half-life and difficulties of preparation. Contrarily, 18F-FET has a long half-life, and the preparation process is relatively easy. In a study of immunotherapy with DC vaccination in GBM patients, 18F-FET PET imaging showed a more accurate identification ability than that of contrast-enhanced MRI initially (38). Although this study had several limitations such as a small sample size, it pointed out that 18F-FET PET had a potential role in monitoring the immunotherapy efficacy of GBM. In addition, Joseph et al. speculated that the PET probe for deoxycytidine kinase (dCK) could be used to distinguish between immune inflammatory response and enhancement foci caused by other factors in contrast-enhanced MRI imaging. They applied DC vaccination and/or PD-1 mAb blockade therapy to mice with orthotopic malignant gliomas model and GBM patients, and then used dCK PET probe and contrast-enhanced MRI for imaging respectively. The ratio of MRI contrast enhancement region to PET probe uptake area (immunotherapeutic response index) was used to describe the immune inflammatory activity in tumors. Finally, it was found that the accumulation of dCK PET probe in tumors and secondary lymphoid organs increased after immunotherapy, indicating that the immunotherapeutic response could be quantified by combining dCK PET probe with MRI imaging, which could be a potential biomarker for monitoring tumor immunotherapy (39).

With the gradual development of PET/MRI, the combination of PET and MRI makes full use of the good soft tissue contrast and multi-parameter evaluation ability of MRI. Compared with PET/CT, PET/MRI has superiority in the diagnosis and characterization of several diseases (88). Researchers have found the potential of PET/MRI in evaluating therapeutic response of GBM (89, 90) and the potential benefit of F-18 fluorothymidine (FLT)–PET/MRI for the diagnosis of melanoma brain metastasis and treatment monitoring of targeted therapy and immunotherapy (91). The ability of PET/MRI imaging in monitoring the treatment response to immunotherapy of GBM needs to be further studied. The future of PET/MRI is bright, and any new techniques need lots of researches to prove its value in clinical application.

APPLICATION OF ARTIFICIAL INTELLIGENCE IN IMMUNOTHERAPY OF GLIOMA

AI has developed rapidly in medical field in the past decade, especially in image identification. Many studies have reported the application of AI in diagnosis, grading, curative effect evaluation, and overall survival prediction of glioma, showing the great superiority of AI technology (92–99). Among them, radiomics is a new field that uses automatic data mining algorithm to transform a large number of image data into high-dimensional feature space. In the identification of treatment response and tumor progression in glioma, studies have investigated that the diagnostic performance of multiparameter radiomics model is better than single parameter model. The former can find more hidden information in the image data of glioma and improve the treatment of patients (100). The expression status of genes related to the prognosis of GBM can also be predicted from the features extracted from radiomics (101). Furthermore, radiogenomics, which combines imaging features with genome maps, is also helpful to find prognosis-related immune biomarkers (102). Despite the rapid development of AI, there are still some problems. Any algorithm needs to provide a large amount of high-quality data, and many researches on AI have a small amount of data, poor quality, and lack unified standards. These are the problems that need to be solved in the future.

CONCLUSION

Pseudoprogression is the main problem that needs to be tackled in the treatment process of GBM, and the identification of which is also essential for the follow-up treatment. However, current assessment of treatment response of immunotherapy is still in the exploratory stage and does not meet the standard of routine clinical use. Fortunately, establishing a standard imaging scheme is the key to reverse this situation. The advanced imaging techniques have been widely studied and used as a tool to evaluate the therapeutic response in GBM. A large number of studies supported that the combination of various advanced imaging techniques can improve the diagnostic accuracy, expanding our prospective to the development of multimodal imaging. As for now, however, these imaging methods need to be further verified in multicenter and large sample clinical trials to

drive them to truly become powerful diagnostic tools in the future.

AUTHOR CONTRIBUTIONS

YL and YM provided the draft, evaluated the literature, and wrote the review. MW, BS, SL, and LC revised the review. RX, FZ, and HC contributed to the review. All authors contributed to the article and approved the submitted version.

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T Cell-Mediated Autoimmunity in Glaucoma Neurodegeneration

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Glaucoma as the leading neurodegenerative disease leads to blindness in 3.6 million people aged 50 years and older worldwide. For many decades, glaucoma therapy has primarily focused on controlling intraocular pressure (IOP) and sound evidence supports its role in delaying the progress of retinal ganglial cell (RGC) damage and protecting patients from vision loss. Meanwhile, accumulating data point to the immune-mediated attack of the neural retina as the underlying pathological process behind glaucoma that may come independent of raised IOP. Recently, some scholars have suggested autoimmune aspects in glaucoma, with autoreactive T cells mediating the chief pathogenic process. This autoimmune process, as well as the pathological features of glaucoma, largely overlaps with other neurodegenerative diseases in the central nervous system (CNS), including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. In addition, immune modulation therapy, which is regarded as a potential solution for glaucoma, has been boosted in trials in some CNS neurodegenerative diseases. Thus, novel insights into the T cell-mediated immunity and treatment in CNS neurodegenerative diseases may serve as valuable inspirations for ophthalmologists. This review focuses on the role of T cell-mediated immunity in the pathogenesis of glaucoma and discusses potential applications of relevant findings of CNS neurodegenerative diseases in future glaucoma research.

Keywords: glaucoma, T cell, autoimmune, neurodegenerative disease, immune modulation therapy

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INTRODUCTION

The retina is an extension of the central nervous system (CNS) in the eye. Retinal ganglial cells (RGCs) are the primary sites of pathology in glaucoma (1). Their cell bodies reside in the inner retina, while their axes extend a long way through the optic nerve into the brain. Thus, glaucoma is considered a leading neurodegenerative disease that is estimated to affect 79.6 million people worldwide in 2020 (2). The pathogenic mechanisms of RGC loss in glaucoma are multifactorial, including elevated intraocular pressure (IOP), aging, oxidative stress, excitotoxicity, and mitochondrial dysfunction, but the full picture of glaucoma remains elusive due to its nature of high complexity and chronicity (3, 4). The role of immune-mediated neurodegeneration in glaucoma has been established in recent decades and is regarded as an important component in the pathogenesis of glaucoma.

Activation of residential immunocompetent cells in the retina (microglia and macroglia) and infiltration of peripheral immune cells (T cells, B cells, macrophages, monocytes, *etc.*) are found to be pathogenic and associated with the RGC loss (5, 6). Based on the discovery of autoantigens and self-reactive T cells, some scholars have proposed glaucoma to be an autoimmune disease with T cells playing a central role (7–9). Meanwhile, the essential roles of autoreactive T cells in the maintenance of tissue homeostasis and restriction of inflammation in the retina and CNS have also been elucidated in animal studies (10, 11). Evidence of either neurotoxic or neuroprotective roles of autoreactive T cells indicates the complexity of neuroinflammation in the progression of glaucoma. The role of autoreactive T cells is multifaceted, and an imbalance in T cell/microglia interactions is considered to be the culprit of glaucoma. Based on this, immunomodulation therapy has been proposed as a potential strategy for glaucoma (12).

As a representative of neurodegenerative disease in the eye, glaucoma shares many common pathological features with other CNS neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) (13–15). Most importantly, they are all characterized by compromised barrier function and chronic neuroinflammation toward self-antigens (16–18). As the strategy to harness the immune response is most extensively explored in AD, it may provide valuable insights and experience for the development of a possible immune modulation therapy for glaucoma. In this review, we focus on the role of autoreactive T cells in glaucoma pathology and treatment options and systematically review publications in PubMed, Embase, and the Cochrane Library based on the topics of "glaucoma", "T cell", and "neurodegeneration".

STRESS RESPONSE AND GUT DYSBIOSIS ACTIVATE PERIPHERAL T CELLS WITH AUTOIMMUNE BEHAVIORS IN GLAUCOMA PATIENTS

Heat Shock Proteins in Stress Response

In glaucoma eyes, chronic stress challenges such as elevated IOP, oxidative stress, glutamate excitotoxicity, deprivation of neurotrophic factors, and ionic imbalance are considered to be primary triggers for neuroinflammation and RGC loss (19, 20). These stress stimuli can be sensed by multiple mechanical and nonmechanical stress receptors expressed on RGCs, including pannexin-1 (Panx1), P2X7 receptor (P2X7R), and transient receptor potential vanilloid isoform 4 (TRPV4), which leads to the production of danger signals (21). Heat shock proteins (HSPs) are one of such signal and have been recognized as principal autoantigens involved in the pathogenesis of glaucoma in both animal disease models and patients (22–25). HSPs are a group of highly conserved proteins that are ubiquitous in cellular organisms and are classified based on their molecular weights into 7 major families (small HSPs, HSP40, HSP60, HSP70, HSP90, HSP100, and HSP110) (26). Under physiological and stressful conditions, HSPs can serve as molecular chaperones to help refold misfolded proteins, enhance the survival of cells, and resist apoptosis (27, 28). Thus, HSPs are innate protectors of cells in the stress response. In addition, although most HSPs are constitutively intracellular components, under stress challenges, their expression is upregulated, and some HSPs can be released into the extracellular space to provoke immune reactions (29). Complex autoantibody patterns, including antibodies for small HSPs (HSP27, B-crystallin, and vimentin) and HSP70 in the aqueous humor have been detected in patients with various subtypes of glaucoma (30, 31). HSPs can induce both innate and adaptive immunity (32). In addition, a high local level of HSP27 itself is also found to be sufficiently pathogenic for glaucoma neural damage (33). However, it should be noted that HSPs should not be solely considered as proinflammatory factors but may induce immune suppression at suitable concentrations (34). Some HSPs also have neuroprotective effects. For example, induction of endogenous HSP72 in rats protects against neurodegeneration in acute IOP elevation (35, 36). Ongoing clinical trials are even applying HSPs to suppress the overactivation of the immune system in patients with rheumatoid arthritis and COVID-19 (37, 38). Thus, in patients with glaucoma, it is generally believed that aberrant production of HSPs under stress conditions and dysregulated autoimmune response in the long term, such as in the case of gut dysbiosis, may tilt the balance and result in uncontrolled neuroinflammation (39).

Gut Microbiota Shapes the Immune System

Recently, researchers have revealed a potential link between gut dysbiosis and glaucoma, with the immune system as the connecting bridge. The gut microbiota is a critical factor that shapes the peripheral immune system and has been found to contribute to the activation of autoimmune T cells in glaucoma and other neurodegenerative diseases (40–42). The gut microbiota serves as the major source of bacterial HSPs in the human body, which cross-react with the highly conserved human HSPs to provoke autoimmunity and RGC damage (40, 43).

The pathological roles of gut dysbiosis in glaucoma are found to be chiefly mediated by T cells rather than autoantibodies or humoral immunity. In a mouse ocular hypertension (OHT) model induced by microbead injection, transient elevation of IOP resulted in a prolonged activation and infiltration of interferon-γ-secreting CD4-positive T cells in the RGC layer and subsequent neurodegeneration. The activated T cells were reactive to HSP27, which, along with its autoantibody, were found to increase in the serum after the OHT challenge. This prolonged autoimmunity to the retina, however, was absent in *TCRβ*$^{-/-}$ mice with deficit T cell immunity but not in *Igh6*$^{-/-}$ mice with deficit B cell immunity (40). On the other hand, although the infiltration of plasma cells in the retina and deposition of autoantibodies are also evident in the retina of glaucoma animal models, their causative roles in glaucoma remain elusive based on the finding that inhibition of B cells or autoantibody deprivation brings no significant benefits of RGC protection (44–46). Moreover, mice raised in a germ-free environment were found to be resistant to chronic OHT challenge and did not result in similar T cell infiltration in the

retina, indicating the fundamental role of preexisting gut microbiota modulation of the immune system in the trigger of glaucomatous retinal damage (40).

The exact mechanisms of how gut microbiota shapes the host systemic immune system remain to be further elucidated, and the local gut mucosal immune system, as well as the compromised gut vascular barrier (GVB), have been suggested to play a role. Studies have found that primary open-angle glaucoma (POAG) patients have different compositions of gut microbiota compared to healthy individuals, with a higher abundance of *Prevotellaceae, Enterobacteriaceae, Escherichia coli*, and decreased number of *Megamonas* and *Bacteroides plebeius* (47). *Helicobacter pylori* infection was also linked to POAG and normal-tension glaucoma (NTG) in a recent meta-analysis (48). The opening of the GVB and dislocation of bacteria and bacterial elements not only affects autoimmune T cells, but may initially result in an overall chronic shift from the anti-inflammatory microenvironment to the pro-inflammatory one locally, systemically, and even at far target sites such as the brain and retina by activating the innate immune system (49). In a mouse experiment, a single peripheral administration of the bacterial element lipopolysaccharide (LPS) resulted in prolonged activation of brain resident microglial cells for over 10 months, resulting in progressive neurodegeneration (50). In the retina, resident microglia can recognize microbial pathogen-associated molecular pattern (PAMP) molecules *via* Toll-like receptors (TLRs), and upregulate the secretion of proinflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α), as well as major histocompatibility complex (MHC) II, an essential element for antigen presentation to T cells during peripheral bacterial infection (51, 52). The chronic activation of innate immunity in the retina, in turn, can facilitate the homing and infiltration of peripheral primed T cells into the retina. Studies have found that peripheral T cells responsive to microbial antigens in the intestine through autoreactive T cell receptors (TCRs) can break the blood-retinal barrier (BRB) and infiltrate the retina, which has previously been considered an immune-privileged site (53). This so-called "dual-hit hypothesis" (first hit in GVB and second hit in BRB) proposed by Braak et al. was first used to describe the potential role of gut dysbiosis in the pathogenesis of PD but is also supposed to be suitable for glaucoma and other neurodegenerative disorders (**Figure 1**). Interestingly, in patients with POAG, dislocation of gut *Helicobacter pylori* is even found in the trabecular meshwork (54). However, it is not clear whether the dislocation of gut bacteria itself is pathogenic or essential for glaucoma or is it just an epiphenomenon of GVB impairment. In addition, recent evidence also suggests that an imbalance in the oral microbiota may also contribute to the pathogenesis of glaucoma, which needs further investigation (55, 56). Above all, emerging evidence suggests that gut dysbiosis may result in the activation of both innate and adaptive immune systems. Through the cross-reaction of human and bacterial HSPs, peripherally-activated T cells may break the BRB and result in RGC damage under stressful conditions.

Recently, modulation of the gut microbiota to reshape the systemic immune response has become an emerging therapy for glaucoma. A study found the beneficial roles of fermented maize slurry and its supernatant rich in probiotic bacteria to reshape gut microbiota in rats in the modulation of retinal immune reaction and protection of RGCs (57). Further studies, especially clinical trials, to illuminate the crosstalk between the retina and gut may provide more valuable information on the pathogenesis of glaucoma and explore the possible intervention methods.

COMPROMISED BRB IS ESSENTIAL FOR T CELL INFILTRATION IN GLAUCOMA EYES

The neural retina is a delicate visual transmission system and is firmly protected from the BRB, which controls the entrance of peripheral immune cells under normal conditions (58). Recently, some scholars have proposed the breakdown of BRB as an essential pathogenic step in glaucoma (59). This theory, although lacks definite evidence, is primarily supported by the observations of retinal T cell infiltration in different animal models of glaucoma (40, 60–62). In addition, the breakdown of blood-brain barrier (BBB) has been observed in some other CNS neurodegenerative diseases. Their common pathogenic features with glaucoma and the similar structures of the BBB and BRB also suggest the potential role of BRB breakdown in glaucoma development (13, 63–65).

The neural retina is isolated from the systemic circulation by the inner BRB (iBRB, formed with nonfenestrated retinal capillary endothelium, vascular basement membrane, pericytes, astrocytes end-feet, and microglial cells) and from the leaky choroidal vessels by the outer BRB (oBRB, formed with tightly connected RPE that stands on the Bruch's membrane) (66). Acute IOP elevation is sufficient to alter the protein levels of tight junctions and adherens junctions of the RPE and subsequently affect the integrity of the BRB in glaucoma animal models (67). In dogs with primary glaucoma, disruption of PRE and extravasation of T cells and plasma proteins suggest the breakdown of both iBRB and oBRB (60). Acute induction of OHT in rats results in a significant reduction in pericyte coverage after 7-10 days, but the extent of vascular leakage remains unchanged (68). However, in patients with POAG and NTG, neurodegeneration is a slowly progressive event, and the breakdown of the BRB is likely associated with the long-standing retinal parainflammation otherwise. For example, aging as a contributing factor of glaucoma is associated with the progressive loss of BRB integrity and a low level of retinal inflammation (69). Some degenerative proteins such as amyloid-beta (Aβ) and hyperphosphorylated tau (p-tau), which are hallmarks of CNS neurodegenerative diseases are also found in the retina of glaucoma patients (14). These degenerative proteins contribute to the local inflammatory response and compromise BRB function by damaging tight junctions of the RPE and activating retinal microglial cells (70–72). The parainflammation state makes the retina vulnerable to the attack by adaptive autoimmune cells of peripheral origin.

The infiltration of T cells into retinal parenchyma includes 2 major steps: first, the extravasation from retinal vessels and then the breaching of glia limitans formed by astrocyte end-feet. The whole process is a continuous action that relies on the close interaction of T cells with multiple local factors and cells, as discussed in detail below.

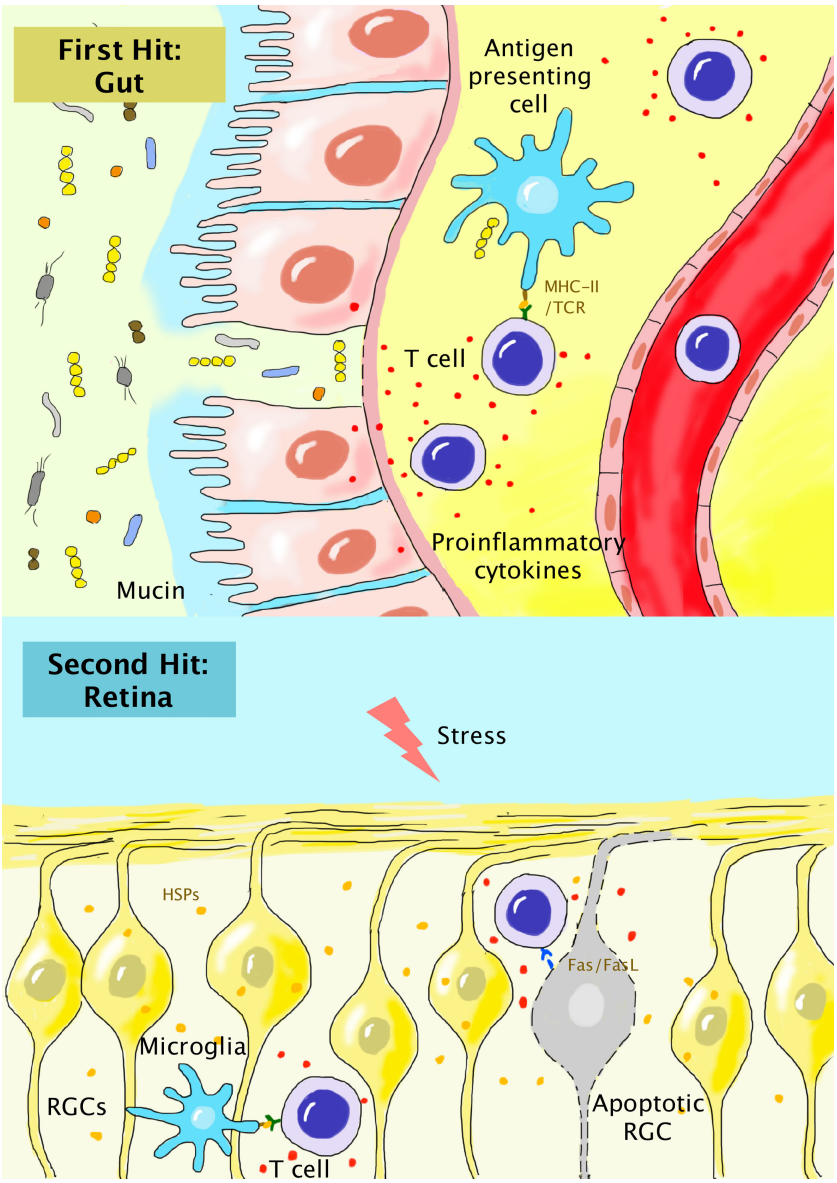

FIGURE 1 | "Dual-hit hypothesis" for the association of peripherally-activated T cells during gut dysbiosis with glaucoma. In gut dysbiosis, the alteration of the components of gut microbiota, compromised mucin and gut epithelium, and dislocation of bacteria result in chronic local inflammation. Autoreactive T cells primed by microbial HSPs are generated and enter the systemic circulation (first hit). In the retina, the chronic stress response results in the release of HSPs and activation of residential microglia. Autoreactive T cells breach the compromised BRB and become reactivated in the retinal parenchyma (second hit). T cells can further induce the apoptosis of RGCs *via* the interaction of Fas/FasL.

T Cell Extravasation

As retinal capillaries are nonfenestrated, T cells have to escape through the tightly-connected vascular endothelium to reach the paravascular space in the retina (**Figure 2**). The extravasation of T cells into the retina is decided by the state of T lymphocyte activation, the state of retinal vascular endothelium, the microenvironment of the neuroretina, and local blood flow, which all affect the lymphocyte-endothelium interaction (73, 74). A study of T cell subsets in glaucoma patients revealed a significant shift in the T cell population and a greater stimulation response (75). When the BRB is intact and vascular endothelial cells are nonactive, peripherally-activated T lymphocytes can also cross the BRB and scan the retina for immune surveillance. Nevertheless, intravenous infusion of 5×10^6 activated ovalbumin-specific T cells in rats only results in the transient opening of the BRB and activation of resident retinal microglial cells that subsides within 3 days (76). This indicates that intact and quiescent BRB can endure the challenge of systemic inflammation. However, when the retina is inflamed, activated vascular endothelium becomes highly adhesive for circulating

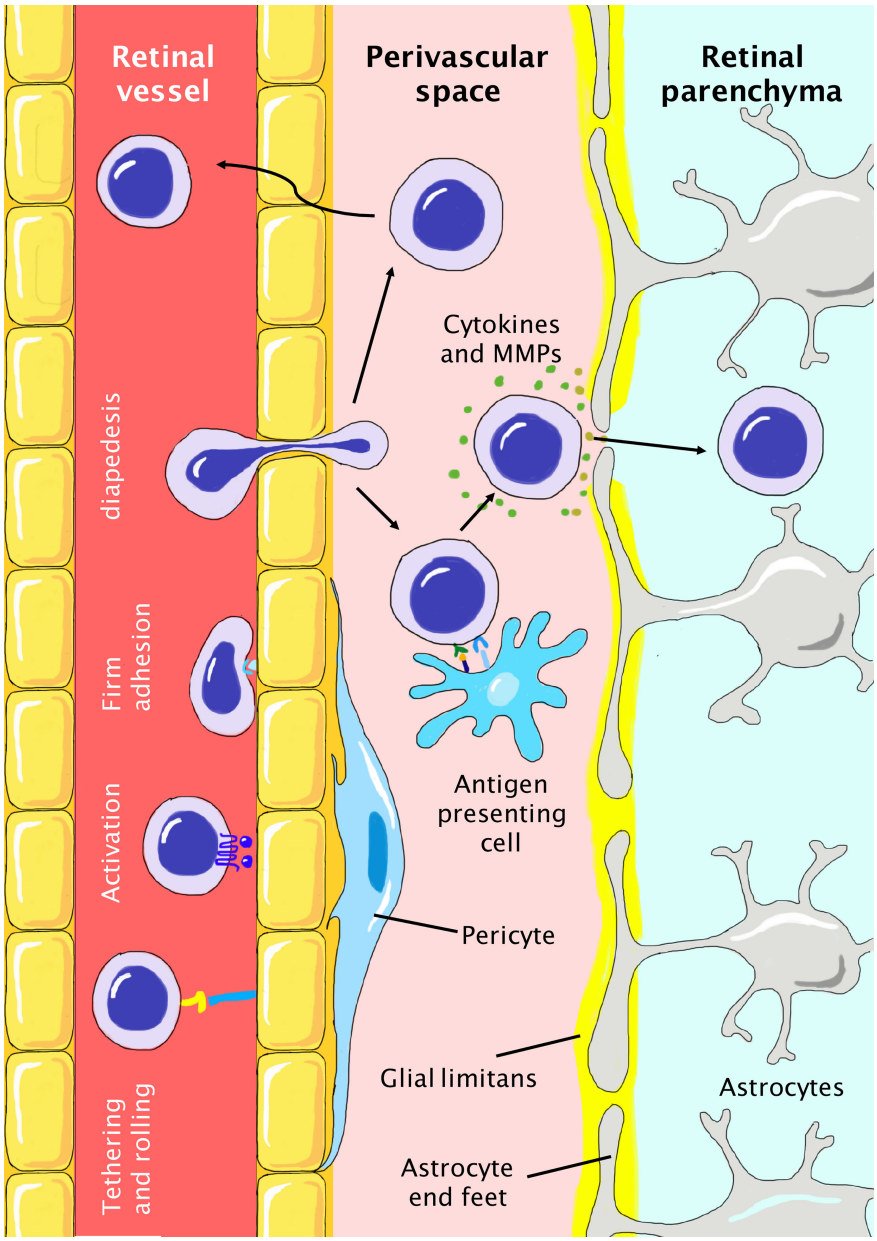

FIGURE 2 | Infiltration of peripheral T cells into the retinal parenchyma during the pathogenesis of glaucoma. The BRB is tightly protected with nonfenestrated vascular endothelium, basement membrane, pericytes, and glia limitans formed by astrocyte end-feet. T cells need to first reach the perivascular space and then breach the glia limitans to finally infiltrate the retinal parenchyma. T cell extravasation includes steps of initial tethering and rolling, activation, firm adhesion, and final diapedesis. In the perivascular space, T cells need to be reactivated by antigen-presenting cells and secrete cytokines and MMPs to breach the glia limitans. Otherwise, T cells have to travel back to the vessel. In glaucoma, the local proinflammatory microenvironment favors the infiltration of T cells due to the higher abundance of molecules (cytokines, selectins, and chemokines) and cells (microglia, dendritic cells) involved in these processes.

lymphocytes by the upregulation of the expression of surface adhesion molecules, and a higher level of T cell extravasation is expected. The whole process of T cell trafficking across the retinal vascular endothelium is a complex action and includes 4 steps: tethering and rolling on the luminal surface, activation, firm adhesion on the vascular endothelium, and diapedesis (**Figure 2**) (74). In the initial step, the interaction of selectins and integrins on the surface of lymphocytes and endothelial cells helps capture circulating immune cells to roll slowly and finally become arrested at the luminal surface (77). This initial attachment is not firm enough, and subsequent activation *via* chemokines presented by endothelial cells is needed to induce clustering and conformational changes of integrins to improve their affinity and avidity (78). T lymphocytes, now tightly

grasped by endothelial cells, can break tight junctions and crawl out of blood vessels *via* transcellular or paracellular pathways (79–81).

In patients with glaucoma, the alterations of both T cell states and induced expression of local cytokines/chemokines may collectively contribute to the elevated extravasation of T cells. Some proinflammatory cytokines, particularly interferon-γ (IFN-γ), tumor necrotizing factor-α (TNF-α), and interleukin-1 (IL-1), are elevated in the aqueous humor or tears of glaucoma patients, indicating a globally proinflammatory state (82–84). These cytokines help T cell tethering and rolling by inducing the expression of integrins on the cell surface (85). Under resting conditions, only approximately 5% of lymphocytes derived from peripheral lymph nodes adhere to the retinal vascular endothelium, which doubles when the endothelium is activated by IFN-γ or IL-1 (86). In addition, peripherally activated T lymphocytes are also able to upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) on the retina, a key integrin molecule for T cell rolling (87). In addition, RGCs of glaucomatous eyes demonstrate significantly elevated expression of genes involved in chemokine signaling (88). In the murine retina, the β chemokine and its receptor C-C chemokine ligand 5/C-C chemokine receptor type 5 (CCL5/CCR5) are constitutively expressed and can respond to IOP challenge (89). CCR5, which is inducible on activation, is found to be involved in the recruitment of T-helper 1 (Th1) cells into the mouse retina (90). Another pair of chemokines (C-X-C motif) ligand 10/(C-X-C motif) receptor 3 (CXCL10/CXCR3) are found to be induced by acute IOP elevation and subsequently contribute to the release of proinflammatory cytokines, elevated expression of E-selectin, and infiltration of inflammatory cells (91). In patients with glaucoma, the levels of chemokines of macrophage chemoattractant protein-1 (MCP-1), CXCR3, CCL2, and CCL7 show prognostic value and are correlated with disease progression (92–94). On the other hand, the induced expression of corresponding chemokines and receptors on the T cell surface under inflamed conditions favors their interplay with the vascular endothelium (90, 95). Overall, the activation of both T cells and the vascular endothelium are two arms of inflammatory episodes in glaucoma that promote T cell extravasation.

The Breaching of Glia Limitans and Roles of Antigen-Presenting Cells

The retinal vessels are tightly wrapped with end-feet of astroglial cells that serve as a double barrier for T cell infiltration into the retinal parenchyma after extravasation (**Figure 2**). This physical barrier, named glia limitans, is concentric to the retinal vessels and creates a paravascular space. Tightening glia limitans with induced matrix metalloproteinase-3 attenuates lymphocyte infiltration after optic nerve injury (96). To cross the glia limitans, primed T cells need to be reactivated by the cognate antigen presented by antigen-presenting cells (APCs) or otherwise have to re-enter the retinal circulation. Thus, such APCs serve as major gatekeepers of the BRB, as they decide the fate of extravasated T cells. Compared with the CNS, the retina lacks meningeal layers and choroidal plexus which harbor numerous MHC-II$^+$ APCs such as marginal dendritic cells and meningeal macrophages (97). In the pathogenesis of glaucoma, it is still under debate whether the APCs that initiate retinal inflammation are exogenous or activated resident cells. To serve as initial APCs, candidate cells need to distribute in the paravascular region and constitutively express MHC-II and costimulatory molecules. In a canine model of acute primary angle-closure glaucoma (PACG), infiltration of MHC-II$^+$ phagocytes in the optic nerve and retina is observed within the first 24 hours, along with the infiltration of circulatory immune cells and RGC loss (98). However, it is controversial whether the infiltration of systemic phagocytes precedes the infiltration of T cells or *vice versa*. Some researchers also identified some innate players that may serve as initial APCs in the retina. A special group of microglial cells that are located in the paravascular space instead of retinal parenchyma are found to constitutively express high levels of CD45, MHC-I, and MHC-II (99). However, these CD45$^+$ microglial cells demonstrate only a weak activation effect on primed T cells (100). Thus, the player (s) of initial antigen presentation and associated antigen in glaucoma remain to be determined, and their dynamic change and interplay with T cells during the disease course need to be further investigated. The initial antigen presentation process may serve as a novel therapeutic target to halt the initiation of neuroinflammation in glaucoma.

Currently, the study of BRB breakdown and the pathogenesis of glaucoma is still an emerging research field, and many detailed aspects remain to be determined. As suggested by studies of AD, the breakdown of the BBB seems to be one of the initiating events that contribute to the subsequent tissue deposition of degenerated proteins and immune cell infiltration (101). Acute animal glaucoma models suggest the temporary breakdown of the BRB in response to elevated IOP and a globally proinflammatory state that facilitates T cell infiltration. However, whether BRB breakdown only serves as the initial trigger of glaucoma neuroinflammation or is essential during the full term of disease progression remains to be determined by appropriate animal models and clinical studies. In addition, the spatial-temporal pattern of T infiltration in the glaucoma retina remains largely unknown.

RETINAL GLIOSIS AND INTERPLAY WITH T CELLS

The retinal parenchyma harbors a group of endogenous immunocompetent cells including microglial cells, astrocytes, and Müller cells, and the latter two are also called macroglial cells. Under physical conditions, retinal glial cells closely interact with neurons and are highly versatile, participating in functions of mechanical support, metabolite transport, nourishment, tissue remodeling, and immunosurveillance (102). As key players of the retinal immune system, glial cells are distinct from conventional immune cells in the systemic circulation, as they are sequestered by the BRB and have no access to lymph nodes for lymphocyte

priming. Nevertheless, the compromised BRB of glaucoma enables their contact with myeloid-derived peripheral immune cells that infiltrate the retina. These glial cells serve as sensors, mediators, and effectors of the immune response and are found to be early responders in the pathogenesis of glaucoma (103, 104).

Different States of Retinal Glial Cells

In glaucoma eyes, resident glial cells undergo a dynamic change with close coordination of each other in a time-dependent manner during the disease course (105, 106). This process, so-called "retinal gliosis" contributes to both neurodegeneration and neuroprotection, depending on the severity and chronicity of the reaction. Retinal microglial cells and astrocytes are highly heterogeneous and can be chiefly divided into 2 states after activation, i.e., the neurotoxic form (M1 and A1 phenotype, respectively) and neurotrophic form (M2 and A2 phenotype) (107, 108). For example, retinal microglial cells, as the major effector and APCs, assume an M1 phenotype with amoeboid morphology when stimulated by the proinflammatory cytokine interferon-γ (IFN-γ), and are able to release TNF-α, IL1-β, superoxides, proteases, and reactive oxygen species (ROS). On the other hand, when stimulated by IL-4, microglial cells turn into the M2 phenotype characterized by thin cell bodies with ramified processes. The M2 phenotype is associated with immunomodulatory cytokines such as IL-4, IL-10, IL-13, and TGF-β (**Figure 3**) (5, 109). Animal models of acute OHT suggest that a predominant M1/A1 type of microglial cells and astrocytes in the acute phase contributes to the early proinflammatory state, which may subside thereafter. The dynamic turnover and interplay between microglial and macroglial cells in glaucoma set the background of retinal inflammation, which is reviewed in detail by Zhao et al. (110).

T Cell-Microglial Interplay for Neurotoxicity

In the acute phase, infiltrated T cells interact with neurotoxic glial cells to induce the escalation of inflammation. This process can result in neurotoxicity and damage to RGCs if not properly controlled. Once breaching the BRB, peripherally activated T cells can interact with microglial cells, which serve as the bridge between innate immunity and adaptive immunity. The roles of microglia are versatile. First, microglial cells act as early sensors of the retinal stress response *via* inherent Toll-like receptors (TLRs) on their surfaces (111). They are found to rapidly respond to extrinsic danger signals such as HSPs and oxidative stress and assume a predominant M1 phenotype within 24 hours after the induction of OHT (112–114). For example, extracellular HSP27 can activate TLRs and their downstream nuclear factor-kappa-light-chain-enhancer (NFκB) pathway in microglial cells, which subsequently promotes the release of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) and chemokines (MCP-1, MCP-3, MIP-1α, MIP-1β) to facilitate the recruitment and activation of T cells (115–117). Knockout of TIR-domain-containing adapter-inducing interferon-β (TRIF), an adaptor molecule downstream of the TLR3 pathway, results in reduced microglial activation and preserves RGCs following damage (118). Recently, studies found that microglial cells can also propagate inflammatory signals in a paracrine way through the release of exosomes and activate remote microglial cells (119, 120). Another important function of retinal microglial cells is their role as APCs. In the retinal parenchyma, APCs are responsible for the reactivation of T cells with cognate antigens by the expression of MHC class II molecules and costimulatory molecules (CD40, CD86, B7, *etc.*), which can subsequently result in the clonal proliferation of T cells (121). In glaucoma eyes, activated microglial cells gain a stronger ability of antigen presentation and secretion of proinflammatory immune mediators, which further escalate retinal inflammation (122, 123). Conversely, T helper cells are also able to differentially modify the phenotypes of microglial cells *via* the secretion of proinflammatory or suppressive cytokines (124, 125). $CD4^+$ Th1 cells secrete IFN-γ for M1 conversion, while the Th2 interferon IL-4 is associated with the M2 phenotype. Adoptive transfer of primed T cells from hereditary glaucoma mice leads to focal activation of $Iba1^+$ microglial cells with amoeboid morphology that stand in close proximity to infiltrated T cells (126). In tyrosinase T cell receptor (TCR) transgenic mice that spontaneously develop glaucoma, marked infiltration of T cells is observed. T cells, through induction of effector cytokines, lead to a robust increase in glial fibrillary acidic $protein^+$ (GFAP) glial cells that colocalize to T cells in the nerve fiber layer (61). Above all, as demonstrated in **Figure 3**, the interactions of T cells and retinal microglia are reciprocal and collectively shape the inflammatory background in glaucoma.

Both T cells and microglia can directly contribute to RGC loss, but there are also some distinctions in their mechanisms of action, and their relative contributions remain elusive. Histological studies reveal that the neuroinflammation characterized by retinal gliosis and T cell infiltration parallel ongoing neural degeneration spatially and temporally (68, 127, 128). Primed T cells after immunization with HSP27 and HSP60 can induce the apoptosis of retinal ganglion cells by secretion of fas-ligand (FasL) and upregulation of its receptor on RGCs (129). On the other hand, microglial cells can also contribute to RGC loss in other distinct ways such as pyroptosis and complement-mediated synaptic pruning (130). In glaucomatous eyes, both intrinsic and extrinsic apoptosis pathways of RGCs are involved in cell death (116).

T Cell-Microglial Interplay for Neuroprotection

The role of the bidirectional interplay between T cells and microglial cells is multifaceted. Apart from neurotoxicity, it also enhances the clearance of dead neurons *via* phagocytosis, prevents escalation of inflammation, and sets the chance for subsequent tissue remodeling and neural repair, which may benefit the survival of remaining RGCs (131). Damaged neurons can release intracellular components after necrosis, such as DNA and nucleotides, which are highly proinflammatory and trigger the spread of inflammation. Activated microglial cells gain stronger mobility and phagocytic power and quickly move to the damaged site to isolate damaged

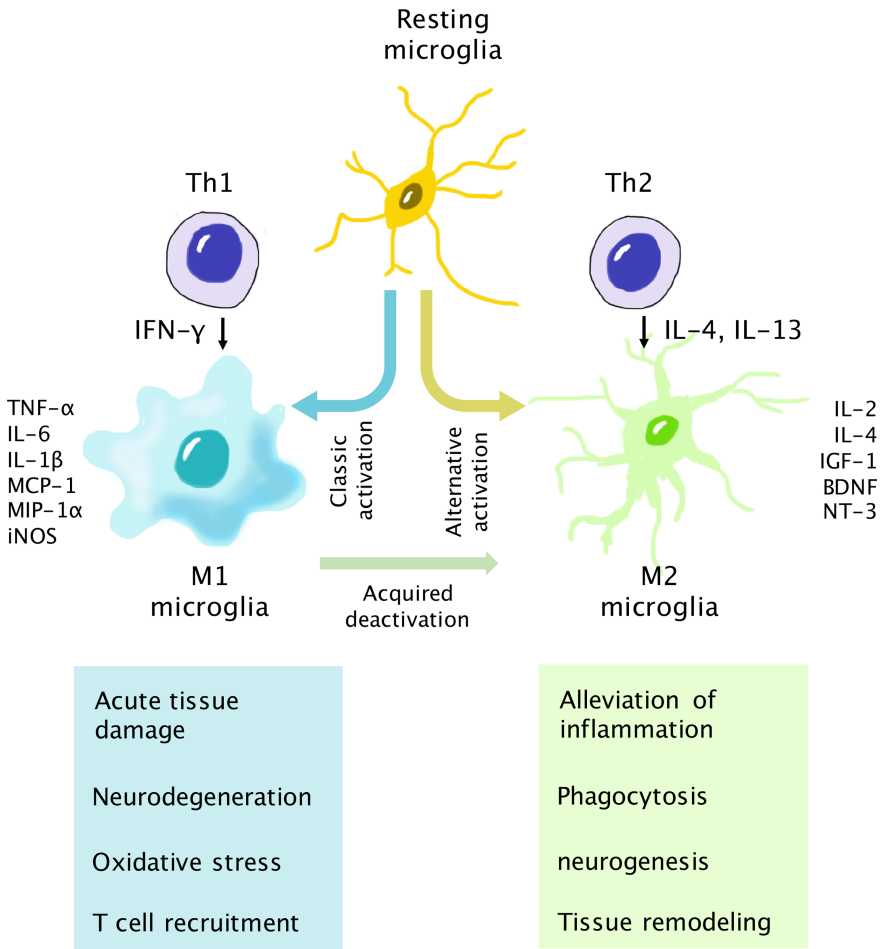

FIGURE 3 | Interaction of T cells and microglia for neurodegeneration and neuroprotection. Upon activation, microglia assume either the M1 (neurotoxic) or M2 (neurotrophic) phenotype. The Th1 cytokine IFN-γ is associated with classic activation of the M1 phenotype. M1 microglia secrete proinflammatory cytokines, chemokines, and inducible nitric oxide synthase (iNOS). The Th1/M1 interaction results in the escalation of retinal inflammation and may lead to neurodegeneration if the reaction is not well controlled. In turn, the Th2 cytokines IL-4 and IL-13 are involved in alternative activation into the M2 phenotype, which secretes anti-inflammatory cytokines and neurotrophic factors. The Th2/M2 interaction results in the alleviation of inflammation and helps neuron survival. In addition, activated M1 microglia can transform into M2 microglia *via* the acquired deactivation process in the presence of IL-10 and TGF-β to control the overactivation of the immune response.

neurons. As found in other CNS neurodegenerative diseases, tissue deposition of Aβ and p-tau is evident in glaucoma eyes (132, 133). These degenerative proteins are pathogenic and involved in progressive neuron death. In the CNS, Aβ can be taken up by activated microglial cells *via* phagocytosis and contribute to T cell activation (134, 135). In turn, primed T cells with Aβ immunization can also enhance the uptake and removal of Aβ in the brain and demonstrate beneficial effects (136, 137). IL-4 and IL-10 secreted mainly by Th2 cells are beneficial for the clearance of tissue deposition and debris by accelerating microglial phagocytic activities (138). In addition, activated retinal microglial cells and infiltrating macrophages secrete proinflammatory IL-6 and TNF-α to stimulate $CD4^+CD25^+$ T cells and suppress T cell-mediated cytotoxicity (139). Following the phase of intense attack, microglial cells, macroglial cells, and T cells coordinate to resolve immune reactions in the retina. In this phase, microglial cells convert from the proinflammatory M1 phenotype to the neuroprotective M2 phenotype and are able to secrete neurotrophic factors to promote neuronal survival. The retina, as an immune-privileged tissue, also possesses multiple negative regulatory circuits to restrict inflammation. For example, retinal microglial cells, as well as neurons and endothelial cells, were found to constitutively express CD200. Through interaction with the CD200 receptor, it attenuates the activation of myeloid cells (140). Microglial cells upregulate the expression of programmed death ligand-1 (PD-L1) at the peak phase of activation and suppress Th1 function *via* interaction with PD1 on their surfaces (141, 142).

Above all, it is generally accepted that the T cell-microglia interaction has both protective and destructive consequences and is related to the disease course. However, it is still under debate how to appropriately modify the immune process and benefit the survival of RGCs in glaucoma patients. Some animal studies in acute OHT explicitly support the beneficial role of neurons by suppressing microglial functions in different ways (143–146). However, this

may not mimic the true chronic nature of glaucoma in most patients. It seems that this prompt immune reaction elicited by the stress response is at least beneficial in the restriction of neuroinflammation, but the longstanding insults of IOP fluctuation may lead to chronic activation of the microglia-T cell axis that exceeds the normal immunomodulation. As suggested by animal models of glaucoma, retinal gliosis is not only an early response but also persists in the chronic phase. Microglial cells remain activated after IOP returns to the normal level, which may potentially explain the ongoing neurodegeneration in patients after IOP has been controlled (147). Autopsy studies in postmortem glaucoma eyes may serve as direct evidence for marked retinal gliosis and infiltration of activated microglial cells in the optic nerve head (111, 148, 149). Glaucomatous eyes of humans also assume a chronic environment of neuroinflammation characterized by IgG autoantibody accumulation and increased levels of proinflammatory cytokines (45). In addition, from the point of view of T cells, there is a lasting imbalance of Th1/Th2 cells that tilts to the proinflammatory side in glaucoma patients (150, 151). These findings support the theory that chronic insults from endogenous (IOP, oxidative stress, *etc.*) and exogenous sources (gut dysbiosis, aging, *etc.*) lead to overactivation and disorder in immune regulation that turns the T cell-microglial axis into a predominant neurotoxic mode in the long term. Above all, this suggests the validity of immune modulation therapy in the treatment of glaucoma, as discussed in more detail in the section below.

THE EMERGING ROLE OF T CELL-BASED IMMUNE MODULATION THERAPY IN THE TREATMENT OF GLAUCOMA - A LESSON FROM CNS NEURODEGENERATIVE DISEASES

As discussed earlier, the immune reactions of T cells and microglia have both beneficial and detrimental effects on RGC survival, depending on whether they are properly evoked and regulated. In glaucoma, the chronic local proinflammatory microenvironment unfavorably shapes the immune reaction to the neurotoxic form and leads to progressive RGC loss. Considering the essential role of immune cells in tissue repair and maintenance of homeostasis, it is generally accepted that immune modulation, instead of simple immune suppression, is a valid option for glaucoma. The term "protective autoimmunity", which refers to the beneficial roles of autoreactive T cells in the protection of neurons in the CNS, has been recognized for more than 2 decades (152, 153). This subsequently leads to the active exploration of T cell-based immune modulation therapy in glaucoma and other CNS neurodegenerative diseases.

Protective Autoimmunity in the CNS and Retina

Under physical conditions, peripherally primed T cells that recognize self-antigens can patrol the retina and brain parenchyma over time. This immunosurveillance is not only helpful for immune detection but also plays a role in maintaining neural homeostasis by actively engaging in multiple physiological activities, including neurogenesis, regulating spatial learning and memory, and assisting neuron survival with neurotrophic factors (154). Deprivation of CNS-specific autoreactive T cells halts the normal development of the CNS system and is involved in cognitive impairment in animal models and exacerbation of CNS injury (155). On the other hand, accumulating evidence suggests that the T cell-mediated immune response may also provide beneficial effects in neuroprotection in the case of acute neural damage. In a rat model of optic nerve crush and contusive spinal cord injury, single low-dose γ-irradiation treatment induces the activation of proinflammatory T cells that ultimately leads to spontaneous recovery, which is absent in mice with T cell deficiency or transferred with regulatory T cells (Tregs) (11). A prior traumatic brain injury evoked protective autoimmunity that was found to prevent RGC loss when the contralateral optic nerve was crushed later in a rat model (10). These protective effects are chiefly dependent on neuron-specific autoimmune T cells, as evidenced by the finding that only transgenic T cells overexpressing the T cell receptor for the CNS-specific antigen of myelin basic protein (MBP) rather than the nonself antigen of ovalbumin demonstrate protective effects on RGC survival after optic nerve injury (156). Thus, the involvement of autoimmune T cells in the protection of neurons during acute CNS insult indicates the validity of the immune modulation approach for the treatment of glaucoma.

Cop-1 Therapy

One investigated approach of immune modulation therapy in glaucoma is active immunization with a weak self-antigen that stimulates a moderate autoreactive T cell response. Autoimmune T cells are expected to regulate the immune response in a beneficial way and coordinate with local and other circulatory immune cells to boost faster tissue repair. Copolymer-1 (Cop-1, or glatiramer acetate), a synthesized analog of MBP, is a suitable candidate because it serves as a weak agonist of numerous self-antigens in the CNS. Immunization with Cop-1 has been tested in many CNS neurodegenerative diseases, and its commercial product Copaxone® has been approved by the US Food and Drug Administration (FDA) for the treatment of MS (157). Glaucoma shares common aspects of pathogenesis with MS, including generation and tissue deposition of common autoantibodies (anti-MBP) and activation of local microglial cells (45, 158). Clinical examination with optical coherence tomography (OCT) also indicates a close relationship between retinal RGC damage and MS neuropathy (15). Thus, Cop-1 as an approved therapy for MS is also explored in glaucoma treatment. Coculture of Cop-1-stimulated T cells and retinal microglia results in their reciprocal activation and the release of insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), TNF-α, and IL-10 *in vitro*, which favors RGC survival *in vitro* (159). Immunization with Cop-1 by subcutaneous injection in a rat glaucoma model results in elevated intraretinal T cell infiltration and prevention of RGC loss, which indicates that the induced T cell response is more

protective than destructive (160). In addition, Cop-1 immunization has been delivered in combination with stem cell transplantation to rescue and replenish RGCs in animal glaucoma models. Cop-1 immunization induces a local favorable environment by balancing the levels of proinflammatory (IFN-γ) and anti-inflammatory (IL-4) cytokines and the secretion of neurotrophic factors (161). Activated T cells also release chemoattractants (such as MCP-1) to facilitate the recruitment of stem cells and progenitor cells to damaged sites (162, 163). The synergic action results in improved landing and survival of transplanted stem cells and alleviates nerve damage in glaucoma animal models (164, 165). Recently, the interim results of an ongoing double-masked clinical trial investigating Cop-1 immunization for the treatment of acute primary angle-closure glaucoma (PACG) have been disclosed (trial No. 01936129). Thirty-eight patients with PACG received either 2 subcutaneous injections of Cop-1 or the placebo without adjuvant, one within 24 hours of onset, and the other one week later. The patients in the Cop-1 group demonstrated improved mean deviation and a trend of a lower mean number of progressing points, but the retinal nerve fiber layer thickness showed no difference after 16 weeks. Due to the small sample sizes in both arms and high interpersonal variation, the researchers could not confirm the protective role of Cop-1 in PACG based on the current evidence (166). In addition, as no serum or immune examination test results were reported, whether the 2 subcutaneous injections of Cop-1 without adjuvant elicit an adequately strong and long-lasting immune response cannot be determined.

Concerns and Further Considerations on Immune Modulation Therapy for Glaucoma

Although immune modulation therapy seems to be a reasonable treatment option for glaucoma and has been well explored in CNS neurodegenerative diseases, in clinical practice, many conditions need to be optimized. Safety is a chief concern. Immunization with R16, a self-peptide from interphotoreceptor retinoid-binding protein, in a mouse model of acute OHT led to neuron protection but induced monophasic autoimmune uveitis in a susceptible mouse strain (167). More experience from CNS clinical trials warns that the stimulation of unwanted detrimental immune responses could lead to adverse events. In a phase II clinical trial investigating the tolerability and efficacy of intramuscular injection of self-antigen Aβ42 with QS-21 as the adjuvant in patients with mild to moderate AD, subacute meningoencephalitis occurred in 6% of patients in the test arm, which led to the termination of the study (168). An aberrant Th1 cell response is speculated to be responsible for the adverse inflammatory response, as QS-21 is a strong inducer of Th1 and infiltration of active T cells was found in an autopsy study (169). Similar meningoencephalitis has been reported in amyloid precursor protein-transgenic (APP/Tg) mice that express limited IFN-γ, but not in other strains. The activation of Th1 cells in a proinflammatory environment and their interplay with microglial cells are causative of neuroinflammation (170). Moreover, the adoptive transfer of Aβ-activated Th1 cells, but not Th2 cells, in mice results in local microglial activation and exacerbation of AD in a mouse model (171). As the patients with CNS neurodegenerative diseases such as AD and glaucoma assume a generally proinflammatory state, the immune response with self-antigen vaccination should be carefully modified, as the induction of proinflammatory Th1 cells may further tilt the balance of immunity. In turn, a vaccination that induces a predominant Th2 response is preferred and helps restore Th1/Th2 imbalance (151, 172). In the study of AD, a dominant Th2-type response has been achieved by multiple means, such as applying Th2-inducing adjuvants (alum) (173), modifying the structures of the self-antigen (174), liposome coating (175), DNA vaccination (176), intranasal immunization (177), and transcutaneous delivery (178), but activation of Th1 cells cannot be fully eradicated and may still be considered a potential risk. Currently, the only safety data of immune modulation therapy in glaucoma patients come from the pilot clinical trial of the Cop-1 vaccine, which shows no other adverse events other than injection site pain (166). In a rat glaucoma model, systemic administration of Cop-1 with complete Freud's adjuvant evokes a strong Th2 response and IL-4 secretion that peaks at 7 days and lasts for more than 31 days, with no obvious Th1 activation (179). In addition, as an old drug in MS that has been monitored for more than 2 decades, subcutaneous injection of Copaxone® daily or three times per week is well tolerated, which is also suitable for children and pregnant women (180, 181). These results support the Cop-1 vaccine as a relatively safe therapy, but its long-term safety in glaucoma should be further elucidated.

Currently, Cop-1 vaccination is the only immune modulation therapy that has been tested in animals and patients with glaucoma, and more clinical data are expected to reflect its validity in patients. In CNS neurodegenerative diseases, many forms of passive or active immune modulation therapy have been explored, including self-antigen vaccination (182), DNA vaccination (183, 184), and transfer Treg cells (185, 186). The highly shared pathological and immunological features of glaucoma with other CNS neurodegenerative diseases support these therapies as rational options for glaucoma, which is worth further investigation. In addition, parallel changes in retinal neurons with the CNS have been observed in animals and patients on immune modulation therapy for CNS disorders (187, 188). However, the development and clinical translation of a mature immune modulation therapy for glaucoma are much more challenging than expected. Experience from clinical trials of AD and other CNS neurodegenerative diseases implicates numerous factors that may affect the efficacy and safety of immune modulation therapy, including antigens for immune induction, the time window of injection, frequency and amount of injection, drug delivery route, adjuvants, drug carriers, and individual factors (age, sex, *etc.*) (153). The development of a suitable regimen for a specific disease should be explored on a case-by-case basis. For example, Cop-1 immunization prior to or on the day of OHT damage significantly prevents RGC loss, while immunization 48 hours later generates no protection (189). In addition, a single Cop-1 immunization with complete Fraud's adjuvant in a mouse acute OHT model is protective, but it dose not work for the optic nerve transection injury or amyotrophic

lateral sclerosis (ALS) (190, 191). On the other hand, daily subcutaneous injection of Cop-1 is most effective for MS, but this regimen causes negative effects on the female mouse ALS model (191). Thus, a careful selection of regimens based on extensive animal and human studies is essential for an immune modulation therapy for glaucoma. Most importantly, researchers need to keep in mind that chronic neurodegeneration is multifactorial and highly complicated, and the efficacy observed on pathology may not translate into clinical improvement. For example, Aβ42 immunization in Alzheimer's patients showed obvious benefits of prolonged plaque removal. Nevertheless, most of the patients progressed to Braak stage V-VI and developed severe dementia before death (192). Thus, a reliable clinical evaluation of drug efficacy is vital for immune modulation therapy of glaucoma and other neurodegenerative diseases.

Above all, the development of a mature immunomodulation therapy for glaucoma needs many more investigations on its safety and practical conditions. Special attention should be given to avoid unwanted exacerbation of the Th1-mediated response. In addition to Cop-1 immunization, more novel therapies, such as DNA vaccination and cell therapy, are also worthy of investigation in glaucoma.

SUMMARY AND CONCLUSIONS

The perspectives on the pathogenesis of glaucoma are continuously updating, and debates are ongoing as new evidence from animal or clinical studies comes out. Although the research, diagnosis, and treatment focus primarily on IOP, T cell-mediated immune attack and its dynamic interplay with retinal microglial cells have been recognized to be the culprit of glaucoma. Researchers are attempting to shape the immune reaction to take advantage of its favorable neuroprotective effect. However, currently, no clear clinical evidence on the beneficial role of immunomodulation therapy in glaucoma patients has been illuminated on patients. Most importantly, signs of activation of autoimmunity and imbalance of immune reactions, including autoantibodies, a shift in the T cell subpopulation, and activation of retinal innate immunity, are clearly involved in the early phase of glaucoma. However, whether these clinical signs and associated biomarkers can assist the early diagnosis and primary prevention of glaucoma remains elusive. In addition, basic research on the autoimmune aspects of glaucoma is still a fresh field. Many theories of immune reactions in glaucoma basically come from findings from other CNS neurodegenerative diseases. In particular, the spatial-temporal breakdown of the BRB and retinal infiltration of peripheral immune cells during the chronic disease course in glaucoma patients are needed to illuminate the real contribution of T cells. As with other neurodegenerative diseases, a chronic animal disease model that mimics the true complexity and chronicity of glaucoma development in humans is lacking. Thus, findings from the acute OHT model may not reflect the whole picture, and more autopsy studies from human glaucoma samples may provide more valuable information. Moreover, the close overlap of glaucoma with other CNS neurodegenerative diseases indicates the need for a closer follow-up of patients in both ophthalmic and neurologic clinics. Their common pathogenesis in immune-mediated neurodegeneration indicates the possibility of their parallel progression and reaction to common therapies. Therefore, the ophthalmic follow-up of patients with CNS neurodegenerative disease going on trials of immunomodulation therapy may also provide valuable insights for glaucoma treatment.

AUTHOR CONTRIBUTIONS

Conception and design – LW and XW. Manuscript preparation – LW. Critical revisions – XW. All authors contributed to the article and approved the submitted version.

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High *TRGV 9* Subfamily Expression Marks an Improved Overall Survival in Patients With Acute Myeloid Leukemia

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Background: Heterogeneous T cells in acute myeloid leukemia (AML) have the combinatorial variety generated by different T cell receptors (TCRs). γδ T cells are a distinct subgroup of T cells containing TCRγ (*TRGV*) and TCRδ (*TRDV*) subfamilies with diverse structural and functional heterogeneity. Our previous study showed that clonally expanded *TRDV* T cells might benefit the immune response directed against AML. However, the features of the *TRGV* repertoire in AML remain unknown. To fully characterize the features of γδ T cells, we analyzed the distribution and clonality of *TRGV I-III* subfamilies (*TRGV II* is also termed *TRVG 9*), the proportions of γδ T cell subsets, and their effects on the overall survival (OS) of patients with AML.

Methods: In this study, the complementarity-determining region 3 (CDR3) size of *TRGV* subfamilies in γδ T cells of peripheral blood (PB) from *de novo* AML patients were analyzed by Genescan analysis. Expression levels of *TRGV* subfamilies were performed by real-time quantitative PCR. The proportions of total γδ T cells and their $V\gamma9^+$ $V\delta2^+$ T cells subsets were detected by multicolor flow cytometry assay. We further compared the correlation among the *TRGV* gene expression levels, the proportion of $V\gamma9^+$ $V\delta2^+$ T cells, and OS in AML.

Results: We first found that the distribution pattern and clonality of *TRGV* subfamilies were changed. The expression frequencies and gene expression levels of three *TRGV* subfamilies in AML samples were significantly lower than those in healthy individuals (HIs). Compared with HIs, the proportions of total γδ T cells and $V\gamma9^+$ $V\delta2^+$ T cells were also significantly decreased in patients with AML. In addition, patients with AML who had higher expression levels of the *TRGV* gene and higher proportion of $V\gamma9^+$ $V\delta2^+$ T cells showed better OS than their counterparts. Furthermore, high expression levels of *TRGV 9* and proportion of $V\gamma9^+$ $V\delta2^+$ T cells were identified as independent protective factors for complete remission in patients with AML.

Conclusions: The restriction of *TRGV* usage might be related to the preference of usage of γδ T cells. Higher expression of *TRGV* subfamilies might be associated with better OS in AML. Higher *TRGV 9* expression and increased Vγ9$^+$ Vδ2$^+$ T cells subfamilies might indicate a better prognosis in patients with AML.

Keywords: acute myeloid leukemia, γδ T cells, *TRGV* repertoire, clonality, prognosis

INTRODUCTION

Acute myeloid leukemia (AML) is a malignant clonal disease originating from hematopoietic stem cells and characterized by genetic and clinical heterogeneity and high mortality (1). Despite considerable progress in treating hematological malignancies, clinical outcomes of patients older than 60 years are unfavorable, and the overall long-term survival in patients with AML remains poor (2). Recent studies have revealed that T cell immunodeficiency is a common characteristic of patients with AML, mainly due to peripheral T cells that restricted oligoclonal T cell repertoires, reduced thymic output function, and lower activation and response to antigens (3, 4).

T cells recognize specific ligands by specific T cell receptors (TCRs), which are heterodimers consisting of either αβ and γδ chains. Genes encode for the variable domains of *TRG* (γ chain) and *TRD* (δ chain), which are assembled by somatic recombination from variable (V), diversity (D, only for *TRD*), and joining (J) segments and compose three hypervariable or complementarity-determining regions (CDR1, CDR2, and CDR3) that occur during T cell differentiation (5, 6). The *TRG* gene contains several different functional variable (*TRGV*) segments belonging to four subgroups (*TRGV I–IV*), and the *TRD* gene contains at least eight functional *TRDV* segments that are subdivided into eight *TRDV* subfamilies (*TRDV* 1–8) (5–9). Previous studies showed that *TRGV IV* was a pseudogene, which was a simple combination between *TRGV IV* and *TRGC* segment lacking *TRGJ* segment and there was no any rearrangement in CDR3 by sequencing (10, 11). Hence, the analysis of *TRGV* repertoire was acquired in three *TRGV* subfamilies in the present study. Nowadays, according to their *TRD* (TCRδ) chain usage, human γδ T cells are mainly divided into 2 major subsets including Vδ1 and Vδ2 in peripheral blood (PB). Several functional *TRG* (TCRγ) gene segments are generally divided into Vγ2, γ3, γ4, γ5, γ8, γ9, γ10 (also termed *TRGV 2, TRGV 3, TRGV 4, TRGV 5, TRGV 8*, *TRGV 9* and *TRGV10*, respectively) (12, 13). The V-genes of *TRGV 2-5 and TRGV 8* have a relatively high sequence similarity, which are different from *TRGV 9* sequences. Different TCRγ chains and TCRδ chains can be combined to form different types of γδ T cells (14). Although Vδ1 T cells are predominantly associated with the Vδ1 comprising *TRGV 2, TRGV 3, TRGV 4, TRGV 5, TRGV 8*, which belonging to *TRGV I* subsets, the majority of Vδ2 T cells express an invariant TCR harboring *TRGV 9*, which belonging to *TRGV II* subsets (15). In addition, *TRGV 10* belongings to *TRGV III* subsets (12). In the PB of healthy individuals (HIs), there is a predominant expression in the γδ T cell population, which is the cell expressing Vγ9 together with Vδ2, termed Vγ9$^+$ Vδ2$^+$ T cells (15, 16). The roles of some T cell subgroups in cancer are controversial because they have been suggested to play both an anti-tumor role and a pro-tumor role. The heterogeneous T cells in AML have the combinatorial variety generated by different TCRs, which might explain why some special T cell subsets have a controversial role in cancer immunity. Although PD-1$^+$Vβ5.2$^+$ and PD-1$^+$Vβ12$^+$ CD8$^+$ T cells were thought to be related to poor prognosis in AML (17), our previous study found that clonally expanded *TRDV* T cells might benefit the immune response directed against AML (18). However, the features of the *TRGV* repertoire in AML remain unknown, and the cellular immunity characteristics of AML have yet to be fully elucidated. To further understand the heterogeneity of γδ T cells, in this study, we first analyzed the distribution pattern and clonality of *TRGV* subfamilies and further investigated correlation between expression levels of *TRGV* subfamilies and proportion of Vγ9$^+$ Vδ2$^+$ T cells and their clinical relevance in patients with AML.

Abbreviations: AML, acute myeloid leukemia; TCRs, T cell receptors; CDR, complementarity-determining region; PB, peripheral blood; HIs, healthy individuals; β_2M, β_2 microglubin; qPCR, real-time quantitative PCR; WBC, white blood cell; RBC, red blood cell; PLT, platelet; BM, bone marrow; FAB, French-American-British; CR, complete remission; OR, odds ratio; CI, confidence interval; OS, overall survival; HR, hazard ratio; ITP, immune thrombocytopenic purpura; GVHD, graft-versus-host disease; MM, multiple myeloma; HSCT, hematopoietic stem cell transplantation.

MATERIALS AND METHODS

Samples

PB samples were collected from 75 patients with *de novo* AML (42 males and 33 females, median age 48 years, range 18–88 years) from January 2015 to December 2021. A total of 51 HIs (29 males and 22 females, median age 45 years, range 25–83 years) served as controls. Among the total samples, there were 56 patients with AML and 33 HIs were used to analyze the expression levels of *TRGV* subfamilies. Of the 56 patients identified, 50 patients with both available *TRGV* gene expression data and outcome information were eventually included in the survival analysis. In addition, the PB of extra 19 patients with AML and 18 HIs were analyzed by flow cytometry. Of the 19 patients, 18 patients with both available flow cytometry data and outcome information were also included in survival analysis. The clinical information was showed in **Table 1** and **Supplementary Table 1**. Informed consent was obtained from all participants. The protocol of all experiments was approved by the Ethics Committee of First Affiliated Hospital, Medical School of Jinan University.

TABLE 1 | Clinical characteristics of AML patients.

Factor	AML
Number	75
Age (median; range)	48 (18-88)
Gender (Male/Female)	42/33
WBC ($\times10^9$/L), (median; range)	23.10 (1-325.42)
RBC ($\times10^{12}$/L), (median; range)	2.51 (1.28-5.67)
PLT ($\times10^9$/L), (median; range)	44.1 (4-632)
BM blast cells (%), (median; range)	63 (20-94)
FAB subtype (n=75)	
M0	6
M1	2
M2	16
M3	12
M4	9
M5	18
M6	/
M7	/
Undetermined	12
Gene mutation	
FLT3 (+/-)	12/63
NPM1 (+/-)	9/66
PML/RARA (+/-)	8/67
MLL (+/-)	6/69
TP53 (+/-)	4/71
AML1/ETO (+/-)	5/70
Others (+/-)	9/66
Unknown (+/-)	29/46
Cytogenetic abnormality	
Normal (+/-)	6/69
Abnormal (+/-)	25/50
Unknown (+/-)	44/31
Treatment	
Chemotherapy (+/-)	66/9
HSCT (+/-)	9/66

AML, acute myeloid leukemia; WBC, white blood cell; RBC, red blood cell; PLT, platelet; BM blast cells, bone marrow blast cells; FAB, French-American-British; M0, minimally differentiated AML; M1, AML without maturation; M2, AML with maturation; M3, acute promyelocytic leukemia; M4, acute myelomonocytic leukemia; M5, acute monocytic leukemia; M6, pure erythroid leukemia; M7, Acute megakaryoblastic leukemia; HSCT, hematopoietic stem cell transplantation; /, unknown.

Mononuclear Cell Isolation and γδ T Cell Sorting

The Ficoll–Hypaque gradient centrifugation method was used to isolate mononuclear cells from fresh PB. The γδ T cells were sorted by γδ monoclonal antibodies and MACS magnetic cell sorting technique (Miltenyi Biotec, Germany) (19). All samples were freshly obtained and subjected to immediate preparation.

RNA Isolation and cDNA Synthesis

According to the manufacturer's recommendations, total RNA of γδ T cells was extracted by Trizol (Invitrogen, USA). Superscript II Kit (Gibco, USA) was used to synthesize the first single-strand complementary DNA (cDNA). Subsequently, the quality of cDNA was confirmed by RT-PCR for β2 microglubin (β2M) gene amplification (the primers of β2M gene for RT-PCR were list in **Table 2**) (20).

RT-PCR for *TRGV* Subfamily Amplification and Genescan Analysis for *TRGV* Subfamily Clonality Analysis

Three sense *TRGV* primers and a single *TRGC* reverse primer were used in unlabeled PCR for the amplification of the *TRGV* subfamilies. Runoff PCR was performed with fluorescent primers labeled at the 5' end with the FAM fluorophore (Cγ-FAM) (TIB MOLBIOL GmbH, Germany). A DNA thermal cycler (BioMetra, Germany) was used to perform this reaction process. The primers are listed in **Table 2**. PCR was performed as described in our previous report (19–21). Aliquots of cDNA (1 μL) were amplified in 20 μL reactions with one of the three Vγ primers and a Cγ primer. The final reaction mixture contained 0.5 μM sense primer and antisense primer, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 1.25 U Taq polymerase (Promega, USA). After 3 min of denaturation at 94°C, 40 PCR cycles were carried out (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a final elongation for 6 min at 72°C). All PCR products were stored at 4°C and ready for Genescan analysis (22).

TABLE 2 | Sequences of primers used in RT-PCR and qPCR.

Primer	Sequence
TRGV I	5'-TACCTACACCAGGAGGGGAAG-3'
TRGV 9	5'-GGCACTGTCAGAAAGGAATC-3'
TRGV III	5'-TCGACGCAGCATGGGTAAGAC-3'
Cγ	5'- GTTGCTCTTCTTTTCTTGCC-3'
Cγ-FAM	5'-FAM-CATCTGCATCAAGTTGTTTATC-3'
β2M-for	5'-TACACTGAATTCACCCCCAC-3'
β2M-back	5'-CATCCAATCCAAATGCGGCA-3'

Aliquots of the unlabeled PCR products (2 μL) were subjected to a cycle of runoff reaction with a fluorophore-labeled Cγ-FAM primer. The labeled runoff PCR products (2 μL) were heat-denatured at 94°C for 4 min with 9.5 μL of formamide (Hi-Di Formamide, ABI, USA) and 0.5 μL of size standards (GENESCAN™-500-LIZ™, Perkin Elmer, USA). The samples were then loaded on 3100 POP-4™ gel (Performance Optimized Polymer-4, ABI, USA) and resolved by electrophoresis in an ABI 3100 DNA sequencer for size and fluorescence intensity determination using Genescan software (23).

Real-Time Quantitative PCR (qPCR) for *TRGV* Gene

The gene expression levels of the *TRGV* subfamilies in cDNA of γδ T cells were determined by qPCR with SYBR Green I technique, and the β_2-microglobulin (β_2M) gene was used as an endogenous reference. The primers are listed in **Table 2**. qPCR was performed as described by Stams WAG et al. and our previous study (10, 24–26). In brief, qPCR was performed in a total volume of 20 μL with approximately 1 μL cDNA, 0.5 μM of each primer (one of the three *TRGV* sense primer and the antisense primer Cγ for *TRGV* amplification, β_2M-for and β_2M-back primers for β_2M gene amplification), 2x RealMastrMix 10 μL (Tiangen, China). After 2 min of denaturation at 95°C, 40 PCR cycles were carried out (95°C for 15 s, 58°C for 20 s, and 72°C for 30 s). At the end of each run, melting curve analysis was performed starting at 65°C up to 95°C with an increase of 1°C per 2 s to verify primer specificities. Specific amplification of PCR products was analyzed by melting curve analysis. qPCR was repeated in at least three separate experiments. The following equation was used to calculate the relative expression level to the β_2M gene for each target PCR. Relative mRNA expression = $2^{-\Delta Ct} \times 100\%$ [$\Delta C_t = C_{t(TRGV\ \text{subfamilies})} - C_{t(\beta2M)}$] (15).

Flow Cytometry

The following monoclonal antibodies APC/Cy7 anti-human CD3 (clone SK7), PE/Cy7 anti-human TCR γ/δ (clone B1), PerCP anti-human TCR Vδ2 (clone B6), and APC anti-human TCR Vγ9 (clone B3; Biolegend, USA) were used for cell surface staining following the manufacturer's instructions (27). The stained cells were examined with BD FACS VERSE flow cytometer (BD, USA), and data were analyzed by Flowjo software (Flowjo LLC, USA).

Statistical Analysis

In this study, data were presented as median. Fisher's exact test was used to compare expression frequencies of three *TRGV* subfamilies between AML patients and HIs. Kruskal–Wallis test was used for comparison of different gene expression levels from different *TRGV* subfamilies in AML and HIs. Differences in mRNA expression level of *TRGV* between two groups were analyzed using the Mann–Whitney U test. Pearson correlation analysis was used to analyze the correlation of mRNA expression levels of *TRGV* subfamilies between two groups. Binary logistic regression analysis was performed to determine associations between expression levels of three *TRGV* subfamilies and clinical outcome of the AML patients. Through Kaplan-Meier method and cox regression analysis the effect of *TRGV* expression and the proportion of $V\gamma9^+$ $V\delta2^+$ T cells on prognosis of AML were analyzed. All analyses included the following variables: including gender, age, white blood cell (WBC), red blood cell (RBC), platelet (PLT), bone marrow (BM) blast cells, French-American-British (FAB) subtype, gene mutation and treatment in patients. Only values with $P < 0.05$ was regarded as statistically significant. All results were analyzed by SPSS 25.0 and GraphPad Prism 8.4.

RESULTS

Expression Pattern and Clonality of the *TRGV* Repertoire in Patients With *De Novo* AML

In this study, the CDR3 region of three *TRGV* subfamily genes was analyzed by Genescan analysis in γδ T cells from 30 patients with *de novo* AML and 10 HIs to assess the spectral pattern visually. Diversity and clonality of TCR repertoire demonstrated the ability of specific amplifications to respond to antigen stimulation. Based on the CDR3 TCR rearrangement lengths, the clonality of γδ T cells was characterized as multipeaks and oligopeaks responding to polyclonality and oligoclonality. Polyclonality of the *TRGV* subfamily genes displayed a Gaussian distribution consisting of three or more peaks, and oligoclonality was a skewed spectral profile showing a single dominant peak. In this study, all patients with AML had a significantly skewed TCR repertoire with 16–21 of the three *TRGV* subfamilies (*TRGV I*, *9*, and *III*) detected in each patient. Among AML samples, the most frequently expressed subfamily members were *TRGV III* (70%, 21/30) and *TRGV 9* (66.67%, 20/30). *TRGV I* from patients with AML was detected only in 16 cases (53.33%, 16/30; **Figures 1A–D**). All of the three *TRGV* subfamilies could be detected in γδ T cells from HIs. The expression frequencies of the *TRGV I* and *TRGV 9* subfamilies in patients with AML were lower than those in HIs (*TRGV I*: $P = 0.007$, *TRGV 9*: $P = 0.043$), whereas the *TRGV III* subfamily in AML was similar to that in HIs ($P = 0.081$; **Figures 2A–C**).

The deviation from the Gaussian profile could indicate a clonally expanded pattern. The PCR products produced only one peak, which represented that CDR3 lengths were identical, named oligoclonal pattern. We further analyzed the different clonotypic expansion patterns in HIs and patients with AML. Oligoclonal expansion was detected in the *TRGV* subfamily from six out of 30 cases in patients with AML (**Figure 1D**). The expression frequencies of clonally expanded *TRGV* subfamilies in the patients with AML were as follows: *TRGV III* (17%, 5/30), *TRGV I* (10%, 3/30), and *TRGV 9* (10%, 3/30). However, there were no clonally expanded *TRGV* subfamilies that could be identified in HIs. Based on the clonally expanded pattern, we divided the clonal expansion frequency of the three *TRGV* subfamilies into three groups: polyclonality, oligoclonality and negative groups. The results showed a significant difference between patients with AML and HIs, and the clonal expansion frequencies of the *TRGV* subfamilies were statistically higher than those of HIs (*TRGV I*: $P = 0.004$; *TRGV 9*: $P = 0.040$; and *TRGV III*: $P = 0.028$; **Figures 2D–F**).

Gene Expression Level of the *TRGV* Subfamily

Subsequently, we focused on detecting expression levels of *TRGV* subfamilies by qPCR, so we expanded the samples' quantity, and further collected extra 26 AML samples on the basis of the original 30 samples. Therefore, three *TRGV* genes expression levels in a total of 56 patients with AML and 33 HIs as control were detected in our study. Results showed significant differences of expression levels in the *TRGV* subfamilies of HIs ($\chi^2 = 9.998$, $P = 0.007$) between *TRGV I* and *TRGV 9* ($P = 0.158$), *TRGV 9* and *TRGV III* ($P = 0.002$), and *TRGV I* and *TRGV III* ($P = 0.082$; **Figure 3A**). There were also significant differences in the *TRGV* subfamilies of AML ($\chi^2 = 7.208$, $P = 0.027$) between *TRGV I* and *TRGV 9* ($P = 0.679$), *TRGV 9* and *TRGV III* ($P = 0.014$), and *TRGV I* and *TRGV III* ($P = 0.032$; **Figure 3B**). We further compared the gene expression levels of the *TRGV* subfamilies in patients with AML and HIs. The gene expression levels of the three *TRGV* subfamilies in AML were lower than those in HIs ($P < 0.001$, $P < 0.001$, and $P < 0.001$; **Figures 3C, G**).

We also obtained more insight to investigate the correlation of the gene expression levels of the three *TRGV* subfamilies in HIs and patients with AML. In HIs, a significant positive correlation was found in the expression levels of *TRGV I* and *TRGV 9* ($r = 0.582$, $P < 0.001$), *TRGV I* and *TRGV III* ($r = 0.485$, $P = 0.004$), and *TRGV 9* and *TRGV III* ($r = 0.591$, $P < 0.001$; **Figures 3D–F**). A positive correlation in the expression levels of *TRGV I* and *TRGV 9* ($r = 0.479$, $P < 0.001$), *TRGV I* and *TRGV III* ($r = 0.611$, $P < 0.001$), and *TRGV 9* and *TRGV III* ($r = 0.609$, $P < 0.001$) was also observed in patients with AML (**Figures 3D–F**).

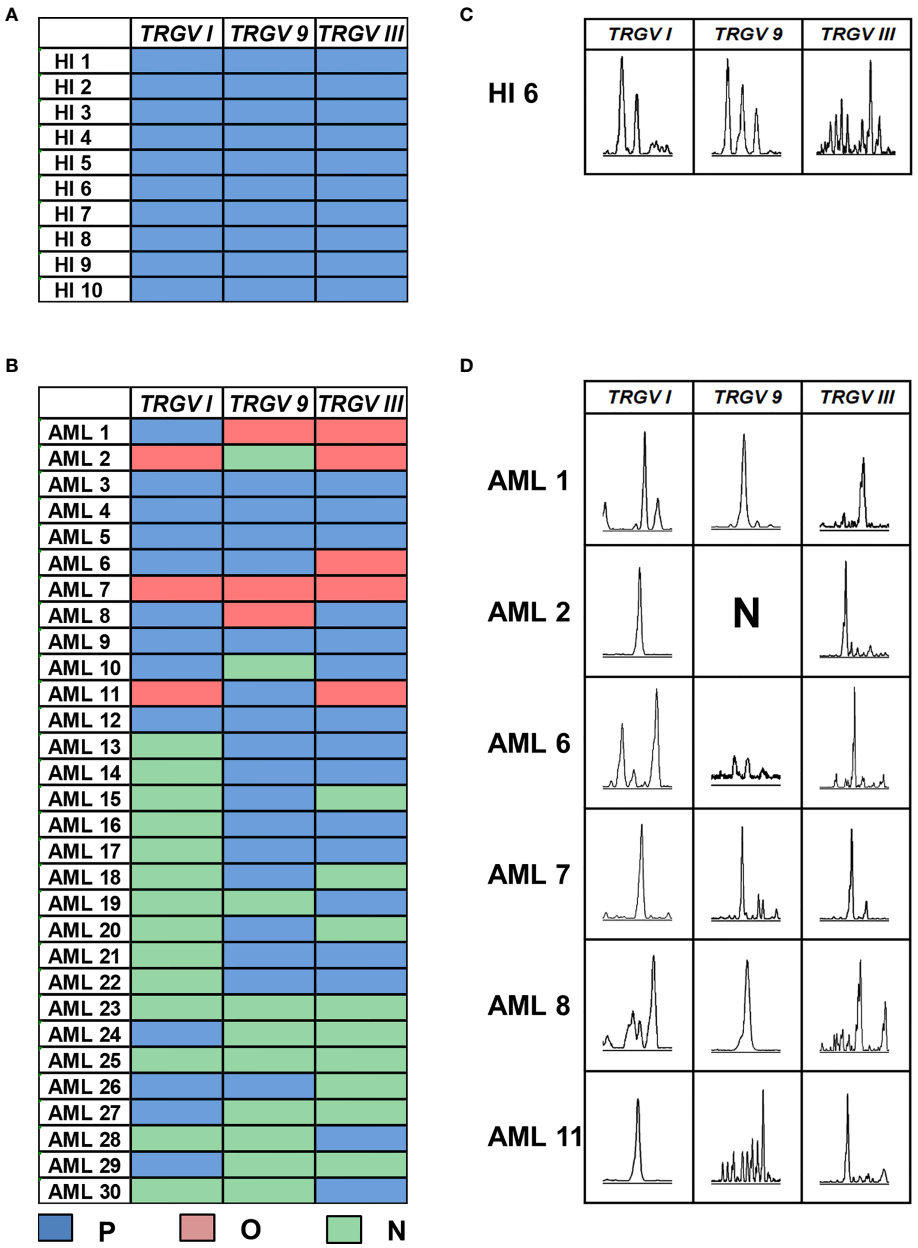

FIGURE 1 | Distribution and clonality of *TRGV* subfamilies in γδ T cells from 10 healthy individuals and 30 patients with AML. The feature of distribution and clonality of *TRGV* subfamilies in healthy individuals and patients with AML **(A, B)**. Expression pattern of *TRGV* subfamilies in one case of healthy individual and six AML patients **(C, D)**. P, polyclonality; O, oligoclonality; N, negative.

Proportions of Total γδ T Cells and Vγ9$^+$ Vδ2$^+$ Subsets in patients With AML

Based on previous finding, we were more interested in proportions of total γδ T cells and Vγ9$^+$ Vδ2$^+$ T cell subsets from PB, so another 19 AML samples and 18 HIs were further collected and analyzed for FACS (**Figures 4A–D**). Compared with HIs, significantly lower proportions of total γδ T cells (median: 4.83% vs. 10.5%) and Vγ9$^+$ Vδ2$^+$ T cells (median: 57.9% vs. 84.25%) were found in patients with AML ($P < 0.001$ and $P = 0.001$, respectively; **Figures 4E, F**).

TRGV Repertoire and Its Clinical Relevance in AML

Despite the increased insight into the phenotype of γδ T cells, whether it correlates with clinical outcome remains poorly understood. To further understand the role of the *TRGV*

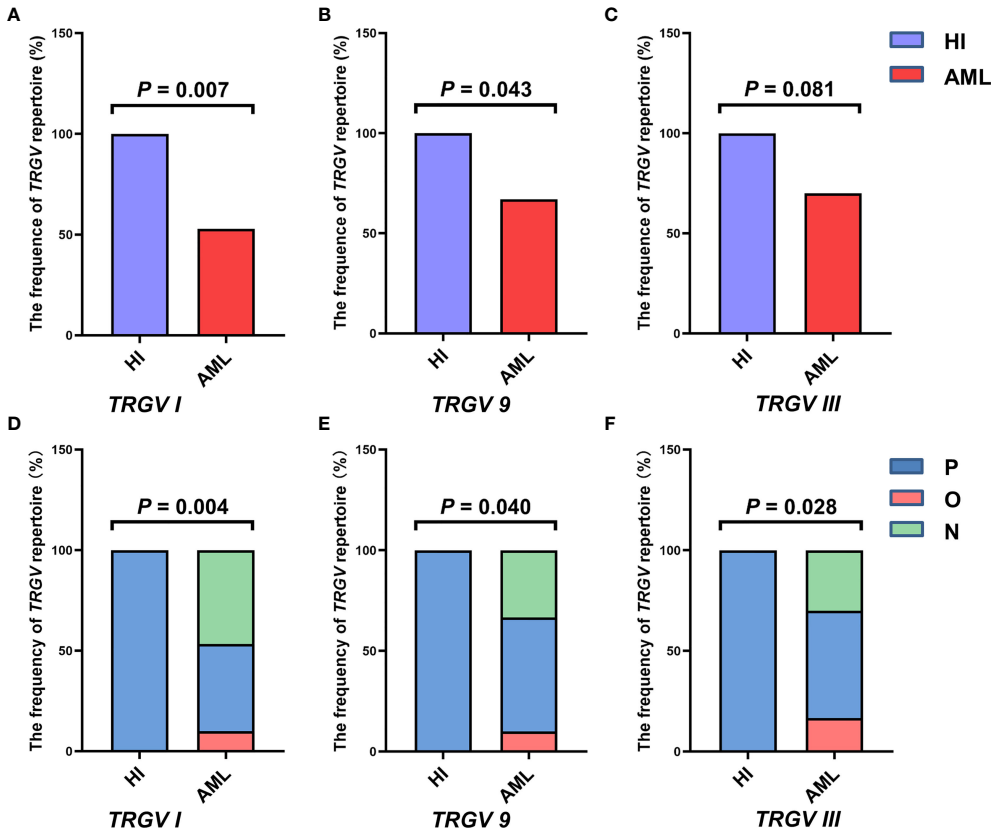

FIGURE 2 | Frequencies of the *TRGV* subfamilies in γδ T cells from 10 healthy individuals and 30 patients with AML. The expression frequencies of three *TRGV* subfamilies in healthy individuals and patients with AML (using the Fisher's exact test) **(A–C)**. The clonal expansion frequency of the three *TRGV* subfamilies in healthy individuals and patients with AML (using the Fisher's exact test) **(D–F)**. P, polyclonality; O, oligoclonality; N, negative.

subfamily and the prognosis of patients with AML, we analyzed correlation between the expression levels of *TRGV* subfamily genes and the frequency of $V\gamma9^+$ $V\delta2^+$ T cells with the clinical prognosis of AML. We first focused on whether expression levels of *TRGV* subfamily genes affected AML clinical prognosis and assessed the clinical prognosis of the 56 AML patients. Due to 6 patients who refused therapy and voluntarily left the hospital, we finally analyzed the prognosis and outcome of 50 AML patients. Univariate and multivariate logistic regression analysis were used to analyze the expressive levels of three *TRGV* subfamilies and other impact factors, including gender, age, WBC, RBC, PLT, BM blast cells, AML subtype, gene mutation and treatment in patients with AML. The patients who followed up after first-cycle chemotherapy were divided into complete remission (CR) and non-CR groups based on BM smears and flow cytometry analysis. Univariate logistic regression analysis demonstrated high WBC counts was an independent risk factor for CR (P = 0.023, odds ratio (OR) = 1.013, 95% confidence interval (CI): 1.002-1.024), whereas high expression levels of *TRGV I*, *TRGV 9*, and *TRGV III* were the significant independent protective factors for CR (*TRGV I*: P = 0.012, OR = 0.211, 95% CI: 0.062-0.711; *TRGV 9*: P = 0.012, OR = 0.211, 95% CI: 0.062-0.711; *TRGV III*: P = 0.003, OR = 0.141, 95% CI: 0.039-0.504),. However, there was no significant difference in gender, age, RBC, PLT, BM blast cells, AML subtype, gene mutation and treatment (P > 0.05). Interestingly, multivariate logistic regression analysis showed that *TRGV 9* expression was an independent protective factor for CR (*TRGV 9*: P = 0.035, OR = 0.079, 95% CI: 0.007-0.831). Besides, we further used univariate and multivariate cox regression analysis to further analyze the relationship between those factors and overall survival (OS) in AML patients. Univariate cox regression analysis showed that high counts of WBC (P = 0.001, hazard ratio (HR) = 1.010, 95% CI: 1.004-1.015) and the AML subtype (non-M3) (P = 0.047, HR = 7.845, 95% CI: 1.028-59.865) was associated with unfavorable OS in AML patients. Importantly, high expression levels of *TRGV I*, *TRGV 9*, and *TRGV III* were associated with favorable OS in AML patients (*TRGV I*: P = 0.018, HR = 0.258, 95% CI: 0.084-0.794; *TRGV 9*: P = 0.004, HR = 0.111, 95% CI: 0.025-0.488; *TRGV III*: P = 0.028, HR = 0.283, 95% CI: 0.092-0.871). Multivariate cox regression analysis also showed that high *TRGV 9* expression could mark an improved OS in patients with AML (*TRGV 9*: P = 0.048, HR = 0.084, 95% CI: 0.007-0.979; **Table 3**).

Furthermore, we also used the same way to access the relationship between the proportion of γδ T cells, $V\gamma9^+$ $V\delta2^+$ T cells and the prognosis of 19 AML patients. Univariate logistic regression analysis showed that the high proportion of $V\gamma9^+$ $V\delta2^+$

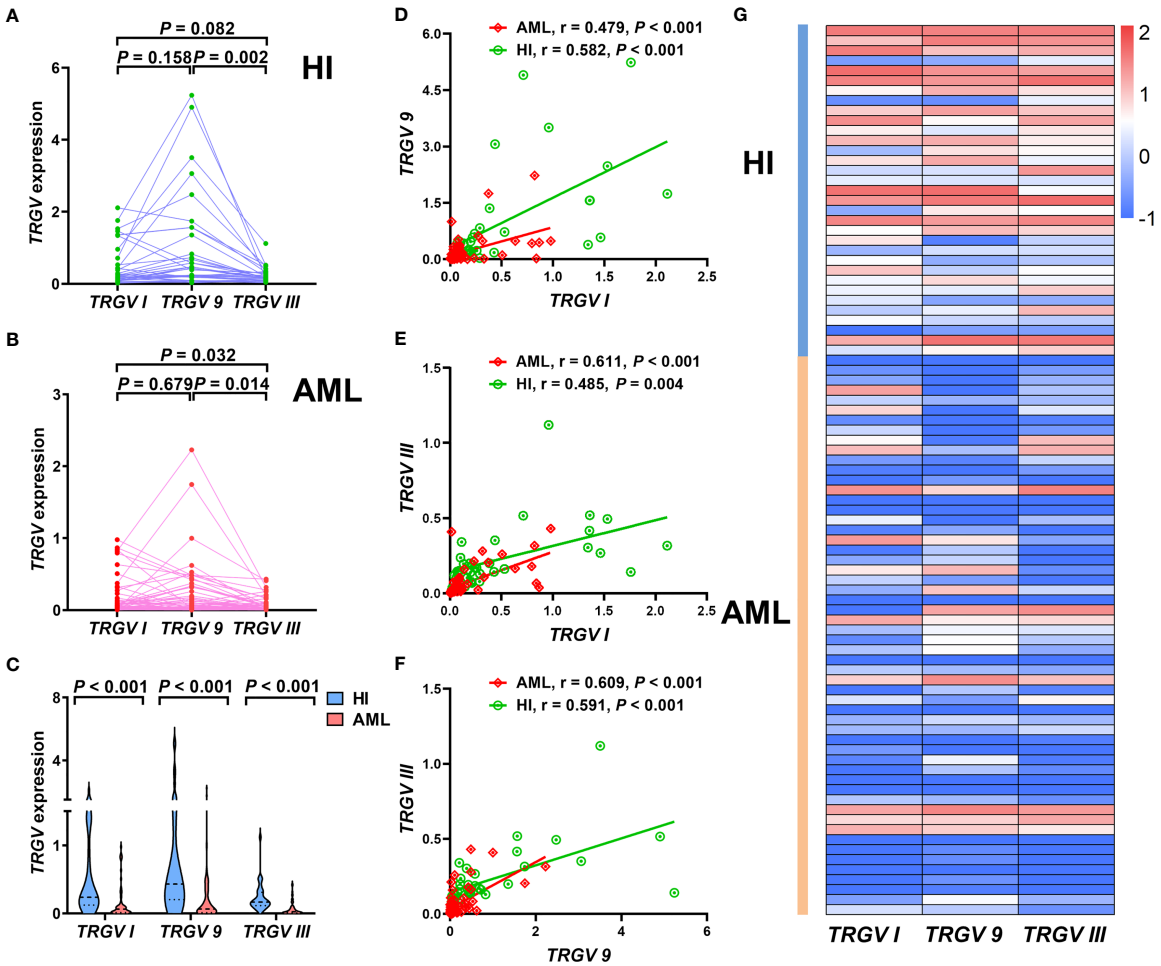

FIGURE 3 | Pattern of expression levels of three *TRGV* subfamilies in γδ T cells from 33 cases with healthy individuals and 56 cases with AML (using the Mann Whitney test) **(A–C)**. Correlations among three *TRGV* subfamilies in 33 healthy individuals and 56 patients with AML (using the Pearson correlation analysis) **(D–F)**. Heatmap representing the expression levels of three *TRGV* subfamilies in 33 healthy individuals and 56 patients with AML **(G)**.

T cells was an independent protected factor for CR ($P = 0.044$, OR = 0.963, 95% CI: 0.927-0.999), and age was an independent risk factor for AML-CR ($P = 0.035$, OR = 1.128, 95% CI: 1.009-1.261), but there was no significant difference in γδ T cells and other factors (gender, age, WBC, RBC, PLT, BM blast cells, AML subtype, gene mutation and treatment) ($P > 0.05$) (data were not showed). Due to insufficient numbers of AML samples, there was no significant difference in multivariate logistic regression analysis. There was one patient was voluntarily left the hospital because of impact of COVID-19 in total 19 AML patients, so we collected outcome of 18 AML patients. Univariate cox regression analysis showed that patients with high proportion of γδ T cells had low risk of death than those with low proportion ($P = 0.008$, hazard ratio (HR) = 0.109, 95% CI: 0.021-0.564), while multivariate cox regression analysis showed no significant difference ($P > 0.05$) (data were not showed).

The Relationship Between *TRGV* Expression and Prognosis in AML Patients

The survival analysis demonstrated that the high expression levels of *TRGV I, TRGV 9* and *TRGV III* were significant related to better OS ($P = 0.011$; $P < 0.001$; $P = 0.019$) (**Figures 5A–C**). To better understand the combination of three *TRGV* subfamilies in predicting the OS of AML patients, we divided patients into the following 3 groups: *TRGV* I^{high} *TRGV* 9^{high} *TRGV* III^{high}, *TRGV I, TRGV 9*, or *TRGV* III^{high} and *TRGV* I^{low} *TRGV* 9^{low} *TRGV* III^{low}. Interestingly, the results suggested that the group of *TRGV* I^{high} *TRGV* 9^{high} *TRGV* III^{high} had longer survival time ($P = 0.001$) (**Figure 5D**). Next, we further access the proportion of $V\gamma9^+$ $V\delta2^+$ T cells from PB with the clinical outcome of AML patients. The OS in high $V\gamma9^+$ $V\delta2^+$ T cells were longer than those in low $V\gamma9^+$ $V\delta2^+$ T cells group ($P = 0.039$) (**Figure 5E**).

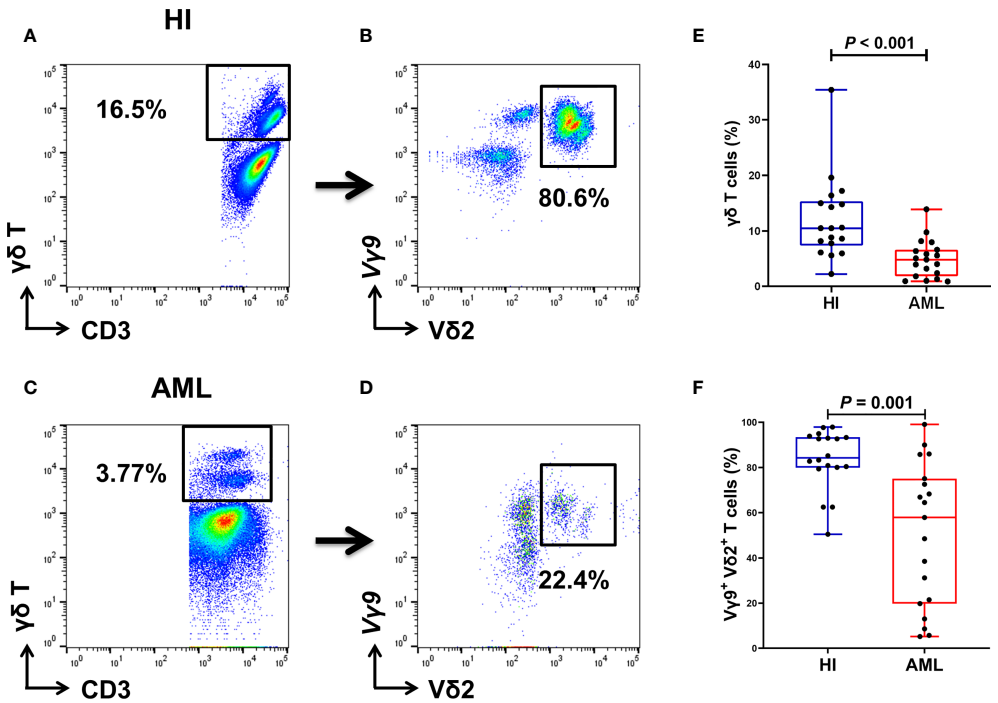

FIGURE 4 | Gating strategy for identifying the percentage of γδ T cells from PB in 18 HIs and 19 patients with AML. Flow cytometry detection of the percentage of $CD3^+$ γδ T cells and $Vγ9^+$ $Vδ2^+$ T cells in HIs **(A, B)** and patients with AML **(C, D)**. The percentage of γδ T cells in HIs and patients with AML (using the Mann Whitney test) **(E)**. Comparison of the percentages of $Vγ9^+$ $Vδ2^+$ T cells in HIs and patients with AML (using the Mann Whitney test) **(F)**.

TABLE 3 | Univariate and multivariate logistic and cox regression analysis in AML patients.

Variables	Univariate logistic regression		Multivariate logistic regression		Univariate cox regression		Multivariate cox regression	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Sex (reference male)								
Female	0.923 (0.297, 2.865)	0.890	0.212 (0.026, 1.697)	0.144	1.602 (0.617, 4.158)	0.333	0.957 (0.314, 2.919)	0.938
Age (year)	1.034 (0.999, 1.070)	0.059	1.064 (1.000, 1.134)	0.052	1.016 (0.985, 1.049)	0.309	1.002 (0.964, 1.042)	0.915
WBC, 10^9/L	1.013 (1.002, 1.024)	0.023	1.010 (0.991, 1.029)	0.325	1.010 (1.004, 1.015)	0.001	1.009 (1.000, 1.018)	0.059
RBC, 10^{12}/L	1.139 (0.599, 2.168)	0.691	4.044 (0.797, 20.528)	0.092	0.896 (0.512, 1.568)	0.700	3.049 (1.093, 8.503)	0.033
PLT, 10^9/L	1.000 (0.994, 1.006)	0.975	1.001 (0.992, 1.010)	0.824	1.000 (0.995, 1.005)	0.984	1.004 (0.996, 1.012)	0.341
BM blast cell, %	1.002 (0.972, 1.033)	0.905	1.024 (0.974, 1.075)	0.354	1.007 (0.981, 1.034)	0.592	1.008 (0.973, 1.045)	0.653
FAB subtype (reference non-M3)								
M3-AML	4.275 (0.816, 22.390)	0.086	1.071 (0.045, 25.315)	0.966	7.845 (1.028, 59.865)	0.047	4.584 (0.197, 106.861)	0.343
Gene mutation (reference non-*FLT3* mutation)								
FLT3 mutation	0.404 (0.073, 2.235)	0.299	0.126 (0.009, 1.711)	0.120	1.475 (0.48, 4.532)	0.497	1.937 (0.373, 10.060)	0.431
Treatment (reference chemotherapy)								
HSCT	0.800 (0.169, 3.793)	0.779	0.904 (0.110, 7.427)	0.925	0.438 (0.099, 1.932)	0.276	0.294 (0.032, 2.656)	0.275
TRGV I	0.211 (0.062, 0.711)	0.012	1.243 (0.086, 17.973)	0.873	0.258 (0.084, 0.794)	0.018	0.552 (0.026, 11.652)	0.703
TRGV 9	0.211 (0.062, 0.711)	0.012	0.079 (0.007, 0.831)	0.035*	0.111 (0.025, 0.488)	0.004	0.084 (0.007, 0.979)	0.048*
TRGV III	0.141 (0.039, 0.504)	0.003	0.069 (0.004, 1.161)	0.063	0.283 (0.092, 0.871)	0.028	1.221 (0.067, 22.238)	0.893

*AML, acute myeloid leukemia; CR, complete remission; OS, overall survival; OR, odds ratio; 95% CI, 95% confidence interval; HR, hazard ratio; WBC, white blood cell; RBC, red blood cell; PLT, platelet; M3, acute promyelocytic leukemia; HSCT, hematopoietic stem cell transplantation. *P < 0.05.*

DISCUSSION

The attractive features of γδ T cells include non-MHC-restricted antigen recognition and abundant cytokine secretion capacity, which have raised expectations for their application in cancer adoptive immunotherapy (28–30). The combinatorial variety generated by different TCRs might be the reason why γδ T cells can exert diverse actions in distinct pathological types of diseases

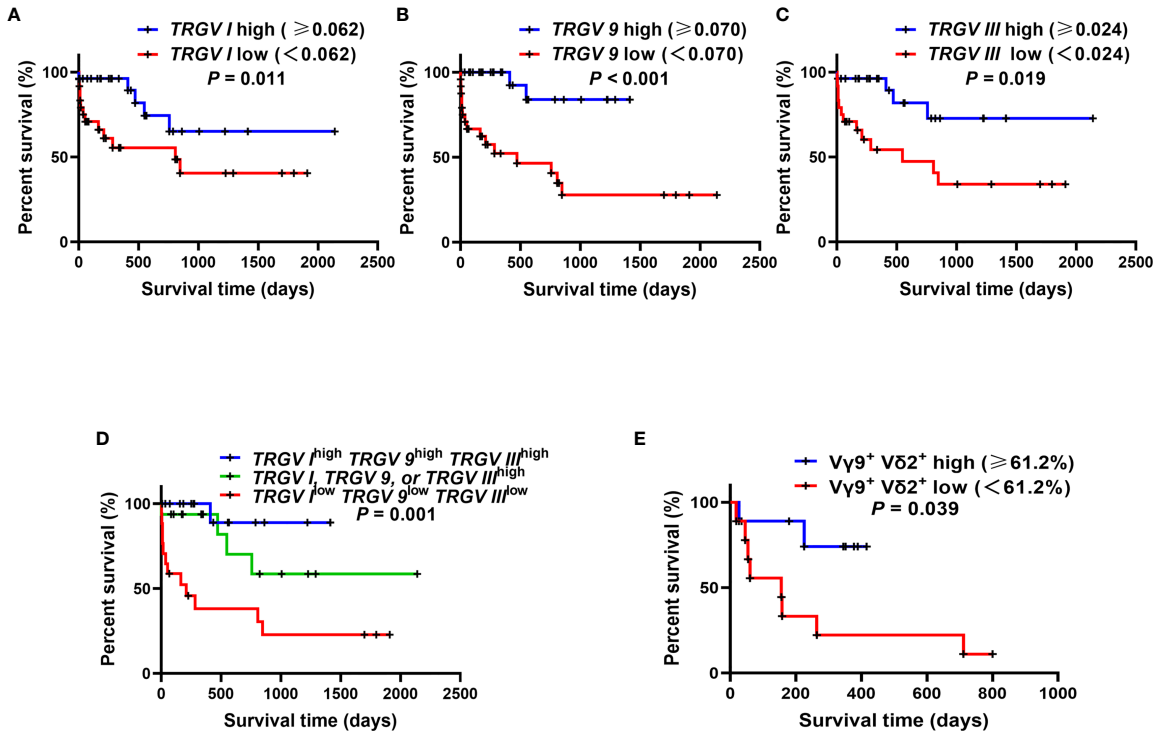

FIGURE 5 | Overall survival (OS) analysis of the gene expression levels of three *TRGV* subfamilies in 50 AML patients and the percentages of Vγ9$^+$ Vδ2$^+$ T cells in 18 AML patients. Kaplan–Meier curves showed the OS for the high (blue line) and low (red line) *TRGV* expression groups **(A–C)**. Kaplan–Meier curves showed the OS for the co-high expression (blue line), single high expression (green line), and co-low expression (red line) of *TRGV I*, *TRGV 9* and *TRGV III* **(D)**. Kaplan–Meier curves showed the OS for the high (blue line) and low (red line) percentages of Vγ9$^+$ Vδ2$^+$ T cells **(E)**.

(31). T cell immunodeficiency is a common feature in different hematological malignancies, including AML, immune thrombocytopenic purpura (ITP), B cell non-Hodgkin lymphoma, and graft-versus-host disease (GVHD) (21, 32–34). Analysis of alterations in the TCR repertoire is a practical approach that can help understand the involved immunological abnormalities and provide guidance for clinics in translational research (19). Analysis of the *TRGV* and *TRDV* repertoire provides a global picture of the distribution and clonal expansion of TCR γδ subfamilies in ITP, multiple myeloma (MM), and GVHD (21, 32, 35, 36). Our previous study also showed the clonally expanded *TRDV* T cells in AML (18). However, the features of the *TRGV* repertoire in AML remain unknown.

In this study, we investigated the expression pattern of TCR Vγ (*TRGV*) subfamilies and characterized the correlation between the expression of *TRGV* and clinical outcome in patients with AML. To further compare the difference in TCR repertoire diversity, three *TRGV* gene spectral profiles were examined by Genescan analysis. In HIs, polyclonal expanded T cells, which showed a small proportion of multiple peaks, were detected in the majority of the *TRGV* subfamily. By contrast, a clonotypic expansion pattern, which included a high peak together with one or a few lower peaks named oligoclonality, was a common pattern for each sample. Skewed expression of the *TRGV* repertoire was an obvious characteristic of patients with AML compared with HIs who expressed nearly all of the *TRGV* subfamilies, which indicated that patients with AML might have low diverse immune responses due to γδ T cell immunodeficiency. The T cell spectra are commonly characterized by a Gaussian distribution containing 6–8 peaks, which are named polyclonality in HIs, representing a repertoire that guarantees sufficiently diverse T cell clones (37). The clonally expanded T cell repertoire was also detected in all samples in this study. Multiple oligoclonal expanded *TRGV* subfamilies were demonstrated in patients with AML who were different from HIs. Thus, the oligoclonal *TRGV* repertoire might be associated with leukemia-associated antigen.

We also found that the gene expression levels of the *TRGV* repertoire in γδ T cells between AML and HIs were different, and lower expression levels were found in *TRGV* genes in AML than in HIs. The change and pattern of *TRGV* subfamilies demonstrated that restrictive *TRGV* usage might be related to the preference of usage of γδ T cells. The biological significance of the difference observed remains unknown, so we attempted to characterize the association between the expression level of the *TRGV* repertoire and clinical patient characteristics. Our previous study showed that TIGIT$^+$ Foxp3$^+$ γδ T cells and TIGIT$^+$ CD226$^-$ γδ T cells were related to the clinical outcome of patients with AML (38, 39). In the present study, we further

analyzed the relationship between the expression of the *TRGV* repertoire and the OS of patients with AML. Our results showed that a higher expression level of *TRGV* subfamilies was associated with better OS in patients with AML, and patients with highly *TRGV I*, *TRGV 9*, and *TRGV III* genes co-expressed had better OS than their counterparts. Moreover, we found that *TRGV 9* was an independent protective factor in AML-CR, thereby indicating that patients with high *TRGV 9* expression may have the better prognosis than those with low expression. In addition, our data showed that increased $V\gamma9^+$ $V\delta2^+$ T cells subfamilies in patients with AML might correlate with better therapeutic effects. Related research showed that γδ T cells played an essential role in cancer (40). Such cells have a long-term disease-free survival advantage to patients with AML and increased γδ T cells following hematopoietic stem cell transplantation (HSCT) (41, 42). The known pleiotropic effects of γδ T cells suggest multiple mechanisms by which γδ T cells might promote survival after HSCT, which were consistent with our findings in patients with AML. Understanding the characteristics of *TRGV* subsets in patients with AML may be helpful for clinical application and promote the treatment of patients. However, these γδ T cell subfamilies exerted certain anti-leukemia effects, so the anti-leukemia potency of γδ T cells could be exhausted due to prolonged antigenic stimulation. In the long run, we should choose a specific anti-tumor γδ T cell subgroup in γδ T cell immunotherapy and try to use a combination of γδ T cell adoptive immunotherapy and immune checkpoint inhibitors.

CONCLUSION

Taken together, in addition to the previously reported clonally expanded *TRDV* T cells in AML (18), our data further provide a detailed profile and feature of the *TRGV* repertoire in patients with AML. Importantly, the patients with AML who had high expression level of the *TRGV* gene or higher proportion of $V\gamma9^+$ $V\delta2^+$ T cells were associated with favorable OS, which may be related to resorting anti-AML γδ T function. Further studies are required to confirm and dissect the detailed mechanisms. These findings could partially explain to promote our understanding of the cellular immune features of γδ T cells, which brings hope for immunotherapy to treat AML patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The protocol of all experiments was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XW and ZJ were involved in experimental design and the concept development. XK and XL conducted the experiments. WW and XJ contributed to data analysis and figure preparation. JC and JL provided all samples and clinical data. ZJ, XK, and XW drafted the manuscript. All authors read and approved the final 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.2022.823352/full#supplementary-material

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REVIEW
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Skin γδ T Cells and Their Function in Wound Healing

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For the skin immune system, γδ T cells are important components, which help in defensing against damage and infection of skin. Compared to the conventional αβ T cells, γδ T cells have their own differentiation, development and activation characteristics. In adult mice, dendritic epidermal T cells (DETCs), Vγ4 and Vγ6 γδ T cells are the main subsets of skin, the coordination and interaction among them play a crucial role in wound repair. To get a clear overview of γδ T cells, this review synopsizes their derivation, development, colonization and activation, and focuses their function in acute and chronic wound healing, as well as the underlining mechanism. The aim of this paper is to provide cues for the study of human epidermal γδ T cells and the potential treatment for skin rehabilitation.

Keywords: γδT cells, wound healing, DETCs, Vγ4, Vγ6, homeostasis

INTRODUCTION

γδ T cells (according to their γδ TCR) were first identified as a novel T-cell subset in the mid-1980s (1). As a gap between innate and adaptive immune response, they participate in regulating carcinoma (2), maintaining antimicrobial barrier (3), wound healing (4), psoriasis (5) and graft rejection (6). γδ T cells represent less than 5% of peripheral lymphocyte population in mice, human and rat (7, 8), whereas it constitutes a relatively large fraction of T lymphocytes in chicken, sheep, cattle and pig (15–50%) (8). In adult mice, γδ T cells are unequally distributed (9); there are less than 5% of total T cells in the lung, approximately 20–40% of the intraepithelial T cells of intestinal, approximately 10–20% of total T cells in the reproductive tracks, approximately 50–70% of skin dermal T cells and approximately 95% of epidermal T cells. In addition, they are divided into Vγ1-7 γδ T subsets according to the γ chain (10). Almost all γδ T cells in epidermis are dendritic epidermal T cells (DETCs: named by its dendritic morphology), expressing an invariant Vγ5Vδ1 TCR (according to Tonegawa's nomenclature, which is adopted in this paper), equal to Vγ3Vδ1 TCR (according to Garman's nomenclature) (11, 12). They maintain a homeostatic population by self-renew and can secrete growth factors such as IGF-1 (Insulin-like growth factor 1) and KGF-1/KGF-2 (keratinocyte growth factor 1/2) etc. (13) Most γδ T cells in dermis are Vγ4 T and Vγ6 Cells, they can secrete IL-17A (interleukin-17A), IFN-γ (interferon-γ) and the growth factors (4).

In humans, γδ T cells are classified based on the presented Vδ gene segment. Until now, there exists three true Vδ genes: Vδ1-3; and seven functional Vγ gene segments: Vγ2-5, Vγ8, Vγ9, and Vγ11 (14). Vδ1 γδ T cells primarily colonized in the dermis, and a small population is distributed in

the epidermis, whereas Vδ2 TCRs are mainly distributed in peripheral blood and dermal (15, 16). Human epidermal γδ T cells play a functionally similar role as DETCs in promoting wound healing *via* secreting insulin-like growth factor 1 (IGF-1) and regulating cutaneous carcinoma (17, 18). However, they are not called DETCs as they do not possess dendritic morphology and take different molecular mechanisms in epidermis homing, antigen recognition and activation.

The skin, which is essential in defencing against external pathogens and environmental factors such as the microbes attack, ultraviolet radiation and heat injury (15, 19), serves as the largest interface between the body and the external environment. On one side, skin needs enough defending power to maintain homeostasis; on the other side, it needs fast and effective responses to repair the injury and restore the integrity upon injury or inflammation. Wound repair mainly contains four overlapping stages, which includes hemostasis, inflammation, proliferation and remodeling (20). Immune cells manage wound repair by secreting cytokines and chemokines to induce inflammatory microenvironment and promote re-epithelialization. DETCs, Vγ4 T cells and Vγ6 T cells are the main subsets of skin T lymphocytes and the equilibrium, coordination and interaction among them significantly affect their effectiveness in wound repair. This review primarily focuses on the discussion the rodent and murine γδ T cells, including their development, differentiation, colonization, activation, their functions and the underlining mechanism in wound healing. In addition, by consolidating the recent research breakthrough in the field, perhaps this article may also provide potential cues for the study of human skin γδ T cells and the potential treatment for skin rehabilitation.

THE DEVELOPMENT AND COLONIZATION OF γδ T CELLS

γδ T cells and αβ T cells originate from the same progenitor in the thymus. When bone marrow-derived hematopoietic stem cells (HSC) migrate into the thymus, Notch receptor 1 (Notch 1) and Delta-like 4 (DLL-4) signaling leads to the generation of T cell progenitors called double-negative cells expressing $CD4^-$ and $CD8^-$ (DNs, $CD4^-$ and $CD8^-$) (19, 21, 22), which commit them to the T-cell fate. Then these immature thymocytes pass through four developmental stages, from DN1 to DN4 (23, 24). DN1 cells are uniformly bipotent, they can give rise to both αβ and γδT cells (25); the next DN2 stage initiates the divergence of αβ and γδ T cells, and in this stage, cells expressing IL-7R and SOX13 (one high mobility group (HMG) box TF) and other unknown factors exhibiting the tendency to γδT cells fate (26, 27). TCR δ, γ and β start to rearrange stochastically (somatic recombination of the V, D, and J genes encoding the V domain of the corresponding TCR proteins) (28–30), and then weak signal strength boosts the divergence of αβ lineage (preTCR: consisting of the invariant pTαchain paired with a full-length β chain), while the strong signal enhances the γδT cells and selectively promotes the precisely rearranged and paired γδ chain (TCR γδ) (28, 29, 31–33), DETCs, IFN-γ-producing V γ1 cells and IL-17A-producing V γ6 cells are markedly depleted in mice with attenuated TCR signaling of their own (34, 35), this process is called the positive selection. The invalidly rearranged cells or validly rearranged cells without sufficient activation signaling from ligand undergo apoptosis similar to the death of the αβ T cells without useful TCR. Whether this phenomenon leads to the successive development characteristic of γδ T cells has to be verified. Partial cells of this stage retain bipotency, whereas other cells just give rise only to αβ or γδ T cells (36). The divergence of αβ and γδ lineage is completed at the DN3 stage, and by this stage, almost all of the cells complete lineage commitment, with a major population exhibiting αβ lineage restriction (25). But the precursor cells with type of TCR (preTCR or γδ TCR) can't dictate the lineage choice, as the γδ TCR and αβ TCR can generate αβ and γδ lineage cells under some special circumstances, respectively (37–39); transitioning into the DN4 stage, the TCRα chain gene-rearrangement begins, which generates double positive($CD4^+$, $CD8^+$) αβ T cells (DP αβ T cells) marking the point of irreversible commitment to the αβ lineage (36, 40). Then the DP αβ cells commit the positive and negative selection and get matured (41). While the subset of immature γδ T cells will develop the effector commitment, the relatively weaker signals enhance the IL-17–producing γδ T cell subset, and progressively stronger signals promote IFN-γ–producing and innate γδ T cells (24). However, there has no direct evidence whether the stronger or weaker signal leads to higher productions of IFN-γ- or IL-17A- V γ4 T cells, respectively. CD24 or heat-stable antigen (HSA) is recognized as the marker of γδ T cell lineage for irreversible commitment. The expression of $CD24^+CD73^+$ indicates that these cells are unable to switch to the αβ T cells (19, 42). Therefore, the TCR signaling operates in sequential developmental windows with distinct outcomes, and it determines the lineage and effector commitment successively (10). In addition, TCR γδ-independent factors are crucial in γδ T cells differentiation, such as the miRNAs, Sox4/Sox13/RORγ axis (SRY-box-containing gene 4/13/retinoid-related orphan receptor γ axis), and Notch signaling (13, 43, 44). Thus, every subset has its own development characteristic.

The development of the γδ subset occurs step by step as follows: T cell commitment–αβ/γδ lineage commitment–γδ subset commitment–effector commitment (**Figure 1**); therefore, the same factor can take different functions during disparate stages. This theory can reconcile some inconsistent research results. For instance, IL-7 and the transcription factor SOX13 promote the survival and development of early precursor cells and are absolutely required for TCRγ gene rearrangement. However, at the later stage, their function mainly promotes the IL-17-producing cells (26, 27, 45, 46). Besides, the same factor can give rise to an identical or a different function for various subsets at the same cross-section in time, just like the PLZF and Egr2/3/id3; the former promotes the development of the Vγ1+ and Vγ6+ cells (47, 48), while the later one takes an opposite function in IL-17- and IFN-γ-producing cells (10).

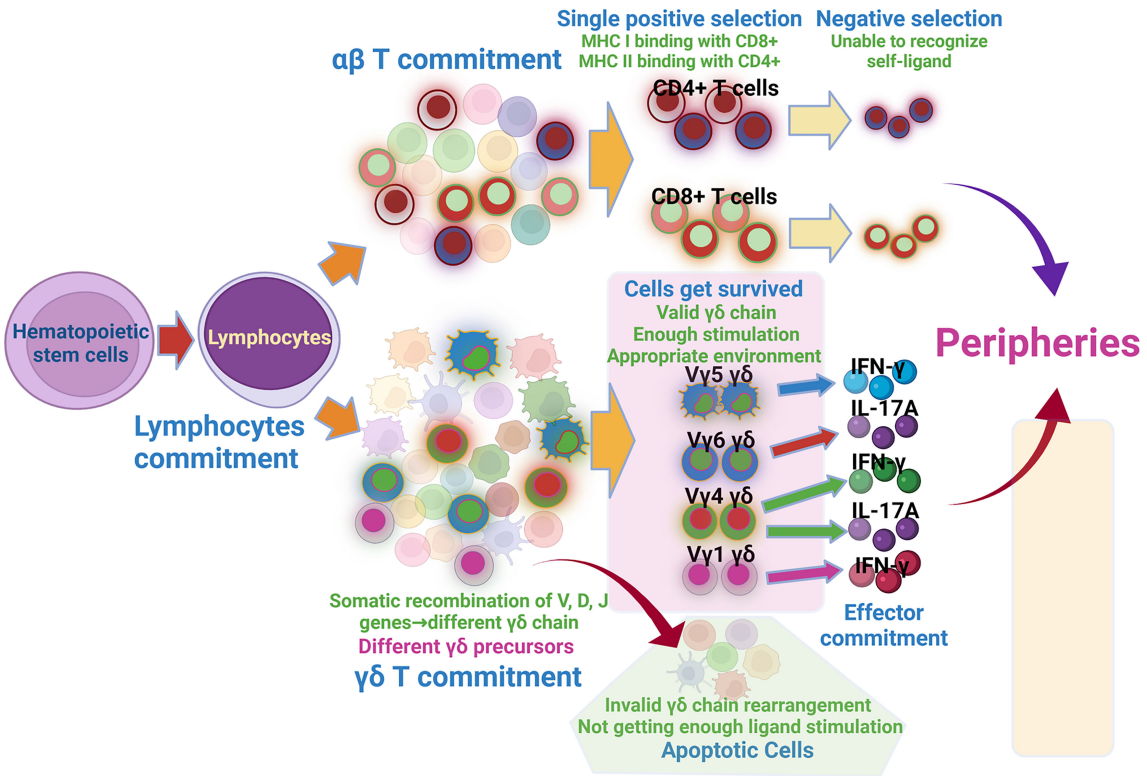

FIGURE 1 | Development of αβ and γδ T cells. Hematopoietic stem cells migrating into thymus get lymphocytes commitment, the lymphocytes then get αβ commitment and γδ commitment. αβ cells passing through sequential single positive selection and negative selection get matured. Somatic recombination of V, D, J genes forms different γδ chain, which produces varied γδ precursors. Among them, cells with valid γδ chain, getting enough stimulation and appropriate environment get survived, cells with invalid γδ chain and getting insufficient ligand stimulation get apoptosis. Survived γδ T cells then undertake effector commitment and get matured.

DETCs expressing a canonical Vγ5Vδ1 TCR are a restricted antigen repertoire and act exclusively as resident T cells in the murine epidermis (12). They derive from DETC progenitors which are restrictedly generated in the embryonic thymus at day 13 to 17 (49), and at E16 and E18 (50), DETCs egress from the thymus and move to the epidermal layer where they self-renew. Existing research have confirmed that the development of DETCs can be influenced by ERK-Egr-Id3 axis (35), Lck (51), Syk (52), ZAP-70 (53), IL-7R/JAK/STAT pathway (54, 55), RunX3 (regulating CD103 and CD122) (56), miRNAs (downregulating CD122/IL-2Rβ and CD45RB expression) (43) and Skint-1 (promoting the selective development of Vγ5+ DETC) in the thymus (35, 57); their skin-homing are affected by the ITK (through promoting CCR10 and S1PR1 expression) (58, 59), SIPR1 (sphingosine-1-phosphate receptor 1, involved in thymic egress) (60), GPR15 (orphan G protein-linked chemoattractant receptor 15, regulating the recruitment of γδT cells to skin) (61), CD103 (62), E, P-selectins ligands (63) (Expressed on DETCs, binding to selectins expressed on the endothelium), CCR10 (64) and CCR4 (63) (binding to CCL27/28 expressed by keratinocytes), Vγ5 T cells have low expression in CCR9 and CCR7, so they will not migrate into lymphoid organ and spleen. Matured DETCs express the markers including $CD27^{-}$, $CD69^{+}$, $T\text{-}bet^{+}$, $NKG2D^{+}$, $JAML^{+}$, $CD100^{+}$, and $CD103^{+}$ (15).

Vγ4 T cells appearing at the late fetal stage(from E16)and afterward (49), are the dominant subset of murine peripheral γδ cells. In addition, Vγ4 T cells exist in peripheral lymphoid organs, blood, liver, lung, spleen and dermis (65). They are divided into two main subsets: $IL\text{-}17A^{+}V\gamma4$ T cells ($CCR6^{+}CD27^{-}$), and $IFN\text{-}\gamma^{+}V\gamma4$ T cells ($CCR6^{-}CD27^{+}$) (66). The majority of γδ T cells in lymph node are IL-17A γδT cells, whereas a large population in splenic is IFN-γ γδ T cells (67); the mechanism leading to this biased distribution is unclear. The development of IL-17A producing cells is also regulated by the comprehensive factors, such as Sox4/Sox13/RORγt/IL-17 axis (68), Notch signaling/Hes-1 axis (44, 69), Wnt signaling pathway/TCF1 and Lef1 axis (70), TGF-β (71), Blk (B lymphoid kinase, a Src family kinase) (72) and IL-7 (45). Moreover, CCR6 is recognized to be critical for their homing to skin, CCR6-deficiency reduced the number of both Vγ4 and Vγ6+ cells in the skin (73). Other research reports that thymic Vγ4 requires extrathymic environment for skin homing, such as getting activated or obtaining CCR6 expression (74). Matured IL-17 producing Vγ4 T cells (thymus-derived) contain variable δ

chain. Most of them express CD3+, CD4-, CD8-, CD44+, CD69+, RORγt+, CCR6+, CD25+, CD27-, Scart2+, CD45RB-, CD122-, CD27-, NK1.1-, T-bet-, IL-23R (31, 66, 75–80). Recent research found that some IL-17 producing γδ T cells are bone derived, and they often just have δ4 chain. In addition, they express CCR2+ and require IL-23 and IL-1β for their reprogramming from CD27+ γδ T cells (81, 82). In addition, IFN-γ-producing γδT cells are affected by ERK-Egr-Id3 axis (10, 34), ThPOK/PLZF/T-bet axis (83), researches have reported thymic γδ T cells with antigen-experience or binding antigen have high affinity in producing IFN-γ (67), matured IFN-γ producing Vγ4 T cells have variable δ chain. Their expression characteristics are CD3+, CD4-, CD8-, CD44+, T-bet+, NK1.1+, CCR6-, CD27+, CD45RB+, CD122+(IL-2/IL-15 receptor β chain) (31, 66, 75–80).

Vγ6 T cells, which exclusively express the Vδ1 TCR chain (74), are generated solely in the thymic second wave around embryonic day E14 (up to the birth) (49). In mice, about half of the dermal γδ T cells are the Vγ6 T cells, while the rest mainly express Vγ4 TCR (4, 74). Vγ6 T cells also localize to uterine epithelia, tongue and meninges, enthesis, pLNs, testis (79, 84–86). Conventionally, dermal Vγ6 T cells are considered bona fide tissue-resident cells that do not recirculate out of the skin and their generation is restricted to the confined window of fetal development. Furthermore, Vγ6 T cells cannot be induced in adult animals with the phenomenon that Vγ6+ γδ T cells become rare in the adult thymus (87, 88). But recent research confirmed that they have a high mobility and can travel between pLNs and tissues (79); however, whether the proliferated Vγ6+ in pLNs or thymus refill the pool of terminally differentiated skin Vγ6 remains to be tested. Their development is affected by IL-7 (45), TGF-β (71), Blk (72), PLZF (47). Matured Vγ6 cells exhibit the expression characteristics of CD27–, IL-23R+, RORγt+, CCR6+, CD69+, CD44+, Scart1+, cMAF+, PLZF+, PD-1 receptor and CCR2 (15, 79).

γδ T CELLS IN MAINTAINING SKIN HOMEOSTASIS

Skin comprises two major compartments, the epidermis and the dermis. The epidermis is mainly composed of keratinocytes (~95%) and residing immune cells (~5%, mainly are Langerhans cells (LC) and T cells) (89). The immune cell composition is subject to species specific differences. In naïve wild type (WT) mice, DETCs dominate the epidermal T cell compartment(~95%). Human epidermis is home to both γδ and αβ T cells, while resident T cells in epidermis show effector functions very similar to that of DETC (90).

The DETCs proliferate and maintain a homeostatic population by themselves, which cannot be reconstituted with bone marrow cells or fetal thymocytes (88). Aryl hydrocarbon receptor (AhR) and Linker for activation of T cells (LAT) are recognized to be the important factors in maintaining DETCs proliferative expansion and self-renewal (91). AHR-KO mice and LAT–deficient mice lack peripheral DETCs neither through affecting the DETCs generation nor skin homing (92). DETCs are characterized with lots of dendrites; most of the dendrites anchor to the apical epidermis where they are immobilized at distal. The remaining dendrites are positioned within the basal epidermis and are highly mobile (93). PALPs (containing prominent co-clusters of TCR and proteins phosphorylated on tyrosine residues) (94) of the apically oriented dendrites contribute the anchoring of DETCs to the squamous keratinocyte junctions, E-cadherin receptor integrin αEβ7(CD103) highly enriched at the ends of apical dendrites modulates the dendrite anchoring, which binds with E-cadherin expressed by keratinocytes. This structure allows the frequent contact of DETCs with the neighbouring cells as well as continuous scanning for antigens in the skin surface (94). Although healthy skin does not appear to express DETC TCR ligand detectable by soluble Vγ5Vδ1 TCR tetramers (95), low grade stresses from outside environment might sustain a basal expression of ligands sufficient for TCR activation but below the sensitivity of currently existed detection method. This presence of agonistic TCR-proximal signals make the DETCs to be a semi-activated state *via* Lck-dependent TCR activation (94), these semi-activated DETCs establish a polarized conduit system for transepithelial cargo transport, which contributes to the accumulation of matured lysosomes and the probe of the epidermal molecular composition (96). Normally, semi-activated DETCs express CD122 and CD69 (marker of pre-activation/semi-activation), their autocrine cytokines can help maintaining steady state of themselves and other cells (93), including IL-13, IGF-1, GM-CSF (**Table 1**). IL-13 plays an important role in regulating epithelial cells homeostasis and maintaining skin integrity through promoting EC (Epithelial cells) maturation and transiting through epidermis, the mice lacking canonical DETCs or IL-13 shows a higher degree of water loss, a poorer barrier function and a declined tolerance to damage compared to the WT skin (97); IGF-1 can protect themselves and keratinocytes from apoptosis (98), while GM-CSF is crucial for LC maturation (92). In turn, the paracrine cytokines by neighboring keratinocytes, fibroblasts and other cells are crucial in keeping the homeostasis of DETCs (96, 99). IL-7 secreted by keratinocytes and fibroblast mesenchymal cells serves as a growth factor for DETCs (100); IL-15 secreted by epithelial cells helps the survival and proliferation of DETCs *via* binding IL-15Ra (CD215) expressed on DETCs (101).

The immune cells residing in the dermis under homeostasis include dermal subsets of dendritic cells (DCs), mast cells, T cells (αβ and γδ T cells), innate lymphoid cells (ILC), B cells, macrophages and NK cells (102). γδ T cells of dermis mainly comprised of Vγ4 and Vγ6 γδ T cells. Vγ6 γδ T cells represent virtually 100% of the dermal γδ T cells in newborn mice, but comprise only about 40% in adult mice, as the Vγ4 γδ T cells in the dermis gradually increase over time (103). The majority of Vγ6+ γδ T cells display tissue residency, but may retain the capability to circulate between tissues, while the Vγ4 T cells display the recirculating characteristic. Recent researches have indicated that both dermal Vγ4 and Vγ6 T subsets are radioresistant (74, 104). Under homeostasis conditions, both subsets can traffic between tissues and lymph nodes at a slow but steady rate (79, 87, 105, 106); a substantial flux of γδ T cells through the skin to draining LNs is observed through analysis of skin-draining lymph in cattle (107).

TABLE 1 | Main cytokines, chemokines, and receptors of DETCs, Vγ4 and Vγ6 T cells in skin homeostasis and wound healing.

Cytokines	Main function	Receptors	Main function
IGF-1	Binding with IGF-1R, promotes keratinocytes survival and regulates their differentiation, prevents the apoptosis of DETCs.	CCR10/CCR4	Mediates DETCs migration and location *via* binding with CCL27/28.
KGF-1/KGF-2	Induces keratinocytes proliferation, differentiation and migration.	CCR6+	Contributes to homeostatic γδ T cells trafficking (Vγ4 and Vγ6).
IL-13	Regulates skin homeostasis and protects against carcinogenesis.	CCR2+	Dominates the trafficking of activated γδ T lymphocytes (Vγ4 and Vγ6).
GM-CSF	Is crucial for LC maturation.	αEβ7(CD103)	Contributes to the anchoring of DETCs.
IL-17A	Induces and amplifies inflammation, induces the migration of inflammatory cells.	AhR	Maintains DETCs proliferative expansion and self-renewal.
IFN-γ	Facilitates anti-tumor and anti-infection response.	IL-15Rα (CD215)	Maintains the survival and proliferation of DETCs and regulates the production of IGF-1 *via* binding with IL-15.
Chemokines	**Main function**	NCRs (NKG2D, TLR, CD100, JAML)	Provides costimulatory signals and participates in antigen recognition and inducing the release of cytokines.
CCL-3/CCL-4/CCL-5	Induces the migration of inflammatory cells.		
Mcp-1	Plays an important role in monocyte migration.		
XCL1	Induces migration of lymphocytes *via* binding with XCR1.		

It is proposed that CCR6-dependent manner contributes to homeostatic γδT17 cell trafficking, CCR6 can bind with CCL20 expressed in mucocutaneous sites and subcapsular region of primate LNs (108), while CCR2-dependent manner dominates the activated trafficking (73), this trafficking characteristic facilitates their immune surveillance function. Upon activated by ligands such as the specific ligands triggered by the imiquimod treatment, the migration will significantly increase. However, it seems that the $V\gamma4^+$ dermal cells are able to migrate more efficiently than the Vγ6+ γδT cells (103, 109). For the resident Vγ6γδT cells, they usually act as persistent effector cells in the skin, high expressions of the anti-apoptotic BCL2A1 protein protects them from activation-induced cell death (79). However, whether the resident Vγ6+ T cells can be refilled by the Vγ6 T cells from pLN and thymus is uncertain, and interesting to be tested. For the Vγ4 cells, they can be reconstituted by thymic $V\gamma4^+$ cells and bone marrow, but they need to go to the periphery and mature before migrating to the dermis (74, 81). The CCR6 expressed on their surface and the CCL20 expressed by epidermal keratinocytes, endothelial cells, and dendritic cells are crucial for their recruitment (82).

Collectively, DETCs exist in epidermis, they maintain a homeostatic population by self-renewal. Under homeostasis, they secrete IL-13, IGF-1 and GM-CSF to help in epithelial cells maturation and proliferation. IL-7 and IL-15 secreted by epithelial cells contribute to the survival and proliferation of DETCs, PALPs of the apically oriented dendrites contribute to the anchoring of DETCs to the keratinocyte junctions. Vγ4 and Vγ6 T are main subsets in the dermis, they traffic between tissues and lymph nodes at a slow but steady rate under homeostasis, CCR6 expressed on their surface combining with the CCL20 expressed in mucocutaneous sites and subcapsular region of primate LNs is an important pathway (**Figure 2**).

THE ACTIVATION OF γδ T CELLS

γδ TCRs have the ability for both innate and adaptive ligand recognition *via* either germline-encoded regions of the receptor, resemble the PRRs or adaptive antigen binding *via* the CDRs, this pattern seems to be distinguished from αβ TCRs (102). Most αβ TCRs bind to MHC I/II (major histocompatibility complexes I/II) which presents small peptide fragments derived from pathogens or pathological tissues. Together with co-receptor engagement of CD4 or CD8 and co-stimulation through CD28, this elicits αβ T-cell activation (110). Similar to αβ T cells, the activation of γδ T cells may require the engagement of both γδ TCR and co-receptors, including junctional adhesion molecule-like protein (JAML) (111), Toll-like receptor (TLR) (112), the semaphorin CD100 (113) and C-type lectin-like stimulatory receptor-natural killer group 2D (NKG2D) (114). As no general restricting molecule could be identified, no effective methods can assess whether the recognition of certain antigens by γδ TCRs is generalized, and the affinity of TCRs to their antigens is typically low, the antigens activating the γδ TCR or γδT cells have not yet been clearly identified up to now. Recent years, many studies have been conducted to explore the antigens. The antigens activating the γδ T cells can be divided into 4 categories (115): First of all, MHC or MHC-like recognition antigen includes MHC-Ib molecule T10/T22 (116), MART-1 (117), MHC-related protein 1 (MR-1) (118). Secondly, there are IG-like recognition of antigens, including Annexin A2 (119), ephrin receptor A2 (EphA2) (120), the human DNA mismatch repair protein MutS-Homologue 2 (hMSH2) (121), heat shock protein (HSP) 60 (122), PE(phycoerythrin) (123). Thirdly, this group contains Phosphoantigen, including 4-hydroxy-3-methyl-but-2- enylpyrophosphate (HMBPP), Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (124). Lastly, there are B7 receptor family-like proteins, including BTNLs (BTNL1 and BTNL6 in mice, BTNL3 and 8 in human) (125, 126). Furthermore, the antigens can be categorized into DAMPs and PAMPs (damage associated molecular patterns and pathogen-associated molecular patterns) according to their derivation, the former ones are generated in cell necrosis (often associated with tissue injury), whereas the controlled cell death, or apoptosis, does not lead to the generation of DAMPs, the latter ones are elicited by

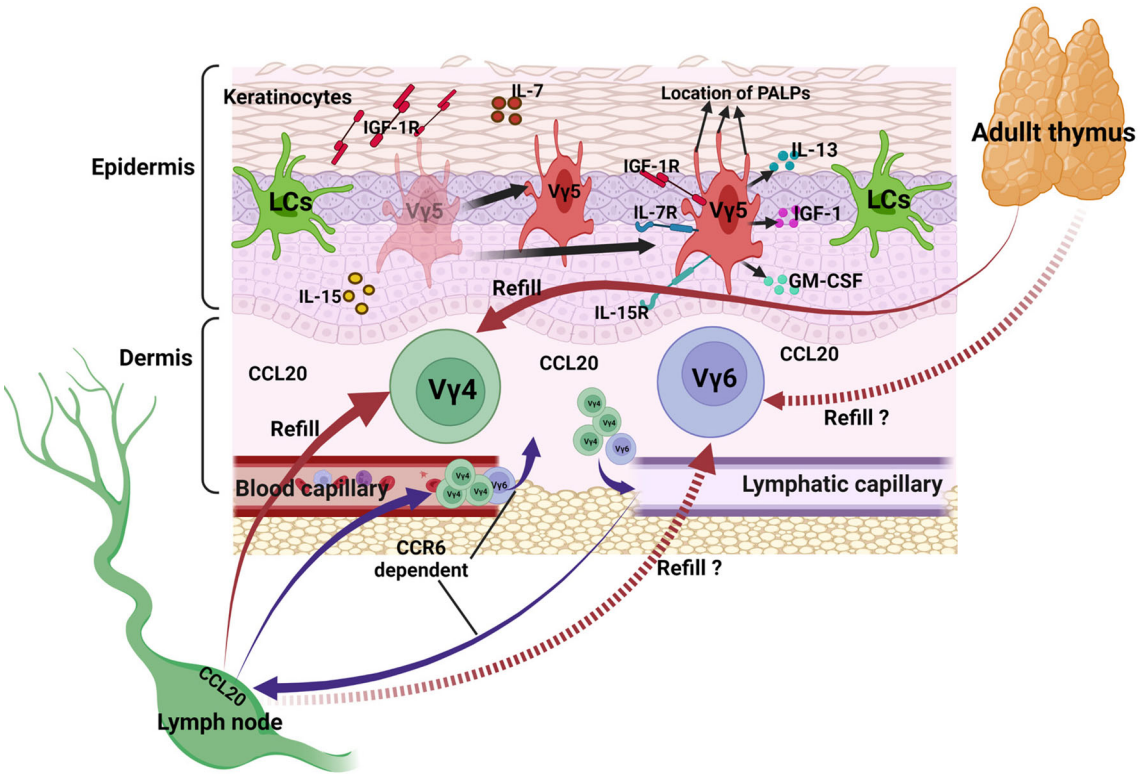

FIGURE 2 | γδ T cells in maintaining skin homeostasis. DETCs in epidermis proliferate and maintain a homeostatic population by themselves, they secrete IL-13, IGF-1 and GM-CSF to help keeping steady state of themselves and other cells. IL-7 and IL-15 secreted by epithelial cells contribute to the survival and proliferation of DETCs, PALPs of the apically oriented dendrites contribute to the anchoring of DETCs to the keratinocyte junctions. Vγ4 and Vγ6 T subsets in the dermis traffic between tissues and lymph nodes at a slow but steady rate, CCR6 expressed on their surface combining with the CCL20 expressed in mucocutaneous sites and subcapsular region of primate LNs is an important pathway.

pathogens (127). In addition, some papers divide the ligands into self ligands and non-self ligands (128).

Shortly after wounding or inflammation, damaged keratinocytes closely adjacent to the lesion quickly and transiently upregulate related stress antigen. The γδT cells of epidermis and dermis get complete activation *via* recognizing the antigens by TCR and co-stimulatory receptors. Activated epidermal γδT cells retract their dendrites and round up within 24 h after wounding (129). Within 48 h, epidermal γδ T cells secrete cytokines and growth factors to regulate inflammation and proliferation, such as KGF-1, KGF-2, IL-13, IFN-γ, TNF-α, IGF-1, IL-2, and IL-17 (**Table 1**), epidermal γδ T cells restore their dendritic morphology 5 days post wounding (4, 129). For the Vγ4 T cells, they are most commonly found early post wounding, accounting for half of the IL-17A$^+$ cells on the third day (130), firstly, they get activated, proliferate and secrete IL-17A, IFN-γ, IL-17F, IL-22 and other cytokines to regulate the inflammation promptly. Secondly, the keratinocytes close to the lesion upregulate the production of CCL20, which increases the epidermal infiltration of dermal γδ T cells by binding their CCR6 (130, 131), in the absence of CCR6, fewer γδ T cells is observed at the wound site leading to 4-day delay in wound closure, this indicates a key role for CCR6 in efficient wound repair (132). The CCL20–CCR6 axis of dermal T cell recruitment occurs similarly in the human epidermis, resulting in Th17 cell infiltration (133). Thirdly, the migration of resident γδ T cells into the local draining lymph nodes increases, the traffic manner is CCR7-independent (105), and Vγ4$^+$ cells homing from inflamed skin to sLNs during psoriasis predominantly lack CCR6 expression (109). It likely occurs *via* afferent lymph draining from dermis, but the definite pathway involved is undetermined. Fourthly, the γδ T cells specific expressing Vγ4Vδ4 in lymph nodes selectively expand promptly (105, 109), the reason leading to the selective expansion is uncertain, cytokines may play a crucial role in this process. Lastly, general γδ T cells and expanded Vγ4Vδ4 γδ T cells infiltrate back into inflammatory skin *via* S1P1 and CCR2 (82, 134), however, whether CCR2 up-regulation promotes the recruitment of thymus-derived Vγ4 T cells to inflamed tissue is unclear. Importantly, the re-filtrated Vγ4 Vδ4 T cells persist for months and respond more rapidly like the memory-like cells in the imiquimod (IMQ)-induced mice model (82). Activated Vγ6 T cells show very similar traits with Vγ4 T cells, CCR2 and CCR6 expressed on their surface are also crucial for the migration in homeostasis and inflammation state (73); however, it seems like their efficiency is lower than the Vγ4 cells (135).

Taken together, the antigens activating the γδ T cells can be divided into 4 categories: MHC-like recognition antigens, IG-like recognition of antigen, phosphoantigen and B7 receptor family-like proteins; they can also be categorized into DAMPs and PAMPs. The binding of these antigens with the γδ TCR and co-stimulatory receptors helps in the complete activation of γδ T cells. Activated γδ T cells secrete chemokines, cytokines and growth factors to regulate inflammation and proliferation. Activated Vγ4 T cells migrate to epidermis *via* CCR6-CCL20 pathway, in addition, the traffic of Vγ4 and Vγ6 T subsets between skin and lymph nodes increases, the traffic from skin to lymph nodes is CCR6/CCR7-independent, while that from lymph nodes to skin is CCR2-dependent (**Figure 3**).

γδ T CELLS IN ACUTE WOUND HEALING

The skin, the largest organ by surface area is susceptible to injury in shielding our internal tissues from microbial infection, temperature variation, radiation and mechanical damage (136). Recognizing the mechanism underlining the wound healing is valuable for regulating the healing effectiveness. Theoretically, both cells residing in skin and cells capable of trafficking to the skin as the keratinocytes, neutrophils, macrophages, T lymphocytes, mast cells, dendritic cells, endothelial cells, fibroblasts, myofibroblasts and epidermal stem cells, can influence the healing result (137–139). To observe their functions, a great number of surgically constructed models of skin injury in rodents have been established. In particular, murine models are used most often. It is well-established that appropriate inflammation and vigorous re-epithelization are crucial in wound healing, immune cells are essential in constructing inflammatory microenvironment and regulating re-epithelization (140). γδT cells as the major immune cells of skin, we sought to discuss their significant functions, and the related mechanism in wound healing below.

Recruitment of Inflammatory Cells

Efficient Infiltration of inflammatory cells including neutrophils and macrophages are crucial for wound repair. Neutrophils are usually recruited as "first responders" from the bone marrow in response to "find me" signals on the day following injury, they clean debris and bacteria to provide a good environment for wound healing, as well as to modulate inflammation by producing ROS, chemokines (CXCL2, CXCL8) and MCP-1 (monocyte chemoattractant protein 1), different cytokines

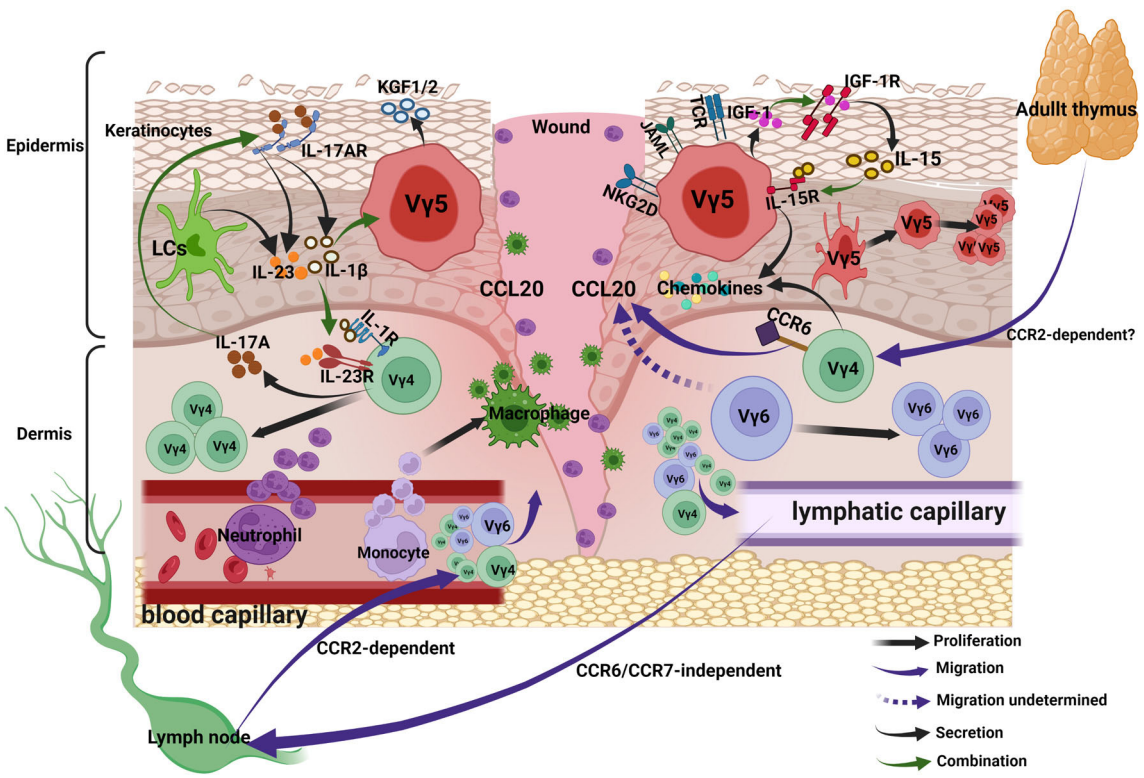

FIGURE 3 | γδ T cells in acute wound healing. Upon activation, DETCs and Vγ4 T cells secrete chemokines to recruit neutrophils and macrophages into lesion site. Activated Vγ4 T cells migrate to epidermis *via* CCR6-CCL20 pathway, in addition, the traffic of Vγ4 and Vγ6 T subsets between skin and lymph nodes increases, the traffic from skin to lymph nodes is CCR6/CCR7-independent, while that from lymph nodes to skin is CCR2-dependent. Keratinocytes-derived IL-15 and DETCs-derived IGF-1 forms a positive feedback loop and promotes re-epithelialization. The positive feedback loop between wound-derived IL-1β/IL-23 and Vγ4-derived IL-17 can amplify the local inflammation, the IL-1β/IL-23 suppresses IGF-1 production of DETCs.

(IL-6, IL-1β, IL-10) (141). The accumulation of macrophages is usually seen within the 24-48 h at the site of injury, and their local accumulation actively participates in all stages of wound healing, including facilitating phagocytosis of bacteria and damage tissue, determining the duration of inflammation and promoting keratinocyte migration and ECM synthesis (142). Studies have confirmed that depletion, deletion, or excessive infiltration of these cells can result in delayed wound healing, keloids or hypertrophic scars (137, 143–146). γδ T cells participate in the recruitment of inflammatory cells in skin wounding. γδ TCR-deficient ($\delta TCR^{-/-}$) C57 male mice exhibit reduction in the cellular infiltration upon injury, including macrophages, αβ T lymphocytes, neutrophils (104, 147, 148). Activated γδ T cells, including DETCs and Vγ4 T cells express CCL-3 (MIP-1α), CCL-4 (MIP-1β), CCL5 (Rantes), MCP-1, and XCL1 (lymphocyte chemokines), IL-17, which induce the migration of inflammatory cells (19, 106, 149–152). In addition, they indirectly affect cells infiltration *via* regulating other cells, such as DETCs-induced hyaluronan production by epithelial cells increases the migration of macrophages (153).

Wound-Derived IL-1β/IL-23 and Vγ4-Derived IL-17 Loop for Inflammatory Responses

As the first line of defense, keratinocytes can recognize ligand by pattern-recognition receptors (PRRs) (154), which lead to the subsequent activation of distinct signaling pathways and the production of different cytokines and chemokines (138). TLR (Toll-like receptor) activation is a critical element in initiating and amplifying inflammation after skin injury, including TLR-1, -2, -3, -4, -5, -6, and -9, which are upregulated in wounds (155), The activation of keratinocytes increases the production of IL-1β, IL-23, IL-15, IL-1α, TNF-α, IL-8, CCL2 (156). Together with the IL-1β produced by platelets, neutrophils and macrophages (157, 158), as well as the IL-23 produced by LCs and DCs (159), the IL-23 and IL-1β induce the resident and infiltrated Vγ4 T cells secreting IL-17A (160, 161), which can bind with the up-regulated IL-17RA expressed on the keratinocytes. The binding enhances the production of epidermal IL-1β and IL-23 (130). Thus, this process creates a positive feedback that the IL-1β/IL-23-IL-17 loop amplifies local inflammation after skin injury. IL-17A, mainly produced by the immune cells, including γδT cells and Th17 cells, is required for efficient skin wound healing. $IL\text{-}17a^{-/-}$ mice exhibit defects in wound repair (3); however, Rodero et al. reported that blocking IL-17A with an IL-17A-neutralizing antibody significantly promotes skin wound repair (162). To reconcile this conflicting result, Li et al. confirmed that different IL-17A levels play a distinct role in wound healing; both low and excessive levels of IL-17A have a negative impact on skin wound repair, while a moderate level of IL-17A is required for efficient skin wound healing (130). They concluded that Vγ4-derived IL-17A indirectly delayed the wound healing through upregulating of IL-1β and IL-23 by keratinocytes, which inhibits IGF-1 production by DETCs through NF-κB signal pathway (130). However, the underlining reason of different levels of IL-17A leading to variant effectiveness was not distinctly explicated in their study.

As we all know, IL-17A participates in inflammation through different pathways (163), we propose that the IL-17A—IL-1β/IL-23—IGF pathway impedes wound healing; whereas the IL-17A—β-defensin3/S100A8/Reg3γ/AMP (3, 164) and other pathways [through driving the production of VEGF by epithelial and fibroblastic cells to stimulate angiogenesis (165, 166)] promote wound healing. Under an excessive expression, the impeding pathway is markedly activated; therefore, IL-17A hinders the wound repair. Similarly, in the IL-17A-depleted mice, the promoting pathway is severely retarded, thus the wound healing is delayed. However, under a moderate expression, the promoting pathway is noticeably activated, IL-17A hence accelerates wound healing. It is worthy to explore these related molecular mechanisms for the details.

Moreover, we deliberate that these dual roles coexist at the same time, depending on the concentration gradient between the central injury tissue and the surrounding wounding tissue, reminiscent of the oxygen gradient in the wounding site (167). Moderate accumulation of IL-17A in the peripheries is beneficial for wound closure; while excessive accumulation of IL-17A at the excessive level in the center of injury leads to delayed repair, which leaves adequate time for inflammatory cells to create a good repair microenvironment. This process confirms the sequential order in repair, from the bottoms up and from the peripheries to the center (168). Further research is needed to justify this inference.

DETCs-Derived IGF-1 and KGF-1-2 for Re-Epithelialization

During homeostasis, DETCs constitutively generate IGF-1, which binds to IGF-1R (IGF-1 receptor) expressed on "keratinocytes and DETCs" and triggers phosphoinositide 3-kinase and mitogen-activated protein kinase pathways to prevent them from apoptosis (98, 169). Meanwhile, keratinocytes secrete IL-15, which helps the survival and proliferation of DETCs (170). Upon injury, the production of IL-15 is upregulated by activated keratinocytes and Langerhans cells (170, 171), increased IL-15 enhances the IGF-1 production of DETCs through binding to their IL-15R (IL-15 receptor). The up-regulated IGF-1 causes an increase in phosphorylated IGF-1R levels at wound margins 24 h after injury (98). This in addition protects keratinocytes from apoptosis in damaged areas (98), also directly stimulates keratinocytes to produce more IL-15, partly through the mTOR-dependent pathway (172). This positive feedback loop of keratinocytes-derived IL-15 and DETCs-derived IGF-1 contributes to the significant accumulation of IGF-1, which exhibits a significant function in promoting re-epithelialization. Impaired epidermal to DETCs signaling slows wound repair (173), and it has been found that the insufficient activation of DETCs upon injury leads to abnormal wound healing in diabetic mice, the insufficient activation partly attributes to the impaired production of IGF-1. Exogenous supplement of IL-15 can rescue the defective IGF-1 expression (93). Whether there is another feedback loop between DETCs and other cells such as LCs, or other signaling deeply involved in the regulation of IL-15 expression is still unknown.

In addition to IGF-1, activated DETCs aid in skin repair by secreting KGF within 24 hours of injury, including KGF-1 and KGF-2 (174). However, they don't secrete KGFs under homeostasis (129). When binding to the KGF receptor (KGFR) expressed on keratinocytes, KGF accelerates the migration and proliferation of keratinocytes by activating the downstream signaling pathways, including mTOR, ERK-MAPK, P13K/Akt (87, 96). KGF plays a commendable function in regulating keratinocytes, but since DETCs do not express KGFR, no positive feedback loop has been identified.

Taken together, upon activation, DETCs and Vγ4 T cells secrete chemokines to recruit neutrophils and macrophages into lesion site. Keratinocytes-derived IL-15 and DETCs-derived IGF-1 forms a positive feedback loop and promotes re-epithelialization. The positive feedback loop between wound-derived IL-1β/IL-23 and Vγ4-derived IL-17 can amplify the local inflammation, whereas the IL-1β/IL-23 suppresses IGF-1 production of DETCs (**Figure 3**).

γδ T CELLS IN CHRONIC WOUND HEALING

Common features of chronic non-healing wounds include repeated infection, tissue necrosis, continuous exudation, defective re-epithelization, reduced angiogenesis and overproduction of ROS (175, 176). They are usually observed in elderly people suffering from pathological conditions, like obesity, diabetes mellitus and vascular disease (177). Chronic wound healing is characterized by the prolonged presence of myeloid cell populations, such as macrophages, neutrophils and monocytes. In the late stage of inflammation (137), incessantly activated γδ T cells participate in the chronic wound healing through inducing persistent inflammatory microenvironment *via* the main pathways mentioned above. For re-epithelialization, the robust activation of EPSCs (Epidermal stem cells) and efficient recruitment of their progeny towards an epidermal lineage are crucial, a stage which facilitates the re-establishment of an intact keratinocyte layer during wound healing (178, 179). For this process, the balance of proliferation of pluripotent EPSCs and their differentiation into terminally differentiated cells are pivotal (**Figure 4A**) (168, 180). In chronic or refractory wound, persistent inflammatory condition leads to excessive proliferation and differentiation, with the sacrifice of subsequent loss of the stem cell reservoir (181–183) and the balance is broken (**Figure 4B**). Supplementing sufficient EPSCs for restoring balance is the effective method to accelerate the wound healing (184–186). Our previous study found that DETCs-derived IGF-1 promotes the proliferation of EPSCs (187), while the IGF-1 secretion is regulated by Vγ4-derived IL-17A (130). So, we therefore hypothesize that the γδ T cells participate in regulating the differentiation and proliferation balance of EPSCs in refractory wound, the potential mechanism seems to be the continuous secretion of IL-17A by Vdifleads sustained inflammation which promotes the excessive differentiation, while suppresses the level of IGF-1 produced by DETCs beneficial for the proliferation of EPSCs (**Figure 4C**). Further research needs to be conducted in this regard.

Collectively, the differentiation and proliferation balance of EPSCs is crucial in wound healing, disordered immune microenvironment constructed by lymphcytes will break this balance in chronic and refractory wound. Given that the isolation and ex vivo expansion of various γδ T cell subsets is feasible (188), upon the molecular and cellular interations between γδ T cells and EPSCs being elucidated, precisely supplementing or clearing certain γδ T cell subsets, cytokines or chemokines in local will be an effective method to restore balanced microenvironment, which is expected to improve the effectiveness of clinical treatments for refractory wounds.

ROLE OF γδ T CELLS IN OTHER SKIN DISEASES

Fibrosis is essential for wound healing and tissue repair, which is characterized by the accumulation of extracellular matrix (ECM) components mainly produced by myofibroblasts. T lymphocytes, macrophages and other inflammation cells cooperatively regulate fibrotic process (189).

Studies have found γδ T cells play critical roles in fibrosis and fibrotic diseases of many tissues, including hepatic, lung, kidney and heart. IL-17/IL-22 producing γδ T cells can protect the liver from excessive fibrosis *via* inducing HSCs (hepatic stellate cells) apoptosis (190). Besides, IFNγ-producing γδ T cells also show protective effect in liver fibrosis, these cells have direct cytotoxicity against activated HSCs (191). For lung, Vγ6Vδ1 γδ T cells protect it from pulmonary fibrosis by secreting IL-22 (192). However, some researches demonstrate γδ T cells accumulation tends to promote fibrosis, IL-17-producing γδ T cells induces myofibroblast activation and ECM deposition in kidney injury model and myocardial infarction model of mice (193, 194). So, it is more likely that their function in regulating fibrosis is tissue-specific.

Up to now, researches related to the γδ T cells in skin fibrosis is inadequate, Ohtsuka found the human skin fibroblasts stimulated by γδ T cells supernatant showed elevated proliferation and collagen synthesis (195), another study demonstrated the activated γδ T cells in systemic sclerosis (SSc) play an important role on fibrosis (196). In addition, Meyer demonstrated epidermal γδ T cells induces profibrotic response of fibroblasts *via* mice in chronic inflammation, this phenotype of mice lacking fibroblast growth factor bears continuous inflammatory response (197). Recently, Shook (198) found CD301b-expressing macrophages activated the proliferation of wound bed adipocyte precursors (APs) through IGF-1, these Aps become fibrotic after injury. DETCs secreted sufficient IGF-1 upon skin injury, whether they can play equivalent effect deserves further study.

For immune-mediated skin diseases, psoriasis, atopic dermatitis (AD) and contact dermatitis (CD) are all chronic and prevalent (15). The prevalence of psoriasis is about 2% to 3% (199), γδ17 T cells have been proved to be critical in imiquimod-

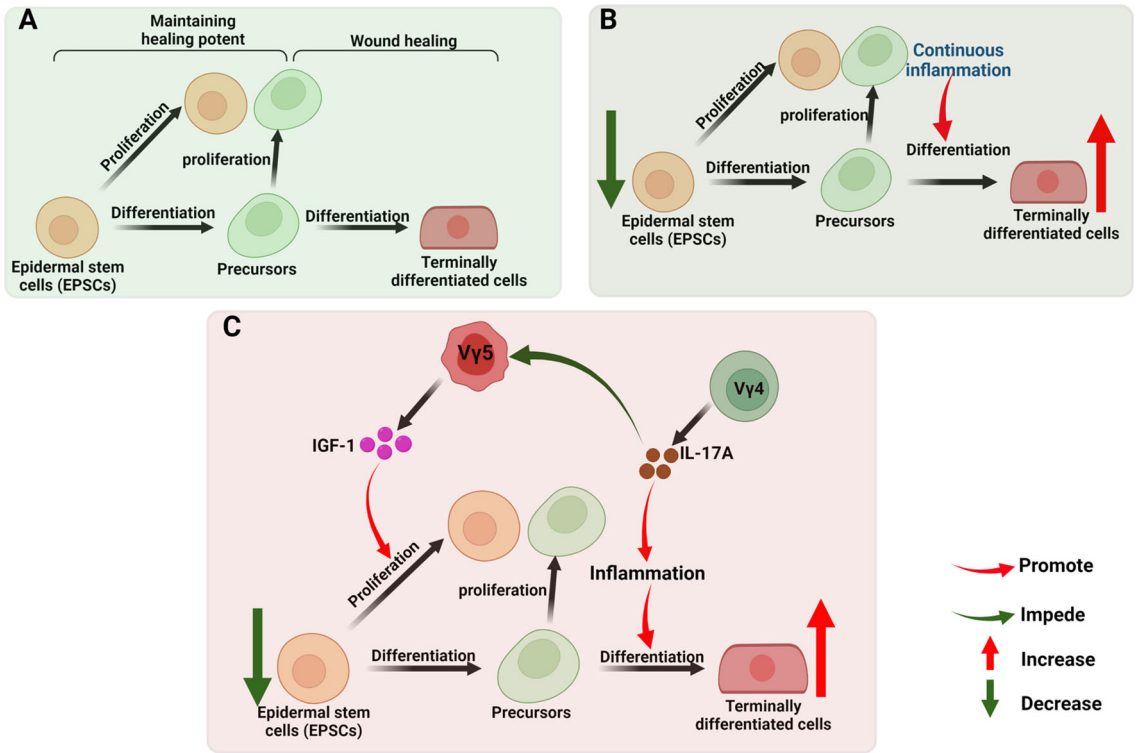

FIGURE 4 | γδ T cells in chronic wound healing. **(A)** The robust activation of EPSCs and efficient recruitment of their progeny towards an epidermal lineage are crucial in the re-establishment of an intact keratinocyte layer during wound healing. The balance of proliferation of pluripotent EPSCs (maintaining healing potent) and their differentiation into terminally differentiated cells (wound healing) are pivotal; **(B)** In chronic or refractory wound, persistent inflammatory condition leads to excessive proliferation and differentiation, with the sacrifice of subsequent loss of the stem cell reservoir. **(C)** In chronic or refractory wound, continuous secretion of IL-17A by Vγ4 leads sustained inflammation which promotes the excessive differentiation, while suppresses the level of IGF-1 produced by DETCs beneficial for the proliferation of EPSCs, this inference is worthy to be tested.

(IMQ) or IL-23-induced psoriasis of mice, both Vγ6 and Vγ4 are clearly pathogenic in these models (131), memory-like dermal Vγ4 γδ17 T cells accumulated in inflamed skin and peripheral lymph nodes lead to faster and stronger responses upon secondary challenge (82). STAT 3 and STAT 4 facilitate the complete effector functions of γδ17 T cells (200). PD-1 and CD109 exert protective role in psoriasis (201, 202), while LAT1 and CD69 exert opposite function (203). In humans, patients with psoriasis also display increased accumulation of γδ T cells (Vγ9Vδ2) in the skin, effective therapy can decrease the numbers, indicating their role in the disease (204). AD is a T cell-mediated chronic skin disease, affecting up to 20% of children worldwide, its onset is associated with skin barrier dysfunction and immune disorder (205), it is characterized by highly expanded dermal αβ T cells which produce IL-17 and IL-22 (206), patients suffered from AD also present decreased proportion of γδ T cells (207). However, children with AD display higher frequency of Vγ9Vδ2 T cells (208). So the specific role and underlined mechanism of γδ T cells in AD is worthy to investigate. CD is the most frequent immune-mediated skin disease, its prevalence is about 95%, which is caused by chemical and allergens (209). The role of DETCs in CD is controversial (15), IL-17 secreted by Vn CD is controversialsed by chemicalproinflammatory role (106), however, their respective role in CD needs to be evaluated in depth.

DISCUSSION AND CONCLUSION

γδ T cells are important components of the skin immune system and DETCs(Vγ5), Vγ4 and Vγ6 T cells are their major subsets. DETCs are particularly generated in the embryonic thymus and implanted in the epidermis where they maintain a homeostatic population by themselves. Vγ4 T cells appearing in the late fetal stage can be generated in the adult thymus, and they possess the recirculating characteristic which can be refilled by newly generated Vγ4 cells from thymus and pLN. Vγ6 T cells are generated solely in the thymic second wave around embryonic day E14 (up to the birth), and they mainly display tissue residency, but retain circulating capability, whether they can be refilled by circulating cells is uncertain. The development and differentiation of γδ T cells are regulated by both TCRγδ-dependent and TCRγδ-independent factor. The combined effect of various factors leads to the differentiation of γδ T cells.

Their functional development is accomplished step by step as follows: T cell commitment–αβ/γδ lineage commitment–γδ subset commitment–effector commitment.

Under homeostasis, γδT cells participate in maintaining skin integrity with the help of paracrine and autocrine factors, traffiking between tissues and lymph nodes of Vγ4 and Vγ6 T cells at a slow rate in the steady state which plays an important role in immune surveillance. Besides, these cells are radioresistant, for mice receiving lethal irradiation, 100% of DETCs (V0%+) remained of host origin, while 90% of Vγ5-γδ T cells in dermal remained host-derived (104). Upon injury or inflammation, antigens including MHC-like recognition antigens, IG-like recognition of antigen, Phosphoantigen or B7 receptor family-like proteins are upregulated. The binding of these antigens with the γδTCR and co-stimulatory receptors helps in the complete activation of γδT cells. Initially, activated γδT cells secrete chemokines to recruit the inflammatory cells, including neutrophils and macrophages etc. Subsequently, they secrete IGF-1, KGF-1/KGF-2, IL-17 to regulate inflammation and re-epithelialization. Injury provide an opportunity for microorganisms to enter into the wound tissues, including microorganisms constituting the skin microbiota and residing in the environment.

It is noteworthy to mention that the positive feedback loop of DETCs-derived IGF-1 and keratinocytes-derived IL-15 leads to the accumulation of IGF-1 in wound bed, on one hand, it protects keratinocytes and epidermal γδ T cells from apoptosis, on the other hand, it exhibits a significant function in promoting re-epithelialization, γδ T cells in the epidermal of both mice and humans show equivalent function. In the dermal, the wound-derived IL-1β/IL-23 and Vγ4-derived IL-17 feedback loop can amplify the local inflammation. IL-17A participates in regulating wound healing by either promoting pathway (like the IL-17A—IL-1β/IL-23—IGF pathway) or impeding pathway (like the IL-17A—β-defensin3/S100A8/Reg3γ/AMP pathway). Different doses affect each pathway to different degrees, both low and excessive levels of IL-17A have a negative impact on skin wound repair, while a moderate level of IL-17A is required for efficient skin wound healing, suggesting that IL-17A plays a varied role in wound healing. For chronic and refractory wounds, they provide a lot of opportunities for microorganisms to enter into the wound tissues (210), including commensal microbiota residing in the skin and microorganisms existed in the environment, pathogenic interaction of microorganisms with the skin cells will induce pathogenic immune response (177, 211). In this process, abnormal accumulated γδ T cells or their disordered function contribute to unbalanced immune microenvironment, which breaks the differentiation and proliferation balance of EPSCs, restoring balanced microenvironment is expected to improve the effectiveness of clinical treatments for refractory wounds. Further research needs to be conducted in this regard.

In addition, γδ T cells play critical roles in fibrosis and fibrotic diseases of many tissues, their protective or deleterious function in fibrosis is more likely tissue-specific. Up to now, researches related to the γδ T cells in skin fibrosis is inadequate, investigating their role in keloids and hypertrophic scars forming is valuable. For immune-mediated skin diseases, both Vγ6 and Vγ4 are clearly pathogenic in imiquimod-induced psoriasis, their function in atopic dermatitis and contact dermatitis needs to be evaluated in depth.

AUTHOR CONTRIBUTIONS

WGH and RS wrote the manuscript. JY and CC participated in the project discussion. ZL made some valuable suggestions about manuscript structure. GPL helped to design the manuscript structure and edited the language. WFH and GXL evaluated and reviewed manuscript structure, ideas and science. All authors contributed to the article and approved the submitted version.

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Bibliometric Analysis of γδ T Cells as Immune Regulators in Cancer Prognosis

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γδ T cells are one of only three immune cell types that express antigen receptors that undergo somatic recombination, and they contribute to immune responses to infection, cellular transformation, and tissue damage. As a "bridge" between the innate and adaptive immune systems, γδ T cells have been noted to be involved in various immune responses during cancer progression. The purpose of our study was to review current published information on γδ T cells and investigate their functions in different types of malignancy using bibliometric and bioinformatic methods. Our results indicated that studies on γδ T cells and cancer progression increased from 2014, and the number had peaked by 2021. We discovered that there is international cooperation in the performance of studies among 26 countries, where China was identified as the most productive with the highest citations. Using keyword co-occurrence analysis, we found that among all the cancer types investigated, gastric and breast cancers were most closely related to γδ T cells. Furthermore, interleukin (IL)-17 and IL-2 were the most common cytokines linked to γδ T cells and our investigation of their potential involvement in the prognosis of gastric and breast cancers, identified their different roles in various malignancies. Thus, we concluded that γδ T cells might influence the progression of different cancers in diverse ways.

Keywords: bibliometric analysis, γδ T cells, cancer prognosis, immune regulators, IL-17, IL-2

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INTRODUCTION

T lymphocytes play a critical role in the response and regulation of human immune functions (1). Human T lymphocytes can be divided into αβ (such as CD4 and CD8) and γδ T cells according to the T cell receptor (TCR) structures. In human peripheral blood lymphocytes, αβ T cells are the dominant cells, whereas γδ T cells generally account for only 1% to 5% (2). The functions of these two types of cells and their response mechanisms in the immune response are also different. For example, the recognition of antigens by αβ T but not γδ cells depends on major histocompatibility complex (MHC) molecules (3).

Human γδ T cells have numerous unique biological functions. Based on their distribution and ability to recognize antigens, this population of cells is considered a "bridge" between innate and adaptive immunity (4). According to the structural differences between the γ and δ chains, γδ T cells can be divided into two main subgroups, namely Vδ1 and Vδ2, with distinct functions (5). For

example, the Vδ2 cell subset inhibits bacterial infection and tumor progression (6, 7). In human peripheral blood, 50–90% of γδ T cells express Vγ9Vδ2 receptors (8). An increasing number of studies have shown that Vγ9Vδ2 T cells have a very important inhibitory effect on the occurrence and development of tumors by significantly inhibiting the growth of tumor cells (9).

However, a pro-tumor role for interleukin (IL)-17-producing γδ T cells has also been reported in human cancers. Specifically, Vδ1 T cells are the major source of the IL-17 involved in chronic inflammation in colorectal cancer (10). These results indicate that γδ T cells might be a "double-edged sword" in cancer treatment. In this study, we aimed to summarize the current knowledge of γδ T cell research and investigate their functions in different subtypes of malignancy using bibliometrics and bioinformatics methods.

METHODS

Scopus Search

Scopus (Elsevier, Amsterdam, The Netherlands) was chosen as the main database for our literature search (11, 12). The following search formulas were used in Scopus: TITLE-ABS-KEY ("γδT cell" and "cancer") AND PUBYEAR>1993 AND PUBYEAR<2022. To avoid citation duplication, the literature search and extraction were completed on a single day, January 10, 2022 and the result yielded 239 studies.

The titles, abstracts, and keywords of the 239 studies were scanned and filtered manually. Full texts were further examined where necessary. To achieve precise and non-duplicated results, we set the inclusion criteria as (1) a clear correlation between γδT cell and cancer; (2) human, mouse, or cell-based studies; and (3) document type as "article". Finally, 190 studies were included and summarized in a csv file for the subsequent analysis.

Analysis Using VOSviewer

We uniformed "γδt cell, γδt cells, γδt-cell, γδt-cells, gdT cells, gamma delta T cell, gamma delta T cells, and gamma delta T lymphocyte" to "γδ T cell". In addition, "natural killer cell, natural killer cells, nkt cell, and nkt cells" were uniformed to "nk cells". Then, the csv file was uploaded into VOSviewer to conduct co-occurrence analysis of authors, countries, and keywords. The minimum number of occurrences of each keyword was set to five and the total intensity of co-occurrence bonds to other keywords was also derived.

Overall Survival Estimation of γδ T Cell in Different Malignancies

The Kaplan–Meier plotter (13) is an online tool for assessing the correlation between the expression of 30 k genes (mRNA, miRNA, and protein) and survival in 25 k+ samples from 21 tumor types. We conducted a survival analysis of γδT cells with different subtypes of cancer. Based on the median transcription level of each target gene, patients were allocated to the high and low expression groups, and Kaplan–Meier plots were generated accordingly. The hazard ratio (HR) with the 95% confidence interval and log-rank p-values were also calculated. Statistical significance was set at $p < 0.05$.

RESULTS

Our literature search identified 190 studies on the relationship between γδT cells and cancer conducted from 1993 to 2021. The results indicated that before 2006, there were few studies on γδ T cells and cancer progression annually, whereas the number increased from 2014 and had peaked by 2021 (**Figure 1A**). There was international cooperation in conducting studies among 26 countries, and China had the most publications as well as the highest citations, followed by Japan and the US (**Figure 1B**). The top-20 cited publications are listed in **Table 1**. The paper "*Enterococcus hirae* and *Barnesiella intestinihominis* facilitate cyclophosphamide-induced therapeutic immunomodulatory Effects" by Daillère et al. (13) had the highest citations at 312 times. The publication "γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer" by Wu et al. (10) had the second highest number of citations at 301 times.

Furthermore, we also conducted the co-occurrences analysis of keywords with the software VOSviewer, in order to figure out the relationship between γδT cells and other important scientific issues. The minimum number of occurrences of a keyword was set to 5 and 14 were finally identified among all 564 keywords. The hotspots in γδT cell functions are presented in the overlay visualization map scaled by occurrences (**Figure 2**). Accordingly, γδT cells were shown to be responsible for the immune response and communicated with dendritic cells and NK cells. We also found that among all the types of cancer, gastric and breast cancer were most closely linked to γδ T cells. Furthermore, IL-17 and IL-2 are the most common cytokines linked to γδ T cells.

IL-17 production by certain γδ T cell subsets has been reported to recruit immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) or small peritoneal macrophages, which can promote angiogenesis, tumor cell growth, and inducible regulatory T (Treg) cell differentiation (7). IL-2 could augment the γδT-17 response in favor of short-lived effectors with limited plasticity, particularly in the presence of IL-1β and IL-23 (33). Taken together, these results led us to conclude that γδ T cells could have a role in cancer development *via* IL-2 or IL-17. Consequently, we investigated the effects of IL-17 and IL-2 on gastric and breast cancer, and identified their distinct roles in different malignancies.

In breast cancer, high expression levels of IL-17 and IL-2 indicated a promising prognosis. The median survival of patients with breast cancer with low and high expression levels of IL-2 was 43 months and 56 months, respectively (HR = 0.86, p = 0.0031, **Figure 3A**). Furthermore, the median survival of breast cancer patients with low and high expression levels of IL-17 was 216.66 and 228.85 months, respectively (HR = 0.8, $p < 0.001$, **Figure 3B**). However, IL-2 and IL-17 played a dinstinct role in

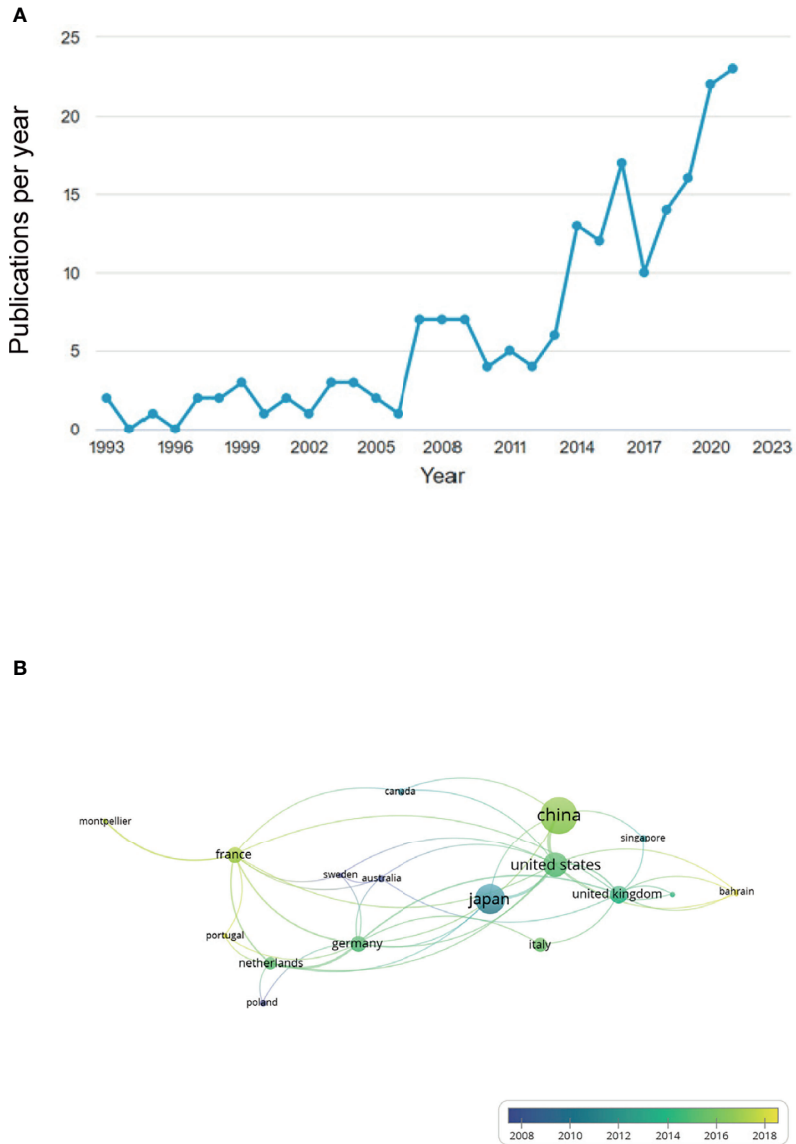

FIGURE 1 | Annual number of publications related to γδ T cell and cancer increased from 2014 to 2021 and international cooperation in related research occurred between different countries. **(A)** Before 2006, there were few studies on γδ T cell and cancer progression, annually, but number of studies increased from 2014 and peaked by 2021. **(B)** Numerous national connections were established in this field of study, with China, Japan, and the US emerging as the most productive countries.

gastric cancer prognosis. Our results also showed that the median survival of patients with gastric cancer who had low and high expression levels of IL-2 was 35.4 and 22 months, respectively (HR = 1.58, $p < 0.001$, **Figure 3C**). In addition, for patients with gastric cancer with low and high expression levels of IL-17, the median survival was 34.7 and 19.5 months (HR = 1.6, $p < 0.001$, **Figure 3D**).

Furthermore, we conducted a subgroup analysis of these two types of cancers. For breast cancer, we examined the status of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). As shown in **Figure 4**, the median survival of ER-negative (ER-) patients with low expression of IL-2 was 18 months, whereas that of patients with high expression was 25 months (HR = 0.79, $p = 0.015$, **Figure 4B**). For HER2- patients, the median survival of patients with low expression of IL-2 was 50 months, whereas that of patients with high expression was 61.92 months (HR = 0.86, $p = 0.011$, **Figure 4F**). The expression of IL-2 did not influence survival in ER-positive (ER+), HER2+, PR+, or PR- subgroups (**Figures 4A, C–E**).

As shown in **Figure 5**, for ER+ patients, high expression of IL-17 was significantly correlated with a longer survival time (HR = 0.85, $p = 0.0095$, **Figure 5A**). For ER- patients, the median survival of those with low and high expression of IL-17 was 18 and 28.75 months, respectively (HR = 0.73, $p = 0.0009$, **Figure 5B**). The

TABLE 1 | Top 20 cited publications studying relationship between γδ T cell and cancer.

Authors	Title	Year	Source title	Cited by	PubMed ID
Daillère et al.	Enterococcus hirae and Barnesiella intestinihominis Facilitate Cyclophosphamide-Induced Therapeutic Immunomodulatory Effects	2016	Immunity	312	27717798 (14)
Wu et al.	γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer	2014	Immunity	301	24816404 (10)
Daley et al.	γδ T Cells Support Pancreatic Oncogenesis by Restraining αβ T Cell Activation	2016	Cell	168	27569912 (15)
Sato et al.	Cytotoxic effects of γδ T cells expanded ex vivo by a third generation bisphosphonate for cancer immunotherapy	2005	International Journal of Cancer	136	15756684 (16)
Schnurr et al.	Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and γδT cells	2002	Cancer Research	127	11956095 (17)
Kong et al.	The NKG2D ligand ULBP4 binds to TCRγ9/δ2 and induces cytotoxicity to tumor cells through both TCRγδ and NKG2D	2009	Blood	120	19436053 (18)
Alexander et al.	Isopentenyl pyrophosphate-activated CD56+ γδ T lymphocytes display potent antitumor activity toward human squamous cell carcinoma	2008	Clinical Cancer Research	105	18594005 (19)
Sakamoto et al.	Adoptive immunotherapy for advanced non-small cell lung cancer using zoledronate-expanded γδT cells: A Phase I clinical study	2011	Journal of Immunotherapy	102	21304399 (20)
Laurent et al.	The engagement of CTLA-4 on primary melanoma cell lines induces antibody-dependent cellular cytotoxicity and TNF-α production.	2013	Journal of translational medicine	97	23634660 (21)
Grose et al.	The role of fibroblast growth factor receptor 2b in skin homeostasis and cancer development	2007	EMBO Journal	95	17304214 (22)
Capsomidis et al.	Chimeric Antigen Receptor-Engineered Human Gamma Delta T Cells: Enhanced Cytotoxicity with Retention of Cross Presentation	2018	Molecular Therapy	77	29310916 (23)
Okada et al.	Origin of CD57+ T cells which increase at tumour sites in patients with colorectal cancer	1995	Clinical and Experimental Immunology	77	7554383 (24)
Cui et al.	Combination of radiofrequency ablation and sequential cellular immunotherapy improves progression-free survival for patients with hepatocellular carcinoma	2014	International Journal of Cancer	69	23825037 (25)
Sudam Patil et al.	IL17 producing γδT cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients	2016	International Journal of Cancer	67	27062572 (26)
Legut et al.	The promise of γδT cells and the γδT cell receptor for cancer immunotherapy	2015	Cellular and Molecular Immunology	64	25864915 (27)
Marcu-Malina et al.	Redirecting αβT cells against cancer cells by transfer of a broadly tumor-reactive γδT-cell receptor	2011	Blood	60	21566093 (28)
Ni et al.	Breast cancer-derived exosomes transmit lncRNA SNHG16 to induce CD73+γδ1 Treg cells	2020	Signal Transduction and Targeted Therapy	55	32345959 (29)
Fisher et al.	Neuroblastoma killing properties of Vδ2 and Vδ2-negative γδT cells following expansion by artificial antigen-presenting cells	2014	Clinical Cancer Research	52	24893631 (30)
Holderness et al.	Select plant tannins induce IL-2Rα up-regulation and augment cell division in γδ T cells	2007	Journal of Immunology	45	17982035 (31)
Hu et al.	Tumor-infiltrating CD39+ γδTregs are novel immunosuppressive T cells in human colorectal cancer	2017	OncoImmunology	43	28344891 (32)

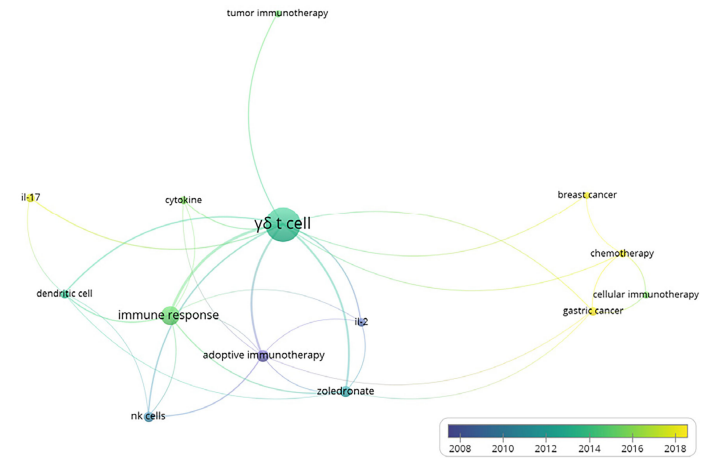

FIGURE 2 | Overlay visualization map of author keywords co-occurrence analysis. Map shows that γδ T cells mediated immune responses. Of all investigated cancers, gastric and breast cancers were most closely linked to γδ T cells. Interleukin (IL)-17 and IL-2 were identified as the most common cytokines linked to γδ T cells.

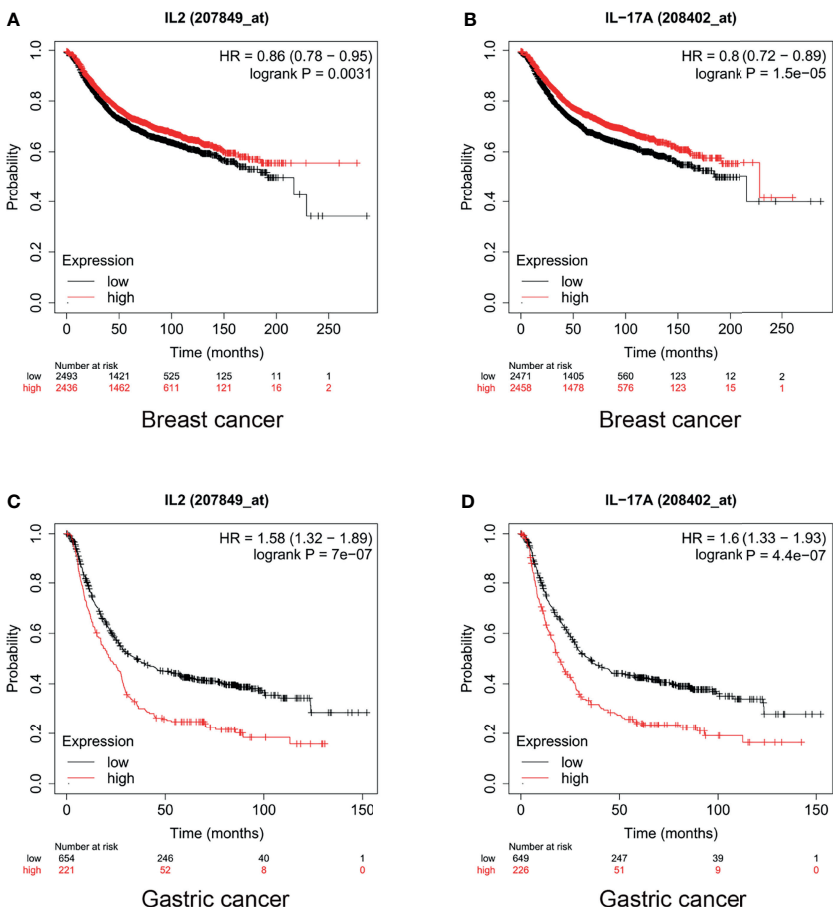

FIGURE 3 | Levels of interleukin (IL)-2 and IL-17 played different roles in breast and gastric cancer prognosis. High IL-17 and IL-2 expression indicated **(A, B)** promising prognosis in breast cancer and **(C, D)** poor prognosis in gastric cancer.

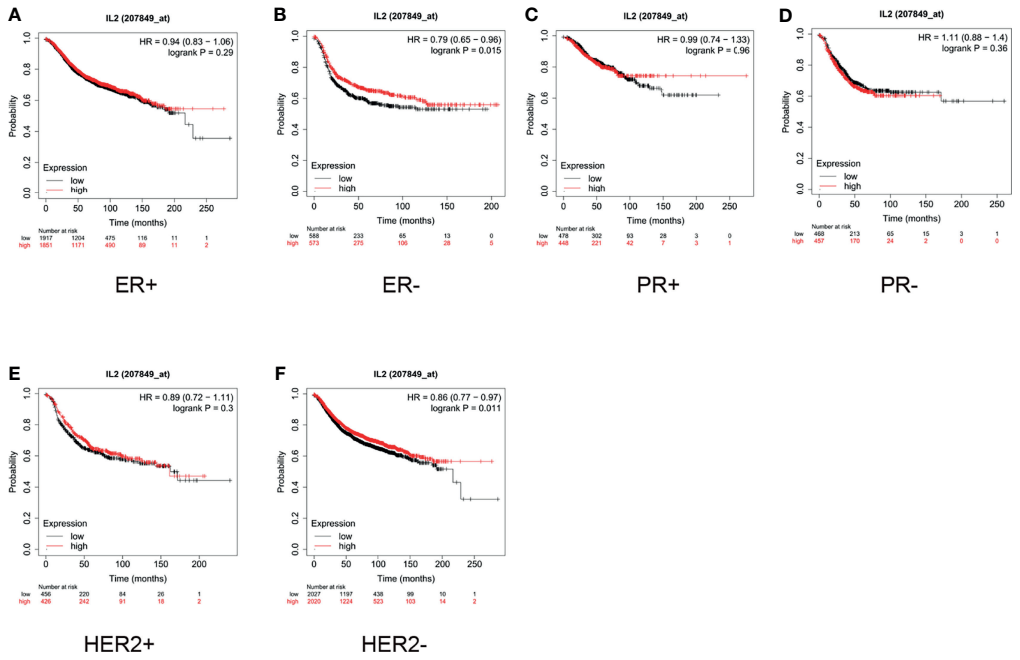

FIGURE 4 | In patients with breast cancer, high interleukin (IL)-2 expression only indicated better prognosis in estrogen receptor-negative (ER-) and human epidermal growth factor receptor 2 negative (HER2-) subgroups. **(A)** High expression of IL-2 **(A)** did not correlate with better prognosis in ER+ patients (hazard ratio [HR] = 0.94, p = 0.29) and **(B)** indicated better prognosis in ER- patients (HR = 0.79, p = 0.015). **(C, D)**. IL-2 expression did not significantly influence survival in progesterone receptor-positive (PR+; HR = 0.99, p = 0.96) or PR- (HR = 1.11, p = 0.36) subgroups. High expression of IL-2 **(E)** did not correlate with better prognosis in HER2+ patients (HR = 0.89, p = 0.3) and **(F)** indicated better prognosis in HER2- patients (HR = 0.86, p = 0.011).

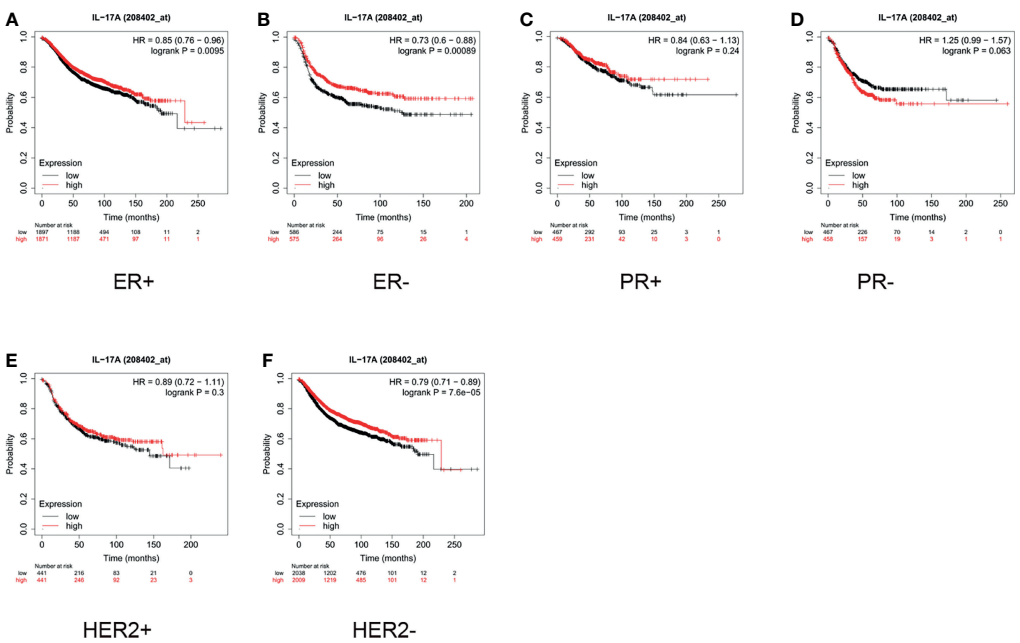

FIGURE 5 | In breast cancer patients, high expression of interleukin (IL)-17 only indicated better prognosis in estrogen receptor-positive (ER+), ER-negative (ER-), and human epidermal growth factor receptor 2 negative (HER2-) subgroups. High expression of IL-17 indicated better prognosis in **(A)** ER+ (hazard ratio [HR] = 0.85, p < 0.001) and **(B)** ER- (HR = 0.73, p < 0.001) patients. **(C, D)** IL-17 expression did not significantly influence survival in progesterone-positive (PR+; HR = 0.84, p = 0.24) or PR- (HR = 1.25, p = 0.063) subgroups. **(E)** High expression of IL-17 did not correlate with better prognosis in HER2+ patients (HR = 0.89, p = 0.3). **(F)** High expression of IL-2 indicated better prognosis in HER2- patients (HR = 0.79, p < 0.001).

expression of IL-17 did not significantly influence the overall survival, regardless of whether the patients were PR+ or PR- (**Figures 5C, D**). As demonstrated in **Figure 5E**, high expression of IL-17 did not correlate with better prognosis in HER2+ patients (HR = 0.89, p = 0.3). For HER2- patients, high expression of IL-17 indicated a better prognosis than low expression did (HR = 0.79, p < 0.001, **Figure 5F**).

For gastric cancer, we used the Lauren classification to divide the cohort into three subgroups: intestinal, diffuse, and mixed. As shown in **Figure 6**, the analysis of the expression of IL-2 in different subtypes of gastric cancer showed that low expression of IL-2 indicated a better prognosis in both intestinal and diffuse patients than high expression levels did (**Figures 6A, B**). However, the difference was not statistically significant in patients with mixed conditions (**Figure 6C**). Moreover, the median survival of intestinal patients with low expression of IL-17 was 123.8 months, whereas that of patients with high expression was only 23.4 months (HR = 2.02, p < 0.001, **Figure 6D**), and the difference did not have statistical significance in diffuse and mixed subgroups (**Figures 6E, F**).

DISCUSSION

Our results showed that the number of studies on γδ T cells has increased since 2014, suggesting that most were likely novel. The examination of the top-20 cited publications revealed that numerous studies concentrated on the role of γδ T cells in cancer treatment. In addition, the co-occurrence analysis revealed that γδ T cells were more closely related to breast and gastric cancers than they were to the other investigated malignancies. IL-2 and IL-17 are the two most important cytokines related to γδ T cells; therefore, we investigated their influence on breast and gastric cancers.

The two cytokines, IL-2 and IL-17, which we focused on in this study, play different roles in breast and gastric cancers, where they promote the development of gastric cancer but inhibit the progression of breast cancer. The results of the subgroup analysis further clarified this finding. For intestinal gastric cancer, low expression of IL-2 and IL-17 indicated a promising prognosis, whereas for diffuse and mixed gastric cancers, the expression of these cytokines did not significantly affect survival. The cases of intestinal gastric cancer were deemed to be early stage and,

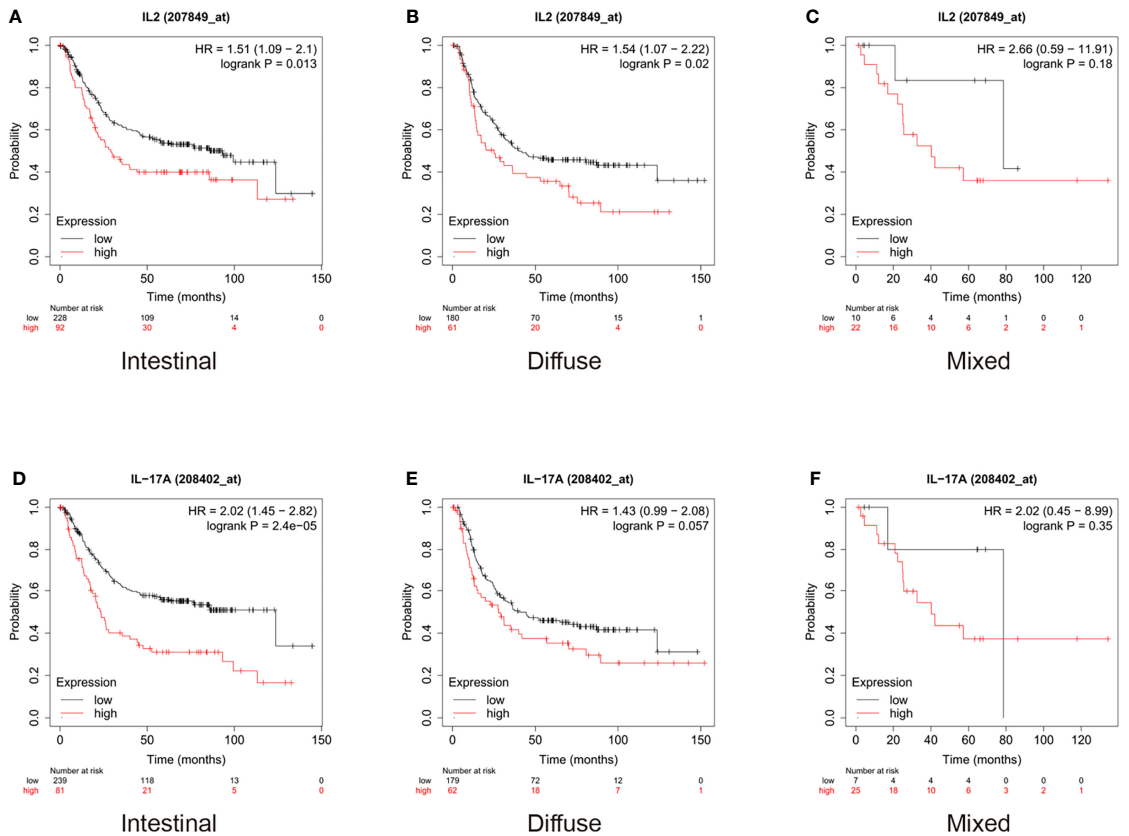

FIGURE 6 | In gastric cancer patients, high expression of interleukin (IL)-2 indicated worse prognosis in patients with both intestinal and diffuse conditions and high expression of IL-17 indicated worse prognosis only in patients with intestinal condition. **(A, B)** High expression of IL-2 indicated worse prognosis in patients with both intestinal (hazard ratio [HR] = 1.51, p = 0.013) and diffuse (HR = 1.54, p = 0.3) conditions. **(C)** High expression of IL-2 did not significantly influence survival in mixed patients (HR = 2.66, p = 0.18). **(D)** High expression of IL-17 indicated worse prognosis in patients with intestinal conditions (HR = 2.02, p < 0.001). **(E, F)** High expression of IL-17 did not significantly influence survival in patients with diffuse (HR = 1.43, p = 0.057) and mixed (HR = 2.02, p = 0.35) conditions.

therefore, we speculated that γδ T cells might affect the prognosis of gastric cancer at the disease onset.

In breast cancer, ER, PR, and HER2 status have been verified as important prognostic factors; therefore, based on these different statuses, we also investigated how the expression of IL-2 and IL-17 influenced patient survival. Our results showed that regardless of ER status, high expression of IL-17 indicated a longer survival time, but only a better prognosis in HER2- patients than low expression did. In addition, high expression of IL-2 indicated a better prognosis in patients with ER- or HER2- breast cancer than low expression did. However, regardless of whether PR was positive or negative, the expression of IL-2 and IL-17 did not influence the survival of patients with breast cancer in this subgroup analysis. Based on these results, we hypothesized that HER2 might play an important role in breast cancer treatment *via* γδ T cells.

IL-2 and IL-17 have opposite roles in the development of breast and gastric cancers and, therefore, we postulated that γδ T cells have distinct functions in different malignancies. Furthermore, we also discovered that even in the same malignancy, γδ T cells might have distinct functions in different forms, implying some underlying mechanism may exist between γδ T cells and cell receptors. The abundant cytokine secretion and non-MHC-restricted antigen recognition capacity of γδ T cells has encouraged the investigation of their application in cancer adoptive immunotherapy (34). Currently, evidence has accumulated from studies in numerous cancers, and the results demonstrate that γδ T cells could be well tolerated in the treatment of cancer (3, 34–36). Studies have also revealed that γδ T cells can exert anticancer activity through various mechanisms, such as eliminating tumor cells *via* the perforin-granzyme pathway (37), binding to TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) (38), *via* antibody-dependent cellular cytotoxicity (ADCC) (39), or by secreting interferon (IFN)-γ and tumor necrosis factor (TNF)-α (40, 41). In addition to these direct antitumor effects, specific γδ T cell subsets also exert an indirect antitumor effect, which is complemented by interactions with other immune cells such as B cells, DCs, αβ T cells, and NK cells (42). However, recent studies claim that γδ T cells could stimulate cancer development (43–45) by impairing the antitumor ability of immunocytes or enhancing the function of immuno suppressive cells (10, 46, 47). For instance, γδ T17 cells are a major source of IL-17 in the cancer microenvironment (48), and IL-17 contributes to cancer development by supporting angiogenesis in several malignancies, such as gallbladder cancer, gastric cancer and non-small-cell lung cancer (26, 49, 50).

The limitation of the current study was that we only chose Scopus as our database and did not use databases such as Pubmed or Google Scholar. Besides, as we used the Kaplan Meier plotter as our database to achieve survival data, this database collected information from many independent datasets, which might cause bias in our analysis.

In conclusion, using bibliometric analysis, we identified IL-17 and IL-2 as the most common cytokines linked to γδ T cells. Furthermore, based on our investigation of the role of IL-17 and IL-2 in the prognosis of gastric and breast cancer, we discovered that they play different roles in various malignancies. Moreover, in the same malignancy, the expression levels of certain genes or different variations could impact the function of γδ T cells (**Figure 7**). Finally, we concluded that γδ T cells might influence the progression of different cancers in diverse ways.

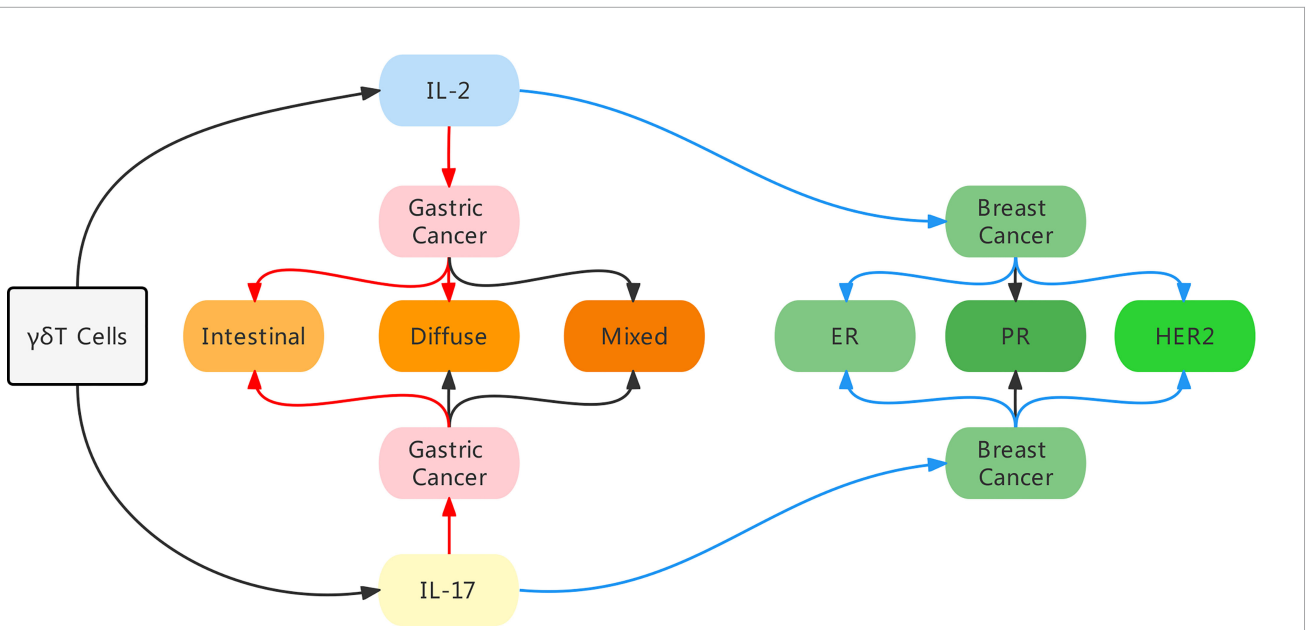

FIGURE 7 | An illustration of the main findings in our study. By bibliometric analysis, we identified IL-17 and IL-2 as the most common cytokines linked to γδ T cells, and these two cytokines influenced the prognosis in different manners in gastric cancer and breast cancer. In breast cancer, high expression of IL-2 and IL-17 indicated a better prognosis, especially in ER negative and HER2 negative patients. While in gastric cancer patients, high level of IL-2 indicated a worse prognosis in intestinal and diffuse conditions, and high level of IL-17 was merely predictive of a poor prognosis in intestinal condition. Red arrow indicates a poor prognosis, blue arrow indicates a good prognosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

BL conducted the data search and paper writing, XH and YW contributed to the bibliometric analysis, J-wH and Y-bZ revised the figures and tables, YL and L-gL designed the study and revised the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Engineering γδ T Cells: Recognizing and Activating on Their Own Way

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Adoptive cell therapy (ACT) with engineered T cells has emerged as a promising strategy for the treatment of malignant tumors. Among them, there is great interest in engineered γδ T cells for ACT. With both adaptive and innate immune characteristics, γδ T cells can be activated by γδ TCRs to recognize antigens in a MHC-independent manner, or by NK receptors to recognize stress-induced molecules. The dual recognition system enables γδ T cells with unique activation and cytotoxicity profiles, which should be considered for the design of engineered γδ T cells. However, the current designs of engineered γδ T cells mostly follow the strategies that used in αβ T cells, but not making good use of the specific characteristics of γδ T cells. Therefore, it is no surprising that current engineered γδ T cells in preclinical or clinical trials have limited efficacy. In this review, we summarized the patterns of antigen recognition of γδ T cells and the features of signaling pathways for the functions of γδ T cells. This review will additionally discuss current progress in engineered γδ T cells and provide insights in the design of engineered γδ T cells based on their specific characteristics.

Keywords: γδ T cells, engineering, stimulation, dual recognition, tumor

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1 INTRODUCTION

Immunotherapy has become one of important pillars of cancer treatment, as it can trigger and augment the power of patients' immunity to attack malignant cells. Among immunotherapy strategies, adoptive cell therapy (ACT) with engineered T cells, such as chimeric antigen receptor (CAR)-T and T cell receptor (TCR)-T cells, has gained considerable attention (1, 2). A good example is that CAR-T

Abbreviations: ACT, Adoptive cell therapy; AMPK, AMP-activated protein kinase; BTN2A1, Butyrophilin 2A1; BTN3A1, Butyrophilin 3A1; CAR, chimeric antigen receptor; CD3 CC, CD3 conformational change; DETCs, dendritic epidermal T cells; EphA2, ephrin type-A receptor 2; FDA, Food and Drug Administration; FPPS, farnesyl-diphosphate-synthase; GD2, disialoganglioside 2; GVHD, graft-versus-host disease; HVGA, host-versus-graft activities; iNKT, invariant natural killer T; IPP, Isopentenyl pyrophosphate; Klrk1, killer cell lectin-like receptor K1; MART-1, melanoma antigen recognized by T cells 1; MHC, major histocompatibility complex; MICA/B, MHC I chain-related molecules A and B; MR1, MHC-related protein 1; MSCP, melanoma cell surface chondroitin sulfate proteoglycan; MUC1, Mucin 1; NCRs, NK cytotoxicity receptors; NKG2D, Natural killer group 2D; NKG2DL, NKG2D ligand; NKRs, NK cell receptors; P-Ag, phosphoantigens; RAG, recombination activating gene; PRS, proline-rich sequence; Rae1, retinoic acid early transcripts-1; RCC, renal cell carcinoma; scFv, single-chain fragment variable; TAA, tumor associated antigen; TCR, T cell receptors; ULBP, UL16-binding protein; ZOL, zoledronate.

therapy has advanced the furthest in clinical development and three CAR-T products (Kymriah, Yescarta, and Tecartus) have gained commercial approval in the United States.

Over the past decades, a variety of researches of γδ T cells have added to the established understanding in highlighting conspicuous roles of γδ T cells in cancers. Although some researches point the potential tumorigenic effector functions of γδ T cells (3, 4), increasing translational researches have shown great interest in the therapeutic use of certain subsets of γδ T cells, especially engineered γδ T cells. In fact, the momentum of engineered γδ T cell therapy may have been generated, as U.S. Food and Drug Administration (FDA) has cleared investigational new drug application for ADI-001 that comprises CD22-allogenic γδ CAR-T cell therapy in 2020.

Activation of γδ T cells is in a TCR-dependent process as similar as that of αβ T cells, yet in independence of major histocompatibility complex (MHC). In addition to γδ TCR signals, γδ T cells mediate multiple responses *via* receptor-ligand interaction of innate signals, similar to NK cells. They bear a variety of NK cell receptors (NKRs) such as NKG2D and NK cytotoxicity receptors (NCRs) including NKp30, NKp44, and NKp46 (5). These receptors may fine-tune the γδ T cell activation threshold, enhance γδ T cells to recognize tumor target, prompt γδ T cells to mediate an immediate immune reaction against tumor target, and release cytotoxic granules such as perforin and granzyme B. In cancer, the down-regulation of MHC-I may prompt 'missing-self recognition', which unlock the binding between MHC-I and inhibitory receptors on γδ T cells, making γδ T cells unhindered to attack tumor cells in a NK-like manner (6). Dual recognition and stimulation system endows γδ T cells distinct anti-tumor effect. However, current design of engineered γδ T cells is a me-too engineered αβ T cells, such as using the same single-chain fragment variable (scFv) and co-stimulation molecules which are proved to help kill tumor cells effectively in αβ T cells but not completely confirmed in γδ T cells. This kind of design may take some advantages of γδ T cells such as GVHD absence, however, this raises the question that how to make the best use of dual recognition and stimulation system of γδ T cells to endow engineered γδ T maximum anti-tumor effect.

In this review, we provided a comprehensive and deep summary of the unique patterns of γδ T recognition and signaling pathways. Based on these underlying mechanisms, this review further discussed valuable insights in the design of engineered γδ T cells. It is promising that an intelligent design that considers the specific characteristics of γδ T cells will be beneficial for the utility of engineered γδ T cells.

2 γδ TCR ANTIGEN RECOGNITION

Like αβ T cells and B cells, γδ T cells generate their specific T cell receptors (TCRs) *via* recombination activating gene (RAG)-mediated V(D)J recombination, which contributes to the high diversity up to 10^{17} theoretically possible combinations of TCR repertoires (7). Since γδ T cells have been discovered in 1980s, what antigens γδ TCRs can recognize remains an outstanding question in this field. Nowadays, it has been known that γδ TCRs antigen recognition pattern is unrestricted by MHC. The ligands that they can recognize include self-antigens, such as MHC-like molecules, B7-like molecules, and foreign-antigens, such as haptens, virus protein, phycobiliproteins (8–12). Recent researches have shown that γδ TCRs most likely take part in complicated mechanisms that involves multiple ligands on the tumor cells, as well as the sensation of spatial and conformational changes through the γδ TCRs and potentially associated molecules.

The comprehensive description of antigen recognition by γδ TCRs has already been summarized in published articles (13, 14). Here, we only briefly review tumor-related antigens recognized by γδ TCRs, which are summarized in **Figure 1**.

Vγ9Vδ2 T cells recognize phosphoantigens (P-Ag) modified Butyrophilin 2A1 (BTN2A1)-Butyrophilin 3A1 (BTN3A1) complex in an MHC-independent, but TCR-dependent manner. In tumor, the dysregulation of mevalonate pathway accounts for the accumulation of phosphorylated mevalonate metabolites, such as Isopentenyl pyrophosphate (IPP) that was identified as a kind of P-Ag, and thus activate the Vγ9Vδ2 T cells (15–17). On the other hand, zoledronate (ZOL) can inhibit IPP-metabolizing enzyme, farnesyl-diphosphate-synthase (FPPS), and increase IPP level, which contributes to an enhanced IPP-induced γδ T cell activation (16, 18). ZOL has been widely used in cancer therapies such as renal cell carcinoma (RCC) and prostate cancer (19, 20). Many clinical trials have found it significantly inhibit the cancer progression and even completely cure cancers.

However, the defined molecular mechanisms of Vγ9Vδ2 T cell activation by phosphoantigens still remains to be discovered. The binding of P-Ags and the intracellular B30.2 domain of BTN3A1 leads to the conformational changes of the BTN3A1 extracellular domain, which can take part in the activation of $V\gamma9V\delta2^+$ TCRs (9, 21, 22). BTN2A1, a BTN molecule that is associated with BTN3A1 in extracellular and intracellular domains, directly binds to Vγ9 domain of the TCRs, potentiating Vγ9Vδ2-mediated P-Ag sensing. In addition, another Vγ9Vδ2 TCR direct interaction, mediated by BTN3A1 or an unknown ligand, is also essential in the response of Vγ9Vδ2 T cell to P-Ag (23, 24).

In addition to BTN3A molecules, the recognition of MHC or MHC-like molecules by γδ T cells has been intensively studied. γδ T cells can recognize molecules such as HLA-A24, HLA-B27 and HLA-A2 and may specifically recognize certain MHC molecules in tumor cells (13). For example, the engineered αβ T cells, which expressed $V\gamma5V\delta1^+$ TCRs, could be activated by HLA-A*24:02$^+$ tumor cells and significantly decreased the tumor burden and enhanced survival rate of HLA-A*24:02$^+$ tumor-bearing mice (25). On the other hand, some MHC-like molecules, such as MHC-related protein 1 (MR1), CD1, has a preference to specifically bind to Vδ1 TCRs in most cases (8, 26–29). Interestingly, loading different lipids may have different influences on the binding affinity of CD1d/CD1c and γδ TCRs, suggesting the loaded lipids on CD1 molecule contribute to γδ TCR antigen recognition (8, 28). MR1, another MHC-like molecule can also be recognized by γδ TCRs. γδ T cells co-cultured with MR1-transduced cells, MR1-restrict γδ TCRs transduced Jurkat-76 cell lines, can be activated with up-regulating CD69 and ERK1/2

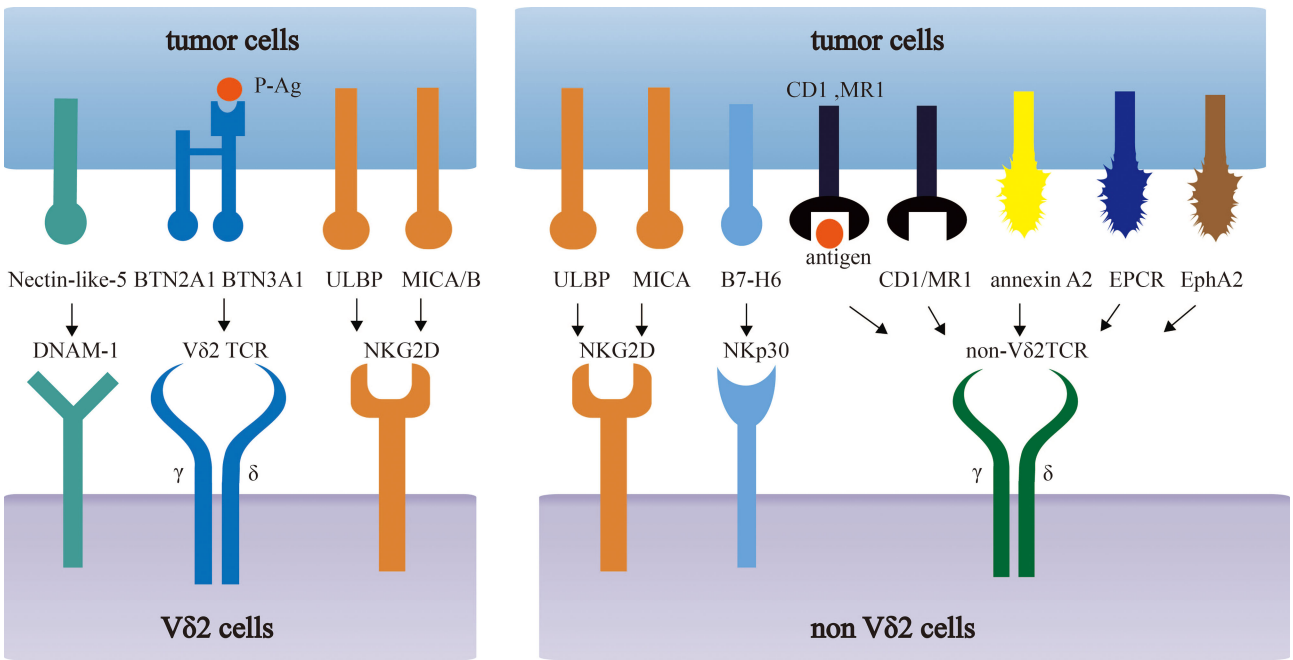

FIGURE 1 | Recognition of tumor-associated antigens (TAAs) by different subsets of γδ T cells. Vδ2 T cells recognize phosphoantigens (P-Ag) modified Butyrophilin 2A1 (BTN2A1)-Butyrophilin 3A1 (BTN3A1) complex. Non-Vδ2 TCRs recognize CD1 family members and MR1 in either antigen dependent or independent manner. Besides, NK receptors (NKG2D, NKp30, DNAM-1) in γδ T cells recognize MICA/B, ULBP and B7-H6, Nectin-like-5 respectively.

phosphorylation (29). Although CD1/MR1-restricted NKT or αβ T cells were reported to induce specific tumor killing ability in many kinds of tumor cells (30–32), there is no evidence that γδ T cells can also lead to antitumor activity by recognizing CD1 or MR1 molecules. The role of CD1 or MR1-restricted γδ T cells in cancer immune surveillance still needs to be further studied.

Recently, more novel tumor-associated molecules that can be recognized by γδ TCRs have been revealed, including annexin A2 (33), EPCR (an MHC-like molecule) (34, 35), and ephrin type-A receptor 2 (EphA2) (36, 37). The expression of EphA2 is up-regulated in cervical cancer and colon cancer cells, which is mediated by the metabolic changes (AMP-activated protein kinase (AMPK)–dependent metabolic reprogramming) in tumor cells. γδ T cells play increasing tumor-killing ability by recognizing EphA2. This ability can be reduced by blocking EphA2 in endometrial carcinoma cells or knockout of *EPHA2* gene in renal and colon tumor cells, which indicates the interaction of EphA2 and γδ T cells play an important role in enhancing the susceptibility of γδ T cytotoxic reactivity (36, 37).

3 THE CHARACTERISTICS OF γδ TCR AND THE RELATED CO-STIMULATION SIGNALS IN γδ T CELLS

3.1 γδ TCR Signal

Since γδ T cells eliminate tumor cells *via* recognizing a variety of tumor-associated antigens, γδ TCR signals play a key role in regulating γδ T cell activation. Like conventional αβTCR, γδ TCR is a complex of a clonotypic heterodimer TCRδ/TCRγ, two CD3 dimers (CD3δε or/and CD3γε), and a ζζ dimer (38). The CD3ε-deficient patients had complete deficiencies in peripheral T cells, suggesting that the ε subunit plays a pivotal role in the αβ T cell development (39). However, some CD3 molecules may play different roles in the functions of γδ T cells. For example, $CD3\delta^{-/-}$ mice have normal numbers of γδ T cells (40, 41). In addition, mouse γδ TCRs, which are naturally CD3δ-deficient, can induces calcium mobilization and ERK activation (42). On the contrary, if CD3δ is deficient in human or mice, the development of αβ T cells are failed (40, 41), and did not induce signaling events by the engagement of CD3δ-deficient αβ TCRs (43). Another important CD3 molecule, CD3γ, only blocks, but not significantly impairs the development of γδ T cells in human, as *CD3δ* gene may rescue the γδ T cell development (44). Current researches reported the function of TCR/CD3 complex components in signaling transmitting in αβ T cells, which was applied in engineered αβ T cells and engineered γδ T cells. For example, CD3ζ chain was determined to transmit signals in the absence of CD3 γ, δ, and ε in αβ T cells (45), which was widely used to deliver a major activation signal in both αβ T cell and CAR-T cells. In addition, in absence of CD3ζ chain, the CD3 γε/δε, or CD3ε alone were also able to independently activate αβ T cells (46, 47). However, the specific signaling function of TCR/CD3 complex components have not been precisely reported in γδ T cells, which needs to be explored in the future. As a whole, signals transmitted by TCR in αβ T cells and γδ T cells are not always the same. A clinical test of

60 samples from hospitalized and healthy individuals demonstrated that human γδ T cells constitutively expressed higher density of TCR/CD3 complex (2.12 ± 0.33 fold) than that in αβ T cells (48). Furthermore, by analyzing the ability to induce calcium mobilization, ERK activation, and cellular proliferation in mouse γδ T cells, it revealed a superior effect on γδ T cells in the aspect of signal transduction than that in αβ T cells with the same stimulation using immobilized anti-CD3 monoclonal antibody (mAb), which revealed that γδ T cells have a better signal-transducing complex than αβ T cells (42). Interestingly, in steady state, compared with αβ T cells, mouse γδ T cells have higher phosphorylation levels of ERK1/2 and stronger proliferation ability. Therefore, it suggested that γδ T cells possess a more "primed for action" status at baseline even in the absence of any external stimulation (49).

TCR conformation also influences the signal. CD3 conformational change (CD3 CC), which takes advantage of the increased accessibility of a proline-rich sequence (PRS) in the CD3ϵ cytoplasmic tail, was required for T cell activation (50, 51). In αβ T cells, cholesterol bound to the transmembrane region of TCRβ keeps the TCR in a resting and inactive conformation that cannot be phosphorylated by active kinases. Only αβ TCRs that spontaneously detached from cholesterol could switch to the active conformation (termed primed TCRs) and then be phosphorylated (52). Moreover, αβ TCR signaling could be inhibited by cholesterol sulfate, suggesting an important role of cholesterol in the conformation of αβ TCR (53). But γδ TCRs does not bind to cholesterol, accounting for a higher percentage of γδ TCRs in the active conformation compared to αβ TCRs (52). In addition, the CD3 CC in Vγ9Vδ2 T cells induced by anti-CD3ϵ mAb stimulation, which dramatically enhanced target cell lysis of the pancreatic tumor cell line Panc89 (54). The better reactivity of γδ T cells provides a better application of engineered γδ T cell therapy.

3.2 Co-Stimulation Molecules

Apart from TCR-dependent stimulation, the co-stimulation signals are also important and widely applied into the 2nd generation of CAR-T therapy. To date, almost all engineered γδ T cells follow the co-stimulation design in αβ T. However, whether these co-stimulation signals are applicable to engineered γδ T cells requires further investigation. The comparison of co-stimulatory molecules and their induced effector functions between αβ T cells and γδ T cells is summarized in **Figure 2**.

3.2.1 CD28

Almost all engineered γδ T cell, especially CAR-γδ T cell, utilize CD28 as a co-stimulation molecule. CD28 is an important co-stimulation molecule that express on most $CD4^+$ and half of $CD8^+$ αβ T cells. It has been widely accepted that CD28 mediates costimulatory signal to amplify signaling generated by TCRs ligation, promoting proliferation, survival and cytokine production of αβ T cells (55). Thus, CD28 has been widely applied in CAR-αβ T cells to help exert better effect. However, co-stimulatory function of CD28 in γδ T cells is still under debate. Some studies indicated that CD28 functioned as a costimulatory molecule in γδ T cells. $CD28^+$ γδ T cells have the better activation, proliferation, survival and production of IL-2 when they were stimulated with anti-CD28 mAb (56–58). It also showed that almost no γδ T cells, especially $CD69^+$ γδ T cells, could expand in CD28-deficient malaria mouse model. Along this line, $CD28^{-/-}$ γδ T cells failed to produce cytokines, such as IFNγ and IL-17. In human, the blockage of CD28 ligand led to the impairment in γδ T cell proliferation and survival (56). However, since CD28 signal is extremely important for αβ T cells, including $CD4^+$ T help cells, neither the $CD28^{-/-}$ mice infection model nor the CD28 ligand blockade experiment can exclude the possibility that blocking CD28 signal reduced the function of $CD4^+$ T help cells, thereby affecting γδ T cells indirectly. On the contrary, some studies disagreed with the co-stimulatory function of CD28 in γδ T cells. Some researchers found that CD28 was not expressed in resting mouse splenic, intestinal intraepithelial, and vaginal γδ T cells, revealing the dispensable role of CD28 in mouse (57, 59, 60). In addition, the proliferation of γδ T was unchanged when they were stimulated with anti-CD3 mAb with or without anti-CD28 mAb (42). Consistently, $CD28^{+/+}$ and $CD28^{-/-}$ mice were revealed to have equivalent increases in the percentage and quantity of the γδ T cells and IL-$17A^+$/IFNγ^+ γδ T cells in a listeria model of Infection (61). In human, although 40-60% freshly isolated human γδ T cells expressed CD28, this subset was diminished to 10% during *in vitro* culture, and even disappeared in long-term culture (62, 63). Moreover, human Vγ9Vδ2 T cells produced TNFα *via* direct TCR-induced p38 kinase and MEK/ERK activation pathway, but irrelative with CD28 (63). As is discussed above, since the co-stimulatory function of CD28 remains controversial in different stimulating conditions or infection models, it is still unclear whether γδ T cell function requires transient or continuous CD28 signals. Comprehensive studies to investigate the role of CD28 signals in γδ T cells are required, which will benefit for the better design of engineered γδ T cells.

3.2.2 4-1BB

4-1BB, also known as CD137, is an inducible T cell costimulatory molecule. It can be detected after stimulation and reaches the peak of expression at 48h in human αβ T cells, and functions as a conventional co-stimulatory molecule (64). 4-1BB has been widely applied in engineered αβ T cells, but not in γδ T cells currently. Many researches pointed that 4-1BB preferred to help expand memory CTLs, up-regulated NKG2D expression and rendered enhanced cytolysis. More importantly, the 4-1BB provided a stronger cytotoxicity than CD28 in some experiments (65). However, no research has specifically evaluated the advantages and disadvantages of 4-1BB in engineered γδ T cells, which raises the question of whether 4-1BB can exert as an efficient co-stimulator in engineered γδ T cells. Existing researches have revealed the co-stimulation function of 4-1BB in γδ T cell in different disease models. For example, 20% 4-$1BB^+$ Vγ9Vδ2 T cells were observed in influenza virus infection model, and most importantly, such cell subset showed an enhanced *ex vivo* effector function such as more intensive granule release, more cytokine production (e.g. IFNγ), and superior cytotoxic activity towards virus-infected cells comparing to the 4-$1BB^-$ counterparts. Furthermore, the

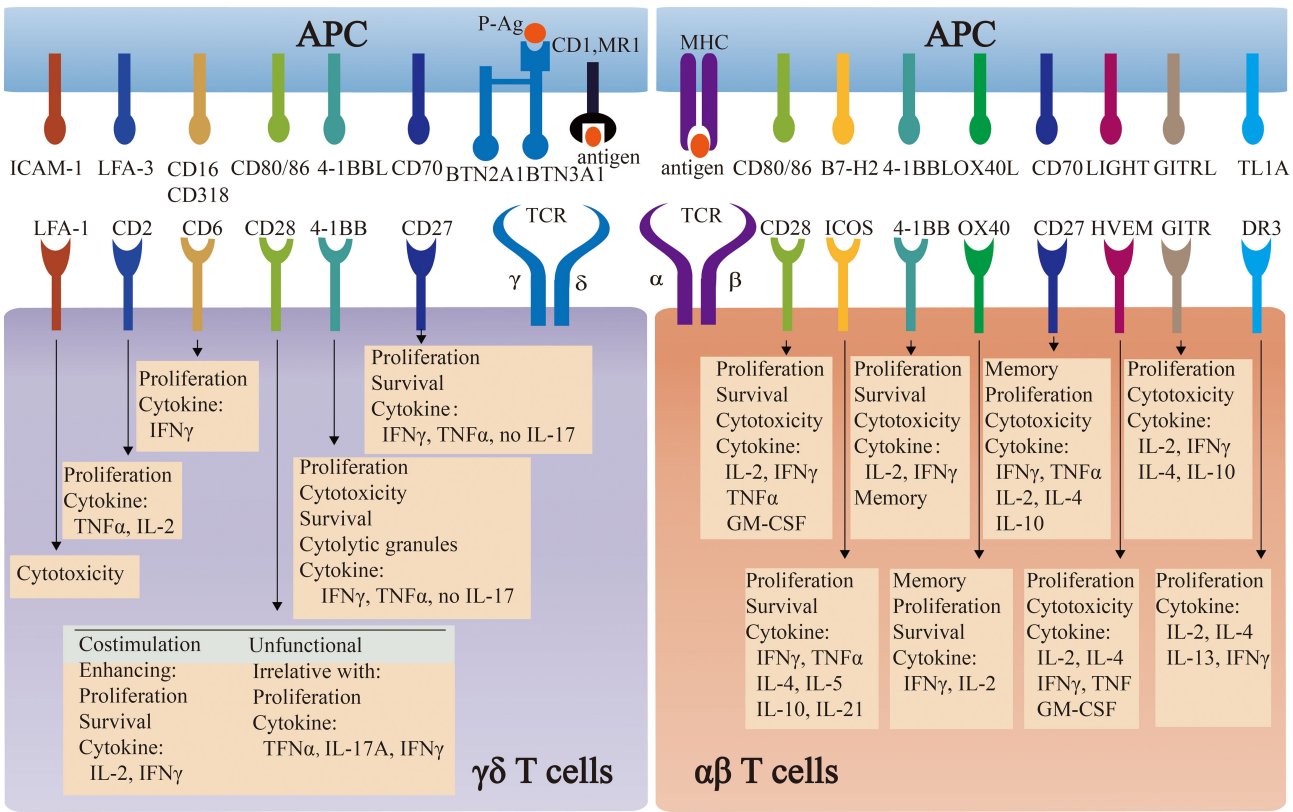

FIGURE 2 | The comparison of co-stimulatory molecules and their induced effector functions between αβ T cells and γδ T cells. Co-stimulatory molecules induce the effector functions of αβ T cells and γδ T cells, by engaging respective ligands and counter-receptors on APCs. The specific functions of some molecules are commonly well-understood in αβ T cells, whereas they are controversial in γδ T cells.

co-stimulation effect of 4-1BB was determined to induce better proliferation and enhance the survival of Vγ9Vδ2 T cells (66). On the other hand, in influenza virus infection mouse model, the transfer of 4-1BB$^+$ γδ T cells was beneficial to maintain the body weight, enhance the survival rate, and reduce virus titers. With the co-stimulated of 4-1BB, only γδ T cells, but not other subsets of PBMCs, had improved therapeutic outcome in this disease model (66). In addition to influenza infection model, *Listeria Monocytogenes* infected mouse model indicated that compared to γδ T cells without 4-1BB stimulation, γδ T cells with 4-1BB stimulation showed the decreased bacterial load *in vivo* and enhanced survival. To be more specific, anti-4-1BB treatment in adoptive γδ T cell treatment, rather than adoptive αβ T cell treatment, significantly increased the cytokine production such as IFNγ and TNFα, and the augmented number of γδ T cells (67).

3.2.3 CD27

CD27 is also a stimulatory molecule in αβ T cells, which interacts with CD70 and induces the activation, proliferation, and survival of αβ T cells (68). It has been applied in CAR-αβ T which can promote the proliferation, anti-tumor effect, and survival both *in vitro* and *in vivo* (69). However, it has not been applied in engineered γδ T cells. CD27 has been found to be widely expressed in γδ T cells. It is expressed in 70-90% of γδ T cells in mouse spleen and lymph nodes (70), 81% of activated Vγ9$^+$ T cells, and even some Vδ1$^+$ T cells in peripheral blood in human (71). Many researches have showed its co-stimulation function as it can promote proliferation, survival and cytokine production in γδ T cells (56, 71). Interestingly, CD27 was used to distinguish mouse γδ T cells with different cytokine production. In this case, CD27$^-$ γδ T cells produce IL-17, whereas CD27$^+$ γδ T cells produce IFNγ. In addition, 90% IFNγ and 70% TNFα-producing cells were CD27$^+$ γδ T cells in naïve and malaria-infected mice (70). Apart from these *in vitro* researches, γδ T cells failed to expand in CD27 deficient mice when infected with MuHV-4, compared to that in WT mice. Moreover, the deficiency of CD27 related to the anergy of IFNγ production (72). In human, comparing to CD27$^-$ γδ T cells, CD27$^+$ Vγ9Vδ2 T cells showed higher level of proliferation and up-regulation of BCL2A1 gene after being cultured with HDMAPP. Vγ9Vδ2 T cells had a stronger ability of proliferation and IFNγ, LT-α secretion under sCD70 stimulation, and CD70 blockade prevented efficient expansion of Vγ9Vδ2 T cells and reduced production of TNFα and LT-α (71). As summarized, CD27 may be a potential co-simulation molecule that can be applied in engineered γδ T cells.

3.2.4 Potential Co-Stimulation Molecules

Recent researches have revealed some other potential co-stimulation molecules. For example, CD6 (ligands to CD166 and CD318), a costimulatory receptor, is expressed on virtually all T cells, especially activated human γδ T cells (73–75). After stimulated by CD166, human $V\delta2^+$ T cells showed increased proliferative capability and IFNγ production. In addition, both CD6 and CD166 were observed to locate at center of synapses in activation process (75). In αβ T cells, a CAR with CD6 showed increased release of IFNγ and enhanced anti-tumor effect when compared with the CAR without CD6 (76). However, CD6 has not been applied in engineered γδ T cell therapy. In addition to CD6, CD2 and LFA1, as adhesion molecules, also have costimulatory function in activated αβ T cells (77, 78). Ligation of CD2 and its ligand was applied in first-generation CD19-specific CAR to drives IL-2 production (79).There are several researches about its costimulatory function in γδ T cells. The stimulation by anti-CD2 mAb promotes IL-2 secretion and/or proliferation of γδ T cells (80). Correspondingly, the blockage of CD2 or LFA1 inhibited the effector function, especially reduced TNFα production, of $V\delta2^-$ T cells (34). However, LFA1 and CD2 signals affected the function of Vγ9Vδ2 T cells differently. CD2 blockade strongly inhibited proliferation of γδ T cells and release of TNFα/IL-2, but had no effect on the lytic activity of γδ T cells, whereas LFA-1 blockade had no effect on cell proliferation and cytokine production, but could effectively inhibited target cell lysis (81). Consequently, CD2/LFA1 co-stimulation may differently influence the effector function of engineered γδ T cells.

4 THE CHARACTERISTICS OF NK CELL RECEPTOR SIGNALS IN γδ T CELLS

The expression of a variety of NK cell receptors, including NKRs and NCRs, is an important feature of γδ T cells, which endows γδ T cells innate immune characteristics like NK cells. Also, the two kinds of lymphocytes share similar characteristics in the perspective of immune responses. Compared to αβ T cells, involvement in innate immune reaction is beneficial for γδ T cells to recognize a more extended spectrum of antigens on tumor cells, reduce the risk of tumor immune escape by losing single tumor-associated antigen, and provide available chances for novel immunotherapies for cancers that lack tumor specific antigens.

4.1 Natural Killer Group 2D (NKG2D)

As one of the most important receptors, NKG2D is a C-type, lectic-like, type II transmembrane glycoprotein, which is expressed on NK cells, γδ T cells and some narrowed subsets of αβ T cells (82, 83). In human peripheral blood, almost all γδ T cells expressed NKG2D, but compared with NK cells, the expression level of NKG2D is about 10-times lower (82). In addition, the intestinal intraepithelial γδ T cells originally express relatively low level of NKG2D. Interestingly, the expression of NKG2D can be upregulated in response to IL-15 stimulation or 4-1BB signals (84).

4.1.1 NKG2D Recognition

Like NK cells, NKG2D on γδ T cells can also recognize ligands including MHC class I-like molecules [e.g. MHC I chain-related molecules A and B (MICA/B) and UL16-binding protein (ULBP1-6)] in human, and retinoic acid early transcripts (Rae1) α-ϵ, murine UL16-binding protein-like transcript 1 MULT1, H60a, H60b, and H60c in mouse. These ligands can be induced in infected and oncogenic transformed cells (85, 86). Therefore, NKG2D is frequently involved in the tumor cell recognition, induces cytokine release, and triggers degranulation. NKG2D is reported to trigger cytotoxicity of γδ T cells against tumor cells and bacterial or virus-infected cells in a NK-like and TCR-independent manner (87–91). Stimulated by anti-NKG2D mAb or NKG2D ligand protein (NKG2DL), human Vγ9Vδ2 T cells and mouse dendritic epidermal T cells (DETCs) released cytotoxic granules and cytokines such as TNFα. The blockade of NKG2D completely abolished such cytotoxicity to tumor cells induced by Vγ9Vδ2 T cells (83, 87, 91).

4.1.2 NKG2D Signal

The pivotal role of NKG2D in γδ T cells has attracted researchers to explore the underlying signaling molecules and specific signaling pathways. The NKG2D signals are much similar between γδ T cells and NK cells. In NK cells, NKG2D is associated with adapter molecules DAP10 to transmit signals in PI3K or Vav/SOS signaling pathway to trigger cytotoxicity, but without IFNγ production. Alternatively, NKG2D connects to DAP12 to recruit Syk and ZAP70 to downstream signaling events to trigger cytotoxicity, along with the secretion of IFNγ in mice (92–96). In γδ T cells, NKG2D has been observed to act in a PI3K-dependent signaling pathway that responds to target cells in a TCR-independent manner (88, 91, 97). DAP10, rather than DAP12, was reported to strongly express in resting and activated human Vγ9Vδ2 T cells (82, 96), while DAP10/DAP12 constitutively expressed in mouse DETCs (83). Human Vγ9Vδ2 T cells stimulated by ULBP proteins could produce IFNγ, TNFα, and released cytolytic granules usually accompanying PKB (a PI3K kinase substrate) phosphorylation (88, 97). The knockdown of either DAP10 or NKG2D in Vγ9Vδ2 T cells showed the similar impaired anti-bacterial effect, when cocultured with infected macrophages. This indicated that DAP10 is involved in NKG2D signaling during bacterial infection (88). In mouse DETCs, NKG2D could trigger a PI3K-dependent signaling pathway by DAP10 to increase phosphorylation of Akt, trigger degranulation and induce cytotoxicity, which could be completely inhibited by PI3K inhibitor. In addition, in the absence of both NKG2D-S-DAP12 (a shorter protein isoform that is produced by alternative splicing of *killer cell lectin-like receptor K1(Klrk1)*) and TCR signals, only NKG2D/DAP10 signals through the PI3K/Grb2/Vav1 pathway is sufficient to trigger cytotoxicity of DETCs against target cells (91). Of note, although γδ T cells could produce IFNγ and were cytotoxic under the stimulation of anti-NKG2D mAb (83), these cells failed to produce IFNγ, TNFα, IL-13 and induced Syk/ZAP70 activation when stimulated by recombinant NKG2DL protein. It indicated that NKG2DLs may not be able to engage enough activation of NKG2D/DAP12 signaling, which might be weaker

than NKG2D/DAP10 on DETCs in the aspects of triggering Syk/ZAP70 signaling (91).

4.1.3 Signal Difference Between NKG2D and TCR in γδ T Cells

Apart from the signaling mechanism, another interesting question is the different activation level through NKG2D and TCR in activating γδ T cells. Some experiments compared effector function in cytokine production, degranulation, killing ability induced by γδ T cells with specific TCR stimulation to that with NKG2D stimulation. For example, comparison of cytokines production in different stimulation groups, Vγ9Vδ2 T cells induced the similar level of TNFα when stimulated by anti-NKG2D mAb or recombinant MICA-Fc or Daudi cells plus IPP, although which is weaker than that of stimulation with anti-CD3ϵ mAb (87). Furthermore, by stimulating NKG2D or TCRs pathways with antibodies or cell lines respectively, Vγ9Vδ2 T cells showed the similar level of degranulation, IFNγ production and cytotoxicity (87). In the real situation, tumor cells can express ligands that could bind both NKG2D and γδ TCRs. Therefore, researchers conducted inhibition experiment to compare the contribution of NKG2D and TCR to the cytotoxicity of γδ T cells. The inhibitory effect of TCR signal blocking on Vγ9Vδ2 T cell cytotoxicity was much stronger than that of NKG2D signal blocking (87, 97). However, TCR signal or NKG2D signal blockade had similar inhibitory effects on cytotoxicity of DETCs, indicating that the signaling pathways in human and mouse γδ T cells are different (91). Interestingly, some ligands that can be recognized by both γδ TCRs and NKG2D, such as ULBP4, can be killed by Vγ9Vδ2 T cells in both TCRs-based and NKG2D-based activation pathways. Blocking one of these pathways could only induce minor inhibitory effect on degranulation of Vγ9Vδ2 T cells and cytotoxicity to EL4-ULBP4$^+$ cells, but almost completely inhibited IFNγ production by Vγ9Vδ2 T cells. However, blocking both of these pathways could significantly reduce the cytotoxicity of Vγ9Vδ2 T cells (97). In addition, NKG2D signal in γδ T cells could enhance TCR-dependent signals, which increased cytokine production and cytotoxicity of γδ T cells, and extended survival of γδ T cells (98–101). For calcium response, compared with αβ T cells and iNKT cells, which showed a strong and rapid TCR-induced Ca^{2+} response, Vγ9Vδ2 T cells showed a delayed and sustained Ca^{2+} response. However, when NKG2D signal was simultaneously activated, Ca^{2+} responses in γδ T cells induced by TCR signal could be accelerated. Besides, NKG2D signal alone could not induce significant Ca^{2+} activation signals, indicating NKG2D signal could enhance TCR signal in γδ T cells. Furthermore, PKCθ were found to play an important role in the NKG2D mediated costimulatory function. Vγ9Vδ2 T cells have significantly improved cytolytic ability to tumor cells with NKG2D signal, which could be blocked by PKCθ inhibitor. It is worth noting that PKCθ inhibitor could inhibit the acceleration of Ca^{2+} response induced by NKG2D, indicating that NKG2D signal could shape Ca^{2+} response and potentiate antitumor CTL activity of Vγ9Vδ2 T cells in a PKCθ-dependent manner (99). Taken together, regulation of NKG2D on DAP10 and/or DAP12 signals alone or together with TCR signals should be carefully designed for the application of engineered γδ T cell therapies, especially for the characteristic of cytotoxicity, proliferation, exhaustion, memory and cytokines production of engineered γδ T cells.

4.2 Natural Cytotoxicity Receptors (NCRs)

Like NKRs, NCRs are mostly detected on Vδ1 population, and can activate γδ T cells by recognizing ligands on tumor cells (102–104). They exert potent anti-tumor activities in a TCR-independent way (103–105), and sometimes enhance effector function of γδ T cells (106). Similar to NKG2D, NCRs ligated with adaptor proteins, such as CD3ζ, FcRγ and DAP12, to transmit intracellular activating signaling in NK cells. Similarly, NKp44 is detected to couple with DAP12 in stimulated human γδ T cells (106). But the specific function of these adapters associating with NCRs in γδ T cells remains unclear and needs further exploration.

The comparison of NKG2D and NCR signaling pathways between γδ T cells and NK cells is summarized in **Figure 3**.

5 OTHER RECEPTORS DELIVERING SIGNALS IN γδ T CELLS

γδ T cells also express cytokine receptors, like IL-2Rβγ, IL-18R, IL-7Rα, IL23R (107–111), which can deliver activated signals by binding to interleukins. Stimulation of these cytokine receptors can not only enhance the effect function of γδ T cells, but also directly trigger the activation of γδ T cells even in the absence of TCR signal. For example, after initial stimulation by P-Ag or γδ TCR antibody, IL-15, IL-12, IL-2, IL-18, IL-33 and IL-7 could additionally enhance the proliferation, cytokine production, cytotoxic effect of γδ T cells (112–118). Furthermore, some cytokines alone or combination, such as IL-15, IL-2, IL-12, IL-18, IL-7, IL-1 and IL-23, were found to induce proliferation, cytokine production and killing ability in the absence of TCR signal (107, 110, 111, 115). Besides, γδ T-induced effector molecules were impacted by cytokines. IL-2, IL-12, IL-18, IL-15 and IL-21 were found to promote IFN-γ-production of γδ T cell (113, 115, 119, 120), whereas IL-17-production of γδ T cell was driven by IL-1, IL-23 and IL-7 (111, 118, 121). Interestingly, IL-18 could replace IL-1β and cooperate with IL-23 to induce IL-17 production in γδ T cells (108). In addition, toll-like receptors (TLRs) were also reported to deliver activated signals in γδ T cells. The simultaneous stimulation of TLRs (e.g. TLR1/2/6, 3, and 5) and TCR significantly enhanced the activation and effect function of γδ T cells. Furthermore, γδ T cells can also directly respond to TLR2 ligands to act effect function in a TCR-independent manner (122).

6 APPLICATION OF γδ T CELLS IN ENGINEERED T CELL THERAPIES

Although γδ T cells have limited ability to expand and proliferate *in vivo*, which may affect the antitumor efficacy of γδ T cells, γδ T

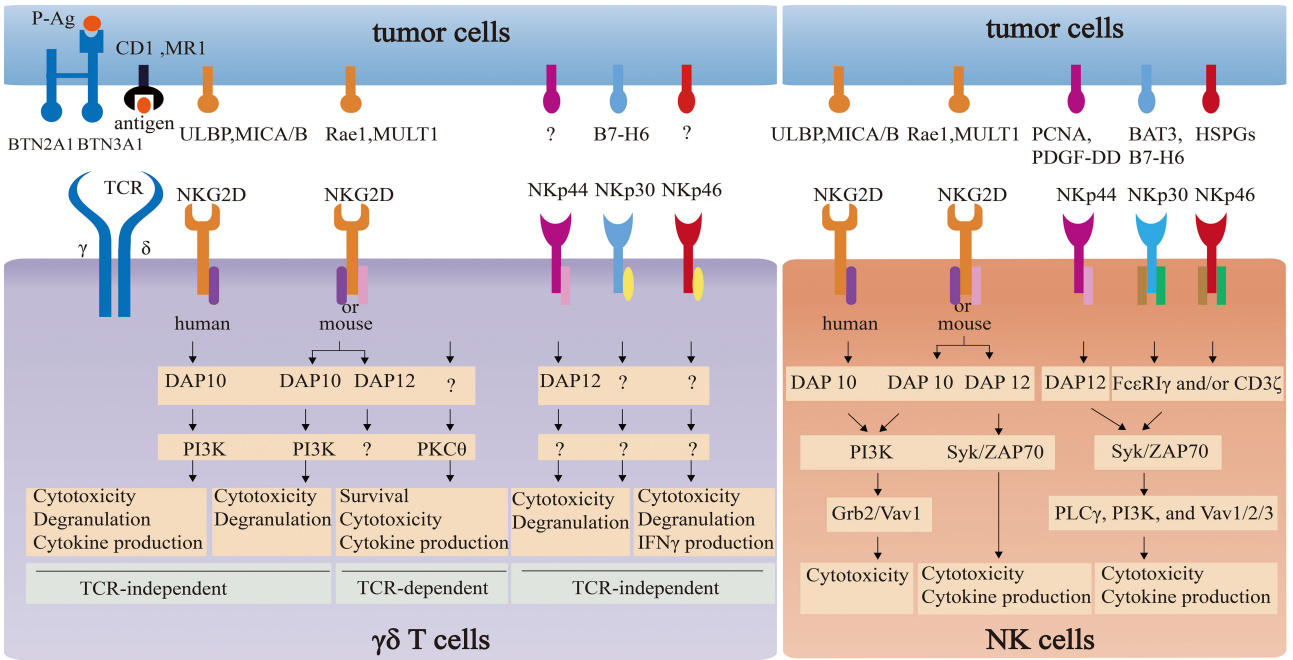

FIGURE 3 | The comparison of NKG2D and NCR signaling pathways between γδ T cells and NK cells. NKG2D in NK cells associates with adapter DAP10 (PI3K and Grb2-Vav1 signaling) or DAP12 (Syk/ZAP70 signaling) to directly induce cytotoxicity and/or cytokine release. NKG2D in γδ T cells not only directly triggers cytotoxicity *via* PI3K-dependent pathway by coupling with DAP10, but also enhances the effector function in a TCR-independent way *via* PKCθ. However, the impact of NKG2D-DAP12 complex on the function on γδ T cells remain elusive. NCRs in NK cells and γδ T cells can induce cytotoxicity activity. However, signaling pathways of NCRs-ligands in NK cells are well-understood, which in γδ T cells remain unclear.

cells remain good candidates for engineered therapies with many advantages. Firstly, since engineered γδ T cells can exploit the endogenous receptors (TCRs and innate immune receptors) and engineered receptors, the current CAR-γδ T therapies induce a significantly stronger potential to kill targeted cells and cytokine production, which contributes to more significant reductions of tumor burden and suppression of tumor growth compared with γδ T cells (123–126). These endogenous receptors enable γδ T cells not only to recognize a myriad of tumor-specific or associated ligands as described above, but also to prevent tumor escape caused by antigen loss or downregulation (127). In this scenario, the downregulation of MHC-I in tumors helps tumor cells to escape surveillance of αβ T cells, but it does not inhibit non-MHC-restrict γδ T cell activation and even enhances the consecutive γδ T cell activation (128). Indeed, compared with CAR-αβ T cells, CAR-γδ T cells targeting CD19 or melanoma cell surface chondroitin sulfate proteoglycan (MSCP) showed a significantly higher cytotoxicity against tumor associated antigen (TAA) negative target cells, or β2-microglobuline-deficient Daudi cells that lacks the expression of MHC-I (125, 129). Secondly, activated human $V\delta2^+$ T cells can present the characteristics of professional antigen-presenting cells like dendritic cells, which can take up, process, and present soluble antigens to αβ T cells. HLA-$A0201^+V2\delta^+$GD2-CAR-γδ T cells can present the epitopes of melanoma antigen recognized by T cells 1 (MART-1) to αβ T cells to promote expansion and cytotoxicity (130). Thirdly, since allogeneic αβ T cell therapies have side-effects of host-versus-graft activities (HVGA) and graft-versus-host disease (GVHD), current engineered αβ T cell products are individualized and have many limitations, such as high cost, time consuming, and unstable quality or quantity of T cells (131). However, engineered γδ T cells with MHC-unrestricted recognition pattern can avoid of GVHD, which makes it possible for engineered γδ T cells to become universal cell products to circumvent many disadvantages of above-mentioned individualized CAR-T cell products. Many clinical cases and trials were trying to assess the safety of allogeneic γδ-TCR T cell therapies to confirm the absence of GVHD by γδ T cells. For example, after receiving allogeneic γδ T cell immunotherapy, a patient with cholangiocarcinoma had improved peripheral immune function, reduced tumor activity, and prolonged life span, and more importantly, without side-effects (132), indicating the safety of γδ T cells and its potential to be universal. Besides, 3 clinical trials (NCT04107142, NCT04735471, NCT04911478) were conducted to evaluate the safety and tolerability of allogeneic CAR-γδ T cells targeting NKG2D ligand (NKG2DL) and CD20. However, HVGA and the persistence remained to be the challenge for engineered γδ T cell products. Taken together, engineered γδ T cells take advantage of recognizing antigens by endogenous receptors as well as engineered receptors, processing and presenting antigens to activate αβ T cells and avoiding GVHD. To sum, γδ T cells

can be a more efficient and wider-applied antitumor candidate to produce engineered products.

6.1 CAR TRANSFER TO γδ T CELLS

CAR-αβ T therapy has shown unprecedented success in hematologic malignancies, but poor efficacy in solid tumors. Many studies found higher infiltration of γδ T cells in solid tumors than that of αβ T cells, and the frequency of infiltrated γδ T cells in solid tumors positively correlated with prognosis (133–135), indicating a promising application of CAR-γδ T cells in solid tumors. Thus, CAR-γδ T cells have been designed to target many solid tumor antigens, such as disialoganglioside 2 (GD2) on neuroblastoma and Ewing sarcoma (136), melanoma chondroitin sulfate proteoglycan (MCSP) on melanoma lesions (137), original or glycosylated Mucin 1 (MUC1) on breast cancer, head and neck squamous cell carcinoma (138, 139). Current ongoing clinical trials involving engineered γδ T products are summarized in **Table 1**.

However, current CAR-γδ T cells fails to show better efficacy of tumor immunotherapy than CAR-αβ T cells. One of reasons is the design of intracellular signaling domain of CAR-γδ T cells is less optimized. The intracellular signaling domains applied in CAR-γδ T cells are almost as same as what used in CAR-αβ T cells. Indeed, CAR-γδ T cells are reported to have a significant effector function against tumor cells. But it is controversial in different studies comparing CAR-γδ T cells with CAR-αβ T cells, particularly in solid and hematologic tumors. Meir Rozenbaum et al. pointed the superiority of CAR-αβ T cells in leukemia *in vivo*. To be more specific, treatment with CAR-γδ T or CAR-αβ T cells led to a respective 5% and 0.1% tumor cell residue in the bone marrow of mice, demonstrating the higher load of leukemia cells in recipients of CAR-γδ T cells compared to the CAR-αβ T treated mice (125). This phenomenon suggested that CAR-γδ T cells with suboptimal design have lower efficiency to eliminate tumor cells than that by CAR-αβ T cells. Furthermore, recent study reported the persistence of CAR-Vγ9Vδ2 T cells was worse than that of CAR-αβ T cells. While CAR-αβ T cells still effectively eliminate all the tumor cells in the fourth round of tumor stimulation, CAR-Vγ9Vδ2 T cells almost lost their cytotoxicity. Fortunately, the cytotoxicity of γδ T cells can be restored by the addition of IL-2 (126). Although compared with CAR-αβ T cells, CAR-Vδ1 and Vδ2 T cells secreted higher levels of granzyme B and cytokines, and exhibited similar or stronger cytotoxicity against some kind of solid tumors *in vitro* (126), the specific effector function against solid tumor *in vivo* should be comprehensively investigated in the future. Therefore, it is extremely important to investigate the optimal use of activation signals for CAR-γδ T cells. Recent studies have made some modifications to simultaneously take advantage of the natural endogenous signal properties of γδ T cells. For example, DAP10 was used in engineered γδ T cells and engaged in the antitumor response. Except for the signal induced by TCRs, GD2-DAP10 CAR transferred γδ T cells used the solitary endodomain derived from the NKG2D adaptor DAP10 to mimic NKG2D co-stimulation, which induced significant cytokine production and equivalent killing as CD28-CD3ζ-CAR-γδ T cells against $GD2^+$ Neuroblastoma and Ewing Sarcoma (140). Interestingly, this example also promoted the utilize of "AND gate" system in engineered γδ T cells to minimize on-target off-tumor toxicity. It was only activated in presence of antigen through γδ TCR and GD2, whereas only GD2 could activate CD28-CD3ζ-CAR-γδ T cells (140).

6.2 αβ TCR Transfer to γδ T Cells

Engineered γδ T cells not only included CAR-γδ T cells, but also TCR-γδ T cells. For example, αβ TCRs were reported to be transferred to γδ T cells, making αβ TCR-γδ T cells sensitive to tumor cells with antigen-negative or tumor escape variants with MHC-downregulating. γδ T cells which expressed an HLA-A*0101 restricted αβ TCR targeting the adenovirus hexon protein of HAdV-species C, released more IFNγ and TNFα than $CD8^+$ αβ T cells with the same αβ TCR, and had comparable cytotoxicity against adenovirus-infected dendritic cells (141). Interestingly, while most γδ T cells lack the expression of the co-receptors CD4 or CD8, some researches transferred the co-receptors along with αβ TCRs to γδ T cells and found the enhanced specific functional activity. Comparing to HA-2-TCR-γδ T cells without the additional transfer of CD8, co-transferring of CD8 and HA-2-TCR to γδ T cells significantly increased IFN-γ and IL-4 production and exerted more efficient cytotoxicity against the HA-2-expressing CML and AML cells (142). In addition, transferring αβ TCRs that recognized the same antigen as endogenous γδ TCRs could improve TCR-γδ T cell antigen recognition and cytotoxicity efficiency. For example, transferring αβ TCRs derived from invariant natural killer T

TABLE 1 | Current ongoing clinical trials of engineered γδ T products.

Clinical Trials/Netherlands Trials Identifier	Phase	Disease	Interventions	Source	γδ T subset
NCT04735471	I	B Cell Malignancies	CD20-CAR expressed on γδ T cells	allogeneic	Vδ1 γδ T-cell
NCT04107142	I	solid tumor.	NKG2D-CAR expressed on γδ T cells	haploidentical/allogeneic	unmentioned
NCT04702841	I	T cell-derived malignant tumors	CD7-CAR expressed on γδ T cells	unknown	unmentioned
NCT03885076	unknown	AML	CD33-CAR expressed on γδ T cells	autogenetic	Vδ2 γδ T-cell
NCT04796441	Not Applicable	AML	CD19-CAR expressed on γδ T cells	allogeneic	unmentioned
NCT02656147	I	Leukemia Lymphoma	CD19-CAR expressed on γδ T cells	allogeneic	unmentioned
NL6357	I	r/r AML, high-risk MDS or MM	a defined γδ T cell receptor expressed on αβ T cells	autologous	/

(iNKT) cells, which recognized glycolipid antigens presented by CD1d, the TCR-γδ T cells were found to respond to CD1d *via* both endogenous γδ TCRs and transferred αβ TCRs, and had increasing antitumor effect against the CD1d positive leukemia cell line K562 (143). Of note, the transfer of αβ TCRs to γδ T cells did not show any mispairing of endogenous and transgenic TCRs (144), which significantly avoided autoimmunity (145, 146). Along this line, in order to obtain better anti-tumor efficacy, CAR-γδ T cells or TCR-γδ T cells can be designed so that endogenous γδ TCR and engineered CAR/TCR can recognize the same antigen, such as CAR-γδ T cell targeting NKG2DL or BTN3A, TCR-γδ T cell targeting HLA-A24, HLA-B27 and HLA-A2, all of which can be investigated in the future.

6.3 γδ TCRs Transfer to αβ T Cells

γδ TCRs transferred αβ T cells was also used to overcome the deficiency of cytotoxicity of particular types of HLA-restricted αβ T cells. This design has several advantages. Firstly, the γδ TCRs could target a broad range of solid and hematological tumors in MHC-independent manner. Secondly, compared with γδ T cells, the mechanism of effects and memory functions of $CD4^+$ and $CD8^+$ αβ T cells are better understood *in vivo* (7). Thirdly, this strategy can avoid the activity of inhibitory receptors like KIRs on γδ T cells. Indeed, αβ T cells expressing the Vγ9Vδ2 TCR clone G115 displayed a γδ T cell-like effector function, such as cytotoxicity against the Daudi cell line, cytokine release, enhanced cytotoxicity using amino-bisphosphonates, and the ability to induce dendritic cell maturation. Surprisingly, endogenous αβ TCRs were down-regulated after the transduction of γδ TCRs, leading to a lack of allo-reactive response (147). Besides, several types of tumor specific CDR3δ-grafted γδ TCRs were also used to modify αβ T cells and exhibited significant antitumor effects (148, 149). Moreover, a novel antibody-TCR (Ab-TCR) modified αβ T cells, combining Fab-based antigen recognition with γδ TCR signaling, showed a similar cytotoxicity and a less cytokine release comparing with CD28/CD3ζ CAR-T cells (150). Recently, TEG001, an engineered αβ T products expressing a defined γδ TCR, was proved to be safe and efficient against tumor models *in vivo* (151), and currently was applied in a first-in-human clinical study (NL6357).

7 CONCLUSION

Currently, increasing studies have confirmed the anti-tumor activities of γδ T cells in targeting various malignancies with their innate and adaptive immunities, which brings hopes to the engineered γδ T cells in cancer treatment. However, current engineered γδ T products almost copy the structure of engineered αβ T cells, owing to the ignore of the specific activating mechanism of γδ T cells. As discussed above, we detailed the activating and stimulating modes of γδ T cells *via* TCR signal, some important costimulatory signals, and innate signals from NK receptors, which were summarized in **Figure 1**. Furthermore, current engineered γδ T products and their characteristics are also depicted. Taken the basics of γδ T cells in previous sections together, this review will shed light on the optimal design of engineered γδ T cell to improve its efficacy. However, there are still numerous problems to be solved. More studies are supposed to be conducted to describe the specific activating mechanism of γδ T cells, which can be applied in engineered γδ T products.

AUTHOR CONTRIBUTIONS

RD and YZ drafted the manuscript. XZ and HX take the primary responsibility for this paper as the corresponding authors. All authors contributed to the article and approved the submitted version.

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The BLT Humanized Mouse Model as a Tool for Studying Human Gamma Delta T Cell-HIV Interactions *In Vivo*

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Gamma-delta (γδ) T cells recognize antigens in a major histocompatibility complex (MHC) independent and have cytotoxic capability. Human immunodeficiency virus (HIV) infection reduces the proportion of the Vδ2 cell subset compared to the Vδ1 cell subset of γδ T cells in the blood in most infected individuals, except for elite controllers. The capacity of Vδ2 T cells to kill HIV-infected targets has been demonstrated *in vitro*, albeit *in vivo* confirmatory studies are lacking. Here, we provide the first characterization of γδ T cell-HIV interactions in bone marrow-liver-thymus (BLT) humanized mice and examined the immunotherapeutic potential of Vδ2 T cells in controlling HIV replication *in vivo*. We demonstrate a reduced proportion of Vδ2 T cells and an increased proportion of Vδ1 T cells in HIV-infected BLT humanized mice, like in HIV-positive individuals. HIV infection in BLT humanized mice also impaired the *ex vivo* expansion of Vδ2 T cells, like in HIV-positive individuals. Adoptive transfer of activated Vδ2 T cells did not control HIV replication during cell-associated HIV transmission in BLT humanized mice but instead exacerbated viremia, suggesting that Vδ2 T cells may serve as early targets for HIV replication. Our findings demonstrate that BLT humanized mice can model γδ T cell-HIV interactions *in vivo*.

Keywords: BLT mice, humanized mice, gamma delta T cells, HIV infection, HIV immunopathogenesis

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INTRODUCTION

Human gamma-delta (γδ) T cells are widely distributed throughout barrier tissues and mediate potent antiviral effects by targeting stressed cells in an MHC-independent manner (1–3). Although human γδ T cells typically makeup <10% of the total T cell population, recognize nonpeptide microbial antigens and play an essential role in controlling various diseases, particularly malaria (4), contributing to both innate and adaptive immune responses (5). While CD4+ T cells are known to be targeted and depleted during the course of HIV infection, there is also a dramatic and immediate impact on γδ T cells, where the normal proportions of the two major subsets of γδ T cells (designated Vδ1 and Vδ2) become inverted due to a selective depletion of Vδ2 T cells expressing the phosphoantigen-responsive Vγ9 chain (Vγ9Vδ2 T cells) (6). Natural history studies of HIV

infection demonstrate an inverse correlation between Vδ2 T cell frequency and HIV viral titers (7), and earlier clinical reports indicated that, unlike most HIV-positive individuals, Vδ2 T cells are maintained at a normal frequency in elite controllers (7). The hypothesis for these observations is that γδ T cells may provide protective immunity against HIV infection by secreting chemokines that compete for HIV entry coreceptors or by promoting the effector activity and recruitment of other immune cells to eliminate infected targets. A few *in vitro* studies demonstrated the direct cytotoxic capacity of Vδ2 T cells against HIV-infected targets (8, 9), but the *in vivo* function and therapeutic potential of Vδ2 T cells against HIV has yet to be fully elucidated.

Non-human primate models of the simian immunodeficiency virus (SIV) dominate the current *in vivo* approaches to understanding the relationship between HIV viremia and γδ T cells. However, SIV contains only about 50 percent of the genetic code of HIV, and there are substantial differences in γδ subset composition and phenotype in monkeys and humans (10). The information we can extrapolate from non-human primate models of SIV becomes limited by the unaltered peripheral Vδ1/Vδ2 T cell ratio in SIV-infected macaques (11) and the genetic differences between SIV and HIV (10). Therefore, an alternate approach is needed to understand the *in vivo* dynamics of γδ T cells in HIV infection. Among the widely used *in vivo* platforms for investigating HIV pathogenesis and therapeutics is the mouse model utilizing bone marrow-liver-thymus (BLT) humanized mice (huMice). Generated *via* peripheral injection of CD34+ hematopoietic stem cells (HSCs) and autologous transplantation of fetal liver and thymic explants into immunodeficient mice, BLT huMice provide both the peripheral immune circulation and human lymphoid microenvironment to study HIV in blood and human lymphoid tissues. Previously it has been shown that human $CD4^+/CD8^+$ T cell ratios before and after HIV infection of BLT huMice are comparable to clinical values seen in natural human infection (12). While in humans Vδ2 T cells become depleted during the early stages of natural HIV infection, often before the $CD4^+/CD8^+$ T cell ratio inverts, the impact of HIV infection on γδ T cells has yet to be fully characterized in the BLT huMouse model.

In the present study, we provide the first reported phenotypic and functional characterization of human γδ T cells in BLT huMice and evaluate how they are impacted by HIV infection *in vivo*, and we assess their therapeutic potential following adoptive cell transfer. We demonstrate that the BLT huMouse model recapitulates the clinical changes in Vδ1 and Vδ2 T cell frequencies in the peripheral blood reported during natural HIV infection in humans, providing for the first time an *in vivo* model relevant for studying human γδ T cell biology and γδ T cell-HIV interactions. We used this *in vivo* model to examine the therapeutic impact of adoptively transferred human Vδ2 T cells on cell-associated HIV transmission and replication (13, 14). Surprisingly, the adoptive transfer of allogenic Vδ2 T cells into BLT huMice enhanced, rather than controlled, HIV replication following cell-associated HIV transmission. This escalation in viral production was accompanied by a marked increase in HIV p24-positive Vδ2 T cells in the blood of BLT huMice, suggesting that the Vδ2 T cells may serve as early targets for HIV infection and replication.

MATERIALS AND METHODS

Construction of BLT HuMice

Non-Obese Diabetic. Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from the Jackson Laboratory and bred in the Division of Laboratory Animal Resources facility at the University of Pittsburgh. The mice were bred and housed under biosafety level 1, pathogen-free conditions according to the guidelines approved by the Institutional Animal Care and Use Committee and were fed irradiated chow (Prolab Isopro RMH 3000 Irradiated, catalog 5P75-RHI-W 22, PMI Nutrition International) and autoclaved water. Human fetal tissues were obtained from the Health Sciences Tissue Bank at the University of Pittsburgh and Advanced Bioscience Resources Inc and processed under biosafety level 2 conditions. Within 12 hours of receiving fetal human liver and thymus, CD34+ hematopoietic stem cells (HSCs) were isolated from the fetal liver as previously described (15) and cryopreserved at -170°C until transplantation. Portions of the fetal liver and thymus tissues were cut into small pieces (<3mm^3) and cryopreserved in Serum-Free Freezing Media (ATCC 30-2600) at -170 C until transplantation. 8 to 10-week-old NSG mice received a radiation dose of 1.50 Gray before transplantation to myoablate the animals and were immediately transferred to biosafety level 2+ animal housing. On the day of operation, the cryopreserved CD34+ HSCs and tissues from two different fetal donors were thawed in a warmed culture medium supplemented with 10% fetal bovine serum. The tissues were minced into ~1-mm^3 fragments, and the irradiated mice were anesthetized using 1.5-3% isoflurane. Autologous human fetal thymus and liver tissue sections were implanted under the kidney capsule, and 150,000 CD34+ HSCs were engrafted *via* retroorbital injection in a volume of 100 uL. Immediately following the procedure, the mice received 150uL injections of carprofen (1 mg/mL) and ceftiofur (1 mg/mL) as an analgesic and antibiotic, respectively. These injections continued once a day for two days for three sets of injections. Successful engraftment was determined by flow cytometric analysis of human CD45 expression on blood cells of mice, now termed BLT huMice. Mice harboring >30% of human $CD45^+$ cells were randomly assigned to groups in further experiments.

Study Participants

Specimens obtained from participants of the Multicenter AIDS Cohort Study (MACS), now the MACS/WIHS Combined Cohort Study (MWCCS), were used in this study. The contents of this publication are solely the responsibility of the authors and do not represent the official views of the funding sources. The authors express their sincerest gratitude to MWCCS Principal Investigators Dr. Charles R. Rinaldo and Dr. Jeremy Martinson (U01-HL146208), William G. Buchanan, and the participants of

the Pittsburgh site of the MWCCS. These participants were HIV-1 infected men who were on ART for a median duration of 12.08 years, who had a median $CD4^+$ T cell count of 620 cell/μl and a viral load of <50 copies/ml. Wherever mentioned, blood products from age-matched HIV-negative individuals were used in the study. Whole blood products from HIV-1-seronegative blood donors were purchased from the Central Blood Bank of Pittsburgh. Written informed consent was obtained from participants before inclusion in the study, which was approved by The University of Pittsburgh Institutional Review Board.

Isolation of Monocytes and Peripheral Blood Lymphocytes

Peripheral blood mononuclear cells (PBMC) were obtained from a buffy coat, or whole blood was isolated by standard density gradient separation using Lymphocyte Separation Medium (Corning). Monocytes were isolated from PBMC by positive magnetic bead selection (Miltenyi Biotec), and CD4+ T cells and γδ T cell subsets (refers to Vδ1 and Vδ2 T cells) were isolated by negative selection (EasySep CD4 T cell, Cat #-17952 and γδ T cell isolation kit, Cat #- 19255) according to the manufacturer's specifications, and the differentially isolated cells were cultured or cryopreserved until use

Flow Cytometry

50-100ul of blood was obtained from the submandibular vein of BLT huMice to check for reconstitution and intermediate infection time points. At 4-6 weeks post-HIV/mock infection, BLT huMice were sacrificed, and the entire blood volume was collected *via* orbital bleed. The murine spleen and the transplanted human spleen and thymus were dissected. Excised tissues were homogenized *via* mechanical dissociation, and single-cell suspensions were retrieved after tissue samples were passed through a 100um filter. Red blood cells were lysed and removed from both blood and spleen samples using ACK lysing buffer (Thermo Fisher) as described by the manufacturer before using samples for flow cytometry. Single-cell suspensions prepared from peripheral blood, splenocytes, and thymocytes from each BLT huMouse were stained with a live/dead fixable aqua dead cell stain kit (Thermo Fisher Scientific). For surface staining, cells were preincubated with 1× PBS labeling buffer containing 2% BSA, 0.1% NaN3, and unfractionated murine IgG (1.0 μg/mL; Sigma-Aldrich Cat# 15381-1MG) to block Fc-receptor binding. Then stained the cells with fluorochrome-conjugated antibodies [anti-human CD45, anti-human CD4, anti-human Vδ2, (BioLegend); anti-human CD8, CD3, PD1, HLA-DR, CD25, CD69, CD45RA, and CD27 (Becton Dickenson); and anti-human Vδ1, (Thermo Fisher Scientific)] and intracellular staining with HIV-p24 (KC57, Beckman Coulter). Cells were fixed using 2% paraformaldehyde, and data were acquired using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software. Gating was done based on Fluorescence minus one (FMO).

Immunohistochemistry

Paraffin-embedded fixed sections were stained with indicated anti-human antibodies (Anti-human TCR δ monoclonal IgG1 κ antibody, Clone H-41, catalog number sc-100289, Santa Cruz Biotechnology; Ultra-LEAF™ Purified Mouse IgG1, κ Isotype Control Antibody, catalog number: 401404, Biolegend). Immunoreactivity of indicated antibodies was determined by incubation with DAB substrate (MACH 2 Detection Kit, Biocare Medical) and counterstained with hematoxylin.

In Vitro Expansion of γδ T Cells

BLT huMice were sacrificed at 4-6 weeks post HIV/mock infection, and fully developed lymphoid tissues were collected, and single cells were isolated following mechanical dissociation. Homogenized spleen and thymus tissues were passed through a 100um filter to obtain single-cell suspensions. Red blood cells were lysed and removed from spleen samples using ACK Lysing Buffer (ThermoFisher) as described by the manufacturer. Cells isolated from the BLT huMice splenocytes were cocultured with allogeneic monocytes (4:1 ratio) from HIV-seronegative human blood bank donors in the presence of nitrogen-containing bisphosphonate zoledronate (ZOL, 5uM) (Zoledronic Acid, Selleckchem, S1314) and recombinant human (rh)IL-2 (Proleukin, 100 IU/mL; Prometheus Laboratories) for ten days as previously described (16). rhIL-2 (100 IU/ml) was subsequently added every three days. The ten-day-cultured γδ T cells were characterized by flow cytometry analysis.

HIV Infection of BLT HuMice

X4-tropic HIV lab strain NL4-3 (17, 18) was generated by transfection of 293T cells (ATCC; ATCC CRL-3216) with a plasmid containing a full-length HIV genome and collecting the HIV containing culture supernatant. The viral titer was determined by HIV-1 p24 AlphaLISA Assay (PerkinElmer, cat. No. AL291F) as described in the manufacturer's protocol (19). Supernatant from uninfected 293T cells was used as a mock control. BLT huMice were anesthetized at 20-22 weeks post-transplantation and inoculated with mock control supernatant or HIV-1 (~1×10^5 infectious units) by i.v. Injection *via* retroorbital delivery.

HIV-1 Genomic RNA Detection

Total RNA was purified from plasma using RNA-Bee (AMSBIO). The RNA was then reverse-transcribed using TaqMan Reverse Transcription Reagents (Invitrogen) and quantitatively detected by real-time PCR using the TaqMan Universal PCR Master Mix (Invitrogen) with primers (forward primer, 5′ - CCCATGTTTT CAGCATTATCAGAA - 3′, and reverse primer, 5′ - CCACTGT GTTTAGCATGGTGTTTAA - 3′) and detection probe targeting HIV Gag gene (5′ - AGCCACCCCACAAGA - 3′) (20). The assay sensitivity/cutoff was ten copies/ml.

Adoptive Transfer of T Cells to BLT HuMice

PBMC derived $CD4^+$ T cells were isolated from HIV-positive individuals using EasySep Human $CD4^+$ T Cell Isolation Kit and activated overnight with Human T-Activator CD3/CD28 Dynabeads (Life Technologies). The next day Dynabeads were separated from the $CD4^+$ T cells by manual dissociation followed by magnet isolation. The activated $CD4^+$ T cells were, washed,

resuspended in PBS, and adoptively transferred into BLT huMice *via* intraperitoneal injection (5 million cells/100µl/mouse). PBMC from the allogenic HIV non-infected donor were cultured in the presence of ZOL and rhIL-2 for ten days to expand the Vδ2 cells. Activated and expanded Vδ2 cells were enriched using gamma delta T cells EasySep negative selection kit (Catalog-19255). This pure gamma delta T cells were adoptively transferred to BLT huMice *via* intraperitoneal injection (10 million/100µl/mouse) at the same time point when $CD4^+$ T cells were injected. The BLT huMice were divided into two treatment cohorts; one that received only activated $CD4^+$ T cells from HIV-infected donor, and the other that received the activated HIV-infected $CD4^+$ T cells as well as *in vitro* expanded allogenic Vδ2 cells.

Statistics

Differences between HIV-infected/uninfected humans and BLT huMice were compared using the two-tailed unpaired Student t-test. Differences among the human or BLT huMice groups were compared using the two-tailed paired students t-test. The normality of the samples was tested using the Shapiro-Wilk normality test. Statistical analyses were performed using the Prism8 (GraphPad Software), and p values <0.05 were considered statistically significant. The sample numbers and statistical analyses used are specified in each figure legend.

Use of Human Fetal Tissue and Biological Agents

We described the approval of the use of human fetal tissue and biological agents in the previous study (21). Briefly, human fetal liver and thymus (gestational age of 18–20 weeks) were obtained from medically, or elective indicated termination of pregnancy through Magee-Women's Hospital of UPMC *via* the University of Pittsburgh, Health Sciences Tissue Bank, or Advance Bioscience Resources Inc. Written, informed consent of the maternal donors was obtained in all cases, under IRB of the University of Pittsburgh guidelines and federal/state regulations. See details in the "Human Ethical Approval and Informed Consent" section.

Approval for Using Animals and Biological Agents for *In Vivo* Experiments

The use of biological agents (e.g., HIV), recombinant DNA, and transgenic animals was reviewed and approved by the Institutional Biosafety Committee (IBC) at the University of Pittsburgh. All animal studies were approved by the IACUC at the University of Pittsburgh and were conducted following the NIH guidelines for housing and care of laboratory animals as well as the ARRIVE guidelines 2.0 for reporting of *in vivo* experiments involving animal research (22).

Human Ethical Approval and Informed Consent

The study was performed following the guidelines of "Ethical Principles for Medical Research Involving Human Subjects" provided by the World Medical Association Declaration of Helsinki (1964) and its subsequent amendments (23). Written informed consents were obtained from the human study participants from the Pittsburgh Men's Study, Multicenter AIDS Cohort Study (PMS-MACS) and the maternal donors of fetal tissues used in the study following the University of Pittsburgh IRB guidelines as well as federal/state regulations. The ethical use of human fetal organs/cells to perform the studies was reviewed before study initiation by the University of Pittsburgh IRB, which determined that the submitted study does not constitute human subject research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c), (e), and (l)]. The ethical use of human hematopoietic stem cells was reviewed and approved by the University of Pittsburgh Human Stem Cell Research Oversight (hSCRO) committee.

RESULTS

Reconstitution of Human γδ and αβ T Cells in BLT HuMice

We first examined the reconstitution of human αβ and γδ T cells in huMice using multicolor flow cytometry (**Figure 1**). Importantly, when denoting γδ T cells in our study, we are referring only to the Vδ1 and Vδ2 T cell subtypes, which together account for ~98% of the total γδ T cell population in human blood (24, 25). We validated the flow cytometry assay for detecting human γδ T cells by demonstrating the presence of

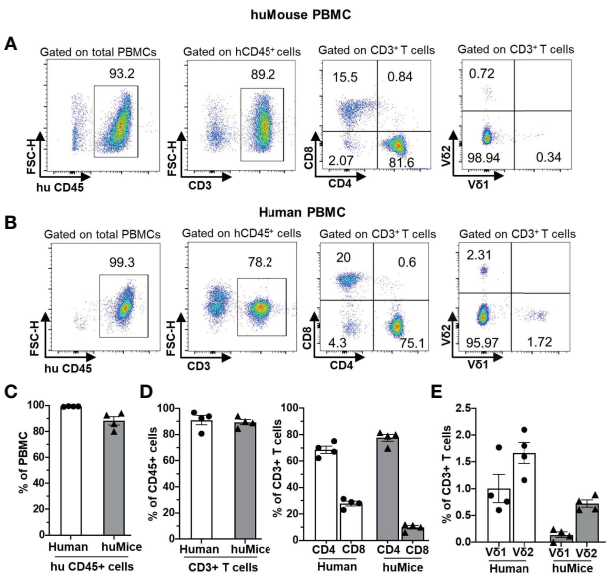

FIGURE 1 | Human αβ and γδ T cell development in the peripheral blood of BLT huMice. **(A, B)** Representative flow cytometry analysis of human immune cell ($hCD45^+$) reconstitution along with lymphocytes subsets, including αβ T cells ($CD3^+$), ($CD4^+$), ($CD8^+$), and γδ T cells (Vδ1 and Vδ2 T cell subsets) in PBMC of BLT huMice **(A)** at ten weeks after transplantation and uninfected human **(B)**. **(C–E)** Quantification of human $CD45^+$ lymphocytes **(C)**, human αβ **(D)**, and γδ **(E)** T cell subsets in PBMCs of BLT huMice and healthy humans (n = 4 biological replicates each).

γδ+ cells in the CD3+ population and the absence of γδ+ cells in the CD3- population of human CD45+ cells from human peripheral blood (**Supplementary Figures S1A–D**). The gating scheme is shown for a representative sample of PBMC derived from a BLT huMouse (**Figure 1A**). We compared these results to PBMC samples from HIV seronegative humans, with data from a representative donor is shown in **Figure 1B**. We observed a high level of reconstitution of human $CD45^+$ cells (~90%) in the peripheral blood of BLT huMice (**Figure 1C**). Approximately 90% of these human $CD45^+$ cells were $CD3^+$ T cells, of which, on average, were comprised of 80% $CD4^+$ T cells and 15% $CD8^+$ T cells (**Figure 1D**). This CD4/CD8 ratio was slightly higher than what is typically seen in humans, as shown with the four donors we tested that displayed a mean of 70% $CD4^+$ T cells and 30% $CD8^+$ T cells (**Figure 1D**). We also analyzed the γδ T cell subsets present in the peripheral blood of BLT huMice and determined a mean of 0.3% and 0.7% of total $CD3^+$ T cells being comprised of Vδ1 T cells and Vδ2 T cells respectively (**Figure 1E**). The relative frequencies of these two subsets are comparable to, albeit lower than, the γδ lymphocyte populations found in the peripheral blood of healthy humans represented in our analysis showing 1% and 1.6% of total $CD3^+$ T cells being Vδ1 and Vδ2 T cells, respectively (**Figure 1E**). To our knowledge, this is the first report to describe the reconstitution of human γδ T cells in BLT huMice. We also examined human immune cell populations reconstituted in the engrafted human thymus and murine spleen of each BLT huMouse (**Figure 2**). The gating scheme is shown for a representative sample of immune cells isolated from the human thymus (**Figure 2A**) and murine spleen (**Figure 2D**). Of the human $CD3^+$ T cells isolated from the thymic tissue, an average of 22% were $CD4^+$ T cells, 16% were $CD8^+$ T cells, and 60% had an immature T cell phenotype being positive for both CD4 and CD8 ($CD4^+/CD8^+$, double-positive) (**Figure 2B**). In the murine splenic tissue, on average, the total T cell population comprised 80% $CD4^+$ T cells and 16% $CD8^+$ T cells (**Figure 2E**). Human γδ T cell subsets (Vδ1 and Vδ2) were also detected in these lymphoid tissues. From the human thymus, an average of 1.5% of the T cells had a Vδ1 cell phenotype, and 0.2% were Vδ2 T cells (**Figure 2C**). We observed a slightly higher prevalence of γδ T cell subsets isolated from murine spleen tissue, with a mean of 2.2% and 0.9% of the total T cell fraction consisting of Vδ1 T cells and Vδ2 T cells respectively (**Figure 2F**). We observed that Vδ2 T cells were predominantly present in the peripheral blood of BLT huMice (**Figure 1E**), while Vδ1 T cells were present primarily in the lymphoid tissues of BLT huMice (**Figures 2C, F**). The murine spleen of the BLT huMouse (hereafter referred to as the "humanized spleen") had an approximate 2-fold higher reconstitution of γδ T cells than what was found in the thymus. This overall distribution of γδ T cell subsets (Vδ1 and Vδ2) in BLT huMice is comparable to

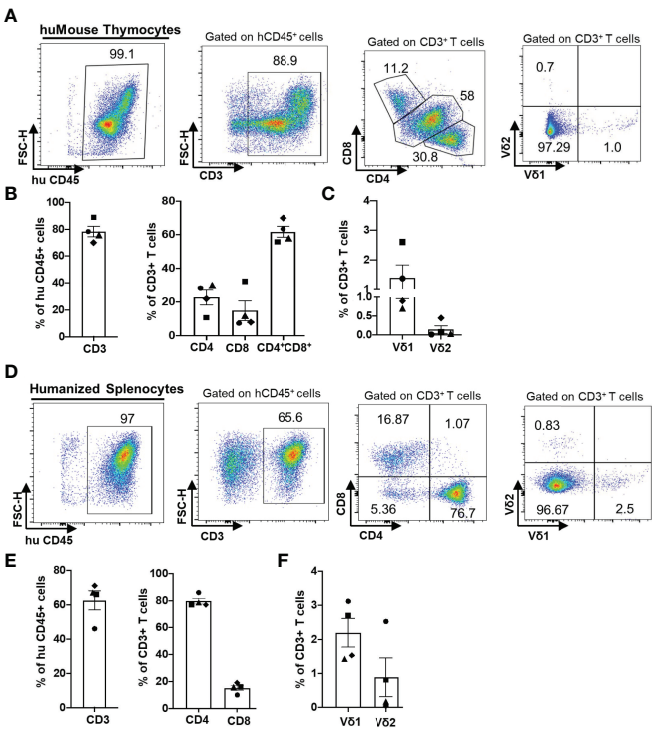

FIGURE 2 | Human αβ and γδ T cell development in lymphoid tissues of a BLT huMouse model. **(A, D)** Representative flow cytometry analysis of human immune cell ($hCD45^+$) reconstitution along with lymphocytes subsets including αβ T cells ($CD3^+$), ($CD4^+$), ($CD8^+$) and γδ T cells (Vδ1 and Vδ2 T cell subsets) in lymphoid tissue [thymus **(A)** and murine spleen **(D)**] of BLT huMice **(A)** at 22 weeks post-transplantation. Quantification of human αβ and γδ T cells in the engrafted human thymus **(B, C)** and murine spleen tissue **(E, F)** of BLT huMice at 22 weeks post-transplantation (n = 4 biological replicates).

those in human peripheral blood and tissue (26, 27). Lastly, we validated the flow cytometry-based detection of human γδ T cells in the lymphoid tissues in the BLT huMouse model *via in-situ* detection using immunohistochemistry (**Supplementary Figure S2**). In summary, these findings demonstrated that BLT huMice sustains physiologically relevant proportions of human αβ and γδ T cells in the periphery, engrafted human thymus, and (humanized) murine spleen.

HIV Infection Alters γδ T Cell Populations in BLT HuMice and Humans

To investigate the impact of HIV infection on γδ (referring to Vδ1 and Vδ2 T cell subsets) and αβ T cell populations, we infected BLT huMice with a laboratory strain of HIV-1_{NL4-3}. Consistent with the previous studies, HIV RNA copies were detected in the peripheral blood of the HIV-infected BLT huMice as early as two weeks post-infection (**Figure 3A**) (12, 21, 28). PBMC from mock-inoculated and HIV-infected BLT huMice were collected before and after HIV infection for further viral load analysis, and these mice were sacrificed for tissue collection four weeks after infection. We first determined the proportion of γδ T cells present in PBMC of HIV-infected and mock-infected BLT huMice before and after HIV infection. Representative flow cytometry analysis plots displaying the percentage of γδ T cells present at pre-and post-infection time points are shown in **Figure 3B**. The total proportion of γδ T cells increases in both mock-infected [p=0.009] and HIV-infected BLT huMice (p=0.001) compared to pre-infection levels, but HIV-infected BLT huMice exhibited 2.3-fold higher levels of total γδ T cells when compared to mock-infected BLT huMice (p=0.009) (**Figure 3C**). We further examined γδ T cell subsets and found that Vδ2 T cell proportions were higher before infection and lowered following infection in BLT huMice (**Figure 3D**). The altered proportion of γδ T cell subsets may be at least partially explained by the depletion of Vδ2 T cells in HIV-infected BLT huMice, though our values did not reach statistical significance (p=0.25) (**Figure 3E**). Contrary to HIV-infected BLT huMice, mock-inoculated BLT huMice exhibited an increase in Vδ2 T cell levels in the blood (**Figure 3E**), which suggests that Vδ2 cells are depleted in HIV infection. Furthermore, depletion of peripheral blood $CD4^+$ T cells in HIV-infected BLT huMice significantly decreases the $CD4^+/CD8^+$ T cell ratio (p =0.049) (**Figure 3F**). These results are consistent with what has been previously reported in human γδ T cell studies (29, 30). We observed similar γδ T cell trends in PBMCs isolated from healthy and ART treated HIV-positive individuals; wherein HIV-positive donors with ART had higher Vδ1 T cell levels and slightly lower Vδ2 T cell levels than healthy donors (**Figure 3G**).

In HIV-positive humans, lymphoid tissues are known to be sanctuaries for the latent HIV reservoir during ART (31). Therefore, we assessed the impact of HIV infection on the lymphocytes derived from lymphoid tissues of BLT huMice by flow cytometry analysis. A representative gating strategy used for this analysis is shown in **Figure 2**. Although not statistically significant, we observed an approximately 3-fold increase in the frequency of Vδ1 T cells in the human thymus (p = 0.058), and an approximately 2-fold increase in the humanized spleen of HIV-infected BLT huMice (p = 0.065) when compared to respective tissues from mock-infected BLT huMice (**Figures 4A, B**, **Supplementary Figures S3A, B**). This suggests that the

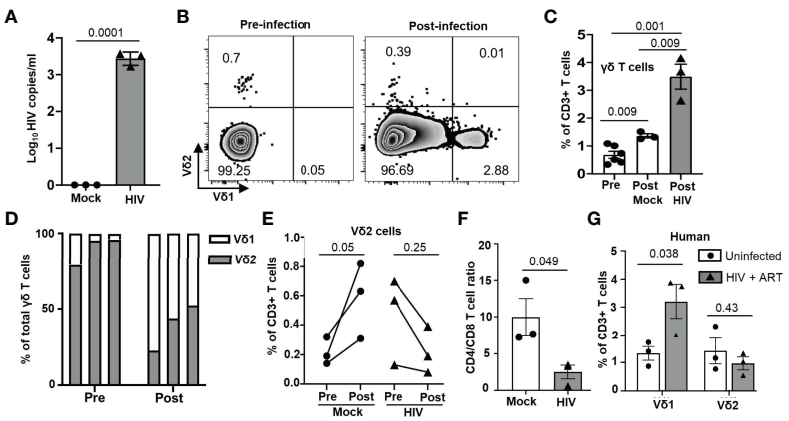

FIGURE 3 | Peripheral blood γδ T cell number is altered in HIV-infected BLT huMice and humans. **(A)** HIV-1 replication (HIV RNA genome copies per ml) in the blood following HIV_{NL4-3} inoculation at 1X 10^5 IU per mouse measured by qPCR (n = 3 biological replicates per group). **(B)** Representative flow plot showing the change in frequency of peripheral blood γδ T cell subsets before and after HIV infection. **(C)** Frequency of total γδ T cells before and after HIV infection in mock and HIV-infected BLT huMice analyzed by flow cytometry (n = 3 biological replicates per group). **(D)** Graphical representation of the change in frequencies of Vδ1 and Vδ2 cells within γδ population before and two weeks after infection. **(E)** Quantitation of changes in Vδ2 T cell frequency before and two weeks after infection in peripheral blood of HIV-infected and non-infected BLT huMice. **(F)** Comparison of changes in $CD4^+/CD8^+$ T cell ratio in peripheral blood of HIV-infected and non-infected BLT huMice analyzed by flow cytometry. **(G)** The frequency of Vδ1 and Vδ2 T cell subsets in the peripheral blood of ART-treated HIV-positive and HIV-negative individuals were analyzed by flow cytometry. Data are presented as a mean value ± SEM. P values <0.05 were considered statistically significant. P values were determined using paired 2-tailed Student's t-test for comparing changes in γδ T cells population within the same cohort at two different time points, whereas an unpaired, 2-tailed Student's t-test was used to compare differences between 2 groups.

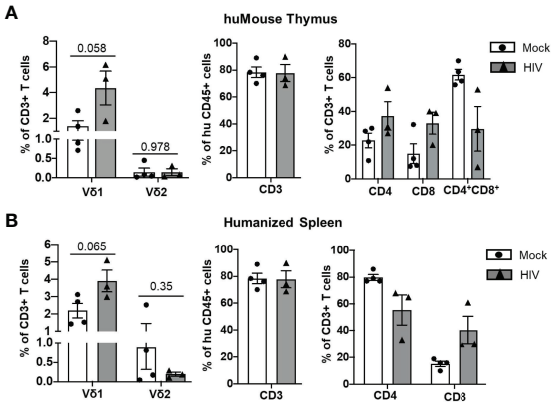

FIGURE 4 | T cell number is altered in lymphoid tissue of HIV-infected BLT huMice. **(A, B)** Quantification of human T cell subsets, γδ T cells and αβ T cells in human thymus and humanized spleen tissue of HIV-infected (n = 3 biological replicates) and non-infected (n = 4 biological replicates) BLT huMice at 4-6 weeks post-infection. Data are presented as mean values ± SEM. P values <0.05 were considered statistically significant as determined using an unpaired, 2-tailed Student's t-test.

frequency of Vδ1 T cells is increased in the lymphoid tissue of BLT huMice during HIV infection. We did not find a significant difference between the Vδ2 T cell population frequencies derived from the lymphoid tissues of HIV-infected or mock-infected BLT huMice. Besides γδ T cells, we found approximately a 2-fold increase in the proportion of CD8+ T cells derived from thymus and humanized spleen tissue of HIV-infected BLT huMice as compared to the mock-inoculated mice, suggesting a rapid proliferation of cytotoxic T cells in response to HIV infection (**Figures 4A, B**, **Supplementary Figures S3C, D**).

HIV Infection in BLT HuMice and Humans Impairs Vδ2 T Cells Responsiveness to Stimuli

To demonstrate the *ex vivo* responsiveness of Vδ2 T cells to activation factors and their potential for therapeutic evaluation, we cultured leukocytes derived from splenocytes of BLT huMice (n = 6), peripheral blood of ART-suppressed HIV-positive (*n* = 5), and age-matched HIV-negative individuals (*n* = 4) and stimulated them with the combination of ZOL and recombinant human Interleukin-2 (rhIL-2). The basal percentage of Vδ2 cells within the $CD3^+$ population of lymphocytes was analyzed by flow cytometry, which revealed a range of inter-individual differences among HIV-negative donors (1.2% - 2.2%), ART-suppressed HIV-positive individuals (0.5% - 1.2%), and BLT huMice (0.2% - 1%). Initially, when we cultured Vδ2 T cells from the peripheral blood or the lymphoid tissues of BLT huMice in the presence of ZOL and rhIL-2, we observed modest expansion of Vδ2 T cells, but it was not optimal. Next, we supplemented the cultures with allogenic monocytes from healthy individuals and obtained higher expansion of Vδ2 T cells. Our results show that Vδ2 T cell expansion from splenocytes of mock-inoculated BLT huMice after ten days was approximately 4-fold higher than HIV-infected BLT huMice (p=0.013) (**Figures 5A, B**). Similarly, we expanded Vδ2 T cells from HIV-positive and HIV-negative individuals and found that Vδ2 T cell expansion was approximately 3-fold higher in HIV-negative individuals than HIV-positive individuals (p=0.001) (**Figures 5C, D**). These results suggest that HIV infection not only reduces the frequency of Vδ2 T cells *in vivo* but it also adversely impacts the ability of these cells to expand in response to stimuli.

The Phenotype of Ex-Vivo Expanded Vδ2 T Cells

The phenotype of expanded Vδ2 cells after ten days of exposure to ZOL and rhIL-2 was analyzed in a subgroup of HIV-positive/HIV-negative individuals and HIV-infected/uninfected BLT huMice by measuring the expression of markers of activation and differentiation by flow cytometry (**Figure 6A**). Surface expression of the inhibitory receptor PD-1 was observed in a mean of 78% and 45% on the cultured Vδ2 cells derived from HIV-infected and uninfected BLT huMice, respectively (p=0.04) (**Figure 6B**). Similarly, the mean percentage of Vδ2 cells expressing PD-1 from HIV-positive and HIV-negative human donors was respectively 40% and 20% (p=0.001) (**Figure 6B**). The activation markers CD69 and CD25 were co-expressed on a mean of 80% and 65% of the Vδ2 cells cultured from HIV-infected and uninfected BLT huMice, respectively. Similarly, CD69 and CD25 co-expression was observed in a mean of 50% and 25% of the Vδ2 T cells from HIV-positive and HIV-negative human donors, respectively (**Figure 6C**). Together, these findings suggest that the expression of activation markers on Vδ2 cells expanded *in vitro* are slightly higher in those derived from HIV-positive humans and BLT huMice than from their HIV-negative counterparts. We also evaluated the differentiation status of the cultured Vδ2 cells based on memory cell phenotypes defined as follows: (CM) central memory ($CD45RA^-CD27^+$), (TDM) terminally differentiated ($CD45RA^+CD27^-$) and (EM) effector memory ($CD45RA^-CD27^-$). Although not statistically significant, we noted an increase in the TDM phenotype and a decrease in the CM and EM phenotypes in the *in vitro* expanded Vδ2 T cells derived from HIV-infected BLT huMice compared to the Vδ2 cells cultured from uninfected BLT huMice (**Figure 6D**).

However, in humans, we found an approximately equal distribution (20-30%) of EM, CM, TDM phenotypes between HIV-positive and HIV-negative individuals (**Figure 6E**).

Adoptive Transfer of Vδ2 T Cells Did Not Control Cell-Associated HIV Transmission and Replication in BLT HuMice

Many *in vitro* studies have demonstrated a protective role of γδ T cells against HIV infection (8, 9, 32). Therefore, we tested the impact of adoptively transferred allogeneic Vδ2 cells in an *in vivo* model of cell-associated HIV transmission and replication using BLT huMice, which is physiologically relevant to HIV transmission in humans. As discussed above, *in vitro* expansion of Vδ2 T cells from HIV-infected individuals was not optimal. We overcame this limitation in our adoptive transfer experiment by utilizing Vδ2 T cells expanded from allogeneic non-infected individuals. A similar strategy was previously demonstrated to be safe and effective in humans (33). Moreover, it is therapeutically relevant because Vδ2 T cells lack functional MHC restriction and pose a minimal risk for developing graft-versus-host complications (34). However, they may serve as targets for an allogeneic response by the engrafted immune cells, albeit graft versus host disease (i.e., alopecia) was not observed during the short duration of this experiment.

BLT huMice were grouped into two different cohorts: one cohort received only activated $CD4^+$ T cells from an HIV-infected human donor (CD4-only cohort) to mimic cell-associated HIV transmission (13, 14), while the other cohort received simultaneous injections of activated $CD4^+$ T cells from an HIV-infected human donor and cultured activated allogenic Vδ2 cells from an uninfected human donor (CD4+Vδ2 cohort). Before the adoptive transfer of CD4 and Vδ2 T cells, we assessed the human immune cell reconstitution in all the BLT huMice, and we observed approximately similar levels of $huCD45^+$ cells and $huCD4^+$ T cells in all the BLT huMice (**Supplementary Figure S4**). Reconstitution of human Vδ2 and $CD4^+$ T cells in the peripheral blood of BLT huMice was examined *via* flow cytometry two weeks after the adoptive transfer procedure. We found that a mean of 50% of all $CD3^+$ T cells was Vδ2 T cells in the peripheral blood of CD4+Vδ2 cohort, whereas less than 1% of all $CD3^+$ T cells were Vδ2 T cells in CD4-only cohort (p=0.03) (**Figure 7A**), which indicated successful engraftment of human Vδ2 T cells in the BLT huMice. Next, we confirmed HIV replication in the plasma of BLT huMice by qPCR two weeks after adoptive transfer. Surprisingly, we observed a viral load in the CD4+Vδ2 cohort was approximately 2-fold higher than the CD4-only cohort (**Figure 7B**) (p=0.042). Hypothesizing that this increase in viral load could be due to HIV-infection of the adoptively transferred Vδ2 T cells, we decided to analyze the $CD4^+$ T cells and Vδ2 T cell subsets in the peripheral blood of both cohorts at two weeks post-adoptive transfer. Representative flow cytometric plots of HIV p24 levels in total $CD4^+$ T cells and Vδ2 T cells from both cohorts are shown in **Figure 7C**. We observed a slightly higher presence of HIV p24 in total $CD4^+$ T cells (p=0.025) and Vδ2 T cells (p=0.10) in the CD4+Vδ2 cohort of BLT huMice compared to the reference CD4-only cohort (**Figure 7D**). Therefore, the adoptive transfer of Vδ2 T cells appears to exacerbate HIV replication in BLT huMice (**Supplementary Figure S5**). Moreover, *in vitro* co-culture of

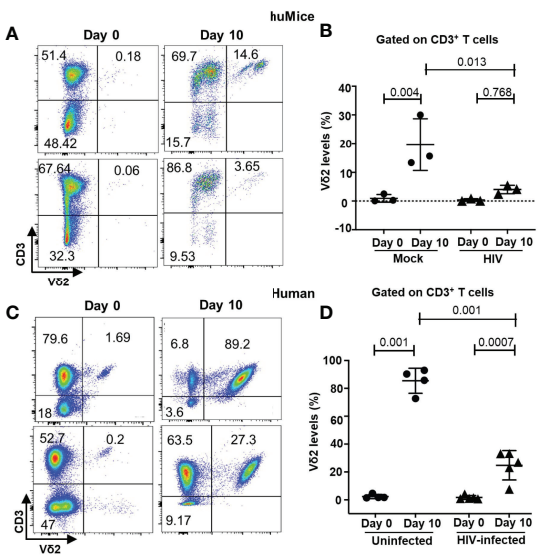

FIGURE 5 | HIV infection impairs the *ex vivo* expansion of Vδ2 T cells. **(A, B)** BLT huMice were sacrificed at 4-6 weeks post-HIV/mock infection, and splenocytes isolated from the humanized spleen of BLT huMice were cultured in the presence of zoledronate IL-2, and uninfected allogenic monocytes (n = three mice per group). **(C, D)** Flow plots represent *in vitro* expansion of Vδ2 cells from HIV-infected and non-infected individuals in the presence of zoledronate and IL-2. Expansion of Vδ2 cell frequency was significantly higher in HIV-negative donors (n = 4) compared to HIV-positive donors (n = 5 biological replicates). Data are presented as mean values ± SEM. P values <0.05 were considered statistically significant as determined using a 2-way ANOVA test.

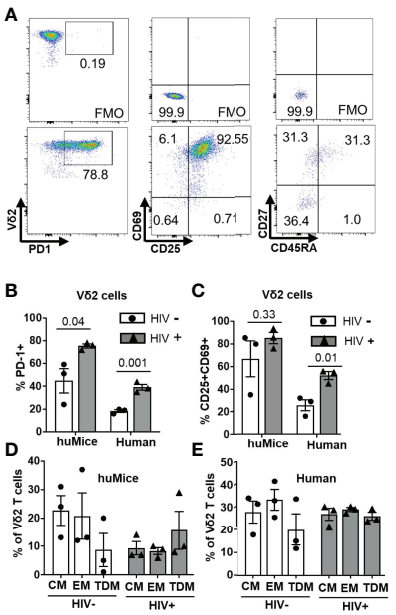

FIGURE 6 | Phenotypic characterization of cultured Vδ2 cells. The phenotype of Vδ2 cells from 6 BLT huMice and 6 HIV-positive/negative individuals after the expansion was analyzed by flow cytometry. **(A)** Representative flow cytometry analysis of expanded Vδ2 cells from splenocytes of humanized mice expressing activation, inhibitory, and differentiation markers. **(B)** Expression of the checkpoint inhibitory marker PD-1 on Vδ2 cells expanded from HIV-infected and non-infected BLT huMice and humans. **(C)** Dual expression of activation markers CD69 and CD25 on Vδ2 cells expanded from HIV-infected and non-infected BLT huMice and humans. **(D)** Percentage of Vδ2 cells defined as central memory (CM) ($CD45RA^{-}CD27^{+}$), terminally differentiated (TDM) ($CD45RA^{+}$ $CD27^{-}$), and effector memory (EM) ($CD45RA^{-}CD27^{-}$) derived from HIV-infected and non-infected BLT huMice. **(E)** Percentage of Vδ2 cells derived from HIV-positive and HIV-negative individuals defined as having EM, CM, TDM phenotypes. Data are presented as mean values ± SEM. P values were determined using two-tailed unpaired t-tests between the two groups.

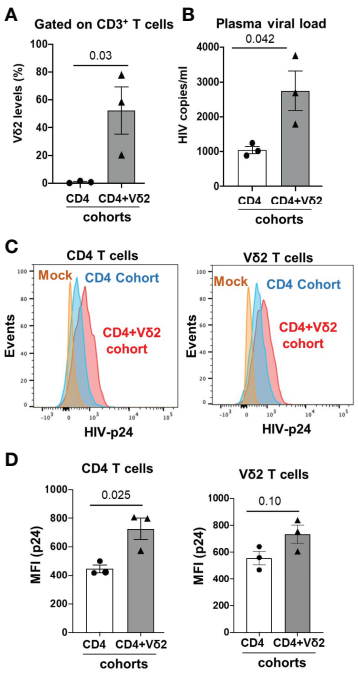

FIGURE 7 | Adoptive transfer of Vδ2 T cells increases HIV replication in cell-associated HIV transmission in BLT huMice. **(A)** Vδ2 cell number significantly increased 2-weeks post-adoptive transfer in peripheral blood of BLT huMice (n = 3 biological replicates per group); analyzed by flow cytometry. **(B)** HIV viral load increased significantly in plasma of Vδ2+CD4-engrafted BLT huMice compared to CD4-engrafted BLT huMice; measured *via* qPCR at two weeks post-adoptive transfer (n = 3 biological replicates per group). **(C)** Representative flow cytometry histogram plots of peripheral blood total $CD4^{+}$ T cells and Vδ2 cells expressing HIV p24 respectively. **(D)** HIV p24 is slightly higher in peripheral blood total $CD4^{+}$ T cells and Vδ2 T cells of BLT huMice that received CD4+Vδ2 treatment compared to the BLT huMice that received only $CD4^{+}$ T cells treatment respectively (n = 3 biological replicates per group). Data are presented as mean values ± SEM. P values were determined using a two-tailed paired t-test within the treatment groups.

HIV-infected $CD4^{+}$ T cells with Vδ2 T cells also suggests that in the presence of Vδ2 T cells, HIV infection increased, and they failed to limit the viral replication (**Supplementary Figure S5**). Additionally, we analyzed the blood and lymphoid tissue associated viral load at four weeks post-adoptive transfer and found no significant difference in the viral levels, which suggests that viral replication plateaued at this time point in BLT huMice of both the cohorts **(Supplementary Figure S6).**

Despite the low or lack of CD4 receptor expression on Vδ2 T cells, our *in vivo* data suggest that these cells can be targets of HIV infection. This is in accordance with a previous study from Sarabia et al., which reported that resting Vδ2 cells act as reservoirs for latent HIV infection (35). We posited that HIV infection could impact the phenotype of Vδ2 T cells to make them more susceptible to direct infection. Since Vδ2 T cells already express high levels of the CCR5 co-receptor, we examined whether the expression of the CD4 receptor on Vδ2 T cells was induced on this cell type during HIV infection. Before adoptive transfer, less than 5% of endogenous (**Figure 8A** and *in-vitro* cultured Vδ2 T cells **(Supplementary Figure S7)** expressed the CD4 receptor, but at two weeks after adoptive transfer, we indeed detected a mean of 30% of Vδ2 T cells expressing the CD4 receptor in both the cohorts (**Figures 8A, B**). Contrary to the previous reports (9, 36) highlighting the protective function of Vδ2 T cells in controlling HIV infection *in vitro*, our result suggests that HIV infection can drive CD4 expression on Vδ2 T cells *in vivo*, priming them to become targets for HIV infection and contributors to viral dissemination.

DISCUSSION

γδ T cells are the first line of defense against many pathogens, but their frequency and functions are severely altered in the setting of many infectious diseases, including HIV (7). Despite long-term ART and viral control, γδ T cells do not reconstitute HIV-infected individuals to their levels set before infection (30). However, in HIV elite controllers, Vδ2 T cell numbers are maintained at normal levels throughout infection, implying

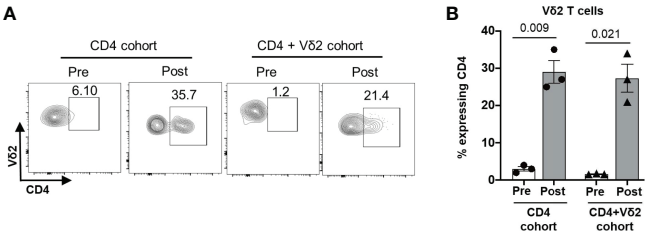

FIGURE 8 | Induction of CD4 expression on Vδ2 T cells *in vivo* during HIV infection. $CD4^+$ T cells from an HIV-positive individuals were administered to BLT huMice with or without co-transfer of *in vitro* activated Vδ2 T cells. **(A)** Representative flow cytometry analysis of CD4 expression on Vδ2 T cells before and after cell transplant. **(B)** CD4 expression on human Vδ2 T cells from the BLT huMice was measured by flow cytometry analysis pre-and post- (2 weeks) cell transplant. Data are presented as mean values ± SEM. P values were determined using a two-tailed paired t-test within the treatment groups.

that Vδ2 T cells play an essential role in HIV infection and control. Thus, a better understanding of Vδ2 T cells during HIV infection will be necessary to be effectively utilized or targeted for therapeutic benefit. While prior studies have demonstrated the protective effect of γδ T cells against HIV infection *in vitro* (8, 9, 32), there is a lack of information available and a gap in knowledge regarding their therapeutic potential *in vivo*.

In this study, we offer the first evidence that clinical trends of γδ T cell subpopulations (Vδ1 and Vδ2) before and after HIV infection can be modeled in BLT huMice. Immunodeficient NSG mice exhibited robust reconstitution of human immune cells, including γδ T cells, by 12 weeks post-engraftment of $CD34^+$ human fetal liver cells and thymic tissues. Flow cytometric analysis of human T cell subsets revealed that $CD4^+$, $CD8^+$, Vδ1, and Vδ2 T cell levels in both the blood and lymphoid tissues of healthy BLT huMice were comparable to those seen in healthy humans. Furthermore, we observed high levels of viremia two weeks following HIV infection, an associated depletion of Vδ2 T cells, and an expansion of Vδ1 T cells in the peripheral blood of BLT huMice. These features have been previously reported in several clinical studies (37–39). Thus, BLT huMice may overcome some of the translational limitations in non-human primate SIV models, which include unremarkable changes in Vδ1/Vδ2 T cell ratios, otherwise common in HIV infection in humans. Our study demonstrating the *in vivo* reconstitution of Vδ2 T cells in the BLT huMouse model also provides a proof-of-concept and basis for the design of future *in vivo* studies that further evaluate the role of human γδ T cells in the setting of HIV infection as well as other chronic diseases such as cancer.

Current HIV cure strategies utilize the effector functions of conventional CD8+ cytotoxic T cell lymphocytes (CTL) to kill the HIV-infected cellular reservoir following the induction of latency reversal (40). Unfortunately, the need to specifically stimulate or target the activation of autologous HIV-antigen specific autologous $CD8^+$ T cells *ex vivo* or *in vitro* on an individual MHC/peptide-specific level and the existence of HIV CTL escape variants within the latent reservoir has challenged the progress of this approach (41, 42). γδ T cells offer an attractive alternative to CTL as a potential therapeutic tool to mediate anti-HIV effector functions. Their lack of MHC restriction may provide added benefits by raising the threshold for HIV to achieve immune escape. Moreover, since they pose a reduced risk of inducing allogeneic graft rejection, they may be considered for application in allogeneic immunotherapy settings. A previous study has shown that γδ T cells mediate inhibition of HIV replication (2), but the natural scarcity of γδ T cells in tissues and circulation indicates that these cells would likely need to be expanded *ex-vivo* for them to have the intended therapeutic effect. Although there are numerous *in vitro* protocols for expanding γδ T cells from bulk PBMC, two major approaches can be considered for targeting γδ T cells for clinical translation. First, both Zoledronic Acid (ZOL) and rhIL-2 can be administered to directly increase the proliferation of endogenous Vδ2 T cells (43). The other approach would be ex-vivo activation and expansion of Vδ2 T cells for adoptive therapy. In the HIV setting, this approach is limited by the substantial loss of Vδ2 T cells during the early stages of the infection cycle, which fail to fully recover after ART initiation. An alternative would be to harvest Vδ2 T cells from healthy donors and expand them *in vitro* using ZOL and rhIL-2 for allogeneic delivery, as has been previously reported in human cancer clinical trials (33, 44) and non-human primate models (45). One of these cancer trials demonstrated that the adoptive transfer of haploidentical expanded Vδ2 T cells from relatives of cancer patients was safe and effective for achieving meaningful responses (33). We attempted to culture and expand Vδ2 T cells derived from PBMC and lymphoid tissue of BLT huMice using ZOL and rhIL-2. Unfortunately, while we could expand these BLT huMice derived cells *in vitro*, we could not collect and generate an adequate number to carry out *in vivo* studies using this method. However, when we supplemented the cultures with allogeneic monocytes from healthy individuals to enhance ZOL-induced phosphoantigen presentation, we achieved a 20-fold increase in Vδ2 T cell expansion. Importantly, this was the first reported evidence that Vδ2 T cells derived from the splenocytes of BLT huMice can indeed be expanded *in vitro*.

Our pilot study examined the therapeutic potential of adoptively transferred Vδ2 T cells in HIV infection of BLT huMice during cell-associated HIV transmission using CD4+ T cells isolated from ART-treated HIV-positive individuals. Cell-associated HIV transmission is a widely reported means

of HIV infection (13, 14). Furthermore, laboratory-derived molecular clones of HIV infection can exhibit different characteristics compared to naturally derived HIV strains (46). Although previous *in vitro* studies described the protective effect of γδ T cells against HIV infection (8, 9, 32), we did not see a therapeutic benefit, namely suppression of viremia, with the delivery of Vδ2 T cells in BLT huMice. Treatment with the activated Vδ2 cells resulted in higher viremia at two weeks post-infection as compared to the HIV-infected BLT huMice that were not co-engrafted with the Vδ2 T cells. Our findings were limited by the study's sample size and low γδ T cell yield from the blood of BLT huMice, but future experiments will focus on the mechanisms of interactions between HIV and Vδ2 T cells. The ability to expand Vδ2 T cells from the murine spleen of BLT huMice provides an additional reservoir of cells for understanding HIV-associated activation or dysregulation of this cell type. Our adoptive transfer experimental data suggest that during the early stages of HIV infection, Vδ2 T cells can transiently upregulate the surface expression of CD4. This is in accordance with a previous study showing that the long-term culture of Vδ2 T cells in the presence of IL2 resulted in CD4 expression *in vitro* (35). Though we see a modest trend in our *in vitro* culture of Vδ2 T cells before the adoptive transfer, CD4 expression in Vδ2 T cells was unexpectedly more pronounced two weeks after transplantation into BLT huMice. While the mechanisms involved remain the subject of future studies, we speculate that this induction of CD4 expression on Vδ2 T cells may be a general inflammatory event in the host, triggered during some viral infections, a phenomenon which has also been noted to occur during COVID-19 infection (47). Moreover, expanded Vγ9Vδ2 T cells can produce pro-inflammatory cytokines that can potentially activate HIV replication. However, we cannot rule out the possibility that allogenic responses induced in the BLT huMice post-adoptive transfer may contribute to the increased HIV viremia associated with the delivery of Vδ2 T cells. Future methods may include introducing fluorescent tags to Vδ2 T cells and fetal HSCs before engraftment for tracking the proliferation, trafficking, or death of Vδ2 T cells during infection. Nevertheless, these findings raise more questions about the role of γδ T cells in the initial sequelae of HIV infection and their potential contribution to the HIV cellular reservoir, as has been previously reported (35).

To our knowledge, this is the first report demonstrating that functional human γδ T cells can be robustly reconstituted in a BLT huMice model. This small animal model provides a platform for future mechanistic studies to explore interactions between HIV and T cell subsets and, more broadly, for *in vivo* evaluation of γδ T cells and γδ T cell-based therapies in the setting of various human diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Biosafety Committee (IBC) at the University of Pittsburgh.

AUTHOR CONTRIBUTIONS

SB, MB, and RM contributed to the experimental and study design. SB and YA performed the experiments. SB analyzed the data and prepared the manuscript. YA, RM, MB, and ML contributed to interpreting the results and critically edited the manuscript. All authors contributed to the article and approved the submitted 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.2022.881607/full#supplementary-material

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REVIEW
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γδ T Cells in Brain Homeostasis and Diseases

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γδ T cells are a distinct subset of T cells expressing γδ T cell receptor (TCR) rather than αβTCR. Since their discovery, the critical roles of γδ T cells in multiple physiological systems and diseases have been investigated. γδ T cells are preferentially located at mucosal surfaces, such as the gut, although a small subset of γδ T cells can circulate the blood. Additionally, a subset of γδ T cells reside in the meninges in the central nervous system. Recent findings suggest γδ T cells in the meninges have critical roles in brain function and homeostasis. In addition, several lines of evidence have shown γδ T cells can infiltrate the brain parenchyma and regulate inflammatory responses in multiple diseases, including neurodegenerative diseases. Although the importance of γδ T cells in the brain is well established, their roles are still incompletely understood due to the complexity of their biology. Because γδ T cells rapidly respond to changes in brain status and regulate disease progression, understanding the role of γδ T cells in the brain will provide critical information that is essential for interpreting neuroimmune modulation. In this review, we summarize the complex role of γδ T cells in the brain and discuss future directions for research.

Keywords: γδ T cell, central nervous system, brain, neuroimmunology, brain diseases

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INTRODUCTION

γδ T cells are a subset of T cells expressing γδ T cell receptor (TCR) rather than αβTCR. γδ T cell was named after discovery of the γ gene in 1984 (1, 2). Initially, γδ T cells were understudied because they constitute a very minor portion of immune cells and are heterogenous. However, recent studies have emphasized the importance of γδ T cells in a number of diseases. Despite some exceptions, γδ T cells are unrestricted to major histocompatibility complex (MHC) and considered innate immune cells (3). In general, the fate of γδ T cells is already programmed from the thymus, and they do not require complex activation mechanisms (3, 4). Therefore, γδ T cells are rapidly recruited and respond to inflammatory cues. Moreover, γδ T cells regulate adaptive immune responses (5), indicating they are an important bridge connecting innate and adaptive immunity.

γδ T cells are found predominantly at mucosal surfaces rather than lymphoid organs (6). Under steady states, they regulate homeostasis and maintain barrier integrity. Upon infection, they are rapidly activated and regulate immune responses. $V\gamma5^+$ dendritic epidermis T cells [DETCs; Tonegawa nomenclature (7)] reside in the skin, $V\gamma7^+$ cells reside in the gut and form

intraepithelial cells (IELs), and Vγ6$^+$ cells are found in the dermis, vagina, and meninges. Vγ4$^+$ T cells have also been observed in the dermis and lung. On the other hand, Vγ1$^+$ and Vγ4$^+$ T cells, which develop after birth, circulate in the blood or lymphatic fluid (6). In humans, Vδ1$^+$ cells usually reside in the mucosal area and Vδ2$^+$ T cells are circulating cells, although there are tissue-resident Vδ2$^+$ T cells and circulating Vδ1$^+$ T cells (8, 9). Although γδ T cells are generally similar across species, murine and human γδ T cells have notable differences (10). Due to the complexity and differences between mouse and human γδ T cells, their investigation is very difficult. For example, classification of murine γδ T cells is dependent on γ chains, whereas human γδ T cells are classified by δ chains (8). In addition, homologous cells for murine Vγ5$^+$ DETCs have not been detected in humans (11). Therefore, many aspects of γδ T cell biology remain unclear and further studies are urgently needed to understand their role in immune system function.

Although most mucosal barriers are in contact with the outside and exposed to potential pathogens, meninges are sterile because they encounter the inner side of the central nervous system (CNS) (12). Classically, the CNS has been regarded as an immune privileged organ. A study showed allografts in the CNS were not rejected, unlike allografts in the skin (13). Though circulating immune cells are strongly restricted to enter parenchyma, recent studies re-discovered meningeal lymphatics that drain waste, including CNS antigens (14, 15). Interestingly, antigen presentation in the meningeal spaces and CNS-draining lymph nodes occurs actively (16). Thus, our immune system actually surveils the CNS. However, there are many things concerning the role of the immune system in the CNS that remain elusive. Surprisingly, current data have shown that various immune cells reside or circulate in the meninges (17). Meningeal cytokines interact with parenchymal neurons, astrocytes, or microglia, though the exact mechanisms underlying these interacts are incompletely understood. Meninges-parenchyma interactions regulate multiple neurological functions under homeostasis (18). In addition, meningeal lymphatics and immune system rapidly respond to CNS status and regulate pathology of neurodegenerative diseases and neuroinflammation. γδ T cells are among the multiple immune cells that reside in meninges (19). Recent studies showed meningeal γδ T cells regulate memory formation and behaviors *via* cytokine release (19, 20). Furthermore, parenchymal infiltration and the immunological role of γδ T cells in multiple CNS diseases, including experimental autoimmune encephalomyelitis (EAE), CNS tumors, and infections, have been discovered (8, 21). Because γδ T cells serve as a "safeguard" for the mucosal barrier, γδ T cells are expected to have an indispensable role in the meninges. However, the exact mechanisms concerning how γδ T cells act is lacking. To help identify directions for future studies, we discuss the role of γδ T cells in homeostasis and disease, with a specific focus on the brain.

γδ T CELLS

T cells are adaptive immune cells that are restricted to MHC-mediated antigen presentation. T cells typically exit from the thymus as naïve cells. Antigen presentation accompanied with multiple inflammatory cues activates T cells and trigger immune reactions (22). However, there are innate-like T cells that have invariant TCRs, such as γδ T cells, natural killer (NK) T cells, and mucosal associated invariant (MAI) T cells (23). γδ T cells are known to be usually independent on MHC-mediated antigen presentation and recognize stress-related molecules, microbial molecules, or phosphoantigens through γδTCR and/or NK receptors, such as NK group 2D (NKG2D) (24). γδ T cells are highly heterogenous and various subsets have been identified. Though some γδTCR ligands have been identified, a comprehensive identification of all ligands is lacking. Functional similarities are shared among multiple γδ T cell subsets and there are two functional subsets. The first functional subset is interferon (IFN)-γ-producing and T helper (Th) 1-like subset and the second functional subset is interleukin (IL)-17-producing and Th17-like subset (**Figure 1A**). The expected roles of γδ T cells are similar to CD4 T cells. IFN-γ-producing γδ T cells are usually antiviral and antitumoral cells, whereas IL-17-producing γδ T cells are antifungal or related to autoimmune diseases such as EAE (8). The detailed functions of γδ T cell subsets are more classified by their circulating capacity. In general, γδ T cells are tissue-resident cells in the mucosal tissues, Vγ5$^+$ cells are DETCs in the skin, Vγ4$^+$ cells are dermis- or lung-resident cells, Vγ6$^+$ cells are residing in vagina, meninges, and dermis, and Vγ7$^+$ cells are gut-resident IELs. On the other hand, Vγ4$^+$ and Vγ1$^+$ cells generated postnatally are circulating cells (6). In humans, Vγ9Vδ2 T cells are predominant circulating γδ T cells, whereas Vδ1$^+$ cells and fetal γδ T cells are commonly tissue-resident cells (8, 9). γδ T cells are usually rapidly reacting innate cells that connect innate immune responses to adaptive immune cells and function as a "safeguard". In addition to their ability to release cytokines, subsets of γδ cells possess NK-like cytotoxicity *via* NK receptors, such as NKG2D (25). However, studying γδ T cells has been technically difficult because of the low number and heterogeneity. Following the recent development of high-throughput analytic tools, such as single cell RNA sequencing, γδ T cell study has progressed tremendously. A number of recent studies have demonstrated the indispensable role of γδ T cells in multiple contexts. Recently, meningeal γδ T cells were identified as a main source of IL-17A in the CNS under homeostasis (19, 20). Currently, cytokines are regarded as neuromodulators because of their ability to directly interact with neurons (18). In addition, IL-17A is one of the most important cytokines for the neurological system and Vγ6$^+$ cells, which reside in meninges, produce IL-17A (19). On the other hand, other γδ T cells can invade into the parenchyma under disease conditions and regulate multiple immune responses. For example, circulating γδ T cells can invade into glioblastoma multiforme (GBM) tissues, leading to antitumor responses (26). Although γδ T cells seem to be critical immune cells in the CNS, many aspects of their biology remained unclear.

DEVELOPMENT AND MAINTENANCE OF γδ T CELLS

γδ T Cell Development

Similar to other T cells, γδ T cells are generated from the thymus (23). Common lymphoid progenitor cells from the

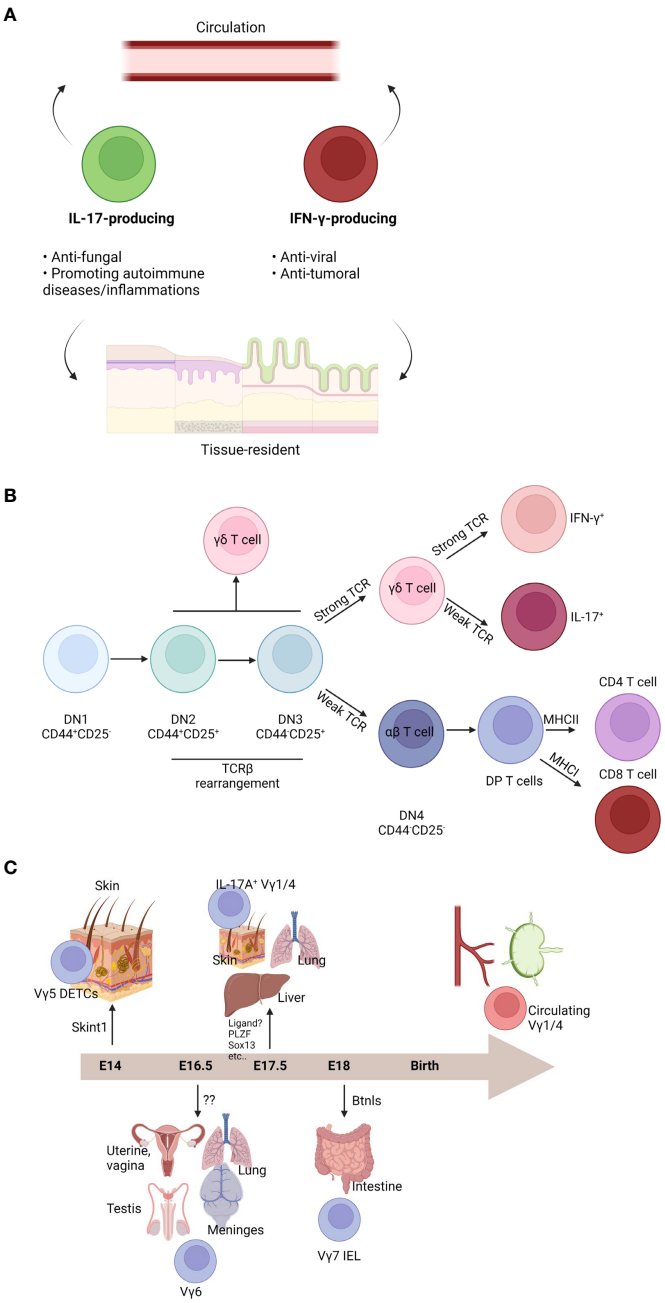

FIGURE 1 | Characteristics and development of γδ T cells. **(A)** γδ T cell subsets are heterogenous. Functionally, γδ T cells can be divided into two groups: one is IL-17-producing cells and the other is IFN-γ-producing cells. IL-17-producing cells are commonly antifungal cells or promoting autoimmune diseases and inflammation. IFN-γ-producing cells are usually antiviral or anti-tumoral cells. Both subsets can be further divided by circulation ability. Although the majority of γδ T cells are tissue-resident cells in the mucosal barriers, some γδ T cells can circulate body. **(B)** T cell development occurs in the thymus. T cell development can be divided by expression of CD44 and CD25 (DN1: CD44+CD25-; DN2: CD44+CD25+; DN3: CD44-CD25+; DN4: CD44-CD25-). Although DN2 or DN3 cells can be γδ T cells, commitment usually occurs after DN3 stage. Strong TCR signal enhances γδ T cell fate. DN4 αβ T cells become CD4+CD8+ DP cells. By their interaction with MHC class I or MHC class II, DP cells become a CD8 T cells or CD4 T cells, respectively. γδ T cells can be IFN-γ-producing cells by strong TCR signal. On the other hand, weak TCR signaling induces IL-17-producing cells. **(C)** Different γδ T cell subsets can be generated in the fetal thymus. At embryonic (E) 14, Vγ5+ dendritic epidermal T cells (DETCs) are generated and migrate into the skin epidermis. SKINT1 is important for Vγ5+ DETC development and selection. At E16.5, Vγ6+ cells can be developed. These cells migrate into the multiple organs such as uterine, vagina, testis, lung, or meninges. They become a tissue-resident cells in those tissues. Cognate ligand for their TCR is not identified. At E17.5, IL-17-producing Vγ1/4+ cells are made. They can migrate into the skin dermis, lung, or liver. They are also tissue-resident cells. Although several factors have been known for their development, cognate TCR ligand is not identified. After E18, Vγ7+ intraepithelial cells (IELs) are generated. They migrate into the gut and become gut-resident cells. BTNL proteins are critical for development and maintenance of Vγ7+ IELs. After birth, Vγ1/4+ cells are further generated. They can circulate and are observed in the blood or lymphoid organs.

bone marrow enter the thymus and become $CD4^-CD8^-$ double negative (DN) T cells. DN T cells are subdivided into four differentiation stages (DN1: $CD44^+CD25^-$; DN2: $CD44^+CD25^+$; DN3: $CD44^-CD25^+$; DN4: $CD44^-CD25^-$) (**Figure 1B**). During the DN stage, pre-TCR are formed when pre-TCRα and TCRβ rearrangement induces progression into the $CD4^+CD8^+$ double positive (DP) stage. Then, DP T cells interact with cortical epithelial cells expressing MHC molecules with self-antigens, which leads to a selection process where too weak signaling induces DP cell apoptosis. Moderately reactive DP T cells become single positive (SP) T cells. Thymocytes that interact with MHC class I become CD8 T cells and cells what interact with MHC class II become CD4 T cells or initial signaling strength determines fates of T cells (27, 28). SP T cells are further selected by negative selection by medullary epithelial cells. Other unconventional T cells, such as NKT cells and MAIT cells, are generated from the DP stages. Uniquely, γδ T cells develop from the DN stages (23). γδ T cell fate is commonly determined at the DN3 stage. However, some γδ T cell subsets are derived from the DN1 or DN2 stages. In mice, γδ T cell development begins in the fetal thymus and γδ T cells constitute the major T cell subset at this early stage due to a lack of αβ T cell development (29). Initial mouse γδ T cell development occurs in the fetal thymus, generating DETCs expressing Vγ5 (**Figure 1C**). At embryonic (E) 14, DETCs are produced and preferentially migrate into the epidermis (30). Interestingly, a study revealed DETCs do not originate from hematopoiesis in bone marrow. However, DETC progenitors were derived from yolk sac like Langerhans cells (31). $V\gamma6^+$ cells are a type of intraepithelial lymphocytes (IELs) of reproductive organs and meninges. $V\gamma6^+$ cells usually express IL-17A and develop at E16.5. $V\gamma4^+$ and $V\gamma1^+$ IL-17A-producing cells develop at E17.5 (32). Development of gut-homing $V\gamma7^+$ IELs begins at E18 and continues postnatally (30). Some intestinal IELs are thought to be developed extrathymically (33). Some IFN-γ-producing liver-resident γδ T cells are extrathymically developed from $Lin^-Sca\text{-}1^+Mac1^+$ hematopoietic stem cells and progenitor cells in the liver (34). Similarly, human γδ T cells arise from the fetal liver (35). $V\gamma9V\delta2$ T cells can be observed at the fetal liver at 5-7 weeks gestation, whereas thymic $V\gamma9V\delta2$ T cells are detected at 8 weeks gestation (36). Fetal $V\gamma9V\delta2$ T cells are relatively invariant and have public clones. Postnatally, $V\gamma9V\delta2$ T cells are rarely generated, whereas $V\delta1^+$ and $V\delta3^+$ T cells are preferentially generated. TCR repertoire of $V\delta1^+$ and $V\delta3^+$ T cells is largely dependent on microbial exposure (37). Although fetal $V\gamma9V\delta2$ T cells slowly turn over and have self-renewal capacity, adult-derived $V\gamma9V\delta2$ T cells can also be generated and be a major source human γδ T cells in the blood (38). Recent observation showed the fetal thymus produces hybrid T cells that expressing both αβTCR and γδTCR (39). These hybrid cells, which can produce IFN-γ, IL-17A, and granulocyte-macrophage colony-stimulating factor, are hyperactive. The hybrid cells underwent positive αβ-selection.

After birth, the majority of newly generated γδ T cells are $V\gamma4^+$ and $V\gamma1^+$ cells. Although both cells can produce IL-17 and/or IFN-γ, $V\gamma1^+$ cells are usually association with IFN-γ production and $V\gamma4^+$ cells are commonly associated with IL-17A production (6). Their fate is determined during thymic development. $CD27^+CD44^{int}$ cells actively secrete IFN-γ, whereas $CD27^-CD44^{hi}$ cells produce IL-17A (8). As this process is not well understood, identifying factors that determine γδ T cell fate has been of great interest. Although various factors can be involved, TCR strength may be the most important factor for determining γδ T cell fate. Before γδ T cell commitment, TCR strength is important for γδ T cell identity. If γδTCR is weak, cells tend to preferentially differentiate into αβ T cells (40). These commitments are known to occur after TCR expression. It was dependent on extracellular signal-regulated kinases (ERKs)-mediated early growth response activation (41). Overexpression of friend leukemia integration 1 (Fli1) prevents progression of DN T cells into DP T cells (42). As a result, Fli1 overexpression may create a preferential environment for γδ T cell development, which was mediated by strong TCR mimicry. Strong TCR activation results in CD73 expression. Although $CD73^-$ γδ T cells retain the potential develop into αβ T cells, $CD73^+$ cells commonly become γδ T cells (43). After γδ T cell commitment, TCR strength may determine whether the γδ T cells become IL-17-producers or IFN-γ-producers. Usually, a strong TCR signal tends to make γδ T cells become a $CD44^+CD45RB^+T\text{-}bet^+$ IFN-γ-producing cells. On the other hand, a weak TCR signal induces $CD44^{hi}ROR\gamma t^+$ IL-17A-producing γδ T cells (44). This mechanism was dependent on the ERK pathway. Mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and Notch signaling also determine αβ/γδ fate *via* metabolism (45). Likewise, metabolic pathways are also important for γδ T cell fate. IFN-γ-producing cells are dependent on glycolysis and IL-17A-producing cells are dependent on oxidative phosphorylation. These dependencies are imprinted from thymic development to peripheral maintenance (46). Environmental cytokines also regulate the function of γδ T cells. For example, IL-1β and IL-23 induce extrathymic commitment of $CD27^+CD122^-$ $V\gamma4^+$ cells to become an IL-17A-producer (47). $V\gamma4^+$ T cells that have never made IL-17A can produce IL-17A *de novo* by IL-1β and IL-23 (48). In parallel, IFN-γ-producing cells can be generated by IL-12 and IL-18 (49). Transcription factors are also important regulators of γδ T cell fate. Fetal-derived γδ T cells may be marked by promyelocytic leukemia zinc finger protein (PLZF) (50, 51). IFN-γ^+ γδ T cells need T-bet, but not Eomes. On the other hand, IL-$17A^+$ γδ T cells need RORγt, but not RORα and BATF (52). Co-stimulatory molecules, such as CD27 or ICOS, also support γδ T cell fate determination (53, 54).

Ligands for γδTCR

As mentioned above, TCR signaling is important for γδ T cell development and maintenance. Thus, identifying γδTCR ligands and their roles is indispensable to further understand γδ T cell biology. Though major subsets of γδ T cells are not dependent on MHC-mediated antigen presentation, γδ T cells are dependent on MHC-like molecules, stress-induced molecules, and phosphoantigens (24). The most well-known γδTCR ligands are selection and upkeep of intraepithelial T cells protein 1

(SKINT1) and butyrophilin-like proteins (BTNL) molecules (**Figure 1C**). Vγ5$^+$ DETCs are dependent on SKINT1 (55). SKINT1 expression is restricted to the thymus and skin keratinocytes. SKINT1-mediated TCR signaling is not only important for development of DETCs, but also epidermal maintenance (56). Likewise, BTNL molecules are important for Vγ7$^+$ IELs. BTNL1 and BTNL6 are necessary for murine Vγ7$^+$ IELs and BTNL3 and BTNL8 are needed for human intestinal Vγ4$^+$ T cells (57). T10/22, a MHC class Ib molecule, is also important for γδ T cell development (58). The most well-known γδTCR ligands in humans are BTN3A1 and BTN2A1. Phosphoantigens induce a conformational change in BTN3A1-BTN2A1 dimers, which binds to Vγ9Vδ2 TCR (59). Endothelial protein C receptor (EPCR)-Vγ4Vδ5 TCR (60), Annexin A2-Vδ2 TCR (61), tRNA synthetases-Vγ3Vδ2 TCR (62), ephrin type-A receptor 2 (EphA2)-Vγ9Vδ1 TCR (63), and R-phycoerythrin-Vδ1 TCR (64), CD1c/d-Vδ1 TCR have been reported (65, 66). Contrary to a number of reports that argued fetal thymus-derived γδ T cells are invariant, adult-derived γδ T cells have relatively variant TCR chains (67). Likewise, there are some γδ T cell subsets that are dependent on MHC-mediated antigen presentation (68). Thus, studying γδ T cells and their ligands is complex. In some cases, γδ T cells can be activated without TCR signaling, but activated by stress-induced molecules, such as MHC class I chain-related protein A/B (MICA/B) or retinoic acid early inducible 1 (Rae-1), *via* NKG2D receptor (8, 69). In conclusion, TCR ligands should be considered in the context-dependent manner to understand the role of γδTCR. A study showed murine γδTCR depletion antibodies could not remove γδ T cells, but made the cells undetectable *via* intracellular uptake of γδTCR (70). Because this system depletes functional γδTCR from cellular surfaces, γδTCR depletion antibodies could be used to investigating the role of γδTCR. Unfortunately, ligands for Vγ6$^+$ cells have not been identified. However, administration of anti-γδTCR inhibits meningeal γδ T cell functions (19). Thus, TCR-mediated signal is required for cytokine secretion in the meninges. Identifying the ligand(s) that regulate meningeal γδ T cell homeostasis and activation is critical to understand the role of γδ T cells in brain physiology.

γδ T CELLS IN BRAIN HOMEOSTASIS

Maintenance and Recruitment of Brain γδ T Cells

Vγ6$^+$ cells, which are enriched in the meninges, reproductive organs, and dermis, are the major γδ T cell subset in these organs (6). In addition, they are a major source of IL-17A; however, they do not express IFN-γ. Although a study claimed ZAP70-deficient mice had less IL-17A-producing γδ T cells, including Vγ6$^+$ cells, compared to wild type (WT) mice (71), previous study has proposed that weak TCR signaling is important for development of IL-17A-producing γδ T cells, including Vγ6$^+$ cells (44). It is important to note that the dispensable role of TCR signaling in thymic development of γδ T cells does not mean that it is also dispensable for peripheral maintenance and cytokine secretion. A series of studies have emphasized that tonic TCR signal from tissue-specific niches is important for maintaining tissue-resident γδ T cells (72, 73). Vγ6$^+$ cells γδ T cells being developing at E.17.5 (23). Furthermore, experiments using bone marrow chimeras demonstrated that adult thymus could not produce IL-17A-producing γδ T cells, suggesting Vγ6$^+$ cells may be fetal-derived, self-renewing, and long-lived cells (32). However, it remains unclear how Vγ6$^+$ cells are recruited into the meninges and maintained. In the uterus, Vγ6$^+$ cells are the dominant γδ T cells in homeostasis (74). However, pregnancy induces recruitment of Vγ4$^+$ cells into the placenta (75). Although the relation of Vγ4$^+$, Vγ6$^+$ cells, or IL-17A to outcomes of pregnancy is controversial, allogenic pregnancy experiments revealed that recruitment of γδ T cells in the uterus is dependent on allotype (75, 76). In parallel, certain inflammatory cues can recruit different γδ T cell subsets in the meninges or brain parenchyma (21, 77). It has shown that brain injury or inflammation can recruit Vγ1$^+$, 4$^+$, 6$^+$ cells in the parenchyma (78–80). CCR6 is important for migration of IL-17-producing γδ T cells (81), and a study showed most meningeal γδ T cells expressed CCR6 (20). However, another study showed meningeal γδ T cells expressed large amounts of *Cxcr6* and *Ccr2*. In addition, *Cxcr6*-deficient mice showed γδ T cell reduction in the meninges (19) and their functions may be dependent on γδTCR, but not cytokines, such as IL-1β or IL-23 under homeostasis (19, 20). However, other factors affecting meningeal γδ T cells should be further addressed. Taken together, meningeal γδ T cells have crucial roles maintaining brain homeostasis and behaviors of animals. However, further study is needed to uncover the exact mechanisms governing how they are recruited, activated, and maintained.

The Role of Meningeal γδ T Cells in the Homeostatic Brain

Decades ago, heat shock protein 70 (HSP70) was the most well-known ligand for human multiple sclerosis (MS) γδ T cells (82). Interestingly, a study observed that oligodendrocytes, postischemic neurons, and microglia express HSP70 under heat exposure (83). This study suggested γδ T cells may be cytotoxic to brain cells. Also, this study revealed that different types of γδTCRs are expressed in the cortex, hypothalamus, and medulla of postmortem samples. Another study showed that normal CNS tissue contains γδ T cells (84). Although this study may have technical limitations, the γδ T cells from normal CNS tissue expressed low CD45RB levels, which may suggest these cells are meningeal IL-17A-producing cells. Currently, many people agree that γδ T cells do not exist in the normal CNS parenchyma. However, a large amount of γδ T cells are present in the meninges (**Figure 2A**) (19). Furthermore, these cells are IL-17A-producing cells, but not IFN-γ-producing or IL-22-producing. Also, these cells are rarely observed in the arachnoid and choroid plexus. This study also showed that meningeal γδ T cells are present three days after the postnatal period (P3). They showed tissue-resident phenotypes that were not derived from circulation. Adult meningeal γδ T cells were not Ki67$^+$ and showed poor incorporation of BrdU, indicating they

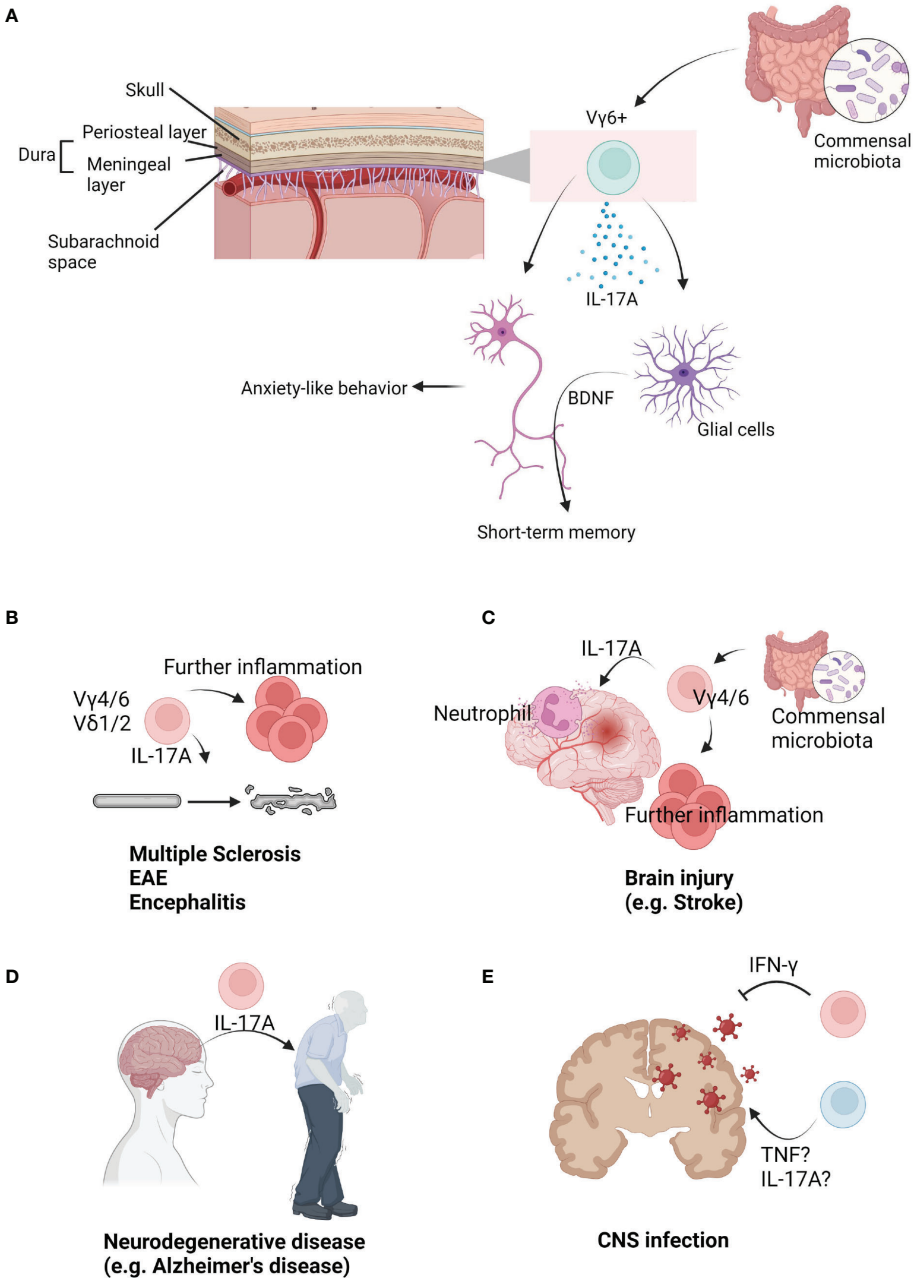

FIGURE 2 | The role of γδ T cells in brain immunology. **(A)** Upon brain parenchyma, multiple layers surround brain. Under skull, dura mater (periosteal layer, meningeal layer) is situated. Under meningeal layer, arachnoid and subarachnoid space exist. In the meninges, Vγ6+ cells are populated. They seem to be affected by commensal microbiota. Under steady state, Vγ6+ cells produce IL-17A. IL-17A from meninges can be delivered into the parenchyma. Direct signal from IL-17A into neurons can regulate anxiety-like behavior. On the other hand, IL-17A can regulate short-term memory via glial BDNF. **(B)** γδ T cells are related to progression and severity of brain autoimmune diseases. Mouse Vγ4/6+ cells or human Vδ1/2+ cells are known to be related to these diseases. Usually, IL-17A from γδ T cells initiate or further promote diseases. **(C)** γδ T cells are involved in injury-induced inflammation in the brain. Vγ4/6+ cells usually produce IL-17A which recruits neutrophils. They are known to be regulated by commensal microbiota. As an early inducer, γδ T cells further promote inflammations. **(D)** γδ T cells are also related to neurodegenerative diseases. IL-17A may be strongly associated with development of diseases such as Alzheimer's disease. **(E)** γδ T cells can infiltrate into the infected brains. Multiple pathogens can infect into the brain. Usually, IFN-γ-producing γδ T cells resolve viral infections. However, TNF or IL-17A is associated with infection-induced inflammation.

are not proliferative and self-renewal. They produce IL-17 under steady states, which may be dependent on TCR signaling. Commensal-derived signaling also contributes to γδ T cell IL-17A production. However, the number of meningeal γδ T cells was not dependent on bacterial signals. This study also revealed that meningeal γδ T cell-derived IL-17A regulates anxiety-like behaviors of mice. Although how meninges-derived cytokines arrive at parenchyma is unclear, IL-17A can directly affect excitatory glutamatergic neurons in the medial prefrontal cortex (mPFC). Notably, IL-17 receptor A (IL-17Ra) is expressed by multiple brain regions. A direct IL-17A signal may promote neurotransmitter release from excitatory presynaptic terminals of mPFC neurons to induce anxiety-like behaviors. However, IL-17A did not affect intrinsic neuronal excitation. This finding may explain how animals can rapidly respond to environmental stresses. On the other hand, *Tcrd*-deficiency did not affect spatial memory task performance, social preference, or foraging behavior. According to an interesting study by the Ribot group, *Tcrd*-deficient mice did not show deficits in exploratory behavior, motor function, and anxiety (20). However, these animals showed impaired short-term spatial working memory, but not long-term memory formation. Critically, these findings were dependent on IL-17A. IL-17A directly signals to glial cells inducing production of brain-derived neurotrophic factor (BDNF) in glial culture system. However, because these phenotypes were not repeated under microglia- or astrocyte-specific deletion of IL-17R, direct evidence linking IL-17A and memory formation is still lacking and should be further addressed. Nonetheless, IL-17A-mediated BDNF seems to be involved in long-term potentiation of neurons during short-term memory formation. Taken together, γδ T cells, as main source of IL-17A, regulate multiple functions of the brain under steady states.

Maternal IL-17A is also important for progeny behavior. Poly I:C-induced maternal immune activation (MIA) mimicking infections showed autism-like behavior of progenies (85). Because *Il17a* expression was not detected in fetal brain at E14.5, IL-17A may be derived from the mother under MIA. MIA resulted in impaired cortex development of offspring. Given the authors showed conditional deletion of *Rorc* using CD4-Cre mice, they concluded CD4 T cells are responsible for IL-17A production. This data excluded participation of γδ T cells, lymphoid tissue inducer cells, and innate lymphoid cell type 3s. In addition, intestinal dendritic cells stimulate CD4 T cells *via* IL-1β, IL-23, and IL-6, which leads to IL-17A production in a maternal microbiota-dependent manner (86). Although they clearly showed CD4 T cells are critical, the contribution of uterine γδ T cells or fetal γδ T cells to behavioral impairment in offspring would be an interesting study to explore. Moreover, dietary salt also induces CD4 T cells to produce IL-17A *via* serum/glucocorticoid regulated kinase 1 (SGK1) (87). Similarly, IL-17A-inhibiting *Lactobacillus murinus* was reversed by salt-uptake, resulting in elevated IL-17A (88). Maternal salt uptake also induces abnormal behaviors of offspring (89, 90). Dietary salt has been shown to induce cognitive dysfunction by gut-initiated Th17 responses (91). Taken together, maternal CD4 T cell-derived IL-17A affects offspring cognitive functions and behaviors. In addition, the role of γδ T cells in MIA-induced autism-like behaviors and cognitive dysfunction under salt uptake or other environmental changes should also be addressed. On the other hand, intrauterine inflammation without systemic inflammation induces neutrophil infiltration into the decidua. In parallel, neutrophils and macrophages were increased in the fetal liver. In the fetal brain, granulocytes and activated microglia were increased. Among immune cells, $Gr1^+$ γδ T cells were the most rapidly responding cells, which produce IFN-γ rather than IL-17A (92). Thus, other kinds of MIA rather than systemic poly I:C should be also considered.

THE ROLE OF γδ T CELLS IN BRAIN DISEASES

Autoimmune Diseases in CNS

In 1991, it was revealed that human peripheral blood-derived γδ T cells can kill fresh human brain-derived oligodendrocytes *ex vivo* (93). Furthermore, γδ T cells were observable in the plaques and cerebrospinal fluid (CSF) of MS patients. This study suggested the possibility of γδ T cell participation in MS progression. Although CD4 T cells are important for chronic MS, γδ T cells were the most activated cells in recent onset MS patients (94), and the activated γδ T cells were oligoclonal. This study suggested γδ T cells can be expanded by MS antigens and are the initiating cells in MS pathology (**Figure 2B**). Demonstrated with a murine EAE model, administration of anti-γδTCR (UC7-13D5) worsened EAE pathology (95). These data suggested the regulatory role of γδ T cells in disease progression. As mentioned above, anti-γδTCR administration does not deplete γδ T cells, rather it inhibits TCR signaling (70). Thus, this finding showed TCR-reactive γδ T cells have regulatory role in the EAE. Another study using a murine EAE model revealed γδ T cells are associated with IFN-γ levels (96). On the other hand, early IL-17A production from γδ T cells promotes later activation of Th17 cells (97), indicating heterogenous γδ T cells participate in MS or EAE. In human samples, $V\delta1^+$ cells were largely observed in the blood and CSF of MS patients. On the other hand, $V\delta2^+$ T cells have strong cytotoxicity against oligodendrocytes (98). Under MS, long-term treatment of IFN-β expands $V\delta1^-V\delta2^-V\gamma9^-$ γδ T cells, which were related to better outcome of MS patients (99). Taken together, human data also suggested a heterogenous role of γδ T cells in the MS progression. In the murine EAE model, γδ T cells infiltrate into the brain parenchyma using integrin beta 2 family, and its expression was rapidly reduced after infiltration (100). Another study showed that gut *L. acidipiscis* reduces $V\gamma4^+$ cells while $V\gamma1^+$ cells were increased. Because gut *L. acidipiscis* was related to better EAE outcomes, $V\gamma4^+$ and $V\gamma1^+$ cells may have opposing roles (101). IFN-γ-producing and IL-17A-producing γδ T cells have been shown to have opposing roles as IFN-γ- or IFN-γR-deficient mice have enhanced EAE (102, 103). It would be interesting to investigate the contribution of meninges-derived IL-17A or $V\gamma6^+$ cells using an EAE murine

model. γδ T cells are also related to Rasmussen's encephalitis (RE) pathology. Although CD8 T cell response is critical for RE inflammation, more innate cell types could be associated with disease initiation (104). This study revealed $V\delta1^+$ cell clonal expansion in the parenchyma of RE patients. Because microglial activation *via* TLRs can enhance IL-17A-producing γδ T cells through IL-1 and IL-23, microglial inflammation can be a trigger for multiple CNS inflammations (105).

γδ T Cells in Brain Injury

Infiltration of γδ T cells in the brain parenchyma is also observable following ischemic injury (106). While CD4 T cells induce tumor necrosis factor (TNF) production by macrophages *via* IFN-γ, γδ T cells promote neutrophil infiltration through IL-17A (**Figure 2C**). IL-17A and TNF synergistically induce CXCL1 expression by astrocytes, which further promotes neutrophil infiltration (107). Another interesting study showed intestinal microbiota regulates outcomes of ischemic stroke *via* γδ T cells. Intestinal microbiota regulates dendritic cells, which promotes γδ T cell activation. IL-17A produced from γδ T cells enhances stroke pathology. On the other hand, antibiotics uptake increases Tregs and reduces γδ T cells resulting in better outcomes for stroke mice (77). Taken together, IL-17A from γδ T cells is a critical cytokine that promotes inflammation after brain injury. Two studies showed IL-17A is predominantly expressed by infiltrating $V\gamma4^+$ or $V\gamma6^+$ cells (79, 108), and CCR6 seems to be important for $V\gamma4^+$ or $V\gamma6^+$ cell migration. Furthermore, the regulatory role of γδ T cells was demonstrated using a NaIO3-mediated retinal pigment epithelium injury model. γδ T cells produce IL-4 and IL-10 to reduce injury in an aryl hydrocarbon receptor (AhR)-dependent manner (109). In the case of perinatal brain injury, injury delays neurophysiological maturation. This was related to gut microbiota, *Klebsiella*, which has been associated with an increase in γδ T cells expressing IL-17A and VEGF-A (110). On the other hand, both the Kipnis group and Colonna group showed that skull bone marrow provides myeloid cells and B cells to the meninges and parenchyma (111, 112). Direct production of immune cells *via* skull bone marrow might be involved in brain injury progression. However, these two studies suggested T cells are derived from the peripheral blood, not the skull bone marrow. It may be due to T cell maturation occurs at the thymus. However, *de novo* development of γδ T cells in the skull bone marrow or meninges should be experimentally tested to clarify this. Also, γδ T cells promote bone regeneration after injury *via* IL-17 (113). Thus, meningeal γδ T cell-derived IL-17 may be able to regulate skull regeneration resulting in recovery after brain injury.

Neurodegenerative Diseases

A number of studies have shown that inflammation is associated with severity of neurodegenerative diseases, including dementia, Parkinson's diseases, and Huntington's diseases (114). Clonal expansion and antigen reactivity of T cells have been observed in multiple neurodegenerative diseases (115–117). Because microglial-intrinsic inflammatory gene regulation can induce T cell infiltration in the parenchyma and neuroinflammation (118), immune reaction may be associated with initiation and development of multiple neurodegenerative diseases. During the initial stage of MS, pioneer cells enter the CNS and initiate further inflammation without pathologies (119). On the other hand, γδ T cell activation, rather than αβ T cells, has been observed in CNS inflammation in early onset MS (94, 106). Thus, γδ T cells may regulate the first wave of neuroinflammation in neurodegenerative diseases, though there is no direct evidence conclusively demonstrating this. *TRG* genes can be detected in both the human brain and blood. The brain has less *TRGV9* clones than the blood. However, the brain contains more *TRGV2, 4*, and *8* genes. In this study, it was shown that aging is known to reduce the *TRG* repertoire. In addition, an Alzheimer's disease (AD)-associated *TRG* pattern was observed among AD patients (120). This study has technical limitations because tissues were not perfused and *TRG* transcript could be expressed by non-T cell lineages (121). Nonetheless, these data suggest a possible relationship between γδ T cells and AD. Consistently, IL-17-producing cells, including γδ T cells, accumulate in the brain and meninges of the 3xTg-AD mouse model (122). This study demonstrated IL-17 triggers AD onset independent of amyloid β and tau pathology (**Figure 2D).** Thus, γδ T cells may be a "pioneer cells" of neurodegenerative diseases. Likewise, γδ T cells were increased in the blood and CSF from Parkinson's disease (PD) patients compared to other neurological diseases (123). In summary, γδ T cells can contribute to progression and initiation of multiple neurodegenerative diseases. Despite the lack of a direct connection, γδ T cells may be related to early trigger of diseases. The diverse roles and mechanisms of γδ T cells in multiple neurodegenerative diseases should be further addressed.

Brain Infections

Microbe infections can also induce neuroinflammation and neurological symptoms. For example, toxoplasma infection can induce toxoplasmic encephalitis. A study showed IL-6 deficiency was associated with more cyst and necrosis of the brain. IL-6 knock out mice have more CD8 T cells and less CD4 T cells and γδ T cells compared to WT mice (124). This suggested γδ T cells may be related to inflammation in toxoplasmic encephalitis. Malaria infection can also induce brain inflammation. Infection by *Plasmodium yoelii* induces brain inflammation of BALB/c mice. However, DBA/2 mice are resistant to infection. IL-2-mediated γδ T cell infiltration in the brain was critical for susceptibility to *Plasmodium yoelii* infection (125). Another study also showed γδ T cell deficiency reduced intracranial mesocestoides corti-mediated neurocysticercosis pathology (126). Thus, γδ T cells contribute to infection-induced brain inflammation (**Figure 2E**).

γδ T cell infiltration was observed following West Nile virus (WNV) infection. The majority of infiltrating γδ T cells were $V\gamma1^+$ and $V\gamma4^+$ cells that produce IFN-γ and TNF, respectively (127). IFN-γ has antiviral functions, whereas TNF was associated with worse symptoms. This study also showed aging increases $V\gamma4^+$ cells but reduces $V\gamma1^+$ cells. $V\gamma4^+$ cells also produce IL-17A following WNV infection (128). According to this study, $V\gamma4^+$ cells also inhibited the $V\gamma1^+$ cell response and associated IL-10 production. Regarding oral herpes simplex virus type 1 (HSV-1) infection, C57BL/6 mice are resistant to infection while BALB/c

mice are susceptible. In C57BL/6 mice, HSV-1 replication is limited to the brain stem. However, HSV-1 replication was observed throughout the whole CNS in BALB/c mice. Although CD8 T cells, NK cells, and NKT cells were crucial for limiting viral infection in the CNS, γδ T cells were important for inhibiting viral spreading in the trigeminal ganglia (129). Epstein-Barr virus (EBV) is one of the most important CNS viruses because it is largely related to MS progression and onset. Longitudinal analysis showed that high prevalence of EBV is related to MS (130). Consistently, a study showed antibodies derived from clonally expanded B cells in MS can bind to EBV Epstein-Barr nuclear antigen 1 (EBNA1) and CNS-derived GlialCAM protein. Furthermore, the presence of EBNA1/GlialCAM antibodies was associated with severe MS (131). A study showed EBV reactivation after hematopoietic stem cell transfer was negatively correlated with $V\delta2^+$ T cells (132). This study showed γδ T cells exhibit cytotoxicity against EBV-infected cells *in vitro*. Thus, γδ T cells may have role in EBV-mediated MS. Likewise, γδ T cells are highly associated with cytomegalovirus (CMV) infection (133). Because herpesviruses such as human CMV or HSV seem to be related to multiple neurodegenerative diseases (134–136), γδ T cells may have critical role preventing CNS viral infection-mediated neurological disorders.

Brain Tumors

Recently, the role of γδ T cells in multiple tumors has been emphasized. A study showed γδ T cell were mostly correlated to better prognosis among multiple tumor-infiltrating immune cells (137). Different subsets of γδ T cells can be identified in the tumor microenvironment (**Figure 3**). Functionally, γδ T cells can be subdivided into IL-17A-producing cells and IFN-γ-producing cells (8). IFN-γ-producing cells tend to be cytotoxic cells, with some exceptions. A recent study showed IL-17A-producing γδ T cells are protumor cells and IFN-γ-producing cells are antitumor cells using subcutaneous murine tumor models (46). This tendency was conserved across multiple tumors (8). Also, our group showed γδ T cells are associated with longer survival of brain tumor patients (138). However, αβ T cells showed the opposite tendency. Meanwhile, using a murine high-grade glioma (HGG) model, we showed depletion of NK cells, γδ T cells, CD8 T cells, or CD4 T cells did not affect survival of HGG-bearing mice. We discovered that hypoxia was positively related to increased glioma grade and negatively related to γδ T cell infiltration. Although further examination should follow, we have concluded γδ T cells are the most HGG-reactive cells, and are suppressed by tumor hypoxia. If we used metformin to block tumor cell respiration, hypoxia-induced suppression of γδ T cells was reduced, which resulted in a recovery of their antitumor functions. Though IL-17A and

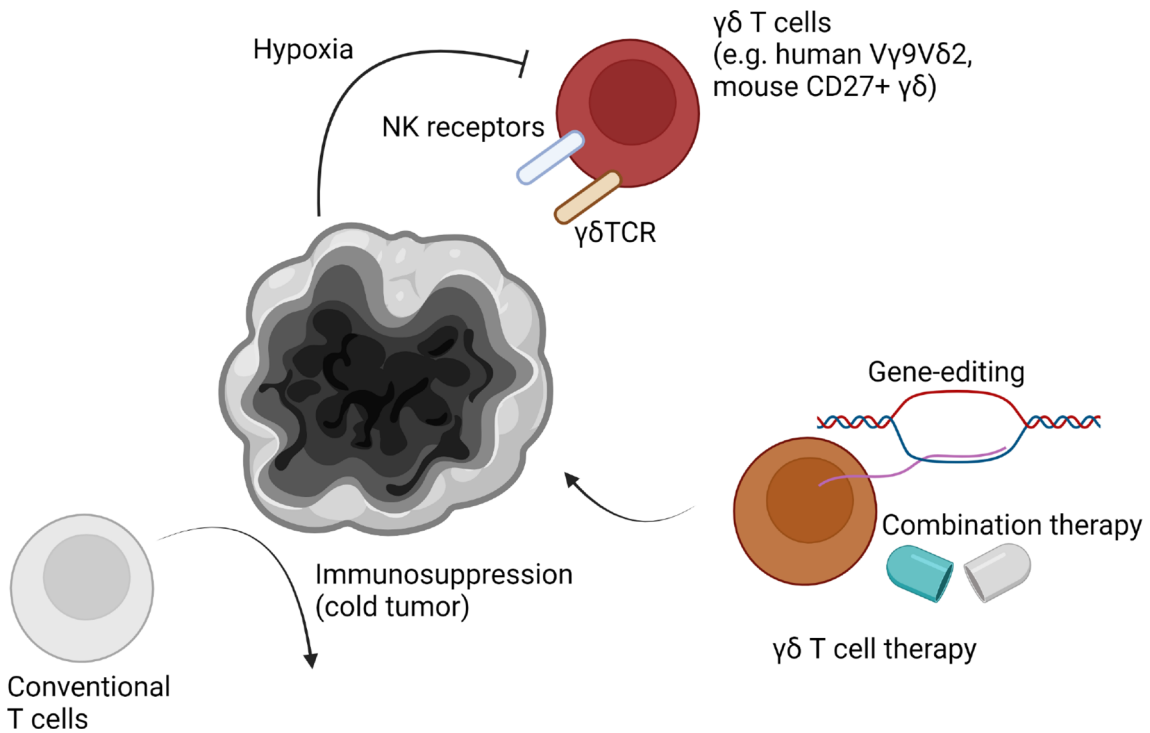

FIGURE 3 | γδ T cells in the brain tumor microenvironment. High-grade brain tumors such as glioblastoma multiforme (GBM) are known to be immunosuppressive "cold tumors". Due to strong immunosuppression mechanisms, conventional T cells are malfunctional. However, γδ T cells are known to be potent strong anti-brain tumor immune cells. Human Vγ9Vδ2 T cells or murine CD27+ γδ T cells are known to infiltrate the tumor microenvironment. They may fight with tumor cells through both of γδTCR and NK receptors, including NKG2D. However, γδ T cell reactivity is suppressed in the brain tumor microenvironment via severe hypoxia. Additionally, other mechanisms such as chemotherapy-induced cell death can be involved in suppression of γδ T cell reactivity. Thus, gene-editing to generate resistant γδ T cells or developing combination therapies can enhance γδ T cell immunity in the brain tumor microenvironment.

IL-17F were not related to survival of HGG mice, NKG2D expression of IFN-γ-producing γδ T cells was critical for anti-HGG immunity. Due to high NKG2D-ligand expression of tumor cells, NKG2D-expressing γδ T cells were the most critical immune cells in the HGG microenvironment. In this study, anti-γδTCR antibody administration also abrogated γδ T cell-mediated antitumor functions. This finding suggested that γδTCR also participates in anti-HGG immunity. Despite lack of a direct connection, this study suggested dual ligation of γδTCR and NKG2D is needed, which could be the reason why other NKG2D-expressing cells, such as NK cells, did not respond to metformin treatment.

γδ T cells have been considered a good target for next-generation anti-brain tumor therapy (139). Among malignant brain tumors, GBM is the most frequent and aggressive tumor type (140). Despite traditional therapies, including surgery, radiotherapy, and chemotherapy, overall survival of GBM patients is around 1-2 years (141). Despite the recent development of immunotherapy, such as anti-PD-1 therapy, clinical trials of immunotherapy to treat GBM showed disappointed results (142). Although it is too early to definitively conclude, these negative results may be due to the poor immune profile of GBM microenvironment. GBM is classified as a "cold tumor," which showing less neoantigen and immune cell infiltration compared to "hot tumors" (143). Thus, modulation of existing immune cells could have limitations. According to our results, γδ T cells could be a better alternative target for anti-GBM therapy (138). In addition, preferential infiltration of Vγ9Vδ2 T cells in the GBM patient tissues was also observed (26). Because pre-existing T cells are not sufficient to eradicate tumors, interest in adoptive cell therapy has gained traction (144). However, adoptive therapy using *in vitro* expanded conventional T cells has shown low effectiveness (145). It may be that expanded conventional T cells are derived from low mutational and neoantigen burden in combination with downregulated antigen processing which resulting in GBM immune evasion despite controversies (146–148). *In vitro* studies have shown γδ T cells have cytotoxicity against multiple GBM cells, but not normal brain cells (149). Vγ9Vδ2 T cells were also able to target glioma stem cells (GSCs). Stereotaxic administration of Vγ9Vδ2 T cells with TCR stimulation by bromohydrin pyrophosphate or zoledronate efficiently controlled GSC-derived brain tumors in animal models (150). However, splenocyte-derived γδ T cell injection did not increase survival period of immunocompetent GL261-bearing mice. Consistently, γδ T cell deficiency did not affect survival of mice (151). The authors of this study suggested that γδ T cells are highly apoptotic in the GBM microenvironment. Consistently, our group has proposed that tumor hypoxia may contribute to γδ T cell apoptosis in the GBM microenvironment (138). Thus, γδ T cell therapy combined with anti-hypoxia strategy could have a beneficial effect. Our study also showed γδ T cell therapy in combination with metformin or pretreatment of HIF1A inhibitor dramatically increased survival of tumor-bearing mice. In addition, chemotherapy-mediated cell death could be another detrimental factor for γδ T cell activity. Thus, engineered γδ T cells which are resistant to chemo/radiotherapy may be an alternative approach (152). Allogenic γδ T cell therapy has a distinct advantage because γδ T cells are not dependent on MHC-mediated antigen presentation. Thus, γδ T cell therapy for tumors, including GBM, is expected to be a "game changer". Because the beneficial effect of γδ T cells in low-grade glioma (LGG) was clearer than HGG (138), γδ T cells may also have antitumor effects against other brain tumors, such as meningioma. Further studies should address the origins of γδ T cells (e.g. meninges, circulation), which ligands γδ T cells recognize, and mechanisms of γδ T cell infiltration (e.g. directly derived from peritumoral blood vessels, leptomeninges, choroid plexus).

CONCLUSION

Several lines of evidence have demonstrated the contribution of γδ T cells to CNS inflammation, antitumor immunity, and maintenance of CNS homeostasis. Under homeostasis, IL-17A-producing γδ T cells are located in the meninges. IL-17A derived from γδ T cells regulates multiple brain functions, including memory formation and behaviors. Brain inflammation also induces parenchymal infiltration of multiple subsets of γδ T cells. Although it is difficult to completely understand due to the complexity of γδ T cell biology, it is clear that γδ T cells play a critical role in a number of brain diseases. Multiple studies have suggested IL-17A-producing γδ T cells are associated with inflammation initiation. On the other hand, IFN-γ-producing γδ T cells are beneficial for removing tumors and pathogens. Furthermore, γδ T cells tend to be associated with early onset of diseases rather than late stages. Thus, γδ T cells can be considered as an early sensor for inflammation and may act as a connecting bridge with further inflammation. Because γδ T cells actively surveil and rapidly respond to brain diseases, understanding their role is important for neuroimmunology research. Further study investigating different γδ T cell subsets in different contexts and at different time points will give critical insights into mechanisms regulating neuro-immune interactions.

AUTHOR CONTRIBUTIONS

JP, IK, and HL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Comparing Mouse and Human Tissue-Resident γδ T Cells

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Circulating immune cell compartments have been extensively studied for decades, but limited access to peripheral tissue and cell yield have hampered our understanding of tissue-based immunity, especially in γδ T cells. γδ T cells are a unique subset of T cells that are rare in secondary lymphoid organs, but enriched in many peripheral tissues including the skin, uterus, and other epithelial tissues. In addition to immune surveillance activities, recent reports have revealed exciting new roles for γδ T cells in homeostatic tissue physiology in mice and humans. It is therefore important to investigate to what extent the developmental rules described using mouse models transfer to human γδ T cells. Besides, it will be necessary to understand the differences in the development and biogenesis of human and mouse γδ T cells; to understand how γδ T cells are maintained in physiological and pathological circumstances within different tissues, as well as characterize the progenitors of different tissue-resident γδ T cells. Here, we summarize current knowledge of the γδ T phenotype in various tissues in mice and humans, describing the similarities and differences of tissue-resident γδ T cells in mice and humans.

Keywords: γδ T cells, tissue-resident γδ T cells, human γδ T cells, mouse γδ T cells, γδ T cells development

1 FUNDAMENTAL CHARACTERISTICS OF γδ T CELLS

Gamma delta (γδ) T cells are a small subset of CD3-positive T cells in the peripheral blood but occur at increased frequency in mucosal tissues in mice and humans (1). Murine and human γδ T cells make up a minor part (1–5%) of the circulating T cell compartment found in the blood and secondary lymphoid organs. However, certain subsets of γδ T cells are present in much higher proportions (10–100%) in epithelial tissues, such as the reproductive tract, skin epidermis, and gastrointestinal tract (2). The mouse γδ T cell subsets are distinguished by different T cell receptor (TCR) Vγ chains, whereas human γδ T cell subsets are often distinguished by Vγ chain usage (2).

Heilig and Tonegawa's nomenclature proposed in 1986 segregated mouse γδ T cells into six distinct subsets: Vγ1, Vγ2, Vγ4, Vγ5, Vγ6, and Vγ7 (3). Meanwhile, the human γ chain locus consists of four subgroups; VγI includes Vγ2, 3, 4, 5, and 8. Among the three other Vγ subgroups, only Vγ9 (from the VγII group) is functional when using the nomenclature of Lefranc and Rabbitts (4). Besides, γδ T cells are reported to bridge the gap between innate and adaptive immune responses in mice and humans. Although γδ bearing cells were shown to constitute a minor proportion of peripheral T lymphocytes, their co-evolution with αβ T cells and B lymphocytes revealed non-redundant functions.

γδ T cells mostly reside within tissues, particularly in epithelial layers, where they might play tissue-protective or inflammatory roles (5). Experiments in mice have demonstrated that γδ T cells are predominantly tissue-resident immune cells (6, 7). From further mouse studies, it is nonetheless becoming increasingly clearer that the γδ T pool residing in a given tissue is the result of the wave of development from fetal to adult life, referred to as layered ontogeny (8). Nevertheless, how the ontogeny of γδ T cells differs between tissues remains obscure. Although the origin of tissue-resident γδ T cells in humans is technically challenging to address, there is evidence that the local γδ T cells pool can partially be replenished by infiltration and *in situ* differentiation of circulating naïve γδ T cells (9).

Reflecting their tissue residency and the impact of the microenvironment on γδ T cell function, recent studies have revealed profound tissue-specific transcriptional signatures for human (9) and mouse γδ T cells (10). Accumulating evidence suggests that γδ T cells are shaped by the microenvironment and exert tissue-specific functions depending on the signals they receive. This review summarizes recent studies on the tissue-specific features of γδ T cells across organs in mice and humans. We discuss the phenotypic differences that contribute to distinct γδ T cell profiles in different tissues, highlighting the similarities and differences between mice and humans. Understanding how various tissue microenvironments impact γδT cells is important for improving therapeutic strategies in pathologies that affect specific tissues.

2 γδ T CELLS DEVELOPMENT IN MICE

αβ T cells and γδ T cells arise from a common progenitor known as a double-negative cell (DN; lacking CD4 and CD8 expression) in the thymus (11). γδ T cells that develop without pre-programming in the thymus and receive the TCR signal in the periphery develop as adaptive types, whereas γδ T subsets that receive the signal in the thymus are innate types, and those which receive the TCR signal in the periphery but during an early phase of life get converted into innate-like γδ T cells (12).

During the development of mouse γδ T cells, γδ T cells are the first T cells to develop in the mouse embryonic thymus and appear as early as embryonic day 15 of gestation. These cells express a monoclonal Vγ5Vδ1 T cell receptor (TCR) and are always located in the skin epidermis. A few days later, by an oligoclonal Vγ6Vδ1 TCR-expressing population, entered multiple peripheral locations, including the tongue, dermis, uterus, testis, abdominal cavity, adipose tissue, and meninges. Semi-invariant $Vγ4^+$ γδ T cells also develop within this time range, and these cells are associated with $Vγ6^+$ cells that have the same functional characteristics. Such $Vγ4^+$ γδ T cells are home to the lungs, the dermis of the skin, and lymph nodes. Subsequent perinatal $Vγ7^+$ γδ T cell waves enter the intestine, followed by polyclonal $Vγ1^+$ and $Vγ4^+$ γδ T cell populations, which are more systematically distributed, including peripheral lymphoid organs, where they exhibit adaptive behavior when activated (revised **Figure 1A**).

On the other hand, mouse γδ T cells can commit to effector cytokine production during thymic development; two main functional subsets have been extensively described: IFN-γ-producing γδ T cells and IL-17-producing γδ T cells. (i) IFN-γ-producing γδ T cells express surface markers, such as CD45RB and CD27. Subpopulations include the fetal and perinatally derived $Vγ5^+$ dendritic epidermal T cells (which is called DETC), which are home to the skin, and the postnatally generated cells that express more polyclonal γδ T cell receptor (TCRs) (mostly $Vγ1^+$ or $Vγ4^+$) and localize to lymphoid tissues. (ii) IL-17-producing γδ T cells lack CD27 expression and include the fetal derived monoclonal and/or oligoclonal $Vγ6^+$ T cells that

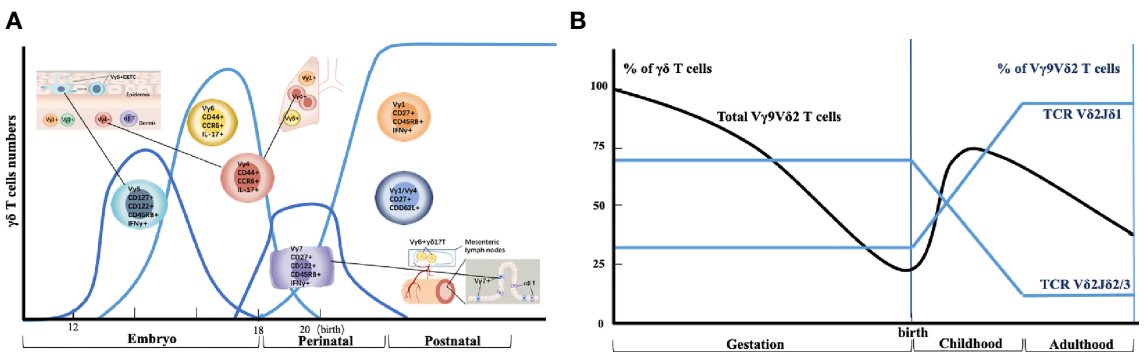

FIGURE 1 | Thymic developmental waves and tissue homing of human and mouse γδ T cell subsets. **(A)** Different waves of γδ T cell progenitor subsets are produced in specific developmental windows in the thymus and selectively home to different organs. **(B)** Schematic depiction of human $Vγ9Vδ2^+$ T cell generation and selection throughout life.

are home to the tongue, dermis, uterus, testis, adipose tissue, and brain meninges, and the Vγ4$^+$ IL-17-producing γδ T cells that express multiple semi-invariant TCRs and are home to the lung, dermis, and lymph nodes.

The development of mouse γδ T cells and their subsets depends critically on IL-7 and IL-15 (5). The growth of dermal γδ T cells preferentially requires IL-7, whereas IL-15 is mandatory for the generation of γδ TCR-expressing intra-epithelial lymphocytes (IELs) (13). IL-7 signaling promotes the development of IL-17-producing γδ T cells, whereas IL-15 and IL-2 induce IFN-γ secretion. Besides, various cytokines have been reported to affect the differentiation of effector γδ T cells. IL-12 and IL-18 promote IFN-γ production, while IL-1β and IL-23 drive them towards IL-17-producing cells (14).

In summary, these very curious 'waves' of mouse γδ T cell development ensure that most peripheral tissues are effectively colonized by long-lived γδ T cells (Revised **Figure 1A**) that are ideally placed to play important roles *in situ*.

3 γδ T CELLS DEVELOPMENT IN HUMANS

Unlike murine γδ T cells, human γδ T cells are usually sub-divided based on the use of one of two variable regions of TCR-δ chains, which is Vδ1 or Vδ2. The Vγ9 and Vδ2 variable (V) gene segments are the first γ/δ chains to undergo rearrangement in development, detected in the fetal liver from as early as at weeks 5~6 of gestation (15) and in the fetal thymus after 8 weeks of gestation (16). By mid-gestation (20~30 weeks), Vγ9Vδ2$^+$ T cells dominate the γδ repertoire (Revised **Figure 1B**). Vδ2 is the largest subset of circulating human γδ T cells in the blood, which gets rapidly recruited to the mucosal surface to participate in the clearance of localized infection (17). Functionally, Vδ2$^+$ T cells exist as naive (CD45RA$^+$CD27$^+$), central memory (CD45RA$^-$ CD27$^+$), effector memory (CD45RA$^-$ CD27$^-$), and terminally differentiated (CD45RA$^+$CD27$^-$) populations (18). By contrast, human Vδ1$^+$ subsets are the major γδ T cells population in the intestine and skin, whereas Vδ3$^+$ subsets are enriched in the liver and gut.

Several features of the Vγ9Vδ2$^+$ compartment suggest similarities to mouse γδ T-cell subsets (19). First, the early fetal wave of Vγ9Vδ2$^+$ production, with the semi-invariant Vγ9Vδ2$^+$ TCR repertoire, mirrors early waves of semi-invariant mouse γδ T cells. Second, the semi-invariant mouse population expresses Vγ4 sequences of restricted length and diversity, analogous to public human Vγ9 sequences (20, 21). Third, consistent with related immunobiology, butyrophilins (BTN3A1 and BTN3A2/3) are important for Vγ9Vδ2$^+$ T cell recognition (22). However, while some semi-invariant mouse γδ T cell populations can become hyporesponsive to TCR triggering following initial strong TCR signaling during development (23), apparently, this does not apply to human Vγ9Vδ2$^+$ T cells. Notably, Vγ9Vδ2$^+$ T cells remain responsive to both pyrophosphate antigens (pAg) and anti-CD3 stimulation, a feature that underlies their potential use in several cancer immunotherapy applications (24), and they also exhibit the potential for further TCR-mediated plasticity (25).

In summary, γδ T cells comprise distinct functional subpopulations. Current views in the field suggest that the functional potential of mouse γδ T cells is related to the use of Vγ, while the functional potential of humans is related to the use of Vδ (26). When assembling TCRs, human γδ T cells express seven bona fide Vγ genes but only three Vδ genes (27).

4 COMPARISON OF THE MODE OF ACTION OF γδ T CELLS IN DIFFERENT ANATOMICAL LOCATIONS IN MICE AND HUMANS

4.1 γδ T Cells in the Skin

γδ T cells localized to the skin are mainly involved in maintaining tissue homeostasis and epithelial repair, maintaining epithelial barriers, and contributing to innate immunity. However, the γδ T subsets in mouse and human skin differ.

4.1.1 γδ T Cells in Mouse Skin

The skin is composed of two major compartments, the epidermis and the dermis, that are populated in the steady-state by distinct γδ T cell subsets. Intraepithelial Vγ5$^+$ and Vγ6$^+$ γδ T cells are present in the dermis (28). In wild-type mice, the epidermal T cell compartment is dominated by a highly specialized γδ T cell subset termed dendritic epidermal T cells (DETCs) (29). DETC precursors that express a canonical Vγ3Vγ1 TCR are the first T cells to develop in the mouse thymus. Vγ3$^+$ thymocytes are generated only during the early fetal stages of thymic development from E13 to E18 and migrate to the epidermis, where a defined homeostatic density is maintained throughout life by self-renewal (30). Moreover, SKINT1 was shown to couple thymic selections of DETC precursors to their functional programming as IFN-γ producers (31). SKINT1, a mouse-specific member of the butyrophilins (BTNs) family that is exclusively expressed in the thymic epithelium and the epidermis, was shown to be essential for thymic selection and skin-specific homing of Vγ5Vγ1 T cell (32).

When the skin is damaged or infected, the γδ T cells that function in the epidermis of mouse skin are the epidermis-localized Vγ5$^+$ DETCs whose dendritic morphology enables them to contact several adjacent cells simultaneously, such as keratinocytes, Langerhans cells and melanocytes, which increase their own susceptibility to tissue stress and pathology (33). The maintenance of steady-state numbers of DETC is dependent on epithelial cell-derived IL-15, insulin-like growth factor I (IGF1) produced by DETC itself, and through the transcription factor aryl hydrocarbon receptor (AHR) ligand (2). Wendy and colleagues have found that the lack of DETCs in Tcrγ$^{-/-}$ (which means the mice lack all γδ T cell subsets) mice also results in increased keratinocyte apoptosis due to a deficiency of insulin-like growth factor 1 (IGF1) (34). Although DETCs are thymically programmed to produce IFN-γ rather than IL-17 in wild-type mice, DETCs on a skint-1-deficient background are primarily committed toward an IL-17 effector phenotype (35, 36). IL-17 release by DETCs can promote DNA repair following exposure

to UV radiation and protect the skin against potential opportunistic infections by releasing keratinocyte-derived antimicrobial peptides (37, 38). However, in the models of psoriasis and dermatitis, IL-17 is detrimental and is produced by dermal Vγ4$^+$ and Vγ6$^+$ γδ T cells rather than by DETCs. Paradoxically, another study showed that IL-17-producing γδ (γδ17) T cells have a beneficial role in steady-state skin physiology, and γδ17 T cells are also necessary for skin homeostasis (Revised **Figure 2**).

4.1.2 γδ T Cells in Human Skin

The composition of T cell subsets in the skin differs between mice and humans. There is no direct equivalent of DETCs in human skin as the immune cell composition of the epidermis is subject to species-specific differences (2). In human skin, γδ T cells dominate in both the dermis and the epidermis, but γδ T cells are present in both compartments (2).

In humans, the subset of γδ T cells localized in human skin is Vδ1$^+$ γδ T cells, which express oligoclonal clonal sequences distinct from circulating γδ T cells (39). Unlike mouse skin epidermal T cells that only contain DETCs, the human epidermis contains both αβ T cells and γδ T cells, and Vδ1$^+$ γδ T cells are localized in both the epidermis and dermis (40). Similar to DETCs, human epidermal T cells produce keratinocyte growth factor (KGF) and insulin-like growth factor 1 (IGF1) and promote wound healing upon activation. It can be seen that DETCs can be regarded as a conserved expression in mouse and human skin and have similar functions, but there are differences in the subgroups of γδ in different species. First, the human γδ T cells subsets rarely secrete IL-17, which was quite different from the mouse γδ T cells subset in the skin. Second, the human γδ T cells subset in the skin is Vγ1 γδ T cells verse DETCs in the mouse skin. Third, the mechanism of how human γδ T cell protects from infection is also different from that of the mouse γδ T cells.

In summary, although the role of DETCs in wound healing in mice has been demonstrated, the functions and roles of human epidermal γδ T cells are just beginning to be elucidated (33) (Revised **Figure 2**). There is an urgent need to explore human γδ T cell functions in future work.

4.2 γδ T Cells in the Lungs

4.2.1 γδ T Cells in Mouse Lungs

Considerable numbers of Vγ4$^+$ and Vγ6$^+$ γδ T cells are present in mouse lungs, but their effect on lung tissue physiology is unclear (28). When lung infection occurs, Vγ1$^+$, Vγ4$^+$, and Vγ6$^+$ T cells proliferate in the lung, and Vγ4$^+$ γδ T cells secrete CXC chemokine ligand 2 (CXCL2; also known as MIP2) and TNF to promote neutrophil recruitment (41). The secretion of IL-17 by γδ T cells may be the main mechanism involved in lung immunity. Studies have shown that infected dendritic cells, through IL-23, can increase the production of IL-17 by Vγ4$^+$ and Vγ6$^+$ T cells and promote granuloma formation. IL-17 production by lung-resident Vγ4$^+$ T cells can also be increased upon secondary attack (42).

4.2.2 γδ T Cells in Human Lungs

In the human lung, both Vδ1$^+$ γδT cells and Vδ2$^+$ γδT cells play vital roles. However, the mechanisms of these two subsets in specific diseases and the comparison of the immune effect need further research. During lung infection, Vγ9Vδ2 γδ T cells are aggregated to produce IL-17 and IFN-γ, the former being the most important cytokine in TB protection (43). Vγ9Vδ2 γδ T cells specifically recognize the phosphoantigen (E)-4-hydroxy-3-methylbutylpyrophosphate (HMB-PP), which is abundantly produced by *Mycobacterium tuberculosis*, and this selective

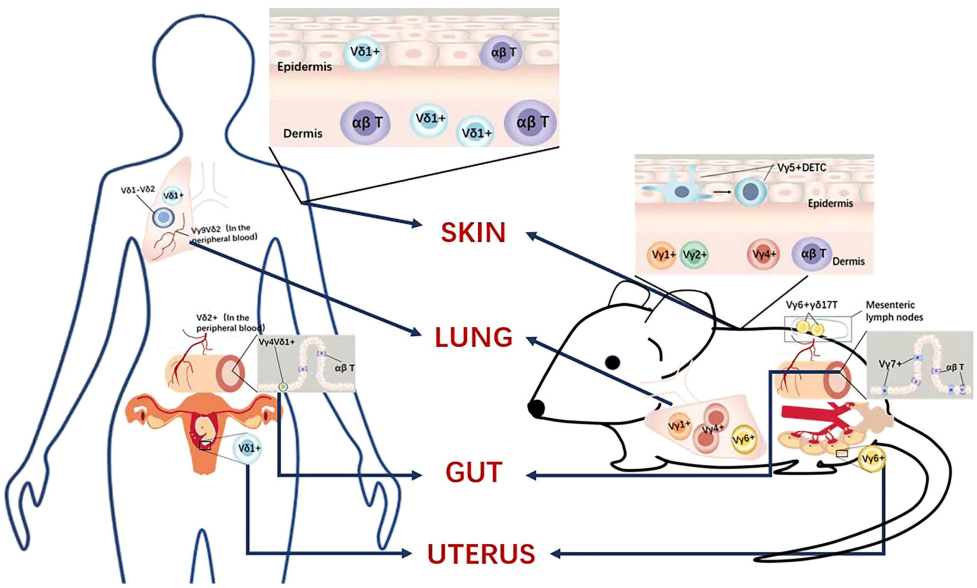

FIGURE 2 | Tissue-resident γδ T cell subsets, comparing humans and mice.

immunity elicits rapid and long-lasting memory, rapidly producing more IL-17 and IFN-γ upon pathogen-specific re-challenge, enhancing bacterial clearance (44). In advanced non-small cell lung cancer, Vδ1 γδ T cells and Vδ1-Vδ2-γδ T cells are the main subpopulations of γδ T cells in the lung, and higher levels of intratumoral Vδ1 γδ T cells is a poor prognosis factor (45). Due to the lack of methods to expand Vδ1 γδ T cells in lung cancer *in vitro*, we have not been able to clarify the role of Vδ1 γδ T cells in the lung (46) (Revised **Figure 2**).

4.3 γδ T Cells in the Uterus

4.3.1 γδ T Cells in Mouse Uterus

Mouse Vγ6/Vδ1 cells are closely associated with the epithelial tissue of the female reproductive tract and account for a major proportion of γδ T cells in uterine tissue (47). Unlike other subpopulations, Vγ6/Vδ1 cells contain a typical Vγ6 TCR amino acid junction. A recent study has reported that the percentages of γδ T cells were significantly higher in the uterus than in peripheral blood, and most γδ T cells in mouse uterus were distributed in the endometrium (48). Further studies indicated that the majority of γδ T cells in the uterus were memory cells with higher expression of CD44 and CD27 but lower expression of CD62L and CCR7 compared to those in the blood (48). In addition, mouse γδ T cells in the uterus were tissue-resident memory γδ T cells expressing CD69 and expressed high levels of CCR6, GranzymeB, and CD107a. Moreover, γδ T cells in the uterus were activated and fully expressed transcription factor RORγt. After a short time of activation, mouse γδ T cells in the uterus significantly expressed high levels of IL-17 but not IFN-γ, promoting the invasion of murine trophocytes.

4.3.2 γδ T Cells in the Human Uterus

In healthy pregnant women, there was an accumulation of $V\delta1^+$ circulating cells, in contrast to women with recurrent abortions where the $V\delta2^+$ circulating cells dominated (47). The ratio of activated γδ TCR^+ cells was significantly increased in normal pregnancies compared to that of recurrent abortions (48). A bias towards circulating $V\delta1^+$γδT cells seemed to be required for a successful normal pregnancy. However, the precise role of circulating γδ T cells in pregnancy is not yet completely established. Although convenient to study the γδ T cells subsets during pregnancy in the peripheral blood, it hardly to study that how the circulating $V\delta1^+$ cells might simply be a spilling over from the fetus-maternal interface.

5 CONCLUDING REMARKS

Recent reports have undoubtedly revealed significant tissue-specific functions of γδ T cells. We highlight the distribution, features, and specific markers of distinct subsets of murine and human γδ T cells (Revised **Table 1**). In humans, γδ T cells in blood display a quiescent state and migratory behavior reminiscent of naiüve T cells. By contrast, γδ T cells in peripheral organs make up a spectrum of

TABLE 1 | Distribution, features and specific markers of distinct subsets of murine and human γδ T cells.

Structural subset	Distribution	Features (mainly cytokines)	specific marker
Murine γδT Cells			
Vγ1	Lymphoid tissue, liver	IFN-γ, TNFα, IL-4 and IL-17	CD27, CD45RB, CD44, CD122
Vγ4	Lymphoid tissue, lung, liver, dermis	IL-17, IFN-γ	CD44, CCR6
Vγ5 - DETC	Epidermis	IFN-γ - Sensing skin keratinocyte damage - Producing KGF and IGF1 to improve wound healing efficiency and participate in the maintenance of epidermal homeostasis - Secreting IL-2, IL-3, granulocyte-macrophage colony-stimulating factor, lymphatic chemokine, etc. to regulate the activation and function of DETCs themselves and keratinocytes and other neighboring cell	CD27, CD44, CD45RB, CD122,
Vγ6	Uterus, Lung, tongue, liver etc.	IL-17, IL-22, IFN-γ	CD44, CCR6
Vγ7	Intestinal mucosa	IFN-γ	CD27, CD45RB, CD122, CD8α
Human γδT Cells			
Vδ1	PBMCs, skin, gut, spleen, liver	In epithelium, some functions are similar to DETCs; produce IL-10, a small amount of IL-2, IL-4 and IFN-γ; exhibit cytotoxicity through FasL, perforin, granzyme, etc. - **Subset γδTreg** Mainly secreting IFN-γ and granulocyte-macrophage colony-stimulating factor. Regulating innate and adaptive immune responses to play an important anti-infective role. - **Subset Tγδ17** Expressing granzyme B, FasL, and CD161, but does not produce IL-22 and IFN-γ; in terms of antigen activation, Tγδ17 cells rapidly induce IL-8-mediated migration and phagocytosis of neutrophils, and are IL-dependent -17 Produces beta defensins	NKR, Toll-Like Receptor, CD8
Vδ2	PBMCs	- Unique Feature:Activated Vδ2γδ T cells acquire APC properties (such as antigen presentation, co-stimulation and expression of adhesion molecules MHC-II, CD80 and CD86) - As circulating γδ T cells, it also possesses cytotoxicity, cytokine and chemokine production and modulation capabilities against infected or tumor cells	NKG2D, Toll-Like Receptor, CD45
Vδ3	PBMCs(very few), Liver	Increasing CD1d recognition and kill CD 1d target cells, releasing Th1, Th2 and Th17 cytokines, and inducing dendritic cells to become APCs, when stimulated by mitogens and IL-2.	CD56, CD161, HLA-DR, NKG2D

activation states that differ depending on the organ. Although human γδ T cells deserve more research, mouse γδ T cells display tissue-specific degrees of IFN-γ and IL-17A production that appear to be regulated by factors present in the tissues, such as cytokines. γδ T cells subsets in different organs show variable means of sensing the microenvironment, particularly regarding cytokines. Finally, the tissue-specific functions of γδ T cells, in terms of tissue retention and response to chemokines/cytokines, are not only related to the organ but also to species. Further elucidation of γδ T cell-mediated tissue immunity, particularly in humans, will be necessary to improve the development of tissue-specific immunomodulatory drugs to be used, for example, in inflammatory conditions and cancer.

AUTHOR CONTRIBUTIONS

GQ and SW helped in drafting the manuscript. ZZ and DJ helped draw the image in **Figures 1**, **2**. JL and AL conceptualized and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Vγ2 x PD-L1, a Bispecific Antibody Targeting Both the Vγ2 TCR and PD-L1, Improves the Anti-Tumor Response of Vγ2Vδ2 T Cell

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The potent cytotoxic property of Vγ2Vδ2 T cells makes them attractive for adoptive T cell transfer therapy. The transfusing of the expanded Vγ2Vδ2 T cells into cancer patients shows well-tolerated, but the clinical response rates are required to be improved, implying that there is still an unmet efficacy with low toxicity for this novel anti-tumor therapy. In this study, we test the anti-tumor efficacy of a Y-body-based bispecific antibody (bsAb) Vγ2 x PD-L1 that preferentially redirects Vγ2Vδ2 T cells to combat PD-L1 positive tumor cells. With nanomolar affinity levels to Vγ2Vδ2 T cells and PD-L1+ tumor cells, Vγ2 x PD-L1 bridges a Vγ2Vδ2 T cell with a SKOV3 tumor cell to form a cell-to-cell conjugation. In a PD-L1-dependent manner, the bsAb elicits effective activation (CD25+CD69+), IFNγ releasing, degranulation (CD107a+), and cytokine production (IFNγ+ and TNFα+) of expanded Vγ2Vδ2 T cells. The activations of the Vγ2Vδ2 T cells eliminate PD-L1-expressing human cancer cell lines, including H1975, SKOV3, A375, H1299, and H2228 cells, but not PD-L1 negative cells including HEK-293 (293) cells and healthy PBMCs. Finally, we show that combining Vγ2 x PD-L1 with adoptively transferring Vγ2Vδ2 T cells inhibits the growth of existing tumor xenografts and increases the number of Vγ2Vδ2 T cells into the tumor bed. Vγ2 x PD-L1 represents a promising reagent for increasing the efficacy of adoptively transferred Vγ2Vδ2 T cells in the treatment of PD-L1 positive malignant tumors.

Keywords: [Vγ2 x PD-L1], Vγ2Vδ2 T cell, PD-L1, adoptive transfer, immunotherapy

INTRODUCTION

Vγ2Vδ2 T cells, a unique fast-acting subset of innate γδ T cells found exclusively in primates (1), have been widely employed for adoptive cell immunotherapy in clinical studies for treating malignancies in past years (2). These cells have NK and cytotoxic T cell features, as well as potential and intrinsic rapid anti-tumor effector capabilities (3, 4), and appear to be a more promising candidate for allogeneic T cell therapy than αβ T cell-based CAR-T cells by

participating in immune surveillance and killing a broad spectrum of cancer cells through a major histocompatibility complex (MHC)-independent activation mechanism (5). Recently, the adoptive transfer of Vγ2Vδ2 T cells to cancer patients has recently been shown to extend the survivals of late-stage liver cancers (23.1 vs 8.1 months) and lung cancers (19.1 vs 9.1 months) (6), and well tolerated as well (7). Yet, this therapy provided moderate clinical benefits with stable disease being the mostly outcome for patients who respond to this therapy (7).One of the reasons for this suboptimal effectiveness is the hostile tumor microenvironment that negatively regulates the anti-tumor functional characteristics of Vγ2Vδ2 T cells by the engagement between the programmed death-ligand 1 (PD-L1) expressed on the tumor cells and PD-1 expressed on the Vγ2Vδ2 T cells (8). Several groups proposed a combination approach of the Vγ2Vδ2 T cell-based adoptive immunotherapy with a PD-1 checkpoint blockade for the immunity against leukemia (9), follicular lymphoma (10), and prostate cancer (11). Likely, the anti-PD-L1 mAb enhances the cytotoxicity of Vγ2Vδ2 T cells against PD-L1^{high} cancer cells by adding ADCC activity (12).

After the success of targeting PD-1/PD-L1 axes, extensive efforts were directed to explore bsAb-based strategies to increase the anti-tumor activity of the adoptively transferred Vγ2Vδ2 T cells (13, 14). As a result, two representative series of Vγ2Vδ2 T cell-targeting bsAbs were constructed, one targeting to Vγ2-TCR and the other targeting to Vδ2-TCR. BsAb [(Her-2)$_2$ × Vγ2] increased the cytotoxicity of Vγ2Vδ2 T cell against Her2-overexpressing pancreatic, ovarian and breast cancer cells showed by *in vitro* assay and in a PDAC grafted mouse model (15, 16). Similarly, Vγ2 x CD123 was created to treat acute myeloid leukemia (17). Lately, Vδ2 x EGFR elicits Vγ2Vδ2 T cell-mediated killing of colon cancer cell line SW480 both *in vitro* and *in vivo* (18), Vδ2 x CD1d for chronic lymphocytic leukemia (19), and Vδ2 x CD40 for b-cell malignancies (20). Moreover, these Vγ2Vδ2 T cell-specific targeting strategies were thought to overcome T cell over-activation induced by current CD3-targeting bsAbs, which could lead to cytokine storm syndrome, a severe side effect due to Treg stimulation. For example, the FDA-approved CD3 x CD19 bsAb, blinatumomab, could increase the numbers of Treg cells, which were correlated with non-responsiveness to blinatumomab in ALL patients (21) and further led to abnormal macrophage activation-dependent cytokine storm syndrome (22). Taken together, T cell engagers designed to activate Vγ2Vδ2 T cells exclusively might represent a feasible approach balanced between efficacy and safety.

Here, we describe the preclinical evaluation of Vγ2 x PD-L1. Our findings reveal that Vγ2 x PD-L1 activates selectively the fresh and expanded Vγ2Vδ2 T cells to kill tumor cells *in vitro*, enhances the migration of the transfused Vγ2Vδ2 T cells into tumor sites, and inhibits the growth of the existing tumors in nude mice. These data suggest that Vγ2 x PD-L1 plus adoptively transferred Vγ2Vδ2 T cells is potential to treat PD-L1 positive solid malignancies.

MATERIALS AND METHODS

Generation of the Recombinant Antibodies

The bsAbs, including Vγ2 x PD-L1 and Vγ2 x Null, were generated similarly to Y111 described previously by Yang et al. (23). Briefly, the expression plasmids for Vγ2 x PD-L1 and Vγ2 x Null were synthesized and verified by sequencing in AuGCT Biotech (Wuhan, China). Then these expression vectors were transfected into cGMP banked CHO-S cells (Invitrogen, Carlsbad, USA) using the Fecto PRO Reagent (Ployplus, New York, USA) according to the manufacturer's protocol, respectively. After a week, the cell culture supernatant was collected and serially purified by Sepharose Fast Flow protein A affinity chromatography column (GE, Milwaukee, USA), Fab Affinity KBP Agarose High Flow Resin (ACROBio systems, Newark, USA), and SP cation exchanged chromatography column (GE, Milwaukee, USA). Finally, the purified proteins were analyzed by SDS-PAGE and size-exclusion chromatograms. The Vγ2 x Null served as the control molecule for Vγ2 x PD-L1, with both molecules sharing the same backbone and Vγ2-targeting scFv part. Similarly, its two parental monoclonal antibodies (Vγ2 mAb (Clone 7A5) and PD-L1 mAb (23)) were produced.

Tumor Cell Lines Culture

Tumor cell lines, including NCI-H1975 (human adenocarcinoma epithelial cell line, CRL-5908), SKOV3 (human ovarian adenocarcinoma cell line, HTB-77), A375 (human malignant melanoma cell line, CRL-1619), NCI-H1299 (human NSCLC metastatic cell line, CRL-5803), NCI-H2228 (human NSCLC adenocarcinoma cell line, CRL-5935), and nonmalignant kidney cell line HEK-293 were purchased from ATCC (Manassas, USA) and used as target cells. These cell lines were first transduced with firefly luciferase gene-containing pseudo-typed lentiviral particles purchased from GeneCopoeia (Shanghai, China), and the stable luciferase-expression cells were then selected under pressure of puromycin (Gibco, New York, USA). CHO-PD-L1 was generated from the parental CHO-K1 cell line (CCL-61, ATCC) through over-expressing human PD-L1. Tumor cells were cultured in RPMI 1640 (Biosharp, Hefei, China), DMEM or F-12K medium (purchased from Hyclone, New York, USA) supplemented with 10% FBS (Excell, Clearwater, USA) and penicillin/streptomycin (Gibco, New York, USA) and maintained in a humidified incubator with 5% CO_2 at 37 °C. All cell lines in use were routinely tested for Mycoplasma infection using a commercial PCR kit (Vazyme, Nanjing, China), and new cultures were established monthly from frozen stocks as described previously (24).

Expansion of Vγ2Vδ2 T Cells

The sampling protocols for human blood and *in vitro* experimental procedures were evaluated and approved by the institutional review boards for human subjects' research and institutional biosafety committees at Hubei Province Food and Drug Safety Evaluation Center (Wuhan, China). All subjects are volunteer adults who signed on the informed consent.

Frozen or fresh human peripheral blood mononuclear cells (PBMCs) were obtained from LeiDeBio (Guangzhou, China) or Milestone (Shanghai, China). The *ex vivo* expansion protocol was described previously (23, 25). Briefly, PBMCs were cultured in RPMI 1640 medium (Gibco, New York, USA) supplemented with 10% FBS (Excell, Clearwater, USA), at 2×10^6 cells/mL with the stimulation of 2.5 μM Zoledronic Acid (Sigma Aldrich, Darmstadt, Germany) and 1000 IU/mL IL2 (Sihuan Pharma, Beijing, China) for 10-14 days. The expanded Vγ2Vδ2 T cells were negatively enriched from the cultures by a TCR γ/δ + T Cell Isolation Kit (Miltenyi Biotech, Teterow, Germany). The purity and quality of the isolated cells were assessed by surface staining Vγ2/Vδ2 and CD86/CD69/HLA-DR as described previously ((23)). Then, the purified Vγ2Vδ2 T cells were maintained in RPMI 1640 medium supplemented with 10% FBS overnight for rest before use. In this study, effector Vγ2Vδ2 T cells were expanded and purified from a total of eight healthy individuals for *in vitro* functional analysis and two healthy donors for *in vivo* anti-tumor evaluations.

Binding Ability of Antibodies to Cells

A flow cytometry-based method was used to determine the affinities of Vγ2 x PD-L1 of its anti-Vγ2 arm to Vγ2Vδ2 T cells and its anti-PD-L1 arm to PD-L1 positive tumor cells. The sorted Vγ2Vδ2 T cells or tumor cells were incubated with serially diluted antibodies (Vγ2 x PD-L1, Vγ2 x Null, Vγ2 mAb, and PD-L1 mAb) for one hour at 4°C. After wash, the cells were stained for 30 minutes at room temperature with APC or PE-conjugated mouse-anti-human IgG Fc antibody (HP6017, Biolegend, San Diego, USA) diluted in 1:100. The cells were then resuspended in 200 μL FACS buffer (PBS with 2% FBS) and analyzed by a BD FACSelesta flow cytometer. For tumor cells, the cell-bound antibodies were quantified by the median fluorescence intensity (MFI) values, and the MFI were plotted against antibody concentrations to obtain the EC_{50}. For Vγ2Vδ2 T cells, APC positive populations were used to determine the specific binding%.

The formation of an in-tans bridge between T cells and tumors cells was accessed by a flow cytometry method. Briefly, SKOV3 cells were stained with 50 nM CFSE, and the PBMC cultures (treated by Zol+IL2 for 10-14 days) were labeled by PKH26 according to the manufacturer's protocol. Then, the CFSE-stained SKOV3 cells were co-cultured with PKH26-labelled PBMC cultures at a ratio of 1:1 with 1 ug/mL of Vγ2 x PD-L1 or Vγ2 x Null at an incubator for 0.5 hours. After washing, the cells were recorded on the FACSelesta (BD, San Jose, USA). The percentages of the $CFSE^+PKH26^+$ double-positive cells among the total cells have represented the ratios of cells engaged in cell-to-cell association.

PD-L1 Blockade Reporter Assay

The assay was carried out following the manufacturer's instructions (Promega, Cat#J1250). Briefly, PD-L1 aAPC/CHO-K1 cells were seeded at 4×10^4 cells/well at 100 μL in white 96-well plates followed by a cultured overnight in an incubator at 37 °C with 5% CO2. The next day, the supernatant was discarded and the PD-L1 aAPC/CHO-K1 cells were incubated with serially diluted antibodies and PD-1 effector cells (5×10^4/well) for 6 h. Then the relative luminescence units (RLU) of each well were determined using a Bio-Luc kit from Vazyme (Nanjing, China).

PD-L1 Expression Scores Determination

Tumor cell lines were incubated with 40 μg/mL Vγ2 X PD-L1 (Target) or Vγ2 X Null (Null) for 1 hour at 4°C, then stained with APC-conjugated mouse-anti-human IgG Fc antibody (HP6017, Biolegend, San Diego, USA) for 30 minutes at room temperature. The APC positive populations and MFI of the APC channel were determined by flow cytometry. The expression scores were defined by $[\log_{10}(\text{Target}_{\text{APC positive populations}} - \text{Null}_{\text{APC positive populations}}) + \log_{10}(\text{Target}_{\text{APC MFI}}/\text{Null}_{\text{APC MFI}})]/2$.

Evaluate T Cell Activation by Surface Staining and Intracellular Cytokine Staining

Flow cytometry was performed to evaluate T-cell activation as described in the other reports (26, 27). Expanded Vγ2Vδ2 T cells were enriched from PBMCs cultures (Zol+IL2 for 10-14 days), and cultured overnight. In parallel, 0.2 million H1975 or SKOV3 cells were plated in a 24-well-plate overnight. For activation assay, 0.2 million expanded and negatively enriched Vγ2Vδ2 T cells were added into either the tumor cell wells or empty wells with 1 μg/mL of Vγ2 X PD-L1 or Vγ2 X Null for 24 hours. Then, the cells were collected for staining FITC-anti-Vδ2 (B6, Biolegend, San Diego, USA), APC-anti-CD25 (M-A251, BD, San Jose, USA), and PE-anti-CD69 (FN50, BD, San Jose, USA) for 20 min at room temperature in dark. After wash, these cells were analyzed using flow cytometry. For intracellular cytokine staining, 0.2 million of the expanded and negatively enriched Vγ2Vδ2 T cells were added into the tumor cell wells or empty wells with 1 μg/mL of Vγ2 X PD-L1 or Vγ2 X Null plus a master mix containing BV510-anti-CD107a (H4A3, Biolegend) and BFA (Golgi Plug, BD, San Jose, USA) for 4 hours at 37 °C in 5% CO_2. Then the cells were stained with Zombie Fixable Viability Kit (Biolegend), incubated with APC-anti-CD3 (SP34-2, BD, San Jose, USA), PE-anti-Vδ2 (B6, Biolegend, San Diego, USA) for 20 min at room temperature in dark. After incubation, cells were washed twice in FACS buffer and permeabilized for 20 min at 4°C (Cytofix/Cytoperm, BD, San Jose, USA). Then, cells were incubated with BV650-anti-IFNγ (4S.B3, Biolegend, San Diego, USA), BV421-anti-TNFα (Mab11, Biolegend, San Diego, USA) in Perm/Wash buffer for 30 min at room temperature in dark. These cells were washed twice with Perm/Wash buffer and collected by a BD FACSelesta flow cytometry. Flow data were analyzed by FlowJo (BD, San Jose, USA).

Antibodies Mediated Cytotoxicity *In Vitro*

Two *in vitro* methods including luciferase-activity based assays and CFSE-PI staining-based assay were developed to access the killing ability of Vγ2Vδ2 T cells mediated by antibodies.

Luciferase-activity based assays: 2 x 10^4 firefly luciferase-expressing tumor cells (Target: T) were co-incubated with

expanded Vγ2Vδ2 T cells (Effector: E) at an E:T ratio of 0.5:1 (or other indicated E: T ratios), or fresh enriched γδ T cells (Effector) at an E:T ratio of 5:1, in the presence of a serial of diluted antibodies for 12 hours in a white 96-well-flat bottom plate. A Bio-Luc kit from Vazyme (Nanjing, China) was used to measure luciferase activity. Then the "Specific lysis" was calculated as follows: % Specific lysis = [1 – (RLU $_{\text{Ab-treated wells}}$)/(RLU $_{\text{Target-only wells}}$)] × 100.

CFSE-PI staining-based assay: Unrelated healthy PBMCs were stained with CFSE according to the manufacturer's protocol. Then these cells were co-cultured with Vγ2Vδ2 T cells at a 1:1 E: T ratio in the presence of various doses of indicated antibodies for 12 hours. Then 1μg/mL of PI (Sigma) was added to the wells. The percentages of $CFSE^+PI^+$ cells among the total of target cells ($CFSE^+$) were defined as "Specific Cytotoxicity%" values.

Measuring Vγ2Vδ2 T Cell Releasing IFNγ

The supernatant was collected from T cell and tumor cell co-culture wells and stored at -80°C until measurement. Human IFNγ were quantified with the ELISA kits from Proteintech (KE00063, Wuhan, China).

Mouse Tumor Model

Female nude mice were obtained from the VITALSTAR (Beijing, China) at age of 6-8 weeks and were used in this study under a protocol approved by the Animal Care and Use Committee from Hubei Province Food and Drug Safety Evaluation Center (#202110191).

Firstly, 5 million SKOV3 cells were subcutaneously inoculated into the right dorsal flank of nude mice on Day 0. After one week, tumor volumes had reached around 200 mm^3, these mice were randomly divided into three groups receiving PBS, 2 million purified Vγ2Vδ2 T cells i.v. through lateral tail vein plus 8 mg/kg Vγ2 X Null i.p. or 8 mg/kg Vγ2 X PD-L1 *i.p.* on Days 7,11,14, and 18 (Q2W, two weeks, four times). After treatment, tumor volumes and mice body weights were measured three times a week. The tumor volume was calculated using the formula: Tumor Volume (mm^3) = (a x b^2)/2, where a is the longitudinal length and b is the transverse width. On day 34, these mice were sacrificed and tumor xenografts were excised for tumor weighting and IHC staining.

IHC Analysis

The tumor tissues were cut into small pieces embedded in 4% paraformaldehyde for fixation. Then these tumor pieces were sectioned and examined by IHC staining using a rabbit-anti-human CD3 antibody (Clone SP7). Tissue sections were then counter-stained with hematoxylin. Positive cells were counted in five randomly selected microscopic fields (magnification 20X) and supplied for further quantification analysis.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 6.0 (La Jolla, USA). Before performing nonlinear regression analysis for *in vitro* assays (cell binding and killing), the antibody concentrations (on the x-axis) were transformed in a log scale. Then, the "log (agonist) vs. response- Variable slope (four parameters)" method was applied to calculate EC_{50}. P values were assessed by one-way or two-way ANOVA, followed by Dunnett test or Tukey multiple comparisons as appropriate. P values <0.05 were considered to be significant. P values were reported in **Supplementary Table 1**

RESULTS

Design, Generation, and Characterization of Vγ2 x PD-L1

We initially designed and constructed four recombinant antibodies, i.e. Vγ2 x PD-L1, Vγ2 x Null, PD-L1 mAb and Vγ2 mAb to test their activities. The structural properties of these generated antibodies were summarized in **Figure 1A**. Firstly, the molecular weights of these recombinant proteins were confirmed through SDS-PAGE under both reducing and non-reducing conditions (**Supplementary Figure 1A**). Then, the SEC results indicated that the purities of the prepared antibodies were more than 95% (**Supplementary Figure 1B**). Next, we used three PD-L1 expression cell lines (CHO-PD-L1, SKOV3, and H1975) to compare antibody binding ability to the cells between Vγ2 x PD-L1 and PD-L1 mAb. The mean EC_{50} values for Vγ2 x PD-L1 binding to CHO-PD-L1, SKOV3, and H1975 were 1.444 nM, 0.594 nM, and 1.687 nM, respectively (**Figure 1B**, **Figure 2**). Both Vγ2 x PD-L1 bsAb and PD-L1 mAb had a similar affinity to the cellular surface PD-L1 (**Figure 1B**), due to these two antibodies having the same variable regions for PD-L1 binding (23). Furthermore, we determined the PD-L1 expression scores for a series of target tumor cells using Vγ2 x PD-L1 bsAb, which confirmed that Vγ2 x PD-L1 exhibited potent affinity toward tumor cells with variable PD-L1 expression levels (**Supplementary Figure 2**). In addition, the binding affinity to the expanded Vγ2Vδ2 T cells of Vγ2 x PD-L1 was about 60-folds weaker than that of the parental Vγ2 mAb, as the mean EC_{50} values for Vγ2 x PD-L1 and Vγ2 mAb were 12.39 nM and 0.21 nM, respectively (**Figures 1C**, **2**). Moreover, Vγ2 x PD-L1 retained the blocking ability as PD-L1 mAb, which was demonstrated in the PD1/PD-L1 cell-based reporter assay (**Figure 1D**). In summary, Vγ2 x PD-L1 bound with nanomolar affinity to the sorted and expanded Vγ2Vδ2 T cells and PD-L1 expressing tumor cells.

Vγ2 x PD-L1 Efficiently Bridges Vγ2Vδ2 T Cells to PD-L1 Positive Tumor Cells

Subsequently, we checked whether the Vγ2 x PD-L1 prompted the formation of the biphasic cell-to-cell conjugates between Vγ2Vδ2 T cells and PD-L1 expressing tumor cells. For this purpose, Vγ2Vδ2 T cells stained with CFSE were co-cultured with PKH26-labelled SKOV3 cells for 30 minutes at 37°C with Vγ2 x PD-L1 or Vγ2 x Null, then the percentages of double-positive cells among total cells were measured to represent the bridging ability. In the presence of Vγ2 x Null at 1 μg/mL, the double-positive cell population (Q2) was 2.21%, while this population was increased up to 20.1% by Vγ2 x PD-L1

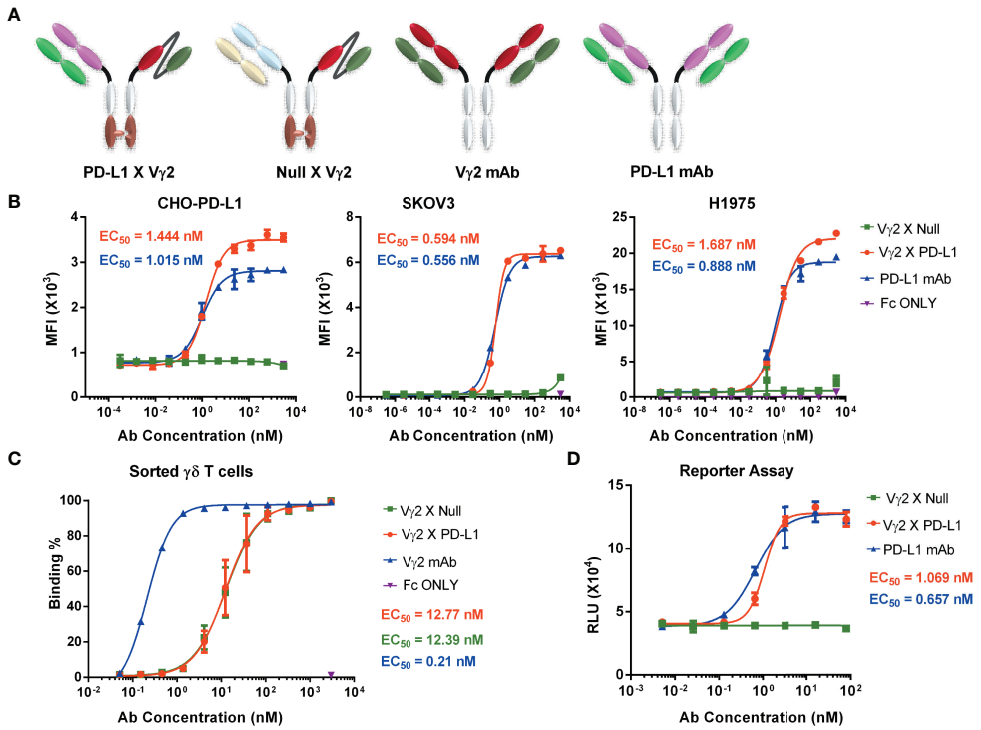

FIGURE 1 | PD-L1 x Vγ2 interacts Vγ2Vδ2 T cells and PD-L1 expressing tumor cells, and blocks the PD1/PD-L1 interaction. **(A)** Structural diagrams of bispecific antibodies, including PD-L1 x Vγ2, Null x Vγ2, Vγ2 mAb, and PD-L1 mAb. A "Knob-into-hole" in Fc region was introduced into the bsAbs (6, 28). Besides, these four antibodies contained a modified silent Fc fragment to abolish Fc-mediated effector functions (6, 28). Please noted that Vγ2 mAb and PD-L1 mAb, targeting Vγ2-TCR and PD-L1, respectively, are parental monoclonal antibodies; Vγ2 x Null, targeting Vγ2 and fluorescein (29), and Vγ2 x PD-L1, targeting Vγ2-TCR and PD-L1. The purity of these prepared antibodies was shown in **Supplementary Figure 1**. **(B)** Binding affinity to PD-L1 positive cell lines. CHO-PD-L1, SKOV3, and H1975 cells were incubated with serially diluted antibodies, followed by PE-labelled mouse-anti-human Fc secondary antibody. Mean fluorescence intensity (MFI) of the PE channel of each sample was measured to determine specific binding ability (EC50). These three cell lines were PD-L1 positive shown in **Supplementary Figure 2A**. **(C)** Antibody binding affinity to Vγ2Vδ2 T cells. Vγ2Vδ2 T cells were negatively enriched from PBMC cultures treated by Zol+IL2 for 14 days. Then, cells were incubated with serial dilutions of indicated antibodies, followed by APC-conjugated mouse-anti-human Fc secondary antibody. APC positive populations were measured to demonstrate specific binding (EC50). The representative flow cytometry plots related **(B, C)** were shown in **Figure 2**. **(D)** The ability of PD-L1 x Vγ2 to block PD1/PD-L1 signaling (EC50) was similar to that of the parental PD-L1 mAb using a cell-based reporter assay. Data were presented as Mean ± SD from n = 3 independent experiments **(B, D)**, pooled from n=1 biological replicate for Vγ2 mAb, n=6 biological replicates for Vγ2 x Null and Vγ2 x PD-L1 **(C)**. Reported EC_{50} values were calculated from non-linear best fits **(B–D)**.

(**Supplementary Figure 3**). In contrast, Vγ2 x PD-L1 failed to prompt the co-binding of Vγ2Vδ2 T cells and HEK-293 cells. (**Supplementary Figure 3**)

Vγ2 x PD-L1 Selectively Activates Vγ2Vδ2 T Cells Exposed to PD-L1 Expressing Tumor Cell Lines

Next, we investigated whether the activation of Vγ2Vδ2 T cells mediated by Vγ2 x PD-L1 was dependent on the presence of PD-L1$^+$ tumor cells. Vγ2Vδ2 T cells were co-cultured with H1975 and SKOV3 cells, the two cell lines that expressed high levels of PD-L1 (**Supplementary Figure 2A**). Vγ2Vδ2 T cells secreted little amount of IFNγ and did not exhibit activation phenotype (measured by CD25$^+$CD69$^+$) in response to the bsAbs treatment alone (**Figures 3A, B**, **Figure 4**). Of note, in the presence of H1975 and SKOV3 cells, Vγ2 x PD-L1, but not Vγ2 x Null, triggered significantly the release of IFNγ and active phenotype of Vγ2Vδ2 T cells (**Figures 3A, B**, **4**). Accordingly, Vγ2 x PD-L1 further enhanced significantly both the IFNγ and TNFα productions and degranulation levels of Vγ2Vδ2 T cells only in the presence of PD-L1 positive SKOV3 and H1975 cells (**Figures 3C-F**, **4**). Moreover, these Vγ2Vδ2 T cells activated jointly by Vγ2 x PD-L1 and PD-L1 tumor cells displayed multifunctional effector phenotypes, which co-expressed IFNγ, TNFα, and CD107a (**Figures 3D, F**, **Figure 4**). In contrast, Vγ2 x Null did not exert agonistic effects on Vγ2Vδ2 T cells even when co-cultured with PD-L1 expressing target cells in the above conditions (**Figures 3C-F**, **4**). Together, these data demonstrated that Vγ2 x PD-L1 revoked robust effector functions of Vγ2Vδ2 T cells, including activation, degranulation, and cytokines secretion, in dependent on the engagement of target tumor cells.

Vγ2 x PD-L1 Induces PD-L1$^+$ Tumor Cell Lysis at a Lower E: T Ratio

Then, we assessed whether Vγ2 x PD-L1 could lysis of tumor cells with variable PD-L1 expressing levels. To this end,

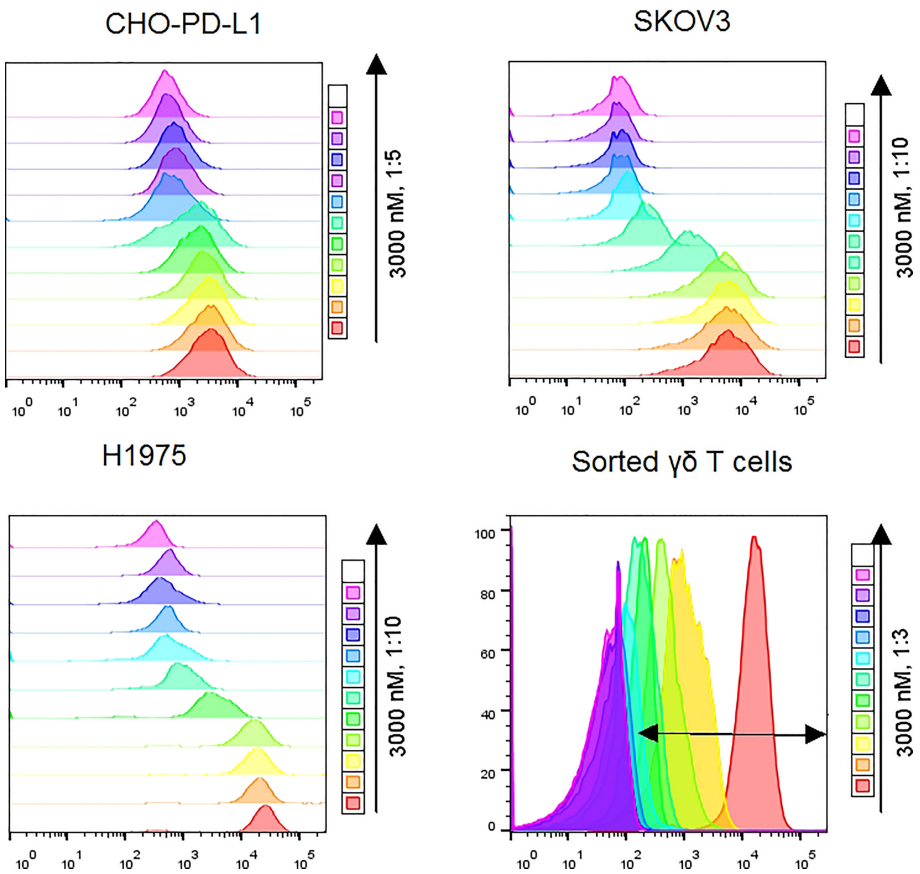

FIGURE 2 | Representative flow cytometry plots showed MFI differences along with doses of Vγ2 x PD-L1 for CHO-PD-L1, SKOV3, and H1975 cells. For sorted γδ T cells, the double-headed arrow indicated the APC positive population.

Vγ2Vδ2 T cells were co-cultured with SKOV3, H2228, and H1299 cell lines in E:T ratios ranging from 5:1 to 0.3125:1 for 12 hours. We selected SKOV3, H2228 for this test as these two cell lines expressed PD-L1 at high or low levels as determined using Vγ2 x PD-L1 staining (**Supplementary Figure 2B**). Vγ2Vδ2 T cells alone showed E: T ratio-dependent cytotoxicity for SKOV3 and H2228 (**Figures 5A, B**). The addition of Vγ2 x PD-L1, but not Vγ2 x Null, significantly enhanced tumor cell death even at the lowest E: T ratio (0.3125:1) for the both cell lines (**Figures 5A, B**). Furthermore, the larger amount of IFNγ was only detected in the Vγ2 x PD-L1 treated cultures, demonstrating that Vγ2 x PD-L1 elicited PD-L1-specific IFNγ production from Vγ2Vδ2 T cells (**Figures 5C, D**). We then evaluated whether Vγ2 x PD-L1 could enhance cytotoxicity towards tumor cells that were resistant and refractory to Vγ2Vδ2 T cells' killing. Indeed, Vγ2Vδ2 T cell alone lysed less than 20% of H1299 cells even at a 5:1 ratio (**Figure 5E**). However, Vγ2 x PD-L1 strongly increased the lysis of H1299 with the increased IFNγ production by Vγ2Vδ2 T cells (**Figures 5E, F**). Importantly, Vγ2 x PD-L1 induced efficient tumor cell lysis, and IFNγ secretion was observed at an E: T ratio as low as 0.3125:1 for these three cell lines (**Figure 5**).

Vγ2 x PD-L1 Potency in Killing PD-L1 Positive Tumor Cell Lines Is Mediated by Both Fresh and Expanded Vγ2Vδ2 T Cell

To confirm whether Vγ2 x PD-L1 could redirect Vγ2Vδ2 T cells to kill a broad spectrum of tumor cells, we took 5 different human solid tumor cell lines expressing PD-L1 for the test. For these PD-L1 expressing tumor cells, Vγ2Vδ2 T cells alone did not exert an appreciable killing effect, nor did the PD-L1 mAb (**Figure 6A**). However, a dose-dependent effective killing mediated by Vγ2Vδ2 T cells was observed with the addition of Vγ2 x PD-L1 irrespective of tumor cells' origin, but not for Vγ2 x Null (**Figure 6A**). As expected, Vγ2Vδ2 T cells exhibited a dose-dependent IFNγ secretion treated with Vγ2 x PD-L1, compared with no such effect with control Abs (**Figure 6B**). We further observed that the Vγ2 x PD-L1-induced Vγ2Vδ2 T cells' cytotoxicity (killing EC_{50}) towards tumor cells was correlated significantly with these tumor cells' PD-L1 expression scores, while the release IFNγ EC_{50} showed a negative trend with the PD-L1 expression scores (**Figure 6C**). Moreover, the viability of PD-L1^{neg} HEK-293 cells remained unaffected in all tested concentrations in the presence of Vγ2 x PD-L1 (**Figure 6D**). In addition, allogeneic PBMCs were used as target cells to check

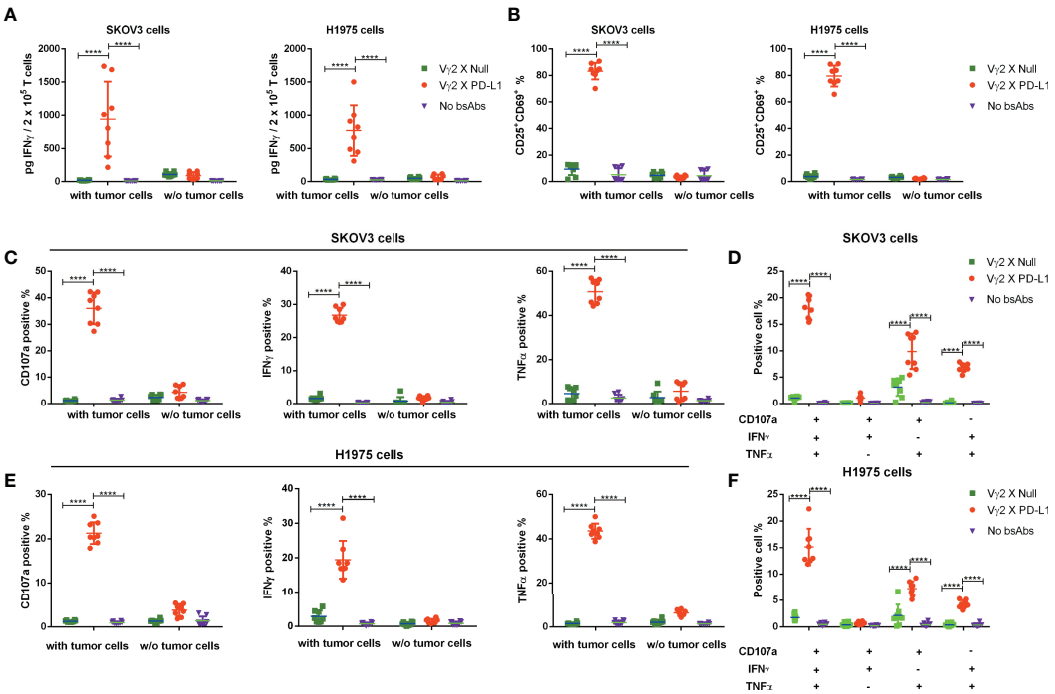

FIGURE 3 | Vγ2 x PD-L1 revoked specifically activation, of the expanded Vγ2Vδ2 T cells in the presence of PD-L1$^+$ tumor cell lines. **(A)** Vγ2 x PD-L1 increased significantly the IFNγ secretion and **(B)** prompted activation of the expanded Vγ2Vδ2 T cells in a PD-L1-dependent fashion. Vγ2Vδ2 T cells were co-cultured with indicated tumor cell lines (SKOV3 or H1975) in the absence or presence of Vγ2 x PD-L1 or Vγ2 x Null (1 μg/mL of each, about 8 nM) at a ratio of 1:1 for 24 hours. Then the supernatant was harvested for measuring the concentration of IFNγ by ELISA **(A)**, and cells were collected for staining CD25+CD69+ double-positive populations **(B)**. **(C–F)** Vγ2 x PD-L1 activated specifically Vγ2Vδ2 T cells to produce IFNγ and TNFα, and degranulate in the presence of PD-L1$^+$ tumor cell lines. Vγ2Vδ2 T cells were stimulated by Vγ2 x PD-L1 or Vγ2 x Null (1 μg/mLof each) in the presence/absence of H1975 **(C, D)** or SKOV3 **(E, F)** cells in a 1:1 ratio for 4 hours. The percentages of T cells positive for CD107a, TNFα, and IFNγ measured by ICS were represented in **(C, E)** and the percentages of multi-functional effector subsets of Vγ2Vδ2 T cells were shown in **(D, F)** Data were presented as Mean ± SD pooled from n=8 biological replicates of three independent experiments. ****$p<$ 0.0001 (Two-way ANOVA, Tukey's multiple comparisons test for **(A, B, C, E)** Dunnett's multiple comparisons test for **(D, F)**.

if the killing activity of Vγ2 x PD-L1 was specific to tumor cells. The Vγ2Vδ2 T cell-mediated killing percentages of allogeneic PBMCs were low even in the presence of Vγ2 x PD-L1, indicating the Vγ2 x PD-L1 activated Vγ2Vδ2 T cells' killing activity was indeed restricted to tumor cells (**Figure 6E**). Moreover, fresh Vγ2Vδ2 T cells enriched from healthy donors also exerted concentration-dependent killing of SKOV3 cells mediated by Vγ2 x PD-L1, but not by Vγ2 x Null or PD-L1 mAb (**Figure 6F**). Taken together, these results demonstrated that Vγ2 x PD-L1 could redirect Vγ2Vδ2 T cells to kill PD-L1+ tumor cell lines with IFNγ secretion, but to leave PD-L1 negative tumor cells and healthy cells un-attacked.

Vγ2 x PD-L1 Enhances the Efficacy of Adoptively Transferred Vγ2Vδ2 T Cells *In Vivo*

We further studied the effect of Vγ2 x PD-L1 on the outgrowth of established PD-L1 expressing tumors. SKOV3 cells were injected into nude mice, and the tumor cells were allowed to grow out and engraft for one week before the mice received twice-weekly *i.v.* injections with human Vγ2Vδ2 T cells, followed by twice-weekly *i.p.* injections with either 8 mg/kg Vγ2 x PD-L1 or Vγ2 x Null, or PBS. The mice were sacrificed at the time of severe disease symptoms (**Figure 7A**). The Vγ2Vδ2 T cells alone, or Vγ2Vδ2 T cells plus Vγ2 x Null did not control the tumor growth (**Figures 7B, C**). In contrast, the combo treatment with Vγ2 x PD-L1 and Vγ2Vδ2 T cells significantly delayed the tumor growth, with lower tumor weights at the end of the study (**Figures 7B-D**) than those of the control groups. After 16 days of treatment, Vγ2Vδ2 T cell counts were significantly higher in the Vγ2 x PD-L1+Vγ2Vδ2 T cells group, compared with the Vγ2 x Null+ Vγ2Vδ2 T cells group or Vγ2Vδ2 T cells group (**Figures 7E, F**).

DISCUSSION

The clinical investigations of PD-1/PD-L1 inhibitors have resulted in a paradigm shift in the treatment of advanced cancer patients, as well as longer overall survival time (30). However, due to the limited efficacy (only 20 to 30% objected response) and resistance to PD-1/PD-L1, there is still an unmet medical need for exploring novel agents to improve PD-L1 targeting therapeutic effectiveness (31). The inadequate

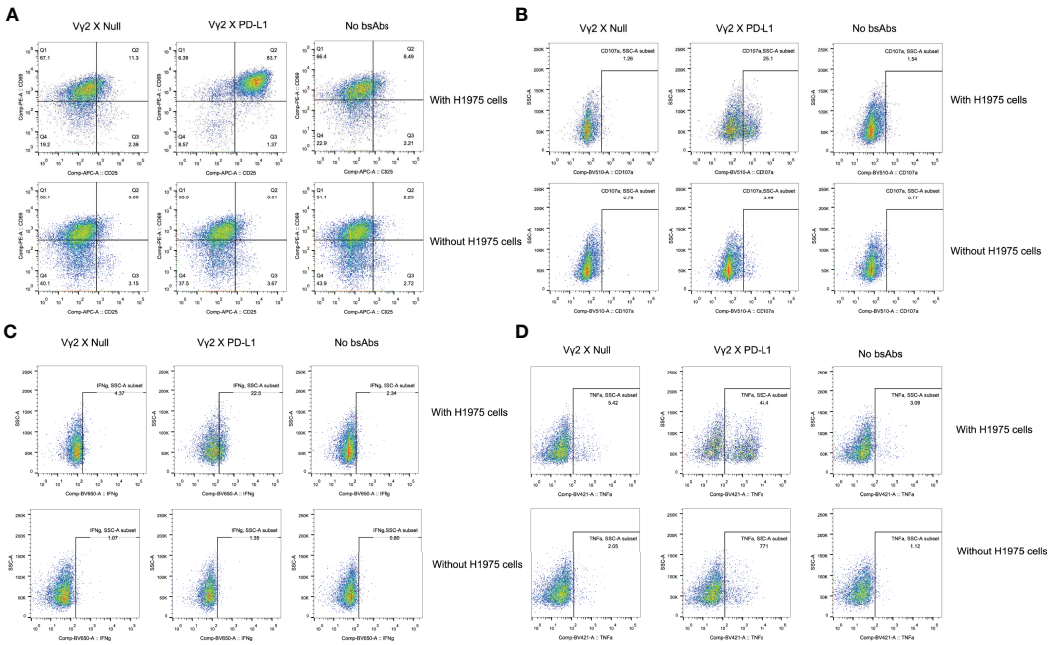

FIGURE 4 | Representative flow cytometry plots. The plots were showed for the activation **(A)**, TNFa and IFNg production **(B, C)**, and CD107a upregulation **(D)** of Vg2Vd2 T cells as under the indicated conditions.

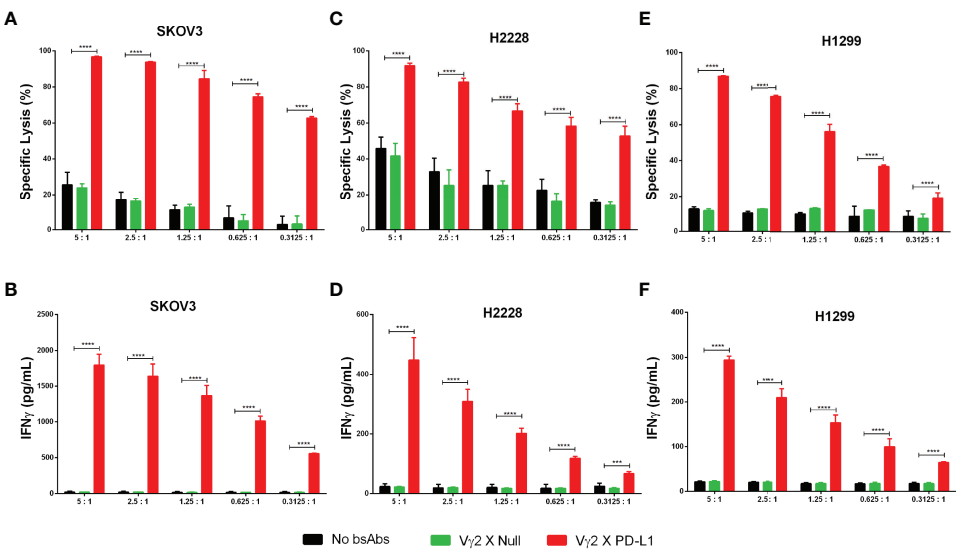

FIGURE 5 | Vγ2 X PD-L1 prompts significantly Vγ2Vδ2 T cell-mediated PD-L1+ tumor cell killing through releasing IFNγ. **(A–F)** Vγ2Vδ2 T cells enriched negatively from Zol +IL2 cultures were co-cultured with tumor targets (luciferase-expressing SKOV3, H2228, and H1299 cells) for 12 hours in the presence of 1 ug/mL (8 nM) Vγ2 X PD-L1 or Vγ2 X Null with serial E:T ratios, ranging from 5:1 to 0.3125:1. The tumor cell killing was measured by recording the RLU of each treated well **(A, C, E)**, and the releasing amounts of IFNγ were determined by ELISA **(B, D, F)**. Data were presented as Mean ± SD pooled from n=4 biological replicates of two independent experiments. ****$p < 0.0001$ (Two-way ANOVA, Dunnett's multiple comparisons test). ***$p<0.001$.

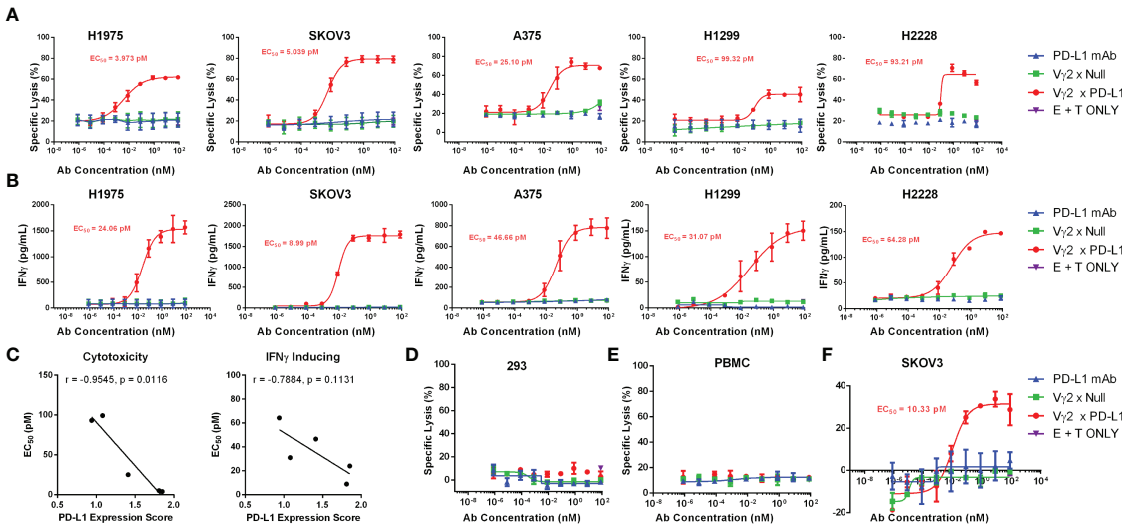

FIGURE 6 | Vγ2 x PD-L1 redirects Vγ2Vδ2 T cells to kill efficiently various PD-L1 positive cancer cell lines *in vitro*, but spared this effect on PD-L1 negative expressing HEK-293 cells or unrelated healthy PBMCs. **(A)** Expanded Vγ2Vδ2 T cells derived from healthy donors' PBMCs (n=3) were incubated with various luciferase-expressing tumor cell lines at a 0.5:1 ratio under the stimulation of serial concentrations of antibodies, including Vγ2 x PD-L1, Vγ2 x Null or PD-L1 mAb, for 12 hours. **(B)** Increased IFNγ secretion in the above co-cultures. **(C)** The cytotoxicity and IFNγ induction of Vγ2Vδ2 T cells revoked by Vγ2 x PD-L1 correlated with the PD-L1 expression score. The spearman's r and two-tailed p values were calculated by GraphPad Prism 6. **(D, E)** The expanded Vγ2Vδ2 T cells derived from healthy donors' PBMCs (n=3) were incubated with CFSE-labelled PD-L1^{neg} HEK-293 **(D)** or allogeneic PBMCs **(E)** at 1:1 ratio as indicated in **(A)**. A CFSE/PI staining-based flow cytometry method to determine the killed target cell percentages. **(F)** Fresh Vγ2Vδ2 T cells enriched from healthy donors (n=2) were incubated with SKOV3-Luc at 5:1 ratio under the stimulation of serial concentrations of antibodies, including Vγ2 x PD-L1, Vγ2 x Null or PD-L1 mAb, for 12 hours.

infiltration of T lymphocytes into the cold tumor is one of the reasons for this therapeutic resistance (32). Several clinical studies showed that transferred Vγ2Vδ2 T cells migrated into the tumor bed, leading to encouraging clinical responses and tumor reduction in treated patients (33). Here, the bispecific antibody and Vγ2Vδ2 T cells transfer combination approach provided a potential strategy to circumvent the PD-L1 blockade therapy limitations. The approach for targeting potent cytotoxicity Vγ2Vδ2 T cells by constructing Vγ2 x PD-L1 on the Y-body platform, based-on which two novel candidate medications are currently on clinical trials, noted as M701 (NCT04501744) and M802 (NCT04501770) (34). Vγ2 x PD-L1 preserved high affinity to PD-L1 as well as the PD1/PD-L1 blocking activity. However, consistent with other reports, the PD-1/PD-L1 blocking activity did not contribute to the killing ability of Vγ2Vδ2 T cells (12), possibly because the PD-L1 mAb used in our study contained silent Fc without ADCC capability. Vγ2 x PD-L1 had a slower affinity for the Vγ2 TCR than Vγ2 mAb, which was desired for clinical use to prevent cytokine release storm (35). Additionally, Vγ2 TCR-targeting Y-body platform allowed for the simple replacement of the PD-L1 Fab to create a sequence of Vγ2 x TAAs, which enabled Vγ2Vδ2 T cells to target a broader spectrum of tumor types and helping a larger population of cancer patients.

In vitro, Vγ2 x PD-L1-activated Vγ2Vδ2 T cells were able to selectively kill tumor cells selectively without killing PD-L1 negative non-malignant cells or normal cells. In fact, the activation, degranulation, and subsequent tumor cell killing mediated by Vγ2 x PD-L1 were all dependent on simultaneous binding to Vγ2Vδ2 T cell and PD-L1 expressing tumor cells, demonstrating the safety of our strategy in comparison to PD-L1 chimeric antigen receptor NK cells (36). In line with these *in vitro* observations, Vγ2 x PD-L1 was found to improve Vγ2Vδ2 T cell mediated tumor growth inhibition *in vivo*. Mechanically, Vγ2 x PD-L1 generated a greater Vγ2Vδ2 T cell infiltration.

Meanwhile, there are several limitations in this study. First, because Vγ2Vδ2 T cells are species specific, we employed an immunodeficiency mouse model to investigate the efficacy of Vγ2 x PD-L1 plus Vγ2Vδ2 T cells, without examining whether this combination therapy could change or reshape the suppressive tumor microenvironment, or the *in vivo* toxicity of combo usage. Second, this combo treatment was not fully curative because tumor volumes did not reach to near zero by the end of treatment. As a small amount of Vγ2Vδ2 T cells and a fixed bsAb dose were used in the current treatment protocol, we intended to improve the present therapy approach involving a modest number of Vγ2Vδ2 T cells and bsAb dosage. Third, we were unable to determine the TCR sequence of tumor bed infiltrating Vγ2Vδ2 T cells, which would provide valuable information for further TCR-T design.

In conclusion, we developed a novel and potential therapeutic T cell engager bispecific antibody Vγ2 x PD-L1, which caused Vγ2Vδ2 T cells to destroy PD-L1 expressing tumor cells efficiently and selectively. Vγ2 x PD-L1 offers promising

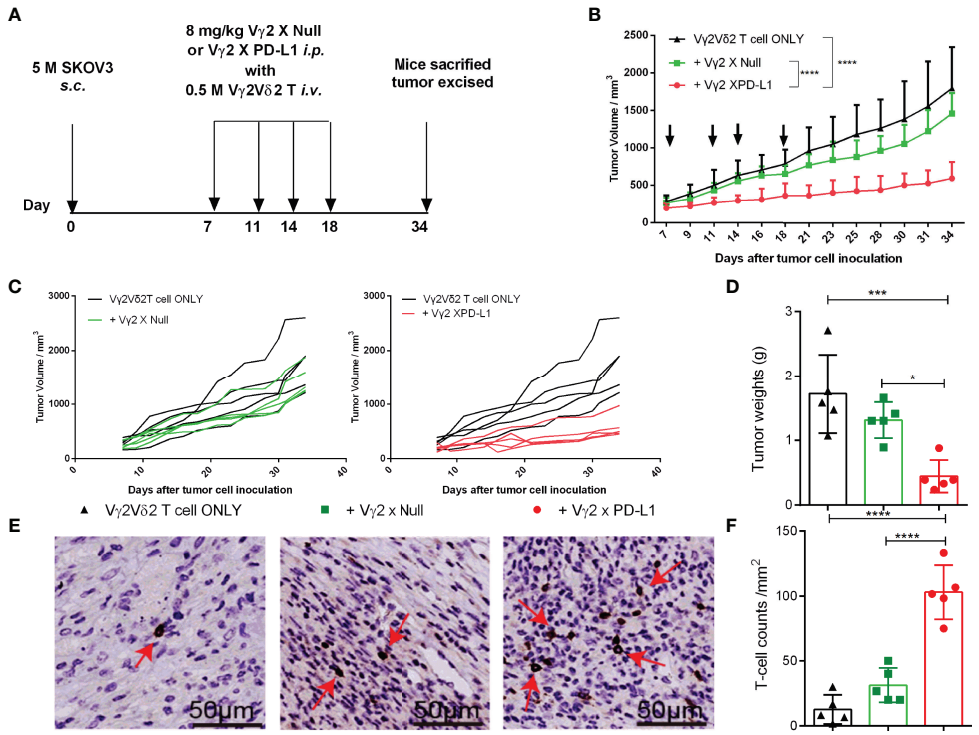

FIGURE 7 | Vγ2 X PD-L1 prompted the survival of inoculated Vγ2Vδ2 T cells in nude mice. **(A)** Experimental schema of evaluating the anti-tumor therapeutic efficacy of Vγ2 X PD-L1. Nude mice were *s.c.* inoculated with 5 million SKOV3 cells on Day 0. After 15 days, mice were treated with *i.v.* Vγ2Vδ2 T cells plus 8 mg/kg Vγ2 X Null or Vγ2 X PD-L1. These treatments were repeated twice a week (Q2W) for 3 weeks. Mice treated PBS only were used as control. **(B, C)** Pooled or individual tumor growth curves. The black arrows indicated the treatment time point. Data are mean ± SD with 5 mice per group, $^{****}p < 0.0001$ (Two-way ANOVA, Dunnett's test), which was determined based on the tumor volumes at the end of the study. **(D)** Tumor weights at the end of the study. Data were mean ± SD with 5 mice per group, $^{***}p < 0.001$, $^{*}p < 0.05$ (ANOVA, Dunnett's test). **(E, F)** Infiltrated and accumulated T-cell counts at the tumor site. Representative IHC figures for the treated group **(E)** and pooled T cell counts **(F)** were presented as mean ± SD, $^{****}p < 0.0001$, (ANOVA, Dunnett's test). Data shown was one of two independent experiments.

therapy options for solid tumors, including ovarian cancer (28, 37, 38), melanoma (38, 39), and non-small cell lung cancer (NSCLC) (38). The infiltrating Vγ2Vδ2 T cells in tumor acted as protective anti-tumor effector population and were linked with positive outcomes. As PD-L1 is a clinically well-established tumor target, its widespread expression pattern suggested that our combination approach might be beneficial for the PD-L1 positive cancer patients who had refractory or relapsed for PD-L1 inhibitor treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review boards for human subjects' research and institutional biosafety committees at Hubei Province Food and Drug Safety Evaluation Center (Wuhan, China). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Care and Use Committee from Hubei Province Food and Drug Safety Evaluation Center (#202110191).

AUTHOR CONTRIBUTIONS

RY, JY, and PZ conceived the ideas and designed the project. JZ, JS, YY, LF and LZ supervised the project. RY, YX, MZ, HW, HZ, CG, XW, FL, XS, ZW, SX, YL, and QN performed the experiments. RY, JY and PZ analyzed the data and jointly wrote the manuscript. All authors read and approved the manuscript. All authors contributed to the article and approved the submitted 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.2022.923969/full#supplementary-material

Supplementary Figure 1 | Biochemical analysis of generated Vγ2Vδ2 T cell-targeting bsAbs. **(A)** SDS-PAGE analysis of purified antibodies under non-reducing (left) and reducing (right) conditions. Molecular weight (MW) was indicated in kDa for protein marker. There were 3 and 2 bands for bsAb and mAb, respectively, under reducing conditions as expected. **(B)** Size exclusion chromatograms of test antibodies (upper, Vγ2 X PD-L1, bottom, Vγ2 X Null). The antibodies were purified by Protein-A and ion-exchange chromatography. The purity of prepared bsAb was more than 95%.

Supplementary Figure 2 | PD-L1 expression scores of various tumor cell lines tested in this study. (A) Representative histogram of PD-L1 expression on CHO-PD-L1, H1975, and SKOV3 cells. Vγ2 X PD-L1 (blizzard blue shade), Vγ2 X Null (pink shade) of two experiments. (B) PD-L1 expression scores of firefly luciferase-transduced tumor cells. The CHO-PD-L1 cells were used as positive control, and HEK-293-Luc cells were displayed null expression of PD-L1 serving as negative control. Various tumor cell lines were incubated with 40 μg/mL Vγ2 X PD-L1 (Target) or Vγ2 X Null (Null) for 1 hour at 4 degree, then stained with APC-hFc for 30 minutes at room temperature. The APC positive populations and MFI of APC channel were determined by flow cytometry. The expression scores were calculated by [$\log_{10}$ (Target $_{\text{APC positive populations}}$ - Null $_{\text{APC positive populations}}$) + $\log_{10}$ (Target $_{\text{APC MFI}}$/Null $_{\text{APC MFI}}$)]/2. Data were derived from one representative experiment of three independent experiments.

Supplementary Figure 3 | Vγ2 x PD-L1 recruited Vγ2Vδ2 T cells to form cell-to-cell conjugates with SKOV3 cells, but not with 293T cells. 0.1 M CFSE-labelled Vγ2Vδ2 T cells were incubated with 0.1 M PKH26-stained SKOV3 cells in the presence of 1 μg/mL (8 nM) Vγ2 x Null (Left) or Vγ2 x PD-L1 (Right) for 30 minutes, then the percentages of CFSE and PKH26 double positive cells (Q2) were depicted as cell-to-cell conjugates. Representative flow cytometric dot plots from three independent experiments (for SKOV3) and three wells (for HEK-293) were shown. Please noted that the upper panel used Vγ2Vδ2 T cells negatively enriched from fresh PBMC cultures treated with Zol+IL2 for 14 days, the bottom panel used Vγ2Vδ2 T cells enriched from cryopreserved PBMC cultures treated with Zol+IL2 for 14 days; and Vγ2Vδ2 T cells were expanded from different donors for SKOV3 and HEK-293.

Supplementary Table 1 | Summary p values for **Figures 2**, **3**, **5**.

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Conflict of Interest: The authors are employees of Wuhan YZY Biopharma Co., Ltd that develops and commercializes antibody therapeutics including bispecific antibodies.

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BTN3A Targeting Vγ9Vδ2 T Cells Antimicrobial Activity Against *Coxiella burnetii*-Infected Cells

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Vγ9Vδ2 T cells have been reported to participate to the immune response against infectious diseases such as the Q fever caused by *Coxiella burnetii* infection. Indeed, the number and proportion of Vγ9Vδ2 T cells are increased during the acute phase of Q fever. Human Vγ9Vδ2 T cell responses are triggered by phosphoantigens (pAgs) produced by pathogens and malignant cells, that are sensed *via* the membrane receptors butyrophilin-3A1 (BTN3A1) and -2A1 (BTN2A1). Here, by using CRISPR-Cas9 inactivation in THP-1 cells, we show that BTN3A and BTN2A are required to Vγ9Vδ2 T cell response to *C. burnetii* infection, though not directly involved in the infection process. Furthermore, *C. burnetii*-infected monocytes display increased BTN3A and BTN2A expression and induce Vγ9Vδ2 T cell activation that can be inhibited by specific antagonist mAb. More importantly, we show that the antimicrobial functions of Vγ9Vδ2 T cells towards *C. burnetii* are enhanced in the presence of an BTN3A activating antibody. This supports the role of Vγ9Vδ2 T cells in the control of *C. burnetii* infection and argues in favor of targeting these cells as an alternative treatment strategy for infectious diseases caused by intracellular bacteria.

Keywords: ***Coxiella burnetii*, Vγ9Vδ2 T cells, butyrophilin, antimicrobial immunity, therapeutic approaches**

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INTRODUCTION

The role of Vγ9Vδ2 T cells in the host immune response to bacterial infection is now well-documented (1). Human Vγ9Vδ2 T cells, which normally represent 2-5% of peripheral blood T cells, are expanded in infected patients to reach up to ≥50% of the circulating T cells (2, 3), as reported for patients undergoing mycobacterial disease, listeriosis, salmonellosis, brucellosis, tularemia, legionellosis and Q fever (4–10). Furthermore, local expansion of Vγ9Vδ2 T cells have also been reported in the bronchoalveolar lavage fluids from patients with active pulmonary tuberculosis and in cerebral spinal fluids from patients with bacterial meningitis (11–13). Two direct antimicrobial actions of Vγ9Vδ2 T cells against various viruses, protozoa and bacteria were reported, including cytotoxic activity to pathogen-infected cells and a cell-mediated non-cytolytic activity based on cytokine production (1, 14–16). *In vitro* studies have shown that Vγ9Vδ2 T cells

are able to effectively kill intracellular pathogens such as *M. tuberculosis, L. monocytogenes*, and *Brucella suis* (17–21).

The butyrophilin 3A1 (BTN3A1) cell surface molecule is involved in cell recognition and the human Vγ9Vδ2 T cells activation (22, 23). Vγ9Vδ2 T cells are activated by small, phosphorylated nonpeptide antigens, called phosphoantigens (pAgs) (14). The production of these metabolites is increased in tumor or stressed eukaryotic cells, and can be naturally produced by several pathogens (11, 24, 25). Among the *BTN3A* isoforms (*BTN3A1*, *BTN3A2*, *BTN3A3*), *BTN3A1* is unique in that its intracellular B30.2 domain binds to pAgs (26, 27), while its juxtamembrane domain performs a critical function in homodimerization and heterodimerization of BTN3A (28). Conformational changes in the juxta-membrane domain, induced by the binding of pAgs to the B30.2 domain, are involved in Vγ9Vδ2 T cell activation (29). More recently, BTN2A1 has been identified as a novel actor in pAg sensing by Vγ9Vδ2 T cells (30–32). BTN2A1 is a direct ligand for the Vγ9 TCR interacting with BTN3A1 to trigger Vγ9Vδ2 TCR activation (30).

Several evidences highlight the key role of Vγ9Vδ2 T cells in Q fever, an infectious disease caused by the intracellular bacterium *Coxiella burnetii*. (**1**) During the acute phase of the disease, the numbers and proportion of Vγ9Vδ2 T cells were found increased (**2**) with a significant increase of the expression of HLA-DR, but not CD25 (10). In this study, we investigated the functional role of Vγ9Vδ2 T cells and the involvement of BTN3A and BTN2A in host defense against *C. burnetii*. Here, we observed that *C. burnetii* infection of healthy monocytes lead to the increase of the expression of these two BTNs. Using a CRISPR-Cas9 knockout model in the THP-1 cell line, we observed that BTN3A and BTN2A are not directly involved in the infection process by *C. burnetii* but play a role in the host immune response to infection. We reported that infected monocytes induced Vγ9Vδ2 T cell activation in a BTN3A and BTN2A dependent manner. Finally, the use of a BTN3A activating antibody enhances the antimicrobial functions of Vγ9Vδ2 T cells against *C. burnetii* infected cells through the production of cytotoxic molecules and large amounts of IFN-γ and TFN-α. Our results highlight the role of Vγ9Vδ2 T cells in the control of *C. burnetii* infection and the therapeutic potential of BTN3A activating antibody in infections.

MATERIALS AND METHODS

Cell Isolation

Blood samples (leucopacks) were obtained from the local French Blood Establishment (*Etablissement français du sang*, EFS), which carries out donor inclusions, informed consent, and sample collection. Through a convention established between our laboratory and the EFS (N°7828), buffy coats were obtained and peripheral blood mononuclear cells (PBMCs) were isolated as previously described (33). Monocytes were purified from PBMCs using anti-CD14-conjugated magnetic beads (Miltenyi Biotec, Bergisch Glabach, Germany) and cultured in Roswell Park Memorial Institute-1640 medium (RPMI, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, Life technologies), 2 mM L-glutamine, 100 U/mL penicillin and 50 µg/mL streptomycin (Life Technologies).

Vγ9Vδ2 T cells were expanded from fresh PBMCs as previously described (34, 35). Briefly, PBMCs were cultured in RPMI-1640 medium supplemented with 10% FBS, interleukin-2 (IL-2, 200 UI/ml) and Zoledronic acid monohydrate (to a final concentration of 1 µM). IL-2 was added every 2 days beginning on day 5 for 12 days and the purity of the Vγ9Vδ2 T cells was assessed by flow cytometry analysis (>85%) and then frozen at -80°C in 10% dimethyl sulfoxide (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 90% FBS.

Lentiviral *Transduction* and CRISPR-Cas9-*Mediated* BTN3A or BTN2A *Knockout*

For all transductions, THP-1 cells were seeded in 12-wells plates ($2.5x10^5$ cells/well), and 25 µL of concentrated lentiviral particles were added to the culture. After 24 hours, cells were washed twice in complete medium, and cultured in their regular culture medium for 48 hours. Optimized CRISPR target sequences targeting the three *BTN3A* gene isoforms and for BTN2A gene inactivation, targeting both *BTN2A* gene isoforms (sequence available upon request) were cloned into the lentiCRISPR-v2 vector (Addgene #52961). For selection of THP-1 transductants, 1 µg/mL puromycin was added to the culture medium (**Supplementary Figure 1**).

Bacterial Production

Coxiella burnetii phase I (Nine Mile (NM) strain, RSA493 and Guiana strain, MST17) were cultured in L929 cells for 10 days, as previously described (36). Briefly, infected cells were sonicated and centrifuged at 10,000*g* for 10 minutes, then washed and stored at -80°C. Bacterial titers were determined using Gimenez staining, and bacterial viability was assessed using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR, USA).

Mycobacterium tuberculosis (H37Rv strain) was cultured in Middelbrook 7H10 (Becton Dickinson, Le Pont de Claix, France) supplemented with 10% oleic acid-albumin-dextrosecatalase (OADC, Becton Dickinson), as previously described (37). Prior to infection, the colonies were resuspended in phosphate buffered saline (PBS, Life Technologies), vigorously vortexed for 10 min using 3 mm sterile glass beads (Sigma-Aldrich) and passed 10 times through a 25 G needle to disperse clustered cells. Calibration was performed at OD 580 nm and confirmed by counting mycobacteria after Ziehl-Neelsen staining.

Cell Infection

Monocytes isolated from healthy donors were infected with *C. burnetii* strains (50 MOI) or with *M. tuberculosis* (5 MOI). After 24 hours of infection, the expression of BTN3A and BTN2A were investigated by qRT-PCR and flow cytometry. For co-cultures experiments, monocytes isolated from healthy donors previously infected 24 hours with *C. burnetii* strains or with *M. tuberculosis* were co-cultured with autologous Vγ9Vδ2 T cells (E:T ratio of 1:1). After 4 hours of co-culture, Vγ9Vδ2 T cell degranulation and cytotoxicity was assessed by flow cytometry and the bacterial

load was measured by flow cytometry and qPCR. Finally, the supernatants of the co-cultures were analyzed for the presence of cytokines and cytotoxic molecules by ELISA assay.

Bacterial Detection

DNA was extracted from *C. burnetii* infected cells using a DNA Mini Kit (Qiagen, Courtaboeuf, France). Bacterial load was quantified using real time quantitative PCR (qPCR) performed with specific primers F (5'-GCACTATTTTTAGCCG-GAACCTT-3') and R (5'-TTGAGGAGAAAAACTGGATTG AGA-3') targeting the *C. burnetii COM-1* gene, as previously described (36).

The presence of *C. burnetii* within cells was also assessed by flow cytometry. Briefly, infected cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). After washing, cells were incubated with a rabbit antibody directed against *C. burnetii* for 30 min and then with an Alexa 647 anti-rabbit antibody (Invitrogen). Data were collected on a BD Canto II instrument (BD Biosciences, Le Pont-de-Claix, France) and analyzed with FlowJo software (FlowJo v10.6.2, Ashland, OR).

For *M. tuberculosis* infected cells, DNA was extracted from infected cells as follows: aliquots of 150 µL were incubated overnight at 56°C with 150 µL of G2 buffer mixed with 15 µL proteinase K (20 mg/mL). After two cycles of mechanical lysis (45 s), the total DNA was extracted using the EZ1 DNA Tissue Kit (Qiagen). *M. tuberculosis* DNA detection was performed targeting the *M. tuberculosis* internal transcribed spacer (ITS) (**Table 1**), as previously described (37).

RNA Isolation and q-RTPCR

Total RNA was extracted from infected cells ($2x10^6$ cells/well) using the RNeasy Mini Kit (Qiagen) with DNAse I treatment as previously described (38). RNAs quality and quantity were evaluated using a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, USA). Reverse transcription was performed using M-MLV Reverse Transcriptase kit (Life Technologies) and oligo(dT) primers. The expression of genes characteristics of M1/M2 macrophage phenotypes, as well as *BTN3A* isoform genes, was evaluated using real time qPCR, Smart SYBR Green fast Master kit (Roche Diagnostics, Meylan, France) and specific primers (**Table 1**). *BTN2A* levels expression was evaluated using real time qPCR, TaqMan® Fast Advanced Master Mix (Applied Biosystems, Life Technologies) and specific probes (**Table 1**). All qPCRs were performed using a CFX Touch Real-Time PCR Detection System (Bio-Rad, Marnes-la-Coquette, France). Results were normalized by the expression of *ACTB* or *GAPDH* housekeeping gene and are expressed as relative expression of investigated genes with $2^{-\Delta Ct}$ where $\Delta Ct = Ct_{target} - Ct_{housekeeping\ gene}$ as previously described (36).

BTN3A and BTN2A Surface Expression

Cells were suspended in PBS (Life Technologies) containing 1% FBS and 2 mM EDTA (Sigma-Aldrich). Cells were labeled with viability dye (Live/Dead Near IR, Invitrogen), mouse anti-BTN3A (clone 103.2) or anti-BTN2A (clone 7.48) Abs or with the appropriate isotype control (Miltenyi Biotech). After 30 min incubation, primary antibody binding was detected with secondary PE anti-mouse antibody (Invitrogen) and data were collected on a Navios instrument (Beckman Coulter) and analyzed with FlowJo software (FlowJo v10.6.2).

Degranulation Assay

Monocytes were co-cultured with Vγ9Vδ2 T cells at effector-to-target (E:T) ratio of 1:1 in presence of mouse anti-BTN2A mAb (clone 7.48) or mouse anti-BTN3A mAb (clones 20.1 or 103.2)

TABLE 1 | Primers used for the response to infection.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ACTB	GGAAATCGTGCGTGACATTA	AGGAGGAAGGCTGGAAGAG
GAPDH	Hs02786624_g1	
M1 genes		
TNF	AGGAGAAGAGGCTGAGGAACAAG	GAGGGAGAGAAGCAACTACAGACC
IL1B	CAGCACCTCTCAAGCAGAAAAC	GTTGGGCATTGGTGTAGACAAC
IL6	CCAGGAGAAGATTCCAAAGATG	GGAAGGTTCAGGTTGTTTTCTG
IFNG	GTTTTGGGTTCTCTTGGCTGTTA	ACACTCTTTTGGATGCTCTGGTC
CXCL10	TCCCATCTTCCAAGGGTACTAA	GGTAGCCACTGAAAGAATTTGG
M2 genes		
IL10	GGGGGTTGAGGTATCAGAGGTAA	GCTCCAAGAGAAAGGCATCTACA
TGFB	GACATCAAAAGATAACCACTC	TCTATGACAAGTTCAAGCAGA
IL1RA	TCTATCACCAGACTTGACACA	CCTAATCACTCTCCTCCTCTTCC
CD163	CGGTCTCTGTGATTTGTAACCAG	TACTATGCTTTCCCCATCCATC
BTN isoform genes		
BTN3A1	TTCCAGGTCATAGTGTCTGC	TGAGCAGCTGAGCAAAAGG
BTN3A2	TGGGAATACCAAGGGA	AGTGAGCAGCTGGACCAAGA
BTN3A3	GAGGGAATACTAAGAAATGGT	GAAGAGGGAGACATGAAAGT
BTN2A1	Hs00924832_m1	
BTN2A2	Hs00950165_g1	
***C. burnetii* gene**		
CB COM-1	GCACTATTTTTAGCCG-GAACCTT	TTGAGGAGAAAAACTGGATTGAGA
MTB *ITS*	CAAGGCATCCACCATGCGC	GGGTGGGGTGTGGTGTTTGA

and fluorochrome-labeled CD107a and CD107b (BD Biosciences). Phorbol 12-myristate 13-acetate (PMA, 20 ng/mL) with ionomycine (1 μg/mL) were used as positive control for Vγ9Vδ2 T cell activation. After 4 hours, cells were harvested and stained with fluorochrome-labeled TCR-specific mAbs (Miltenyi Biotec) and a viability marker (Live/Dead Near IR, Invitrogen). The degranulation was evaluated by flow cytometry as the percentage CD107a/b$^+$ cells in the γδ T cell population (**Supplementary Figure 2**). Data were collected on a Navios instrument (Beckman Coulter) and analyzed with FlowJo software (FlowJo v10.6.2).

Cytotoxicity Assay

Monocytes were labeled with 10 μM Cell Proliferation Dye eFluor® 670 (Invitrogen) and then co-cultured with Vγ9Vδ2 T cells at E:T ratio of 1:1 in presence of mouse anti-BTN3A mAb (clone 20.1) at the indicated concentrations. After 4 hours, cells were stained with CellEvent Caspase-3/7 Green (Invitrogen) to identify dead cells. The cytotoxicity was assessed by flow cytometry as the percentage of Caspase 3/7$^+$ cells in the target cell population (**Supplementary Figure 2**). Data were collected on a BD Canto II instrument (BD Biosciences) and analyzed with FlowJo software (FlowJo v10.6.2).

Immunoassays

Tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), Granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems), granzyme B, perforin, and granulysin (Abcam) levels were quantified in the supernatants of monocyte/Vγ9Vδ2 T cells co-cultures using specific immunoassay kits. TNFα, IFNγ, interleukin (IL)-1β, IL-6, IL-10 and transforming growth factor beta (TGF-β) (R&D Systems) levels were quantified in the supernatants of BTNs KO cells following *C. burnetii* infection. The sensitivity of assays was 6.2 pg/mL for TNFα, 5.7 pg/mL for IFNγ, 1.0 pg/mL for IL-1β, 0.7 pg/mL for IL-6, 3.9 pg/mL for IL-10, 15.4 pg/mL for TGF-β, 3.0 pg/mL for GM-CSF, 20 pg/mL for granzyme B, 40 pg/mL for perforin and 10 pg/mL for granulysin.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism (8.0, La Jolla, CA). After analysis of the distribution of the data with a normality test, the Mann-Whitney *U* test was used as a non-parametric test and the *t* test as a parametric test. Hierarchical clustering of gene expression was analyzed using the ClustVis webtool (39). The limit of significance was set up at $p<0.05$.

RESULTS

C. burnetii Infection Enhances Expression of BTN3A and BTN2A

To assess whether *C. burnetii* infection affected the expression of BTNs, monocytes from healthy donors were isolated and infected with the reference strain NM1 or with the Guiana strain, described to be more virulent (40, 41). After 24 hours of incubation with active or heat-inactivated *C. burnetii* NM1 strain, increases of transcript expression of both *BTN3A1* and *BTN3A2* isoforms, but not of *BTN3A3* were found. Guiana strain infection enhanced the expression of all three isoforms, similar to *M. tuberculosis* infection used as control (**Figure 1A**) (23). Interestingly, significant differences of *BTN3A1* expression were observed between cells infected with active or heat-inactivated *C. burnetii* NM1 (p=0.0374), suggesting that virulence affected *BTN3A1* expression. Indeed, inactivated form of *C. burnetii* are reported to induce a weaker modulation of the expression of the A1 isoform, the essential form for pAg-mediated activation of Vγ9Vδ2 T cells (23). Significant increase of BTN3A protein expression was found for monocytes infected with *C. burnetii* NM1 and Guiana strains (p=0.0021 and p=0.0096, respectively) (**Figure 1B**).

As BTN2A is involved in Vγ9Vδ2 T-cell activation (31), we also investigated whether *C. burnetii* infection affected its expression. After 24 hours of infection, *BTN2A* transcriptional expression for both isoforms (BTN2A1 and BTN2A2) was significantly increased after *C. burnetii* NM1 and Guiana infection (BTN2A1 p=0.0170 and p=0.0021, respectively; and BTN2A2 p=0.0054 and p=0.0463, respectively) compared to uninfected cells and without significant modulation compared to the heat-inactivated form (**Figure 1C**). Regarding BTN2A protein expression, a significant increase was observed for *C. burnetii* infected monocytes (NM1 strain, p=0.0160; and Guiana strain, p=0.0018) compared to uninfected cells, as observed for *M. tuberculosis* infection as control (**Figure 1D**).

Altogether, like *M. tuberculosis* infection, *C. burnetii* infection leads to increased expression of BTN3A and BTN2A in infected cells.

Involvement of BTN3A and BTN2A in *C. burnetii* Infection

Next, we investigated whether BTNs could be involved in the uptake or replication of *C. burnetii*. For this purpose, we performed a CRISPR-Cas9 knockout of the three *BTN3A* genes or the two *BTN2A* genes in the THP-1 cell line. Cells were transduced with a guide targeting either *BTN2A1* and *2A2* (BTN2AKO) or *BTN3A1, 3A2* and *3A3* (BTN3AKO) isoforms or with an irrelevant CRISPR guide (mock). BTN3AKO, BTN2AKO and mock cells were infected with *C. burnetii* NM1, and the bacterial load was assessed by qPCR. No differences were observed concerning the bacterial load (**Figure 2A**) and replication overtime (**Figure 2B**) between BTN3AKO, BTN2AKO and mock cells, suggesting that BTN3A and BTN2A are not directly involved in the process of *C. burnetii* infection.

Involvement of BTN3A and BTN2A in the Inflammatory Response to *C. burnetii* Infection

We then investigated the involvement of BTNs in the host immune response following *C. burnetii* infection in THP-1 cells. As observed in the **Figure 3**, *C. burnetii* infection results in modulation of genes characteristics of both pro-inflammatory

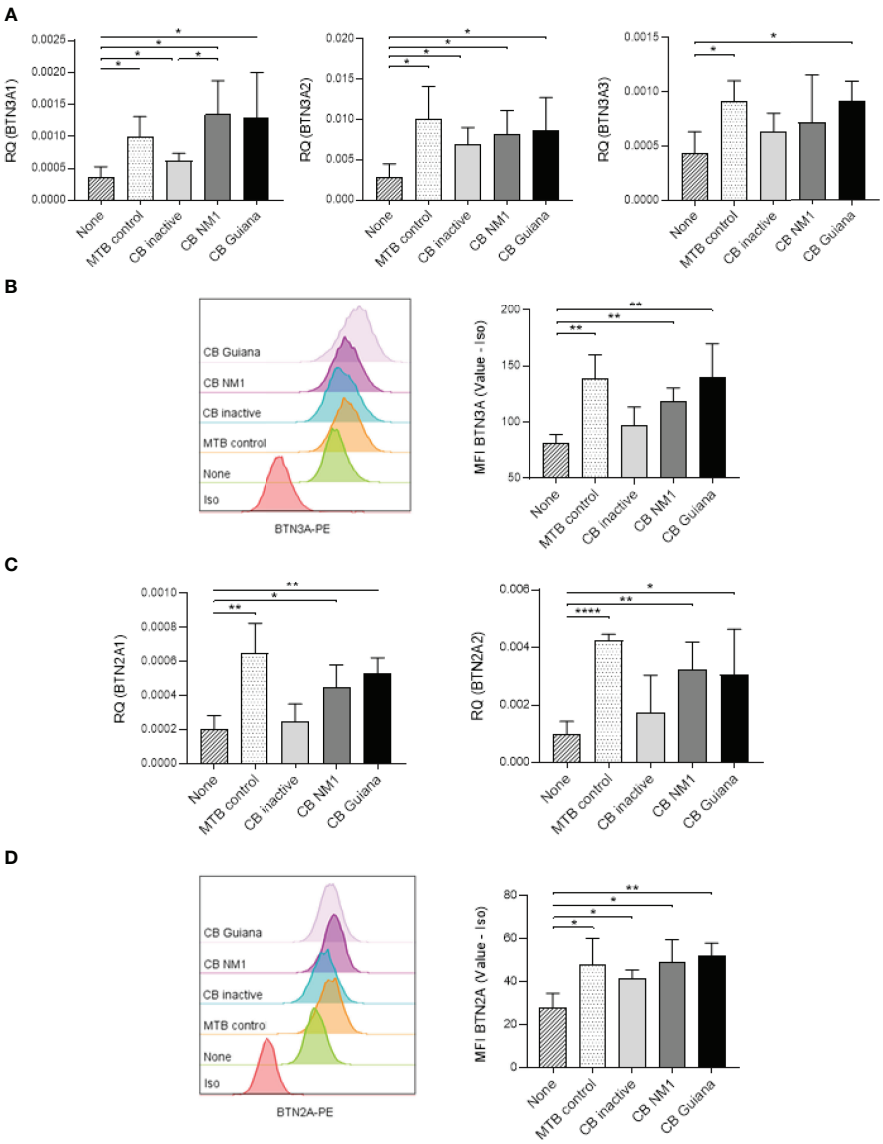

FIGURE 1 | Bacterial infections modulate BTN3A and BTN2A expression. Monocytes isolated from healthy donors (n = 4) were infected with *C. burnetii* strains (50 MOI) or with *M. tuberculosis* (5 MOI) for 24 hours. **(A)** The relative gene expression of *BTN3A* isoforms (A1, A2, A3) and **(B)** the BTN3A protein expression were investigated by qRT-PCR and flow cytometry, respectively. **(C)** The relative gene expression of *BTN2A* isoforms (A1, A2) and **(D)** the BTN2A protein expression were investigated by qRT-PCR and flow cytometry, respectively. Data were analyzed using a normality test and a parametric *t* test. Values represent mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$.

(*TNF, IFNG, IL6, CXCL10, IL1RA* and *IL1B*) and anti-inflammatory (*IL10, TGFVB*and *CD163*) responses in THP-1 cells. Upon infection with *C. burnetii*, the hierarchical clustering based on the expression of the above mentioned genes revealed that BTNs expression correlated with the transcriptional response to infection, as depicted by a separate clustering of BTN3AKO/BTN2AKO cells and mock cells (**Figure 3A**). Indeed, BTN3AKO and BTN2AKO cells displayed significantly decreased expression of inflammatory genes following *C. burnetii* infection, in particular that of *TNF* and *IL1B* (**Figure 3B**). Also, *IL6* transcriptional expression appear to be affected by the BTN3A KO (p=0.0862) but not by the BTN2A KO. Furthermore, the expression of *IL10* transcript was significantly decreased compared to mock cells (p=0.0435) (**Figure 3B**). Consistently, BTN3AKO and BTN2AKO cells presented a significant decrease in TNF and IL-1β release following *C. burnetii* infection compared to mock cells (**Figure 3C**). No significant difference in the levels of anti-inflammatory cytokines such as IL-10 and TGF-β was observed.

Taken together, these data reported that both BTN3A and BTN2A are involved in the inflammatory response to *C. burnetii* infection.

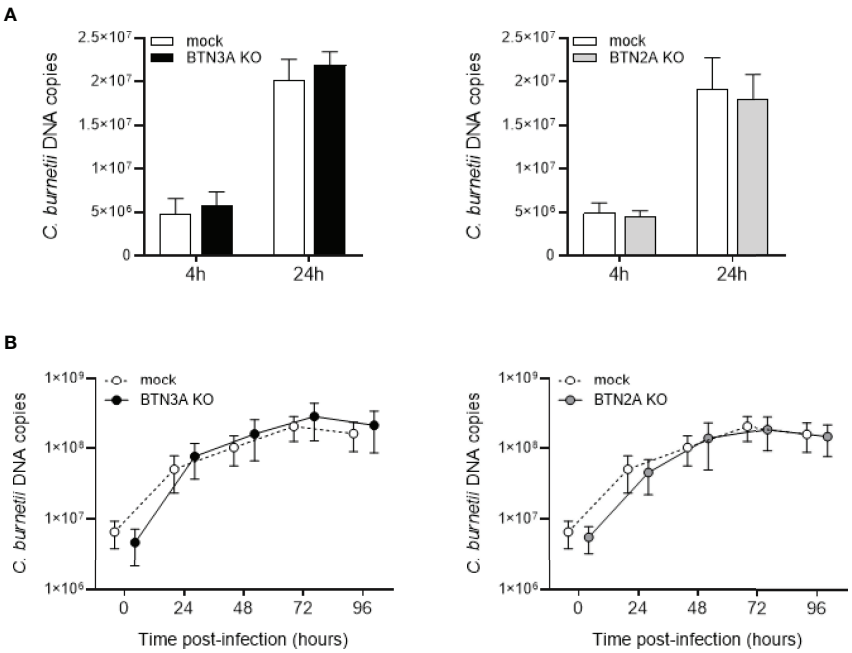

FIGURE 2 | Involvement of BTN3A and BTN2A in *C. burnetii* infection. CRISPR-Cas9-mediated inactivation of BTN3A or BTN2A was performed in THP-1 cell line. THP-1 cells transduced with a guide targeting all *BTN3A* isoforms (BTN3KO) or all *BTN2A* isoforms (BTN2AKO) or with an irrelevant CRISPR guide (mock) for control cells were infected with *C. burnetii* NM1 (50 MOI) (n = 3). **(A)** After 4 and 24 hours of infection, the number of bacterial DNA copies within THP-1 cells was assessed by qPCR. **(B)** THP-1 cells were incubated with *C. burnetii* for 4 h (day 0), then washed to eliminate free bacteria and incubated for 4 days. Each day, the number of bacterial DNA copies was evaluated by qPCR. Values represent mean ± standard deviation.

C. burnetii Infection Leads to Vγ9Vδ2 Cells Activation in a BTN3A and BTN2-Dependent Manner

Since BTNs appeared to be over-expressed in monocytes following *C. burnetii* infection, we hypothesized that it could enhance the Vγ9Vδ2 T cell activation. After 4 hours of co-culture with *C. burnetii* infected monocytes, Vγ9Vδ2 T cell displayed enhanced degranulation as depicted by increased membrane expression of CD107, which also increased with the titer of bacteria used for monocytes infection (**Figure 4A**). We then investigated whether Vγ9Vδ2 T cell activation by *C. burnetii*-infected cells was dependent on BTNs by using anti-BTN3A antagonist (clone 103.2) (26) and anti-BTN2A antagonist (clone 7.48) (30) antibodies. Both antibodies led to significant inhibition of Vγ9Vδ2 T cell degranulation against cells infected with *C. burnetii* NMI or Guiana strains, or *M. tuberculosis* as positive control, suggesting that both BTNs are involved in Vγ9Vδ T cell activation in an infectious context (**Figures 4B, C**) as it was previously shown for malignant cells (30). Taken together, *C. burnetii* infection leads to Vγ9Vδ2 T cell activation in a BTN3A and BTN2A dependent manner.

We next hypothesized that Vγ9Vδ2 T cell activation towards *C. burnetii*-infected cells could be enhanced by an humanized BTN3A agonist antibody (clone 20.1) (26) that activates Vγ9Vδ2 T cells. As illustrated in the **Figure 4D**, we observed that the BTN3A activating antibody leads to increased expression of CD107 (**Figure 4D**) and the cytotoxic activity (**Figure 4E**) of Vγ9Vδ2 T cells towards *C. burnetii* infected monocytes as observed for *M. tuberculosis* after 4 hours of co-culture. A similar effect was observed for all *C. burnetii* strains, to the same extent as *M. tuberculosis*, suggesting that the 20.1 antibody can induce Vγ9Vδ2 T cell activation even towards virulent bacteria. These data show that targeting Vγ9Vδ2 T cells with the 20.1 antibody leads to the activation of their cytotoxicity against *C. burnetii*-infected cells.

Anti-BTN3A Agonist Antibody Increases Antimicrobial Activity of Vγ9Vδ2 T Cells

Since the anti-BTN3A agonist antibody (clone 20.1) increases Vγ9Vδ2 T cell activation, we wondered whether it was able to boost their antimicrobial activity. For this purpose, monocytes were infected with *C. burnetii* NM1 for 24 hours and then co-cultured with Vγ9Vδ2 T cells for 4 hours in presence of 20.1 antibody (0, 0.1, 1 or 10 µg/ml) and the bacterial load was measured by flow cytometry and qRT-PCR. First, Vγ9Vδ2 T cells lead to a significant reduction of *C. burnetii* load from 5.10^7 to 6.10^6 in monocytes in the presence of Vγ9Vδ2 T lymphocytes (p=0.0021) (**Figures 5A, B**), as observed for *M. tuberculosis* infection (**Supplementary Figure 3**). BTN3A activating antibody resulted in a dose-dependent decrease in *C. burnetii* load in monocytes, reaching from 6.10^6 to $4.2.10^6$ (0 *vs.* 10 µg/ml,

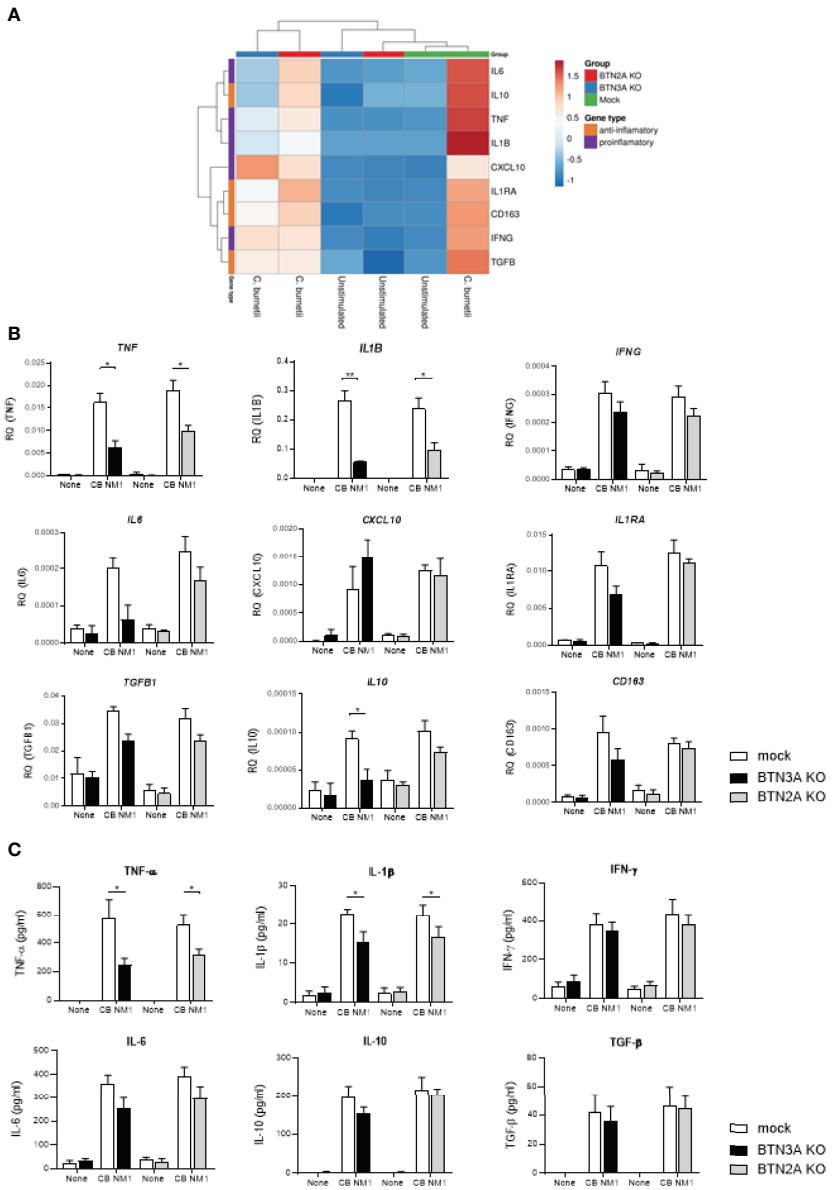

FIGURE 3 | Involvement of BTN3A and BTN2A in the inflammatory response to *C. burnetii* infection. THP-1 cells transduced with an irrelevant CRISPR guide (mock) or a guide targeting all *BTN2A* isoforms (BTN2AKO) or all *BTN3A* isoforms (BTN3AKO) were infected with *C. burnetii* NM1 (100 MOI) (n = 3). After 24 hours infection, the expression of genes involved in the inflammatory (*TNF*, *IL1B*, *IL6*, *IFNG*, *CXCL10*) or immunoregulatory (*IL10*, *TGFB1*, *IL1RA*, *CD163*) response was investigated by quantitative reverse-transcription polymerase chain reaction after normalization with housekeeping actin gene as endogenous control. Data are illustrated as **(A)** hierarchical clustering obtained using ClustVis webtool or **(B)** relative quantity of investigated genes. **(C)** After 24 hours infection, TNF-α, IL-1β, IFN-γ, IL-6, IL-10, and TGF-β release were evaluated in the culture supernatants by ELISA assay. Data were analyzed using a normality test and a parametric *t* test. Values represent mean ± standard error. $^{*}p < 0.05$ and $^{**}p < 0.01$.

p=0.0501) (**Figures 5A, B**). This effect is similar to that observed in the case of *M. tuberculosis*, where the 20.1 antibody resulted in a decrease in the bacterial load in monocytes (0 *vs.* 10 μg/ml, p=0.0158) (**Supplementary Figure 3**). Altogether, BTN3A activating antibody increases the antimicrobial activity of Vγ9Vδ2 T lymphocytes against monocytes infected with *C. burnetii*.

Anti-BTN3A Agonist Antibody Increases the Secretion of Cytokines and Cytotoxic Molecules by Vγ9Vδ2 T Cells

Since treatment with the anti-BTN3A agonist antibody leads to bacterial load reduction, we investigated whether this could be related to the secretion of cytokines and cytotoxic molecules, which are strongly produced by activated Vγ9Vδ2 T cells (17–21). Indeed,

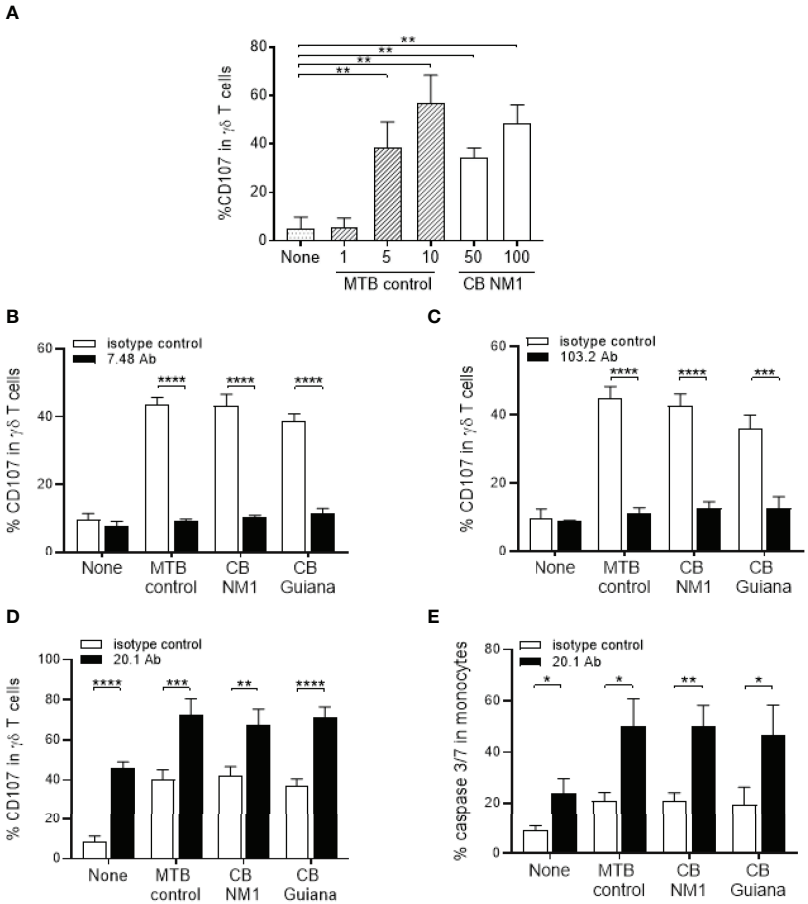

FIGURE 4 | Infection with *C. burnetii* leads BTN3A and BTN2-dependent activation of Vγ9Vδ2 T lymphocytes. **(A)** Monocytes isolated from healthy donors (n = 3) previously infected 24 hours with *C. burnetii* NM1 (50 or 100 MOI) or with *M. tuberculosis* (1, 5 or 10 MOI) were co-cultured with autologous Vγ9Vδ2 T cells (E:T ratio of 1:1). Vγ9Vδ2 T cell degranulation (%CD107ab+ cells) was assessed after 4 hours of co-culture by flow cytometry. **(B–D)** Monocytes isolated from healthy donors (n = 4) previously infected 24 hours with **(C)** *burnetii* strains (50 MOI) or with *M. tuberculosis* (5 MOI) were co-cultured with Vγ9Vδ2 T cells expanded from healthy donor (E:T ratio of 1:1) in the presence of **(B)** anti-BTN2A (clone 7.48), **(C)** anti-BTN3A (clone 103.2) or **(D)** anti-BTN3A (clone 20.1) antibodies (10µg/ml). Vγ9Vδ2 T cell degranulation (%CD107ab+ cells) was assessed after 4 hours of co-culture by flow cytometry. **(E)** The cytotoxicity was assessed by flow cytometry as the percentage of Caspase $3/7^+$ cells in the target cell population after 4 hours of co-culture in presence of anti-BTN3A antibody (clone 20.1) (10µg/ml). Data were analyzed using a normality test and a parametric *t* test. Values represent mean ± standard deviation. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

treatment of Vγ9Vδ2 T cell/*C. burnetii*-infected monocyte co-cultures with the 20.1 mAb increased TFN-α, IFN-γ and GM-CSF secretion in a dose-dependent manner (**Figure 6A**, left panel). Moreover, a significant difference was observed between the 0.1 and 10 µg/ml doses for IFN-γ, TFN-α and GM-CSF secretion (p=0.0260, p=0.0443 and p=0.0265, respectively), in the case of infection with *C. burnetii* Guiana. Regarding cytotoxic molecules, granzyme B and perforin secretion were significantly increased in presence of 10 µg/ml of 20.1 mAb in the case of monocytes infected with *C. burnetii* NM1 and Guiana, *M. tuberculosis* and uninfected monocytes (**Figure 6B**, right panel). On the other hand, the 20.1 mAb showed a less pronounced effect on granulysin secretion, with a significant difference only in the case of *M. tuberculosis* infection (0 *vs.* 10 µg/ml, p=0.0488). It can also be noted that the levels of granulysin appeared to be higher in the case of *M. tuberculosis* infection than with *C. burnetii*. Overall, the presence of the BTN3A activating antibody increases the secretion of cytokines and cytotoxic molecules, both produced by the activated Vγ9Vδ2 T cells.

DISCUSSION

An alteration of circulating Vγ9Vδ2 T cells has been observed in Q fever patients (10). During acute phase of the disease, the proportion of Vγ9Vδ2 T cells is significantly increased in patients (16% *vs.* 4% in healthy donors) (10), indicating the involvement of these cells in the acute immune response to *C. burnetii*. Since human Vγ9Vδ2 T cell responses are triggered *via* an interaction with the BTN2A1/BTN3A1 complex, we first assessed whether their expression was modulated following *C. burnetii* infection. We found that *in vitro* infection of monocytes with *C. burnetii* induced a significant increase in the

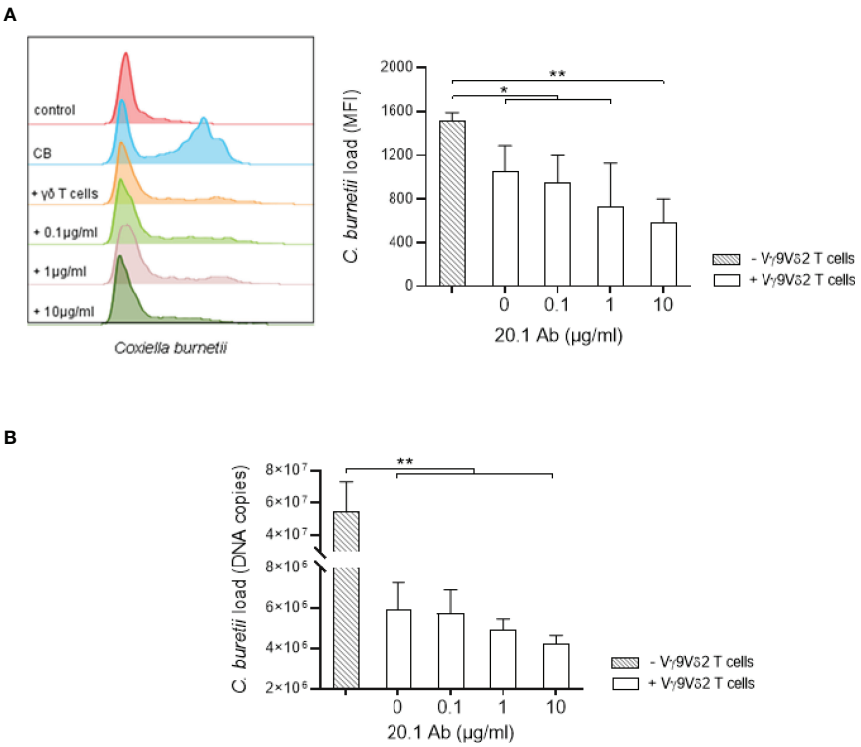

FIGURE 5 | Anti-BTN3A agonist antibody increases antimicrobial activity of Vγ9Vδ2 T cells towards *C. burnetii* infected monocytes. **(A, B)** Monocytes isolated from healthy donors (n = 4) previously infected 24 hours with *C. burnetii* NM1 (50 MOI) were co-cultured with autologous Vγ9Vδ2 T cells (E:T ratio of 1:1) in the presence of anti-BTN3A antibody (clone 20.1) (0-10 µg/ml). After 4 hours of co-culture, *C. burnetii* load was measured by **(A)** flow cytometry and **(B)** qPCR. Data were analyzed using a normality test and a parametric *t* test. Values represent mean ± standard deviation. *$p < 0.05$, **$p < 0.01$.

transcriptomic and plasma membrane expression of these two BTNs. This increase is similar to that observed with *M. tuberculosis* and between *C. burnetii* strains, suggesting that the aggressiveness of the bacteria appears to have limited impact on BTN expression. Similarly, increased expression of these two BTNs has recently been described in red blood cells infected by *Plasmodium falciparum* (42). Our team has recently shown higher expression of BTN3A, but not BTN2A, following SARS-CoV-2 infection of myeloid cells and lung cell lines (submitted manuscript). This may suggest different mechanisms depending on the pathogen.

Using CRISPR-Cas9 gene inactivation in the THP-1 cell line, we found that BTN3A and BTN2A are not directly involved in the infection process of cells by *C. burnetii* but play a role in the cellular immune response to infection. Indeed, THP-1 cells inactivated for BTN3A or BTN2A show a repressed inflammatory response following *C. burnetii* infection, with a significant decrease in *TNF* and *IL1B* gene expression. These results suggest that higher expression of these two molecules on monocytes could favor responses to *C. burnetii* infection.

The fact that both BTN3A and BTN2A, essential for Vγ9Vδ2 T cell activation, are more expressed following *C. burnetii* infection could enhance their activation and antibacterial activity. Using a Vγ9Vδ2 T cell/infected monocyte co-culture model, we observed that monocytes infected with *C. burnetii* strains of different aggressiveness resulted in similar degranulation of Vγ9Vδ2 T cells. Several studies have confirmed that the activation of Vγ9Vδ2 T cells is dependent on BTN3A during infections. Indeed, the anti-BTN3A antagonist antibody 103.2 was able to inhibit the degranulation of Vγ9Vδ2 T cells when they were co-cultured with cells infected with *M. bovis* (BCG), *L. monocytogenes*, *P. falciparum* or Epstein-Barr virus (23, 42–44). In our study, similar results are obtained with the 103.2 antibody but are also observed with an anti-BTN2A antagonist antibody (clone 7.48), underlining the importance of these two BTNs in the activation of Vγ9Vδ2 T cells.

Next, we evaluated the effect of BTN3A on antibacterial activity. For this purpose, we used the anti-BTN3A agonist antibody 20.1 to treat Vγ9Vδ2 T cell/*C. burnetii*-infected monocyte co-cultures. Our results show that the 20.1 mAb increases the antibacterial activity of Vγ9Vδ2 T cells leading to a decreased intracellular load of *C. burnetii*. In our study, Vγ9Vδ2 T cells, whose cytotoxic activity is enhanced by the 20.1 mAb, were both able to kill *C. burnetii*-infected monocytes through the production of lytic granules (granulysin, perforin, granzymes) and at the same time produce large amounts of IFN-γ and TFN-α. These cytokines play an essential role in protection

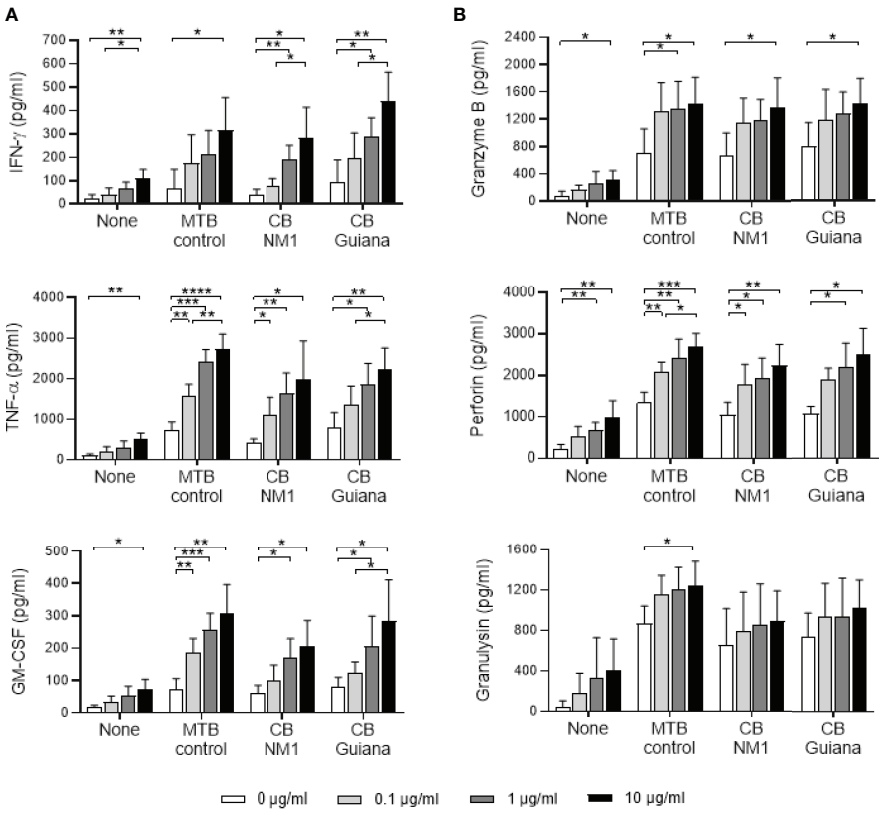

FIGURE 6 | Anti-BTN3A agonist antibody increases the secretion of cytokines and cytotoxic molecules in Vγ9Vδ2 T cell/infected-monocyte co-cultures. Monocytes isolated from healthy donors (n = 4) previously infected 24 hours with *C. burnetii* NM1 (50 MOI) or with *M. tuberculosis* (5 MOI) were co-cultured with autologous Vγ9Vδ2 T cells (E:T ratio of 1:1) in the presence of anti-BTN3A antibody (clone 20.1) (0-10 µg/ml). After 4 hours of co-culture, the culture supernatants were analyzed for the presence of cytokines (**A**, left panel) and cytotoxic molecules (**B**, right panel) by ELISA assay. Data were analyzed using a normality test and a parametric *t* test. Values represent mean ± standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

against intracellular bacteria by activating the antimicrobial machinery of phagocytes. Indeed, IFN-γ induces *C. burnetii* killing by promoting apoptosis of infected monocytes (36, 45), and TNF-α shows an essential role in the control of *C. burnetii* infection like for other pathogens including *M. tuberculosis* or *L. monocytogenes* (46, 47). These data extend previous studies as human Vγ9Vδ2 T cells have already been shown to effectively kill intracellular pathogens, such as *M. tuberculosis*, *L. monocytogenes* and *B. suis*, through the secretion of IFN-γ, TNF-α and cytotoxic molecules such as granzymes, perforin and granulysin (17–21, 48). Some studies have also reported that NKG2D contributed to the anti-infective activity of Vγ9Vδ2 T cells against *Brucella* sp. and *M. tuberculosis* (49, 50). In contrast, in other studies on *M. tuberculosis* or *L. monocytogenes*, NKG2D was not involved (20, 43). These discrepancies may be due to the different expression of NKG2D ligands between infections and between cell populations. Diverse functions of NKG2D ligands could have an impact on the anti-infective activity of Vγ9Vδ2 T cells.

Our data suggest that targeting Vγ9Vδ2 T cells to activate their cytotoxic functions may be considered a promising strategy for the treatment wide range of pathogens like for *C. burnetii*. Indeed, alterations in the phenotype and/or functions of Vγ9Vδ2 T cells have been reported in several infections usually caused by intracellular pathogens. For example, in patients with active tuberculosis, a progressive loss of effector function of circulating Vγ9Vδ2 T cells has been reported, leading to decreased IFN-γ production and granulysin expression (51, 52). This alteration was correlated with disease progression (53, 54), suggesting that a high level of bacteria can lead to chronic stimulation of Vγ9Vδ2 T cells that would result in their apoptosis and/or senescence. Targeting Vγ9Vδ2 T cells in the context of persistent infections could therefore be an attractive strategy. Future phenotypic and functional analyses of Vγ9Vδ2 T cells from patients with Q fever will allow to determine whether their capacity is altered.

Recently, a novel approach has been developed to expand and activate Vγ9Vδ2 T cells besides pAgs. This strategy is based on the development of a new class of molecules called immunoantibiotics, in particular the inhibitor IspH (55). IspH, an enzyme of the isoprenoid synthesis pathway, is essential for the survival of most Gram-negative bacteria and the absence of

IspH causes an accumulation of its substrate HMBPP, which in turn activates Vγ9Vδ2 T cells. Another approach would be to target specifically the ligands expressed on the surface of stressed infected cells, such as BTN3A, which will vehicle activation and cytotoxicity of Vγ9Vδ2 T cells (56). This is the case in a trial in cancer patients where the approach is to activate Vγ9Vδ2 T cells by targeting BTN3A (NCT04243499, ImCheck Therapeutics, Marseille, France) (57, 58).

In addition, we have also explored the effect of the 20.1 mAb in the case of SARS-CoV-2 infection. By activating the Vγ9Vδ2 T cells, 20.1 mAb may affect intracellular SARS-CoV-2 replication *in vitro* in infected cells (submitted manuscript). Future studies should be conducted to elucidate the detailed mechanisms of protective Vγ9Vδ2 T cell activation and how precisely BTN3A is involved in infections. These results highlight that the BTN3A agonist antibody could represent powerful therapeutic tool in infections to overcome the imbalances in immune responses observed in some patients and open new perspectives in Vγ9Vδ2 T-cell-based immunotherapies in infectious diseases.

In summary, this study provided further insight into the role of Vγ9Vδ2 T cells in infections with intracellular bacteria. We demonstrated that *C. burnetii* infection results in modulation of BTN3A and BTN2A co-receptor expression, allowing activation of Vγ9Vδ2 T cells. We report for the first time the role of a BTN3A agonist antibody in the control of intracellular bacterial infection. The latter boosts the cytotoxic functions of Vγ9Vδ2 T cells *in vitro* such as their degranulation, the production of TNF-α and IFN-γ, and killing activity leading to a better clearance of *C. burnetii* load of infected target cells. These results may facilitate new approaches to the treatment of persistent bacterial infections by enhancing Vγ9Vδ2 T cell responses in presence of infected cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

A convention No.7828 was established between our laboratory and the Etablissement Français du Sang (Marseille, France). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LG, MG, and MF performed the experiments and analyzed the data. SM, CC, EF, L.M, J-LM and DO supervised the work. LG, SM, J-LM, and DO participated in the writing of the paper. All the authors read and approved the final manuscript.

FUNDING

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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.2022.915244/full#supplementary-material

Supplementary Figure 1. | Phenotype confirming gene invalidation in THP-1 cell line. CRISPR-Cas9-mediated inactivation of BTN3A1/3A2/3A3 or BTN2A1/2A2 isoforms was performed in THP-1 cell lines. (A) The expression level of BTN3A was assessed by flow cytometry. Data were collected on a BD Canto II instrument (BD Biosciences). (B) The expression level of BTN2A was assessed by flow cytometry. Data were collected on a CytoFLEX S instrument (Beckman Coulter). All data were analyzed with FlowJo software (FlowJo v10.6.2).

Supplementary Figure 2. | Manual gating for Vγ9Vδ2 T cell functional assays. (A) Monocytes were co-cultured with Vγ9Vδ2 T cells at effector-to-target (E:T) ratio of 1:1 and fluorochrome-labeled CD107a and CD107b. Phorbol 12-myristate 13-acetate (PMA, 20 ng/mL) with ionomycine (1 µg/mL) were used as positive control for Vγ9Vδ2 T cell activation. After 4 hours, cells were harvested and stained with fluorochrome-labeled TCR-specific mAbs and a viability marker. The degranulation was evaluated by flow cytometry as the percentage CD107a/b+ cells in the γδ T cell population. (B) Monocytes were labeled with 10 µM Cell Proliferation Dye eFluor® 670 and then co-cultured with Vγ9Vδ2 T cells at E:T ratio of 1:1. After 4 hours, cells were stained with CellEvent Caspase-3/7 Green to identify dead cells. The cytotoxicity was assessed by flow cytometry as the percentage of Caspase 3/7+ cells in the target cell population.

Supplementary Figure 3. | Anti-BTN3A agonist antibody increases antimicrobial activity of Vγ9Vδ2 T cells towards M. tuberculosis infected monocytes. Monocytes isolated from healthy donors (n=4) previously infected 24 hours with M. tuberculosis (5 MOI) were co-cultured with autologous Vγ9Vδ2 T cells (E:T ratio of 1:1) in the presence of anti-BTN3A antibody (clone 20.1) (0-10 µg/ml). After 4 hours of co-culture, M. tuberculosis load was measured by qPCR. Data were analyzed using a normality test and a Mann-Whitney U test. Values represent mean ± standard deviation. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

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Phenotypic Changes of Peripheral γδ T Cell and Its Subsets in Patients With Coronary Artery Disease

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Coronary atherosclerotic heart disease (CAD) is a chronic inflammatory cardiovascular disease with high morbidity and mortality. Growing data indicate that many immune cells are involved in the development of atherosclerosis. However, the immunological roles of γδ T cells in the initiation and progression of CAD are not fully understood. Here, we used flow cytometry to determine phenotypical changes of γδ T cells and their subpopulations in peripheral blood samples collected from 37 CAD patients. The Pearson correlation coefficient was used to analyze the relationship between the clinical parameter (serum LDL-C level) and the changes of immunophenotypes of γδ T cells. Our results demonstrated that the frequencies and absolute numbers of total γδ T cells and $V\delta2^+$ T cells were significantly decreased in CAD patients when compared to healthy individuals. However, the proportion of $V\delta1^+$ T cells was much lower in CAD patients than that of healthy individuals. Most importantly, a significant alteration of the Vδ1/Vδ2 ratio was found in CAD patients. In addition, a series of surface markers that are associated with costimulatory signals (CD28, CD40L, CD80, CD86), activation levels (CD69, CD25, HLA-DR), activating NK cell receptors (NKp30, NKp46, NKG2D) and inhibitory receptors (PD-1, CTLA-4, PD-1, Tim-3) were determined and then analyzed in the total γδ T cells, $V\delta2^+$T cells and $V\delta2^-$T cells of CAD patients and healthy individuals. The data demonstrated that immunological activities of total γδ T cells, $V\delta2^+$T cells, and $V\delta2^-$T cells of CAD patients were much lower than those in healthy individuals. Moreover, we found that there were positive correlations between the serum LDL-C levels and frequencies of $CD3^+\gamma\delta^+$ T cells, $CD69^+V\delta2^+$T cells, $NKG2D^+V\delta2^+$T cells, and $NKp46^+V\delta2^+$T cells. By contrast, there was an inverse correlation between the levels of serum

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LDL-C and the frequencies of $CD69^+V\delta2^-$T cells and $NKp46^+V\delta2^-$T cells. Accordingly, these findings could help us to better understand the roles of γδ T cells in the CAD, and shed light on the development of novel diagnostic techniques and therapeutic strategies by targeting γδ T cells for CAD patients.

Keywords: coronary atherosclerotic heart disease, atherosclerosis, NKG2D, γδ T cells, $V\delta2^+$T cell, $V\delta2^-$T cells

INTRODUCTION

Cardiovascular diseases (CVDs) continue to increase in prevalence and deaths worldwide, and remain the leading cause of severe disease burden and death in the world (1). Coronary atherosclerotic heart disease (CAD) is one of the most common cardiovascular diseases as well as the one of the leading causes of death in middle-aged and elderly people (2). The main pathophysiological change of CAD is coronary artery atherosclerosis (AS), which is a chronic inflammatory disease involved with many large and medium-sized arteries. Severe coronary stenosis and/or unstable atherosclerotic plaque rupture can lead to vascular embolism, myocardial ischemia, angina pectoris, myocardial infarction, arrhythmia, and even sudden death, which seriously threatens human life and health. The occurrence and development of atherosclerotic heart disease are related to a variety of risk factors, and metabolic factors, such as hypertension and hyperlipidemia (1). In order to reduce the occurrence of various complications, premature death and disability caused by coronary artery disease, more studies are required to investigate the underlying mechanisms of occurrence and development of CAD and coronary atherosclerosis and this will help us to explore new strategies for prevention of atherosclerosis and CAD.

The formation of atherosclerosis is complex and multifactorial, in which immunological dysregulation and serious inflammatory response are suggested to be the critical keys to its occurrence and progression. The various immune cells, such as monocytes, macrophages, dendritic cells, NK cells of the innate immune system as well as T cells and B cells of the adaptive immune system, are supposed to be involved in all processes of formation and development of atherosclerosis and rupture of vulnerable plaques. Some immune cells that infiltrate into the atherosclerotic plaque and the interactions of cells induce the secretion of pro-inflammatory cytokines which maintain the atherosclerotic plaque in an inflammatory microenvironment (3). The immunological roles of monocytes, macrophages and $CD4^+$T cells, which are the most common T cells in plaques and atherosclerotic lesions, have been extensively studied. However, the immunological functions of some other immune cells in atherosclerosis are still unclear, such as γδ T cells (4). γδ T cells are innate-like T cells that carry TCR γ and δ chains. Unlike αβT cells, γδ T cells only account for 1-10% of T cells in human peripheral blood but are abundant in mucosal tissues such as skin, intestine, and lung (5). γδ T can recognize, in a major histocompatibility complex (MHC) non-restricted (6) manner, endogenous and exogenous phosphorylated antigens (pAgs) such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and isopentenyl pyrophosphate (IPP) (7) and respond rapidly as the first line of immune defense. γδ T cells can also be activated through their surface-expressed TCRs and natural killer cell receptors, such as NKp30, NKp44, and natural-killer group 2 member D (NKG2D) (6). Activated γδ T cells can exhibit multiple immunological functions by expressing and producing cytotoxins such as TRAIL, Fas/Fas-L, granzyme B and perforin. In addition, γδ T cells can also produce large amounts of cytokines, such as IFN-γ, TNF-α, and IL-17. Human γδ T cells can generally be divided into two major subpopulations based on the expression of the TCR Vδ chain in the peripheral blood: $V\delta2^+$T cells and $V\delta2^-$T cells (8). Accordingly, 65~90% of γδ T cells in adult peripheral blood are $V\delta2^+$T cells, which are almost always paired with $V\gamma9^+$, thus these cells are often referred to as $V\gamma9^+V\delta2^+$T cells (9). $V\gamma9^+V\delta2^+$T cells usually display a cytotoxic phenotype and secrete excessive amounts of IFN-γ and TNF-α, which have the potent killing effects against many hematological tumors and solid tissue tumors (10). While $V\delta2^-$T cells are mainly $V\delta1^+$T cells, with fewer $V\delta3^+$T and $V\delta5^+$T cells (9). Unlike $V\gamma9^+V\delta2^+$T cells, this $V\delta2^-$T cell subpopulation mainly exists in mucosal tissues, and only a small part (less than 30%) exists in peripheral blood. In addition, $V\delta1^+$ T cells mainly play an immunosuppressive role. Previous studies reported that $V\delta1^+$ T cells can induce FoxP3 expression in the presence of TGF-β and IL-2/IL-15, and their immunomodulatory effects are similar to those of Treg cells (11, 12). A large body of evidence indicates that γδ T cells are regarded as a bridge between innate and adaptive immune responses, which play an important role in the human immune response (13).

The presence of γδ T cells in human atherosclerotic plaques was first reported in 1993 (14). However, there are still only a few studies to mention the immunological functions of γδ T cells in atherosclerosis. The evidence which was collected from an experimental model of atherosclerosis demonstrated that lack of γδ T cells decreased plasma total cholesterol levels and reduced atherosclerosis in the aortic sinus of $ApoE^{-/-}$ $TCR\delta^{-/-}$ mice, although these differences did not reach statistical significance (15). Additionally, a significant increase in γδ T cells, which produce IL-17 but not IFN-γ, was also found in the aortic root and arch of ApoE KO mice, and depletion of these cells reduced the size of early atherosclerotic lesions at this site in mice (16). However, another $TCR\delta^{-/-}ApoE^{-/-}$ mouse study found no critical role for γδ T cells in the development of early atherosclerosis in the total aorta of mice after 10 weeks of high-fat diet feeding (17). These studies suggest a pathogenic role for γδ T cells in early atherosclerosis in mice and their effects may be site-specific. Recent studies demonstrated that $IL\text{-}23R^+$γδ T cells are frequently found in the aortic root of $Ldlr^{-/-}Il23rgfp/^+$ mice. Moreover, the absence of this subset of γδ T cells reduces reduced the formation of AS lesions in the aortic root. And the γδ T cells

were confirmed by scRNAseq to be the predominant cells expressing IL-23R and IL-17A in the aorta (18). This suggested that the pathogenic role of γδ T cells in early mouse AS may be related to IL-17A- and IL-23R-mediated immune response. Although some progress has been made with γδ T cells in experimental mouse models, the specific pathogenic mechanisms of γδ T cells in early atherosclerotic lesions and how they change with disease progression have not been fully elucidated, and it is even more challenging to translate these findings in animal models to human disease. At present, the roles of γδ T cells in the development of human atherosclerotic disease are less studied and remain largely unknown. A Multi-Ethnic Study of Atherosclerosis (MESA) cohort study showed that a higher proportion of γδ T cells in older adults was associated with poorer cardiac function in a subclinical state with cardiovascular risk factors but without heart failure (19). Analysis of high-throughput gene expression datasets revealed that the proportion of infiltrating γδ T cells in human AS plaques was decreased and significantly negatively correlated with inflammation-related pathways such as the IL-23 signaling pathway and NOTCH2 signaling pathway (3). This study found that γδ T cells from patients with acute myocardial infarction (AMI) had restricted expression of γδ rearrangement of TCR and higher expression of IL-17A, suggesting that γδ T cells may play an important role in the pathological progression of AMI (20). These results suggested that γδ T cell-mediated inflammatory responses may play an important role in the formation and development of human coronary atherosclerotic heart disease; however, the immunological functions of γδ T cells in advanced atherosclerosis has not been elucidated.

Therefore, based on the pathogenic role of γδ T cells in early atherosclerosis in mice, our study intends to analyze and compare the changes in the absolute number and immunophenotypes of γδ T cells and their subpopulations in the peripheral blood of CAD patients and healthy individuals, as well as the analysis of the correlation between clinically relevant indices and the immunophenotypes in the peripheral blood of CAD patients. In this study, we demonstrated that immunological characteristics of total γδ T cells, $V\delta2^+$T cells, and $V\delta2^-$T cells exhibited significant alteration in CAD patients when compared with the healthy individuals. Most interestingly, our data found that serum LDL-C level had a diametrically opposite correlation with the frequencies of subpopulation cells in $V\delta2^+$T cells and $V\delta2^-$T cells, particularly the cells that expressed CD69. These results could provide us with more clues and hints to reveal the potential functions of γδ T cells in the progression of CAD.

MATERIALS AND METHODS

Patients and Samples

Including 24 male cases and 13 female cases, 37 CAD patients with 37~82 years old were enrolled who have been diagnosed with coronary angiography in the Department of Cardiology, Nanfang Hospital of Southern Medical University from April to June 2021. To assess the phenotypes of peripheral immune cells, 10 mL of heparinized anticoagulant was collected before coronary stenting in CAD patients. The clinical and demographic characteristics of CAD patients were described in **Table 1**. At the same time, 84 healthy individuals aged 40 to 66 years were recruited for this study, including 47 males and 37 females. The exclusion criteria of the healthy individuals included (1) a history of cardiovascular system diseases such as myocardial infarction, heart failure, angina pectoris, and/or cerebrovascular disease (2); hypertension, diabetes, and obesity; (3) severe liver damage, severe infectious diseases, hematological or autoimmune diseases. Similarly, 10 mL of peripheral blood was collected for subsequent immunological assays. Ethical approval was obtained from the Medical Ethics Committee of Nanfang Hospital of Southern Medical University.

Peripheral Immune Cell Phenotype Analyzing by Flow Cytometry

To determine the phenotypic surface markers of γδ T cells and their subpopulations, collected peripheral blood samples were analyzed by flow cytometry. After the collected heparinized anticoagulant whole blood (1 mL) was directly lysed twice with red blood cell lysis solution, the whole blood leukocytes were isolated, and then stained with mouse anti-human fluorescein-conjugated monoclonal antibodies against different markers (**Supplementary Table 1**). After incubation in the dark for 15 mins, the cells were washed twice with PBS. Subsequently, the cells were resuspended in 200 uL PBS, and then added 5 uL of CountBright™ Absolute Counting Beads (Thermo Fisher Scientific). Finally, samples were collected using the instrument FACSVerse (BD biosciences) and data were analyzed using FlowJo 10.5.3 (FlowJo LLC).

Statistical Analysis

Data were processed and analyzed using Microsoft Excel 2019 and Graphpad Prism 8.0.1. Unpaired t-test was used for statistical comparison of immune indexes between CAD patients and healthy individuals, and the Pearson correlation coefficient was used to calculate the correlation between two continuous variables of clinical related indexes of CAD patients and immune indexes in their peripheral blood. Statistical graphs were drawn by Graphpad Prism 8.0.1, all tests were set as two-

TABLE 1 | Clinical and demographic characteristics of CAD patients.

	CAD (n=37)
Age (years)	64.70 ± 9.1
Sex (male/female)	24/13
Smoking	15 (40.5%)
Hypertension	20 (54.1%)
Diabetes	8 (21.6%)
Gensini	39.88 ± 36.89
LDL-C (mmol/L)	2.79 ± 0.93
HDL-C (mmol/L)	1.14 ± 0.28
TG (mmol/L)	1.66 ± 0.83

LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride.

tailed, P<0.05 was considered to be significantly different, and the results in the figure were expressed as Mean with SD.

RESULTS

Changes in the Proportion of γδ T Cells and Their Subsets in Peripheral Blood of CAD Patients

To investigate whether the immunological features of γδ T cells and their subsets were altered in CAD patients. The percentages and absolute numbers of γδ T cells and their subsets in the peripheral blood of CAD patients and healthy individuals were analyzed, respectively. The gating strategy identifying γδ T cells, $V\delta1^+$ and $V\delta2^+$ γδ T cell subsets were shown in **Supplementary Figure 1A**, and the results indicated that the percentages of $CD3^+$T cells (**Figure 1A**), γδ T cells in $CD3^+$ T cells (**Figure 1B**), $V\delta2^+$T cells in γδ T cells (**Figure 1C**) in the CAD patients were significantly decreased when compared to that in healthy individuals. However, the CAD patients had a higher frequency of $V\delta1^+$T cells in γδ T cells than that in healthy individuals (**Figure 1D**). When further analyzing the absolute number of $CD3^+$T cells (**Figure 1E**), γδ T cells in $CD3^+$ T cells (**Figure 1F**), the data indicated that $V\delta2^+$T cells in γδ T cells (**Figure 1G**) were higher in CAD patients than those in healthy individuals. By contrast, there was no significant difference in the absolute number of $V\delta1^+$T cells in γδ T cells between CAD patients and healthy individuals (**Figure 1H**). Moreover, the Vδ1/Vδ2 ratio in CAD patients was elevated (2.326 ± 4.448), when compared to that in healthy individuals (0.411 ± 0.944) (**Figure 1I**).

The Activation Levels of γδ T Cells in the CAD Patients Are Altered

γδ T cells can exhibit multiple immunological functions because this population of cells can express CD28, CD80/CD86, and CD40L. In addition, CD80/CD86-CD28 and CD40-CD40L signaling are involved in regulating different functions of γδ T cells and their subsets. To investigate the functional status of γδ T cells during CAD, we next compared the frequencies of CD28,

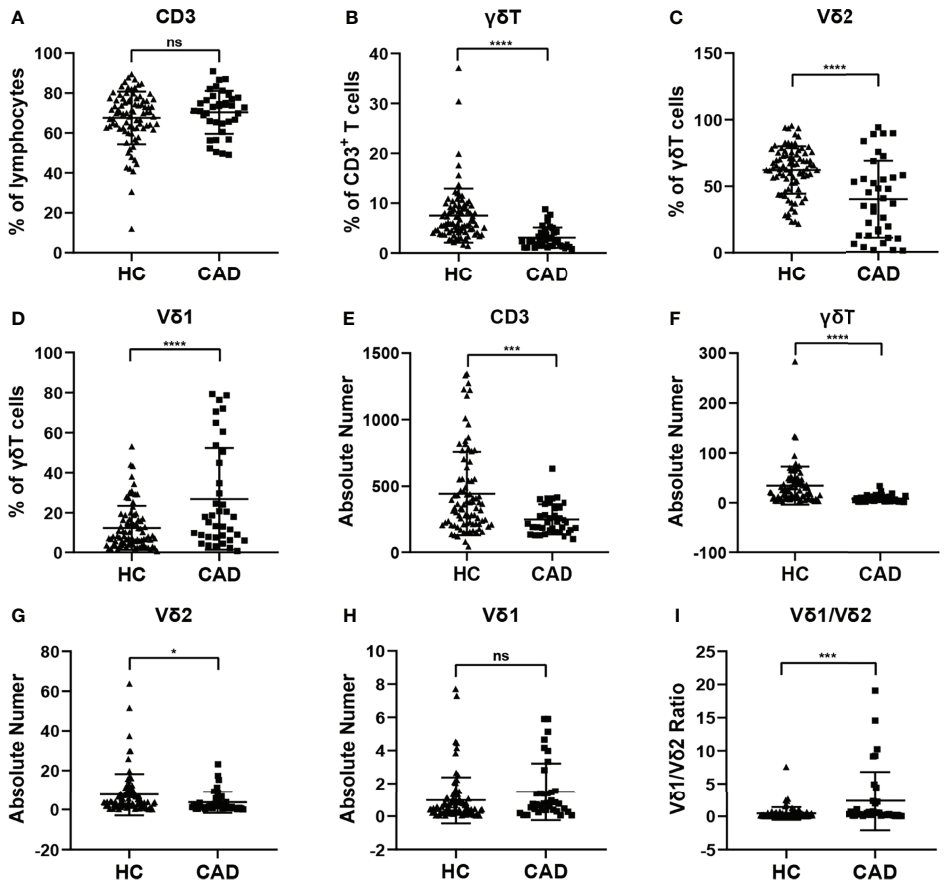

FIGURE 1 | Statistical comparison of the percentages and absolute numbers of γδ T cell and its subsets Vδ1+T cells, Vδ2+T cells, and Vδ1/Vδ2 ratios in the peripheral blood between healthy individuals (HC) and CAD patients. **(A)** $CD3^+$T cells, **(B)** γδ T cells, **(C)** $V\delta2^+$T and **(D)** $V\delta1^+$T cells frequencies as percentages of lymphocytes, $CD3^+$T cells, and total γδ T cells respectively, absolute numbers of **(E)** $CD3^+$T cells, **(F)** γδ T cells, **(G)** $V\delta2^+$T and **(H)** $V\delta1^+$T cells and **(I)** Vδ1/Vδ2 ratios of healthy individuals versus CAD patients. *P < 0.05, ***P < 0.001, and ****P < 0.0001; ns, no significance.

CD80, CD86, and CD40L positive cells in total γδ T cells, and Vδ2$^+$T cells between CAD patients and healthy individuals. The gating strategy for the identification of γδ T cells, Vδ2$^+$T cells, and Vδ2$^-$T cells, and their surface immunophenotypes were presented in **Supplementary Figure 1B**. Analysis of γδ$^+$ CD28$^+$ T cells (**Figure 2A**), γδ$^+$ CD80$^+$ T cells (**Figure 2B**), and γδ$^+$ CD40L$^+$T cells (**Figure 2D**) frequencies demonstrated that there were no significant differences between CAD patients and healthy individuals. However, the frequency of γδ$^+$ CD86$^+$ T cells in total γδ T cells is much higher in healthy individuals compared to CAD patients (**Figure 2C**). In the Vδ2$^+$T cells, the percentage of CD28$^+$ T cells, CD80$^+$ T cells, CD86$^+$ T cells, and CD40L$^+$ T cells were found no significant differences between healthy individuals and CAD patients (**Figures 2E–H**).

To investigate the changes in the activation status of γδ T cells and their subsets in CAD, the activation markers of γδ T cells and their subsets were also determined for evaluating their potential immunological roles. Activated γδ T cells can express several classical markers, including CD69, CD25, and HLA-DR. Additionally, in human peripheral γδ T cells, the majority subset of γδ T cells is Vδ2$^+$T cells, whereas γδ T cells expressing other Vδ elements (Vδ2$^-$T cells) are rare in the blood but they still display some functions. Therefore, the CD69$^+$, CD25$^+$ and HLA-DR$^+$ cells in total γδ T cells, Vδ2$^+$T cells, and Vδ2$^-$T cells were compared between healthy individuals and CAD patients. Our results indicated that the proportions of total γδ T cells, Vδ2$^+$T cells, and Vδ2$^-$T cells that were CD69$^+$ were significantly lower in CAD patients compared to healthy individuals (**Figures 3A, D, G**). In addition, no significant differences were found in the percentages of CD25$^+$ cells in the total γδ T cells and Vδ2$^+$T cells between healthy individuals and CAD patients (**Figures 3B, E**). But in the Vδ2$^-$T cells, the frequency of CD25$^+$ cells were lower in CAD patients than that in healthy individuals (**Figure 3H**). Furthermore, the frequencies of HLA-DR$^+$ cells in total γδ T cells and Vδ2$^+$T cells were lower in CAD patients compared to healthy individuals (**Figures 3C, F**). And there was no significant difference in the percentage of HLA-DR$^+$ cells in Vδ2$^-$T cells between healthy individuals and CAD patients (**Figure 3I**).

According to previous studies, the activating NK cell receptors, such as NKp30, NKp46, and NKG2D, play critical roles in modulating multiple functions of γδ T cells. To determine the phenotypic changes of activating NK cell receptors in γδ T cells and their subsets in CAD, the surface levels of NKp30, NKp46, and NKG2D in total γδ T cells, Vδ2$^+$T cells, and Vδ2$^-$T cells were compared between healthy individuals and CAD patients. The percentages of NKp30$^+$ (**Figures 4A, D, G**), NKp46$^+$(**Figures 4B, E, H**), and NKG2D$^+$ cells (**Figures 4C, F, I**) in total γδ T cells, Vδ2$^+$T cells, and Vδ2$^-$T cells were significantly lower in CAD patients compared to healthy individuals.

Changes in the Expression of Immunosuppressive Molecules on the Surface of γδ T Cells in Peripheral Blood of CAD Patients

Although CAD was previously known as a lipid accumulation-mediated disease, it has now been considered a chronic inflammatory disease. The multiple inhibitory receptors (including PD-1, CTLA-4, PD-1, and Tim-3) are involved in regulating the functions of γδ T cells in the inflammatory response. To better understand the roles of inhibitory signals in γδ T cells during the onset of CAD, the proportions of NKG2A$^+$, Tim-3$^+$, PD-1$^+$, and CTLA-4$^+$cells among total γδ T cells, Vδ2$^+$T

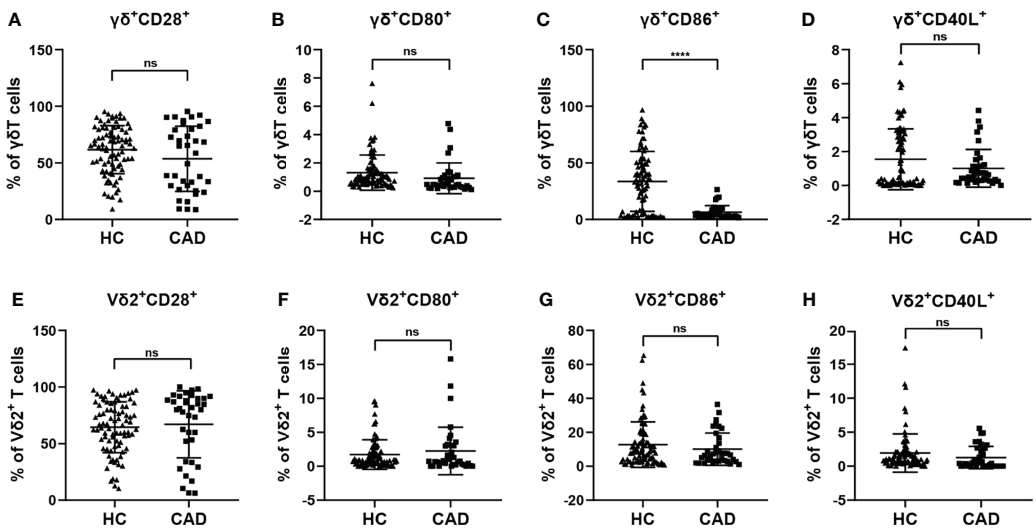

FIGURE 2 | Expression of co-stimulatory molecules on the surface of γδ T cell and its subsets in peripheral blood of healthy individuals (HC) and CAD patients **(A, E)** CD28, **(B, F)** CD80, **(C, G)** CD86, and **(D, H)** CD40L frequencies as percentages of total γδ T cells, and Vδ2$^+$T cells respectively, of healthy individuals versus CAD patients. ****P < 0.0001; ns, no significance.

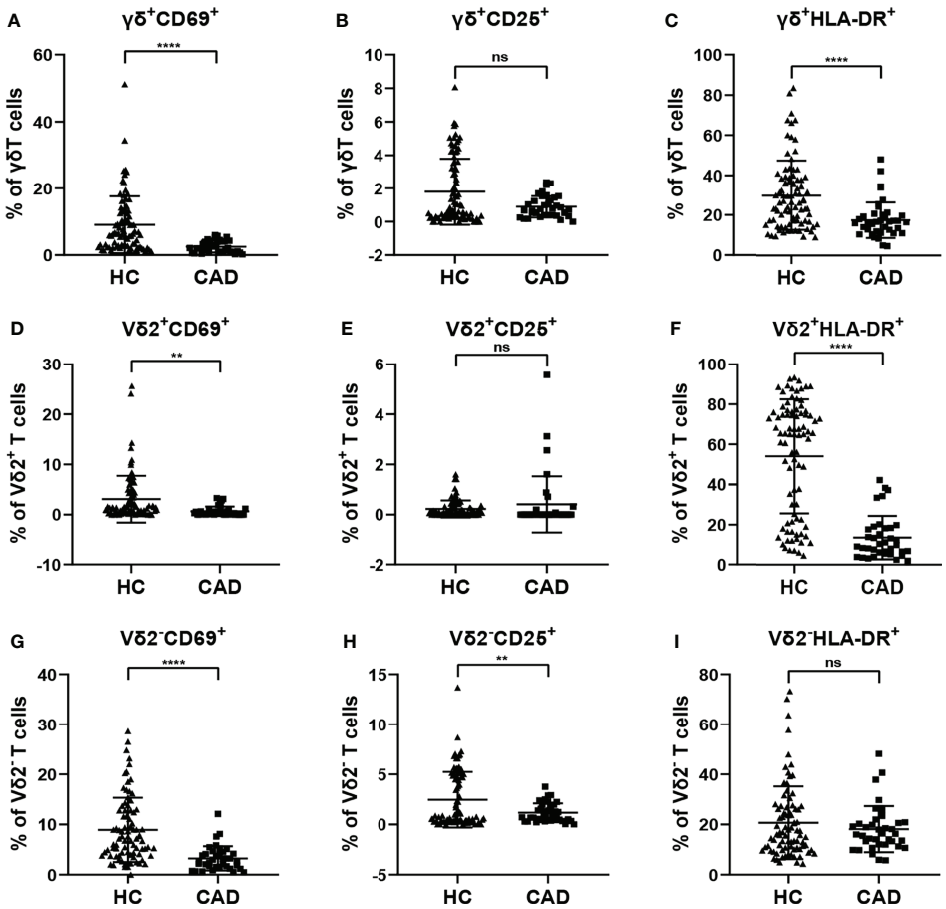

FIGURE 3 | Expression of activation marker on the surface of γδ T cell and its subsets in peripheral blood of healthy individuals (HC) and CAD patients. **(A, D, G)** CD69, **(B, E, H)** CD25, and **(C, F, I)** HLA-DR frequencies as percentages of total γδ T cells, Vδ2$^+$T, and Vδ2$^-$T cells respectively, of healthy individuals versus CAD patients. **P < 0.01 and ****P < 0.0001; ns, no significance.

cells, and Vδ2$^-$T cells were determined. Our results indicated that the frequencies of NKG2A$^+$, CTLA-4$^+$, and PD-1$^+$ cells in the total γδ T cells were significantly lower in CAD patients (**Figures 5A, C, D**), but the frequencies of Tim-3$^+$ cells in the total γδ T cells were significantly higher in CAD patients compared to healthy individuals (**Figure 5B**). In the Vδ2$^+$T cells, the frequencies of PD-1$^+$, and CTLA-4$^+$ cells are much lower in CAD patients than that in healthy individuals (**Figures 5G, H**). And no significant differences in the percentages of NKG2A$^+$, Tim-3$^+$ cells between healthy individuals and CAD patients (**Figures 5E, F**). By contrast, there were no significant differences in the percentages of NKG2A$^+$ and Tim-3$^+$ cells in Vδ2$^-$T cells between healthy individuals and CAD patients (**Figures 5I, J**). However, the frequencies of PD-1$^+$ Vδ2$^-$T cells (**Figure 5K**) and CTLA-4$^+$ Vδ2$^-$T cells (**Figure 5L**) were significantly lower in CAD patients than that in healthy individuals

Correlation of Serum LDL-C With the Immunophenotype of γδ T Cells in CAD Patients

It is well known that elevated low-density lipoprotein cholesterol (LDL-C) level is a major risk factor for CAD. Therefore, we next to further evaluate the correlation of immunological features of γδ T cells and their subsets with the levels of serum LDL-C, which will help us to use these immunological factors as indicators to predict the development of CAD. The results indicated that the level of serum LDL-C in CAD patients was significantly positively correlated with the percentage of γδ T cells in total CD3$^+$ cells (r=0.3994, P= 0.0158) (**Figure 6A**), However, the level of serum LDL-C was significantly negatively correlated with the expression of CD69 (r=-0.4073, P=0.0137) on the surface of γδ T cells in peripheral blood (**Figure 6B**). In addition, the level of serum LDL-C in CAD patients was significantly positively correlated with the proportions of CD69$^+$ cells (r=0.3368, P=0.0446), NKG2D$^+$ cells (r=0.5131, P=0.0014), and NKp46$^+$ cells (r=0.3384, P=0.0435); r=0.3368) in Vδ2$^+$T cells (**Figures 6C–E**). By contrast, there was an inverse correlation between the serum levels of LDL-C and the frequencies of CD69$^+$Vδ2$^-$T cells (r=-0.4357, P=0.0079) and NKp46$^+$Vδ2$^-$T cells (r=-0.4028, P=0.0149) (**Figures 6F–H**). Taken together, our data suggested that activation levels of Vδ2$^+$T cells and Vδ2$^-$T cells might be associated with the development of CAD and these two populations of cells may exhibit different potential functions in CAD.

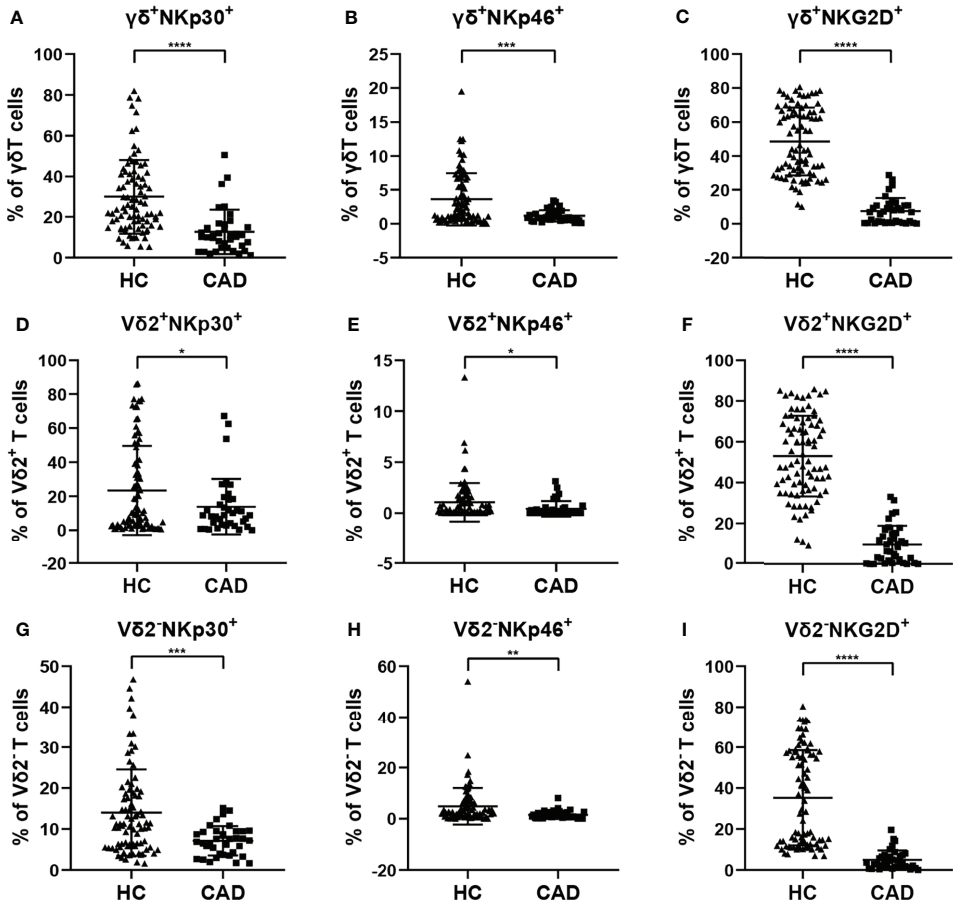

FIGURE 4 | Expression of NK cell-activating receptors on the surface of γδ T cell and its subsets in peripheral blood of healthy individuals (HC) and CAD patients. **(A, D, G)** NKp30, **(B, E, H)** NKp46, and **(C, F, I)** NKG2D frequencies as percentages of total γδ T cells, $V\delta2^+$T, and $V\delta2^-$T cells respectively, of healthy individuals versus CAD patients. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

DISCUSSION

The pieces of evidence collected from the animal models suggest that γδ T cells display a critical pathogenic role in the early stage of atherosclerosis. However, whether human γδ T cells display similar roles in CAD remains unclear. Here, this study aims to preliminarily explore the changes in immunophenotypes of γδ T cells in human coronary atherosclerotic heart disease. In this study, we included 37 patients with a clinical diagnosis of CAD and severe stenosis with greater than 80% arterial vascular involvement as the study group and recruited 84 healthy individuals as controls. Flow cytometry was used to analyze the alterations in the absolute numbers and immunophenotypic changes of γδ T cells and their subsets in peripheral blood between the two groups. Furthermore, we also analyzed the correlation between clinically relevant indicators and the immune cell phenotype in CAD patients. We found that the absolute number of circulating γδ T cells in the peripheral blood of CAD patients was significantly lower than that of healthy individuals, which may be due to the significantly lower percentage of naïve γδ T cells and the marked significantly increase of Fas, which is a molecule that mediates apoptosis on the surface of γδ T cells in the peripheral blood of CAD patients (**Supplementary Figure 2**). The proportion of γδ T cell subsets, such as the $V\delta2^+$T cells and $V\delta1^+$T cells, in the peripheral blood of CAD patients, is unbalanced, which leads to an alteration in the ratio of Vδ1/Vδ2 in CAD patients. Compared with healthy individuals, the expression of activation markers on the surface of γδ T cells and their subsets in the peripheral blood of CAD patients were significantly lower than those of healthy individuals. In addition, the levels of surface immunosuppressive molecules PD-1 and CTLA-4 on the surface of γδ T cells and their subsets in the peripheral blood of CAD patients were also significantly decreased, but the expression of the inhibitory marker Tim-3 on the surface of γδ T cells was significantly increased. We also observed that the serum LDL-C level of CAD patients was significantly positively correlated with the percentage of γδ T cells and was also different significant correlations with the immunophenotypes of different subsets. These results suggest that under the continuous stimulation of the chronic inflammatory environment in CAD and the functions of γδ T cells may be progressively exhausted in a state

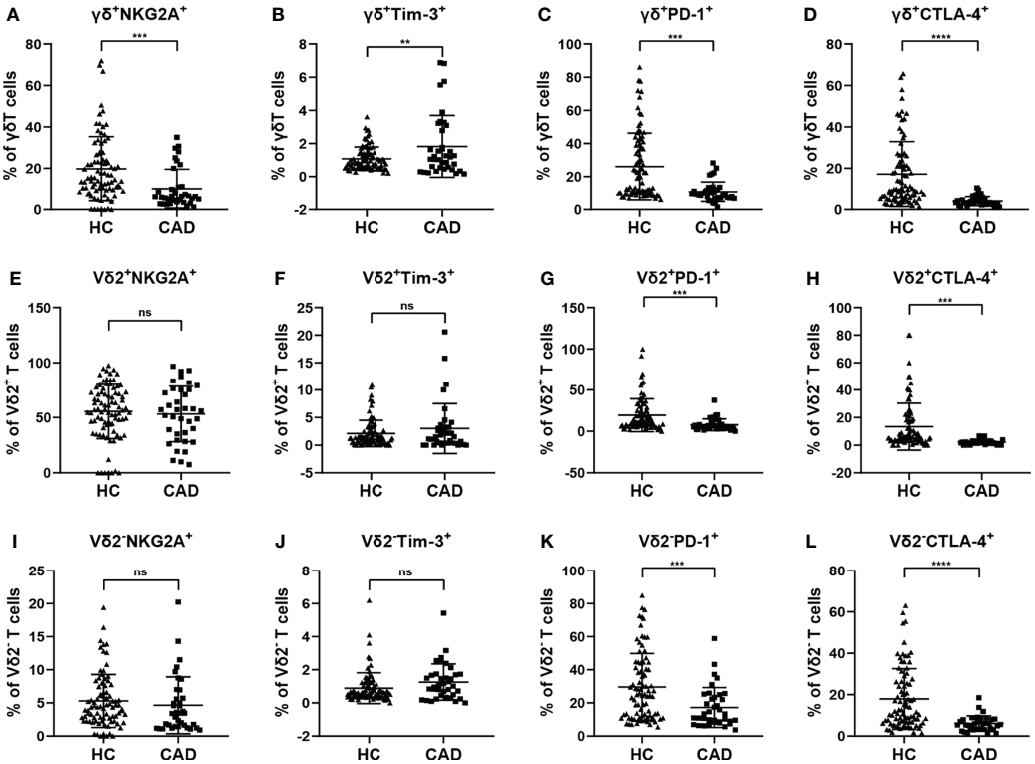

FIGURE 5 | Expression of immunosuppressive molecules on the surface of γδ T cell and its subsets in peripheral blood of healthy individuals (HC) and CAD patients. **(A, E, I)** NKG2A, **(B, F, J)** Tim-3, **(C, G, K)** PD-1, and **(D, H, L)** CTLA-4 frequencies as percentages of total γδ T cells, Vδ2$^+$T, and Vδ2$^-$T cells respectively, of healthy individuals versus CAD patients. **P < 0.01, ***P < 0.001, and ****P < 0.0001; ns, no significance.

of low activation and high inhibition, suggesting an important association of γδ T cells and their subpopulations with the pathophysiological processes of human CAD.

Roman Kleindienst et al. were the first time to report that γδ T cells existed in human atherosclerotic plaques, and the number of γδ T cells was the highest in the early atherosclerotic lesions when there were relatively few CD3$^+$T cells, and its proportion was 9.7% in the transition zone between normal vascular intima and lipid streaks, and then gradually decreased to 6.6% and 4.3% in fat streak and atherosclerotic plaque (14). Using high-

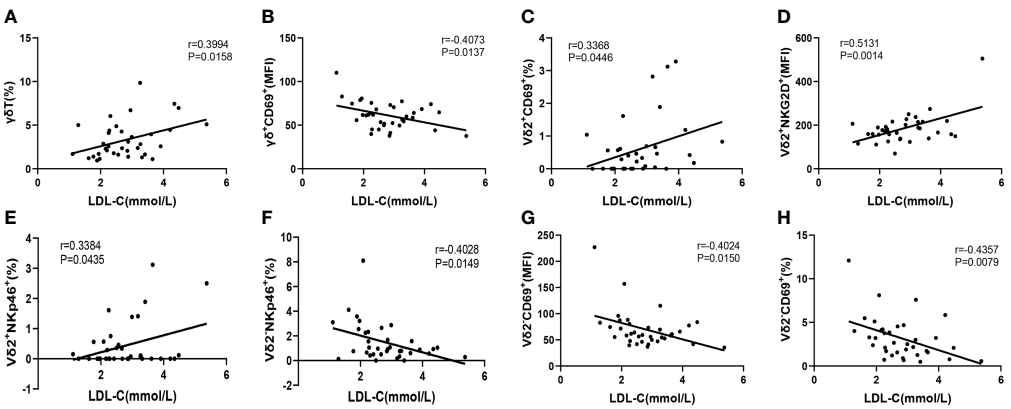

FIGURE 6 | The Pearson correlation between serum LDL-C level in CAD patients and immune cell phenotypes of γδ T cell and its subsets in peripheral blood of CAD patients. **(A)** γδ T cells, **(B)** γδ$^+$CD69$^+$T cells, **(C)** Vδ2$^+$CD69$^+$T cells, **(D)** Vδ2$^+$NKG2D$^+$T cells, **(E)** Vδ2$^+$NKp46$^+$T cells, **(F)** Vδ2$^-$NKp46$^+$T cells and **(G, H)** Vδ2$^-$CD69$^+$T cells.

throughput analysis technology to analyze three gene expression databases related to CAD, a recent study demonstrated that the number of γδ T cells infiltrating human atherosclerotic plaques was lower than that in control groups. Meanwhile, the expression levels of immunological function-related genes including the chemokines (CCL5, CX3CL1, CXCL10) in CAD plaques were higher than those in the control group (3). Previous studies have shown that the phenotypic distribution of immune cells differs between carotid atherosclerotic plaques and blood, and the frequencies of $CD4^-CD8^-$T cells were more abundant in plaques than that in blood. Furthermore, T cells in the blood were mostly quiescent, whereas the coexistence of activated, pro-inflammatory and exhausted T cells in the same plaque suggested a progressive loss of T cell function during chronic and prolonged inflammatory responses (21). Thus, these findings will extend our understanding of the interconnectedness of migration, heterogeneity, and functional alterations of immune cells between atherosclerotic plaques and peripheral blood. Our results demonstrated that the proportion of γδ T cells in the peripheral blood of CAD patients was also significantly lower than that of healthy individuals. We also found that the absolute number of subset $V\delta2^+$T cells in the peripheral blood of CAD patients was significantly decreased compared with it in the healthy individuals, and the number of $V\delta1^+$T cells was significantly increased, resulting in an increased Vδ1/Vδ2 ratio. Generally, since $V\delta2^+$T cells in peripheral blood are the predominant γδ T cells, the Vδ1/Vδ2 ratio in peripheral blood is less than 1. But, the phenotypes of γδ T cell subsets in CAD patients and healthy individuals are so different, indicating that the balance between $V\delta1^+$T cells and $V\delta2^+$T cells is very important for the maintenance of immune function in CAD patients. However, whether the different functions of these two subpopulations of γδ T cells lead to such opposite phenotypes in CAD patients remains to be further investigated.

The functional responses of T cells require the co-existence of antigen-stimulatory signals and co-stimulatory signals, and the integration and transmission of these signals require the participation of activating or inhibitory molecules expressed on the surface of T cells. The immunoglobulin-like CD28 family and the tumor necrosis factor receptor superfamily are the important co-stimulatory molecules on the surface of T cells. And the interaction of CD28 on the surface of T cells and CD80/CD86 on the surface of antigen-presenting cells (APCs) is the critical costimulatory signal for activating T cells. According to the previous studies, the data have shown that the CD28/CTLA-4-CD80/CD86 pathway plays an important role in accelerating the development of atherosclerotic lesions, and was considered as an important potential target in immune regulation of atherosclerosis. For example, the absence of CD80/86 costimulation significantly reduced the development of early hyper-cholesterol-induced atherosclerotic lesions in $Ldlr^{-/-}$ mice. This data suggested that CD80 and CD86 molecules are involved in the regulation of the occurrence of atherosclerotic lesions and the initiation of antigen-specific T cell responses in atherosclerotic lesions (22, 23). In addition, the CD80/CD86 pathway is also a promising biomarker of atherosclerotic plaque vulnerability. Analysis of atherosclerotic plaques from human carotid endarterectomy revealed that the expression levels of costimulatory molecules CD80 and CD86 were closely related to plaque vulnerability (24). In CTLA-4-transgenic/$Apoe^{-/-}$ mice, overexpression of CTLA-4 can significantly reduce the formation of atherosclerotic lesions and significantly inhibit the accumulation of macrophages and $CD4^+$T cells in the plaque, and then regulate atherosclerosis by down-regulating the expression of costimulatory molecules CD80, CD86, and CD28 (25). PD-1 is another regulatory molecule induced and expressed upon T-cell activation that plays an important role in modulating immune responses and autoimmunity by binding to its ligand PD-L1/2. Some studies have shown that deficiency of PD-1/PD-L1 could accelerate the development of atherosclerosis (26, 27). Our results showed that the expression levels of CD86, PD-1, and CTLA-4 in the peripheral blood of CAD patients were significantly lower than those of healthy individuals, indicating that the co-stimulatory signal required for activation of γδ T cells in CAD patients may be down-regulated in the chronic inflammation environment which established in the CAD patients. These findings suggested that abnormalities in CD28/CTLA-4-CD80/CD86 and PD-1-mediated signaling pathways may be involved in the pathogenic mechanism of γδ T cells in the progression of CAD. Previous studies have shown that Tim-3 is highly expressed on the surface of circulating NK cells in patients with atherosclerosis, suggesting that Tim-3 is involved in the occurrence of atherosclerosis and may be associated with the microenvironmental inflammation of atherosclerosis. Furthermore, treatment of statins can reduce the expression proportion of Tim-3 on NK cells in patients with atherosclerosis (28, 29). The treatment of anti-Tim-3 monoclonal antibody in $LDLr^{-/-}$ mice exhibited therapeutic effects in the acceleration of atherosclerotic plaque formation accompanied by an increase in the number of monocytes/macrophages and $CD4^+$T cells and a decrease in the number of regulatory T cells and regulatory B cells (30). Tim-3 is also significantly increased in non-classical immune cells human artery vascular smooth muscle cells (HASMCs) and can inhibit platelet-derived growth factor-BB (PDGF-BB)-induced inflammatory response by inhibiting the activation of NF-kB, and limiting the expression levels of inflammatory cytokines IL-6 and TNF-α, suggesting that Tim-3 as a potential target for controlling atherosclerotic (31). In our study, our results indicated that Tim-3 on the surface of γδ T cells in the peripheral blood of CAD patients is significantly higher than that of healthy individuals, suggesting that the $Tim\text{-}3^+$γδ T cells might be involved in the formation of atherosclerosis, and may be an alternative therapeutic target. Another study found that expression levels of Tim-3 in $CD8^+$T cells in patients with atherosclerosis were significantly up-regulated than those in healthy individuals. Furthermore, the blockade of Tim-3 could lead to a down-regulation in the production of anti-atherosclerotic cytokines, while an increase in the production of pro-atherosclerotic cytokines TNF-α and IFN-γ, which could promote the development of atherosclerotic lesions (32). This evidence reported that expression patterns of CD86, PD-1, and

Tim-3 were altered in the development of CAD, suggesting that these molecules exhibited critical roles in this disease. Accordingly, our results showed that similar patterns were found in the CD86, PD-1, and Tim-3 expressions in γδ T cells of CAD patients. These data will extend our understanding that not only NK cells and $CD8^+$T cells are the effector immune cells but also the putative role of γδ T cells in the pathogenic mechanism in the CAD.

A recent study reported that there was a significant downregulation of CD69 mRNA level in peripheral blood leukocytes in the patient with the progression of atherosclerosis. Thus, CD69 expression was considered as an independent predictor of subclinical atherosclerosis (33). In our study, the expression levels of CD69 on the surface ofγδ T cells $V\delta2^+$T cells, and $V\delta2^-$T cells in the peripheral blood of CAD patients were significantly lower than those of healthy individuals. Moreover, the expression of HLA-DR, which is a marker to indicate a late stage of T cell activation, on the surface of γδ T cells and $V\delta2^+$T cells in CAD patients was lower than that of healthy individuals. Additionally, the expression of CD25, which plays an important role in the formation of high-affinity IL-2 receptors and promotes the proliferation of T cells, on the surface of $V\delta2^-$T cells is lower in CAD patients compared with healthy individuals. Finally, our data showed the expression levels of NKG2D, NKp30, and NKp46 on the surface of γδ T cells and their subsets in the peripheral blood of CAD patients were significantly lower than those of healthy individuals. Similar to our findings, a previous study demonstrated that LDL inhibited the activation and functions of human $V\delta2^+$T cells, in terms of down-regulation of activation markers, such as NKG2D expression (34). Together, these results suggest that the activation levels of γδ T cells and their subpopulations in peripheral blood of CAD patients are significantly reduced in the severe CAD, which may impair the immune function of γδ T cells in atherosclerosis.

Serum LDL-C level is an important risk factor for CAD, and it can be oxidized and modified to ox-LDL *in vivo*. And this reactivity can promote a series of complex pathophysiological processes *in vivo* and plays an important role in the occurrence and development of atherosclerosis. Our results indicated that the level of serum LDL-C in CAD patients was significantly positively correlated with the percentage of γδ T cells in peripheral blood. A previous study demonstrated that cholesterol levels in γδ T cells were much higher than that in αβT cells. And highly activated cholesterol metabolism can regulate immunological activities of γδ T cells, indicating that γδ T cells can quickly reach the cholesterol checkpoint, which did a contribution to the hyper-activated phenotype of γδ T cells (35). Moreover, a previous study also found that human $V\delta2^+$T cells expressed LDL receptor post activation and treatment of LDL-C resulted in inhibition of functions of $V\delta2^+$T cells, which in turn down-regulated the CD69 expression and IFN-γ production (34). In contrast, we found that levels of serum LDL-C were significantly positively correlated with the percentage of γδ T cells in the peripheral blood of CAD patients. In addition, the level of serum LDL-C in CAD patients was also significantly positively correlated with the expression levels of NKp46, NKG2D, and CD69 on the surface of $V\delta2^+$T cells. Therefore, these data indicate that there is still controversy about the link between the cholesterol metabolism and immune functions of γδ T cells, especially in the $V\delta2^+$T cells. Meanwhile, our analysis demonstrated that there was significantly negatively correlated with the expression of CD69 on the surface of γδ T cells and $V\delta2^-$T cells and the expression of NKp46 on the surface of $V\delta2^-$T cells. Here, in line with our findings, one previous study showed that CD69 as an ox-LDL receptor in T cells and CD69 expression in circulating T cells correlate inversely with subclinical atherosclerosis in the patients (33). Accordingly, the evidence collected from CAD patients will help us to completely understand how functions of γδ T cell and its subpopulations are differently modulated by cholesterol metabolism. Most importantly, our *in vivo* data further implies a potential role by which cholesterol differentially regulates the pathogenic mechanisms of γδ T cell subsets in the progression of CAD.

However, the limitation of this study could not be neglected. First, further large sample size study is warranted to validate these findings. And the effects of age, sexual and other factors in impacting the immunological features of γδ T cell should be further evaluated in CAD. Second, in this study, we only determined the phenotypic changes of γδ T cell subsets in the peripheral blood of patients. The immunological features of γδ T cell and its subpopulations are rarely investigated in CAD and should be determined in future studies by using the samples collected from blood and atherosclerotic plaque of CAD patients. Third, we found the phenotypic changes of γδ T cell subsets by comparing the data collected from CAD patients and healthy individuals. In the future study, using samples from each CAD patient at different time points in disease progression will further help us to clarify the clinical significance of phenotypic alterations of γδ T cell subsets.

Taken together, our findings show that immunophenotypes of γδ T cell and its subsets are significantly changed in the CAD patients. In addition, the expression levels of some markers, especially the CD69, in $V\delta2^+$T cells and $V\delta2^-$T cells exhibit different association patterns with the serum LDL level. These data indicate that γδ T cell subsets play an important role in the progression of CAD. And $V\delta2^+$T cells and $V\delta2^-$T cells may display different functions involved in the development of CAD. Therefore, prospective research is needed to confirm the functional diversity of γδ T cell subsets in CAD. We propose that future studies should investigate the link between cholesterol metabolism and pathogenic roles of γδ T cell subsets in the heart diseases, thus evaluating the therapeutic potential and clinical significance of γδ T cells in the clinic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medicine Ethics Committee of Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZY, ZX, and YX designed the study and critically revised the manuscript; YL perform the experiments, data analysis, and manuscript writing; SJ, YYC, YC, YX, HY, and QQ contributed to the collection and processing of experimental samples and data analysis; FY and YZ contributed to the collection of patient samples and management of clinical information of patients. All authors contributed to the article and approved the submitted 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.2022.900334/full#supplementary-material

Supplementary Figure 1 | Overview of gating strategy used in flow cytometric analysis. **(A)** Gating strategy identifying γδ T cells, Vδ1$^+$ and Vδ2$^+$ γδ T cell subsets, **(B)** Gating strategy identifying γδ T cells, Vδ2$^+$ T and Vδ2$^-$ γδ T cell subsets, and their immunophenotypes.

Supplementary Figure 2 | Statistical comparison of naïve γδ T cells and the expression of Fas on the surface of γδ T cells in peripheral blood of healthy individuals (HC) and CAD patients. The percentage of **(A)** γδ$^+$CD45RO$^-$CD62L$^+$ T cells (naïve γδ T cells), and **(B)** γδ$^+$Fas$^+$ T cells of healthy individuals versus CAD patients. $^{***}P < 0.001$, and $^{****}P < 0.0001$.

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Role of Vγ9vδ2 T lymphocytes in infectious diseases

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The T cell receptor Vγ9Vδ2 T cells bridge innate and adaptive antimicrobial immunity in primates. These Vγ9Vδ2 T cells respond to phosphoantigens (pAgs) present in microbial or eukaryotic cells in a butyrophilin 3A1 (BTN3) and butyrophilin 2A1 (BTN2A1) dependent manner. In humans, the rapid expansion of circulating Vγ9Vδ2 T lymphocytes during several infections as well as their localization at the site of active disease demonstrates their important role in the immune response to infection. However, Vγ9Vδ2 T cell deficiencies have been observed in some infectious diseases such as active tuberculosis and chronic viral infections. In this review, we are providing an overview of the mechanisms of Vγ9Vδ2 T cell-mediated antimicrobial immunity. These cells kill infected cells mainly by releasing lytic mediators and pro-inflammatory cytokines and inducing target cell apoptosis. In addition, the release of chemokines and cytokines allows the recruitment and activation of immune cells, promoting the initiation of the adaptive immune response. Finaly, we also describe potential new therapeutic tools of Vγ9Vδ2 T cell-based immunotherapy that could be applied to emerging infections.

KEYWORDS

Vγ9Vδ2 T cell, antimicrobial immunity, infectious diseases, butyrophilin, therapeutic approaches

Introduction

Gamma-delta (γδ) T cells are « unconventional » T lymphocytes that do not require major histocompatibility complex (MHC) presentation of antigen (1). Human γδ T cells are classified into two main subsets according to the expression of T cell receptor (TCR) δ chain (2). Vδ1 T cells are more common in mucosal tissues and are involved in the first line of the immune defense against solid tumors and infections. The Vδ2 T cells, that is a subset uniquely associated with Vγ9 chain (called Vγ9Vδ2), are abundant in the peripheral blood and play a role of immune effector in tumor surveillance and also in

antimicrobial defense (2). Indeed, Vγ9Vδ2 T cells can directly kill infected cells through different mechanisms, and also prime and modulate functions of other innate and adaptive immune cells *via* cytokines, antigen presentation and cell contact to develop antimicrobial immunity (3).

Human Vγ9Vδ2 T cells, typically represent 2 to 5% of peripheral blood T cells, are expanded following infection with a wide range of microbial agents and can represent up to 50% of the peripheral T cell pool (3, 4). This subset of T cells is enriched in the circulation of patients with bacterial infections, including mycobacterial diseases, listeriosis, salmonellosis, brucellosis, tularemia, legionellosis and Q fever (5–11), and with protozoal parasite infections such as malaria, toxoplasmosis and leishmaniasis (12–14). Vγ9Vδ2 T cells are also increased in the bronchoalveolar lavage fluid of patients with active pulmonary tuberculosis or psittacosis (15), and in cerebral spinal fluid from patients with bacterial meningitis (*M. tuberculosis*, *H. influenzae*, *S. pneumoniae*, and *N. meningitidis*); such pattern is corrected by successful antibacterial therapy (16, 17). Bacterial vaginosis is also associated with an increase of Vγ9Vδ2 T cells in the female reproductive tract in women (18, 19). Furthermore, in patients with *P. falciparum* malaria, an increase in Vγ9Vδ2 T lymphocytes in human spleens during infection has also been observed (20). Globally, the rapid expansion of circulating Vγ9Vδ2 T lymphocytes during acute infections as well as their localization at the site of active disease indicate that Vγ9Vδ2 T cells may play an important role in the immune response to infection.

In contrast, it seems that the number of Vγ9Vδ2 T cells in the blood is reduced in patients with a viral infection (21–24). In patients with chronic hepatitis B, the frequency of peripheral and hepatic Vγ9Vδ2 T cells decreases with disease progression. Similarly, the frequency of Vγ9Vδ2 T cells is markedly reduced in the blood and the mucosal tissues of HIV patients, and interestingly is restored with highly active antiretroviral therapy (HAART) (25–27). These observations indicate that Vγ9Vδ2 T cells are activated early after infection but are lost if infection is not controlled. Recently, a decrease in the number of circulating Vγ9Vδ2 T cells has been reported in patients with coronavirus, especially SARS-CoV-2, which was followed by a return to normal levels in recovered patients (22, 23). The aim is to review the mechanisms of Vγ9Vδ2 T cell-mediated antimicrobial immunity and to report the potential therapeutic application of Vγ9Vδ2 T cell immunotherapy to infectious diseases.

Vγ9vδ2 T cell recruitment to the site of inflammation and their implication in tissue repair

The traffic of leukocytes to tissues is an essential step for the development of an immune response that is mainly controlled by the interactions between chemokines and their specific receptors (28). During infection the onset of local inflammation is associated with an increased chemokine production that plays a role in transendothelial migration of Vγ9Vδ2 T cells into the tissues. The majority of circulating Vγ9Vδ2 T cells have the potential to be rapidly recruited in tissues during the course of infection, due to their expression of inflammatory homing chemokine receptor CCR5 and CXCR3 (Figure 1) (28, 29). Indeed, CCR5 expressed on activated Vγ9Vδ2 T cells mediates their migration to influenza virus-infected sites (30). Similarly, high levels of CCR5 and CXCR3 receptors on Vγ9Vδ2 T cells are responsible of transendothelial migration of cells to the lungs in monkeys infected with *M. tuberculosis* or Bacille Calmette-Guerin (BCG) (31). A macaque model showed that Vγ9Vδ2 T cells exhibit trans-endothelial migration, interstitial localization, and granuloma infiltration in response to *M. tuberculosis* infections (32).

In addition to be anti-microbial effectors, Vγ9Vδ2 T cells, once activated locally or recruited to tissue compartments, might also participate to tissue repair or wound healing after post-infectious tissue damage. In acute bacterial peritonitis, Vγ9Vδ2 T cells accumulate rapidly at the site of infection and likely contribute to scarring in the peritoneal cavity, both directly *via* the local release of IFN-γ, and indirectly *via* induction of IL-6 production by mesothelial cells and peritoneal fibroblasts (33, 34). In addition, migrating Vγ9Vδ2 T cells can locally produce fibroblast growth factor-7 (FGF-7), a homeostatic mediator against tissue damages induced by bacterial infections (35). In a macaque model, induced expansion of Vγ9Vδ2 T cells by treatment with 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and IL-2 led to the apparent attenuation of plague lesions in lungs (35). These Vγ9Vδ2 T cells may therefore contribute to immune responses or tissue homeostasis against bacterial infections.

Recognition of infected cells by Vγ9vδ2 T cells

Recognition by phosphoantigens

In humans, Vγ9Vδ2 T cells recognise small pyrophosphate-containing molecules called phosphoantigens (pAgs) present in the malignant target cell or in the infected cells (Figure 1) (29). These small molecules are isopentenyl pyrophosphate (IPP) produced by infected cells or HMBPP produced by certain bacteria (*Mycobacterium tuberculosis*, *Listeria monocytogenes*) and parasites (*Plasmodium falciparum*, *Toxoplasma gondii*). It is important to note that the naturally occurring pAg HMBPP stimulates Vγ9Vδ2 T cells about 10,000-fold more efficiently than IPP (15, 36, 37), this recognition provides a formal basis for the role of Vγ9Vδ2 T cells in anti-infective immunity (38–40). A

recent study showed that Vγ9Vδ2 T cell activation can occur independently of HMBPP produced by the bacteria but *via* the regulation of host cholesterol biosynthesis (41). Indeed, infection of human dendritic cells (DCs) with HMBPP-negative *L. monocytogenes* results in an upregulation of cholesterol metabolism in these cells, leading to increased intracellular IPP levels and direct activation of Vγ9Vδ2 T cells. On the other hand, Vγ9Vδ2 T cells can recognize a mycobacterial glycolipid component, 6-O-methylglucose lipopolysaccharide, which promotes TCR-dependent effector functions of Vγ9Vδ2 T cells against *M. tuberculosis in vitro* (42).

The recognition mechanisms of pAgs by Vγ9Vδ2 T cells involve the butyrophilin (BTN) protein family. The butyrophilin 3A1 (BTN3A1, CD277), expressed by both immune cells and somatic cells (43), directly binds pAg intracellularly through its B30.2 cytoplasmic domain leading to a conformational change in its ectodomain that is sensed by Vγ9Vδ2 T cells (44–46). BTN3A1 interacts at the plasma membrane with another member of the BTN family, BTN2A1 which is a direct ligand for the Vγ9 TCR chain, thus ensuring the synapse between Vγ9Vδ2 T cells and target cells (47–49). Several studies have confirmed that Vγ9Vδ2 T cell activation is dependent on BTN3A during infections. Indeed, the BTN3A blocking antibody (103.2 mAb) was able to inhibit the degranulation of Vγ9Vδ2 T cells when they were co-cultured with cells infected with *M. tuberculosis*, *L. monocytogenes*, *P. falciparum* or Epstein-Barr virus (38, 41, 42, 44, 50).

The expression of these butyrophilins can be modulated by infection in some cases. Indeed, the plasma membrane expression of BTN3A and BTN2A was induced on *P. falciparum* infected red blood cells (iRBCs) (38). In addition, we recently showed that intracellular bacteria, *M. tuberculosis* and *C. burnetii* increased BTN3A and BTN2A expression on monocytes, concomitantly to Vγ9Vδ2 T cell activation (manuscript submitted). In contrast, human immunodeficiency virus (HIV) infection did not appear to enhance BTN3A expression on DCs (51), indicating that basal BTN3A expression maybe sufficient for translating pAgs signal in HIV-infected cells.

Recognition *via* Nkg2d (natural killer group 2 member D) receptor

Other transmembrane activatory receptors, notably the NKG2D receptor, have been implicated in the effective triggering of antimicrobial responses by Vγ9Vδ2 T cells. Indeed, NKG2D can bind to its ligands including MICA/B (MHC class I-related chain proteins A and B) and UL16-binding proteins (ULBP1-4). Besides their expression on tumor cells, these ligands are upregulated on cells infected by Zika virus and EBV (52–55). This is also the case during infection with intracellular bacteria, for instance MICA is upregulated by DCs infected with *M. tuberculosis* (56) and ULBP1 by macrophages infected with *M. tuberculosis* and *Brucella* (57, 58).

Recognition *via* toll-like receptors

Human γδ T cells also recognise danger signals from pathogens *via* TLRs. Vγ9Vδ2 T cells can be activated by TLR3 and TLR4 ligands and exhibit enhanced antibacterial responses (59). On the other hand, TLR8 ligands were shown to inhibit the expansion of Vγ9Vδ2 T cells *in vitro*, while these can be potent co-stimuli for Vγ9Vδ2 T cell activation in a monocyte-dependent manner (60). Hence, Vγ9Vδ2 T cells may recognize infected cells through several different receptors involved in innate immune responses.

Antimicrobial responses of vγ9vδ2 T cells

Vγ9vδ2 T cells Kill infected cells in an innate immune manner

Human Vγ9Vδ2 T cells exert both a direct cytotoxic activity against pathogen-infected cells as well as a cell-mediated non-cytolytic activity based on cytokine production (Figure 1) (Table 1). Regarding direct cytotoxicity, Vγ9Vδ2 T cells have been shown to kill cells infected by *M. tuberculosis*, *Brucella suis*, *Listeria monocytogenes*, *P. falciparum* and influenza virus *in vitro*, through the secretion of cytolytic molecules such granzymes, granulysin and perforin (64–66, 68, 71, 74, 75), similar to their responses to malignat cells. In addition, apoptosis triggered by death inducible receptors, including Fas and tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL), is a major mechanism of Vγ9Vδ2 T cells involved in the elimination of cells infected by Epstein-Barr and influenza virus (78, 79, 84, 93). Furthermore, engagement of NKG2D is sufficient to induce cytokine production and release of lytic granules; it increases TCR-dependent effector functions of Vγ9Vδ2 T cells in *M. tuberculosis* and *Brucella* infections (56, 58). In contrast, in other studies on *M. tuberculosis* or *L. monocytogenes*, NKG2D was not involved (41, 94). These discrepancies may be due to the different expression of NKG2D ligands between infections and between cell populations. On the other hand, NKG2D activation is required for Vγ9Vδ2 T cell cytotoxicity in viral infections with Epstein-Barr, influenza and Zika viruses (82).

Vγ9Vδ2 T cells have also been shown to be able of antibody dependent cell-mediated cytotoxicity (ADCC). Indeed, upon stimulation by pAgs, Vγ9Vδ2 T cells express CD16 (FcγRIIIa), an activatory Fcγ receptor that is constitutively expressed on NK cells and mediates ADCC (95, 96). Although total numbers of Vγ9Vδ2 T cells are decreased during HIV infection, resilient activated CD16+ Vγ9Vδ2 T cells were shown to retain the ability to induce ADCC and exert their antiviral functions in HIV

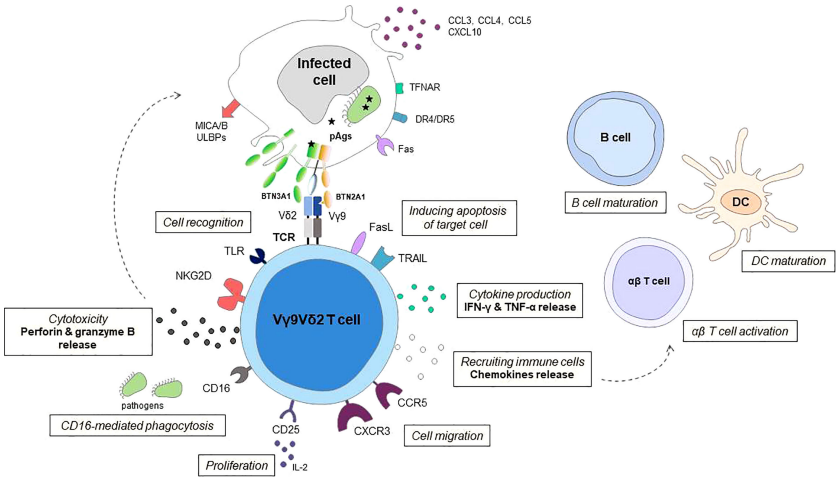

FIGURE 1
Schematic representation of effector mechanisms of Vγ9Vδ2 T cells in response to infection Vγ9Vδ2 T cells can distinguish between infected cells and normal cells using T cell receptor (TCR) and other cellular receptors especially natural killer group 2 member D receptor the (NKG2D) to sense isopentenyl pyrophosphate (IPP) levels and stress signals (such as MICA/B, ULBPs) displayed on target cells. The butyrophilin receptors BTN3A1 and BTN2A1 on target cells act to detect (pAgs) such as HMBPP and as a direct ligand for the Vγ9Vδ2 T cell receptor. Human Vγ9Vδ2 T cells can also recognize danger signals directly from pathogens through Toll-like receptors (TLRs). Following activation, Vγ9Vδ2 T cells kill infected cells by releasing lytic mediators (perforin, granzyme B), and pro-inflammatory cytokines, inducing target cell apoptosis *via* Fas/FasL, TNF-related apoptosis-inducing ligand (TRAIL) and TNF-α pathways, and antibody-dependent cell-mediated cytotoxicity (ADCC) through CD16 expression. In a CD16-dependent manner, Vγ9Vδ2 T cells may also have phagocytic functions. The chemokine receptors, including CCR5, control the ability of Vγ9Vδ2 T cell to migrate to the site of infection. The release of chemokines and cytokines allows recruitment of immune cells, enhance antigen priming of dendritic cells (DCs) and maturation of B cells. Vγ9Vδ2 T cells can display an APC-like phenotype and are able to present Ags and provide costimulatory signals sufficient for strong induction of αβ T cells, promoting the initiation of the adaptive immune response. The survival and proliferation of Vγ9Vδ2 T cells are mostly modulated by different cytokines, such as IL-2.

disease (89). Moreover, Vγ9Vδ2 T cell expression of CD16 is increased in children in malaria-endemic regions, suggesting a potential role for Vγ9Vδ2 T cells in inciting antibody-mediated parasite killing (97). Besides ADCC, a recent study showed that Vγ9Vδ2 T cells destroy *P. falciparum* infected red blood cells (iRBCs) by a CD16-dependent phagocytosis mechanism (38). As a matter of fact, there are data suggesting that Vγ9Vδ2 T cells can phagocytose particles and act as professional antigen-presenting cells (pAPCs). In response to *E. coli*, peripheral human Vγ9Vδ2 T cells transitioned from cytokine-producing bacterial effectors to professional phagocytic killers in a CD16-dependent manner (98, 99). A recent study also showed that Vγ9Vδ2 T cells suppress *P. falciparum* by direct killing and phagocytosis (38).

Regarding cell-mediated non-cytolytic activity, there are abundant data documenting the pivotal role of IFN-γ and TNF-α secretion on Vγ9Vδ2 T cell responses during infection. In Vγ9Vδ2 T cell-depleted humanized mice, decreased resistance to acute lethal infections with *Staphylococcus aureus*, *Escherichia coli*, and *Morganella morganii* correlated with decreased serum IFN-γ titers, a cytokine known to control numerous bacterial infections (100). The release of IFN-γ is part of the effector mechanisms of Vγ9Vδ2 T cells in *M. tuberculosis*, *B. suis* and *P. falciparum* infection (64, 71, 101), and also inhibits influenza virus, HCV and SARS-CoV-1 replication (21, 30, 88, 102). Early in HSV-induced inflammation, activated Vγ9Vδ2 T cells secrete IFN-γ and TNF-α, and chemokines, that may affect the course of inflammation (19). Finally, the production of chemokines MIP-1α, MIP-1β and RANTES by Vγ9Vδ2 T cells has also been shown to block HIV replication *in vitro* by inhibiting the CCR5 co-receptor that is required for HIV entry (90).

Vγ9vδ2 T cells cooperation with immune cells

Vγ9Vδ2 T cells contribute to responses against pathogen infection by modulating indirectly the function of other immune cells. Activated Vγ9Vδ2 T cells can induce recruitment of immune cells by secreting chemokines and stimulating monocytes, neutrophils, DCs, B lymphocytes, and different subtypes of T cells through cytokine secretion, notably IFN-γ (Figure 1) (103–106). In patients with acute bacterial peritonitis, Vγ9Vδ2 T cells that accumulate at the site of infection favor the recruitment of monocytes, neutrophils, and lymphocytes and produce inflammatory cytokines that are controlled by BTN3A, as demonstrated by the inhibitory effect of BTN3A antagonist mAb 103.2 in this process (33). Vγ9Vδ2 T cells may impact DC function during infection. Indeed, Vγ9Vδ2 T cells may enhance

TABLE 1 Summary table of the main involvement of Vγ9Vδ2 T cells in infectious diseases.

	Infections	Human Vγ9Vδ2 T cells	Mechanisms of antimicrobial immunity	Vγ9Vδ2 T cell "memory" responses
Bacteria	*M. tuberculosis*	↑ in blood, bronchoalveolar lavage fluid and cerebral spinal fluid (5, 15, 16) ↓ loss of cytotoxic activity (61–63)	- IFN-γ, TNF-α, perforin, granzymes, and granulysin release (64–66) - NKG2D activation (58)	BCG vaccination: recall expansion in humans and in macaques (67)
	L. monocytogenes	↑ in blood (6)	- IFN-γ, TNF-α, IL-4, IL-17, and perforin release (68)	*L. monocytogenes* secondary infection: recall expansion in macaques and in mice (69, 70)
	Brucella spp.	↑ in blood (8)	- IFN-γ and perforin release (71) - Fas-mediated signals (71) - NKG2D activation (58)	Restore the full functional capacity of *Brucella*-infected DCs (72)
Parasite	*P. falciparum*	↑ in blood and spleens (20) ↓ loss of cytotoxic activity (12, 73)	- IFN-γ, granzymes and granulysin release (74, 75) - phagocytosis (38)	*P. falciparum* sporozoite vaccine: recall expansion associated with protection in humans (76, 77)
Virus	Influenza	Not known	- IFN-γ, perforin and granzymes release (78, 79) - TRAIL and Fas-mediated signals (78, 79) - NKG2D activation (78, 79)	- Help to produce influenza virus-specific Ab (80, 81) - Influenza vaccination: memory responses (82, 83)
	SARS-CoV	↑ in blood after clearing SARS-CoV-1 and SARS-CoV-2 infections (21–23)	- IFN-γ release (21)	Correlation with higher anti-SARS-CoV-1 specific IgG titers (21)
	Epstein-Barr	↑ in blood (50)	- TRAIL and Fas mediated signals (84) - NKG2D activation (50, 84)	Not known
	HBV/HCV	↓ in blood in chronic hepatitis (24) inability of cytotoxic activity (85–87)	- IFN-γ release (88)	Not known
	HIV	↓ in blood and mucosal tissues inability of cytotoxic activity (25–27, 61)	- ADCC mediated cytotoxicity (89) - production of antiviral factors that block HIV replication *in vitro* (90)	- DC maturation and HIV-specific $CD8^+$ T cell responses (91) - HIV Env-specific Ab titers during chronic SHIV (92)

The arrow ↑ represents an increase and the arrow ↓ indicates a decrease in the number of Vγ9Vδ2 T cells.

DC activation through IFN-γ secretion and $CD4^+$ cell responses to *S. aureus* (106). Several intracellular bacterial pathogens including *M. tuberculosis*, *B. suis*, *C. burnetii*, interfere with DC maturation, which results in poor priming of the adaptive immune response (107, 108). *Brucella*-infected DCs trigger Vγ9Vδ2 T cells activation that required cell-to-cell contact. In turn, co-culture with activated Vγ9Vδ2 T cells resulted in maturation of *Brucella*-infected DCs with increased expression of co-stimulatory CD80 and CD86, and enhanced IFN-γ and IL-12 secretion (72). In ten HIV patients naive of antiretroviral therapy, treatment with zoledronate and recombinant IL-2 achieved not only Vγ9Vδ2 T cells expansion and activation but also DC maturation and HIV-specific $CD8^+$ T cell responses, although the eventual interaction between these immune compartments was not explored in the study (91).

Vγ9Vδ2 T cells were shown to induce differentiation and migration of neutrophils through the production of IL-17 during *M. tuberculosis*, *L. monocytogenes* infections and in bacterial meningitis (17, 109). Moreover, Vγ9Vδ2 T cells respond rapidly to neutrophils after phagocytosis of a broad range of bacteria at the site of infection, and in turn mediate the local differentiation of neighbouring neutrophils into APCs for both $CD4^+$ and $CD8^+$ T cells *in vitro* (110).

Vγ9Vδ2 T cells can also promote adaptive-like responses by sharing functions with APCs (111). Indeed, Vγ9Vδ2 T cells promote efficient adaptive immunity through processing and presenting influenza virus-derived peptides to $CD4^+$ and $CD8^+$ T cells (80, 81). In malaria patients, Vγ9Vδ2 T cells presented increased plasma membrane expression of APC markers HLA-DR and CD86. Similarly, in response to infected red blood cells *in vitro*, Vγ9Vδ2 T cells show an APC-like phenotype and are able of Ag presentation and αβT cell activation *in vitro* (112). Vγ9Vδ2 T cells may therefore promote the initiation of the adaptive response despite a possible impairment of conventional APCs. In response to *E. coli* and *L. monocytogenes*, human Vγ9Vδ2 T cells also display APC functions (99, 113). Futhermore, phosphoantigen-activated Vγ9Vδ2 T cells can inhibit IL-2-induced expansion of Tregs and reverse subsequent suppression of mycobacterium-specific T-cell immune responses (114).

Finally, it is well known that γδ T cells have a strong impact on humoral immunity. A subset of human Vγ9Vδ2 T cells isolated from peripheral blood expresses the CXC chemokine receptor type 5 (CXCR5) like T follicular helper cells, and, upon antigen stimulation, they are able to express the costimulatory molecules ICOS and CD40L, to produce cytokines such as IL-2, IL-4, and IL-10, and to help B cells for antibody production (Figure 1) (115, 116).

In addition, Vγ9Vδ2 T cells activated with the phosphoantigen HMBPP and in presence of IL-21 can also influence the localization of B cell inside the germinal center, positioning them into the light zone thanks to the production of CXC motif chemokine 13 (CXCL13) (116). Surprisingly, during chronic Simian-Human Immunodeficiency Virus (SHIV) infection, Vγ9Vδ2 T cell activation boosted HIV Env-specific Ab titres (92). It has also been reported that human Vγ9Vδ2 T cells facilitated H9N2 influenza virus specific IgG production (81), and that the higher number of circulating Vγ9Vδ2 T cells was associated with higher anti-SARS-CoV-1 specific IgG titers (21).

Vγ9vδ2 T cell "memory" responses

The Vγ9Vδ2 T cells may acquire a memory effector phenotype (T_{EM} cells) following several infections, as shown by the expression of the memory and activation markers CD27 and CD45RA. This phenotype has been reported in bacterial (31), parasitic (76), and viral infections (21, 22, 117).

In macaques, a clear memory-type response of Vγ9Vδ2 T cells was detected as early as four days after BCG re-infection and the magnitude of this expansion was 2-9-fold greater than that seen during primary BCG infection (67). A recall expansion of Vγ9Vδ2 T cells was also observed in macaques infected with *L. monocytogenes* or challenged with Salmonella and smallpox vaccines (69, 70, 118). In addition, studies in cattle and pigs showed similar responses to those found in macaques with a rapid γδ T cell proliferation after BCG vaccination (119–123). These observations demonstrate the essential role of γδ T cells in developing a long-term immunity against pathogens.

It is difficult to determine in humans whether a Vγ9Vδ2 T cell expansion observed during an infection represents a primary or recall response. Interestingly, Vγ9Vδ2 T cells induced by BCG or influenza vaccination develop memory responses (83, 124), and the numbers of memory Vγ9Vδ2 T cells correlates with protection in an *P. falciparum* sporozoite vaccine trial in a malaria endemic region (77). These data suggest that immunotherapy based on Vγ9Vδ2 T cells, which contribute to adaptive immunity, represents a great potential for the treatment of infections.

Overall, Vγ9Vδ2 T cells may act as an antimicrobial defense through different molecular mechanisms and also constitute a memory cell population that provides protection against subsequent infection. Hence, human Vγ9Vδ2 T cells may affect the progression and outcome of infectious diseases.

Vγ9vδ2 T cell deficiencies in infectious disease

Alterations of Vγ9Vδ2 T cell phenotype and/or functions have been reported in several infections usually due to intracellular pathogens. Hence, a loss of CD27 expression on circulating Vγ9Vδ2 T cells was reported in patients with active tuberculosis, suggesting an impairment of effector functions (61, 62). Indeed, Vγ9Vδ2 T cell expansion was accompanied by the dramatic reduction of the Vγ9Vδ2 T cells effectors (T_{EM} and T_{EMRA} cells), with decreased IFN-γ production and granulysin expression. This deficiency was restored by successful antimycobacterial therapy. A loss of cytotoxic activity is also observed in lung Vγ9Vδ2 T cells (63). These results suggest that a high bacterial burden leads to chronic stimulation of effector Vγ9Vδ2 T cells that may result in their loss or exhaustion. As a matter of fact, The progressive loss of reactive Vγ9Vδ2 T cells from the blood and bronchoalveolar fluid in pulmonary tuberculosis patients paralleles upregulation of FasL expression on Vγ9Vδ2 T cells resulting in fratricidal killing (1, 125). A progressive attenuation of the Vγ9Vδ2 response was also observed in children with high parasitaemia in malaria (73). Similarly, prophylaxis with antimalarial drug dihydroartemisinin-piperaquine (DHA-P) during early childhood prevents the development of dysfunctional Vγ9Vδ2 T cells (12, 73).

Patients with chronic HBV infection are usually characterized by a population of exhausted T cells, similarly the ability of Vγ9Vδ2 T cells to proliferate and to respond to a chemotactic signal is diminished, which may explain the reduced frequency of Vγ9Vδ2 T cells in the liver of these patients (85). In HIV and chronic HCV patients, peripheral Vγ9Vδ2 T cells are unable to proliferate and specifically to expand the cytotoxic subset (27, 61, 86, 87). In addition, it has been demonstrated that, during HIV infection, myeloid-derived suppressor cells (MDSC) are expanded and their frequency is inversely correlated with the capacity of Vγ9Vδ2 T cells to produce IFN-γ. However, *in vitro* MDSC depletion did not completely restore IFN-γ production by Vγ9Vδ2 T cells from HIV patients (126), suggesting that during HIV infection MDSC are not the unique player in dampening Vγ9Vδ2 T cell response. Finally, in chronic HCV infection and in HIV/HCV co-infection, direct acting antivirals (DAA) fail to restore Vγ9Vδ2-induced IFN-γ production. In contrast to other T cell subsets, Vγ9Vδ2 T cell dysfunction may persist in liver despite a successful HCV treatment for a reason that remains to be elucidated (87).

Overall, these data support a crucial role for Vγ2Vδ2 T cells in infectious diseases, since functional alterations of these cells can have a significant impact on the outcome of the infectious pathology.

Vγ9vδ2 T cell-based emerging therapeutic approaches

Overall, the data summarized above indicate that triggering Vγ9Vδ2 T cell cytotoxicity may be a promising strategy for the treatment of infectious diseases caused by intracellular pathogens. Specifically, proliferative, cytotoxic, and cytokine

responses of human Vγ9Vδ2 T cell subset are induced by bisphosphonates, such as pamidronate (PAM) and zoledronic acid (Zol), through the intracellular accumulation of IPP and its metabolites. The administration of PAM, a common treatment for osteoporosis and Paget's disease, to humanized mice decreases the disease severity and mortality caused by human influenza virus infection and EBV-induced lymphoproliferative disease by enhancing Vγ9Vδ2 T cells immunity (84, 93). On the other hand, Zol, a treatment for bone disease, is broadly used *in vitro* and *ex vivo* to stimulate effector Vγ9Vδ2 T cells (127). Zoledronate affects HCV, HCMV and West Nile virus replication by expanding IFN-γ-producing Vγ9Vδ2 T cells (88, 128, 129). As mentioned previously, low-dose IL-2 synergizes with bisphosphonates and hence, is an effective method to activate and expand Vγ9Vδ2 T cells both *in vitro* and *in vivo*. In HIV patients, Zol along with IL-2 allowed the rapid expansion of CD16-expressing T Vγ9Vδ2 cells *in vitro*, associated with enhanced ADCC cytotoxicity (130). In macaques, HMBPP/IL-2 administration induced remarkable Vγ9Vδ2 T cell expansion and resulted in apparent attenuation of plague lesions in lung tissues caused by *Yersinia pestis* infection (35). Similarly, Picostim (similar to HMBPP except one carbon difference)/IL-2 administration induced activation and expansion of effector Vγ9Vδ2 T cells during both the acute and chronic phases of SHIV infection and also increased resistance to tuberculosis in macaques (131), supporting a rationale to explore Vγ9Vδ2 T cell-targeting as treatment of drug-resistant tuberculosis or HIV-associated tuberculosis. Furthermore, IL-12 and also IL-15 enhance the proliferation and expansion of HMBPP-activated Vγ9Vδ2 T cells with effector functions capable of inhibiting intracellular mycobacterial growth (108, 132). On the other hand, IL-18 enhances the proliferative, cytotoxic and recall response of Vγ9Vδ2 T cells from HIV-1-infected individuals (133). In HIV seropositive individuals, where Vγ9Vδ2 T cells are typically reduced even after effective antiretroviral therapy and CD4 T-cell reconstitution, therapies directed at restoring the antiviral activity of Vγ9Vδ2 T cells represent an appealing potential treatment. This raises questions about the therapeutic use of these cells, including the minimal requirement for eliciting a response and the cytokines required for the boost of immune response. A new strategy for treating influenza virus infection has been suggested using the combination of PAM and CD137 agonist. Indeed, activation of the CD137/CD137L pathway could maintain the survival of Vγ9Vδ2 T cells, this may provide a new solution to avoid Vγ9Vδ2 T cell exhaustion and to increase the efficacy of γδ T cell-based immunotherapy (134). However, the clinical use of bisphosphonates as an anti-infective agent has certain limitations. Indeed, it has been reported that repeated pAg treatment may lead effector cells to a senescent or exhausted phenotype, and even lead to their death (135). Better antigens should be sought to help stimulating Vγ9Vδ2 T cells *in vitro*.

Besides pAg-induced activation of Vγ9Vδ2 T cells, a recently novel approach involved the development of a new class of molecules called immunoantibiotics, notably the IspH inhibitor, has been described as also inducing the expansion and activation of human Vγ9Vδ2 T cells (136). IspH, an enzyme in the isoprenoid synthesis pathway, is essential for the survival of most Gram-negative bacteria and the lack of IspH causes an accumulation of its substrate HMBPP, thus allowing the activation of cytotoxic Vγ9Vδ2 T cells. In a humanized mice model of *E. coli* infection, these prodrugs resulted in Vγ9Vδ2 T cell expansion and a lower bacterial load in the tissues (136). This strategy synergises direct antibiotic action with rapid immune response. In addition, these prodrugs allow the targeting of existing multi-resistant microbes (136), as well as decrease the chances of resistance emerging. Unlike antibiotics derived from natural sources, no IspH inhibitors have been discovered in microorganisms, which justify their therapeutical use (137).

Another approach would be to target specifically the ligands expressed on the plasma membrane of stressed cells, such as BTN3A, which are responsible for activation and effector functions of Vγ9Vδ2 T cells. Indeed, an important tool generated in BTN3A research are activating mAbs including the anti-BTN3A agonist 20.1, that mimics the pAg-induced Vγ9Vδ2 T cell activation (43, 138, 139). After successfully showing proof-of-concept of preclinical efficacy (140), another BTN3A agonist mAb, ICT01, is currently under evaluation in the EVICTION phase I/II clinical trial (NCT04243499) sponsored by ImCheck Therapeutics in patients with solid tumors and hematological malignancies (141, 142).

The activating anti-BTN3A mAb could represent important therapeutic tools in infections to overcome the imbalances in immune responses observed in some patients. In this context, we are currently testing the ability of the agonist anti-BTN3A 20.1 to modulate viral/bacterial replication *in vitro* in co-cultures of infected cells with Vγ9Vδ2 T cells (143). By enhancing Vγ9Vδ2 T cell cytotoxicity against infected cells, anti-BTN3A agonist antibodies could offer an alternative treatment strategy for infectious diseases. Combinations of newly emerging therapy with established treatments could minimize the potential side effects of immune reconstitution in the future.

Conclusion and perspectives

The unique features of Vγ9Vδ2 T cells make these cells ideal candidates that could be targeted to induce protective and durable immunity in the context of infectious diseases. Therapies must be developed to enhance the effector functions of these cells at the site of infection, which would be relevant especially in chronic infections such as HIV infection or tuberculosis where the effector Vγ9Vδ2 T cells are impaired. For the preparation of large number of cells for adoptive cell transfer, it is necessary to identify and develop better antigens, which stimulate the Vγ9Vδ2 T cells expansion *in vitro*. Targeting key receptors such as the BTN3A and BTN2A involved in

activation and recognition of Vγ9Vδ2 T cells emerge as potential therapeutic strategies in infectious diseases. Therefore, further research might shed more light on the in-depth understanding of the underlying mechanisms of the antigen recognition and key factors influencing the Vγ9Vδ2 T cell activation during infectious diseases, which will be pivotal for developping effective Vγ9Vδ2 T cell-based therapies against pathogen infections.

Author contributions

LG, SM, and CC wrote/revised the manuscript. J-LM and DO supervised/revised the manuscript. All authors reviewed the manuscript and contributed to the work.

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Conflict of interest

DO is cofounder and shareholder of Imcheck Therapeutics, Emergence Therapeutics, Alderaan Biotechnology and Stealth. IO. CC, PF and LM are employees and shareholders of Imcheck Therapeutics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

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Systematic pattern analyses of Vδ2$^+$ TCRs reveal that shared "public" Vδ2$^+$ γδ T cell clones are a consequence of rearrangement bias and a higher expansion status

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Background: Vγ9Vδ2$^+$ T cells are a major innate T cell subset in human peripheral blood. Their Vδ2$^+$ VDJ-rearrangements are short and simple in the fetal thymus and gradually increase in diversity and CDR3 length along with development. So-called "public" versions of Vδ2$^+$ TCRs are shared among individuals of all ages. However, it is unclear whether such frequently occurring "public" Vγ9Vδ2$^+$ T cell clones are derived from the fetal thymus and whether they are fitter to proliferate and persist than infrequent "private" clones.

Methods: Shared "public" Vδ2$^+$ TCRs were identified from Vδ2$^+$ TCR-repertoires collected from 89 individuals, including newborns (cord blood), infants, and adults (peripheral blood). Distance matrices of Vδ2$^+$ CDR3 were generated by TCRdist3 and then embedded into a UMAP for visualizing the heterogeneity of Vδ2$^+$ TCRs.

Results: Vδ2$^+$ CDR3 distance matrix embedded by UMAP revealed that the heterogeneity of Vδ2$^+$ TCRs is primarily determined by the J-usage and CDR3aa length, while age or publicity-specific motifs were not found. The most prevalent public Vδ2$^+$ TCRs showed germline-like rearrangement with low N-insertions. Age-related features were also identified. Public Vδ2$^+$ *TRDJ1* TCRs from cord blood showed higher N-insertions and longer CDR3 lengths. Synonymous codons resulting from VDJ rearrangement also contribute to the generation of public Vδ2$^+$ TCRs. Each public TCR was always produced by multiple different transcripts, even with different D gene usage, and the publicity of Vδ2$^+$ TCRs was positively associated with expansion status.

Conclusion: To conclude, the heterogeneity of Vδ2$^+$ TCRs is mainly determined by *TRDJ*-usage and the length of CDR3aa sequences. Public

Vδ2$^+$ TCRs result from germline-like rearrangement and synonymous codons, associated with a higher expansion status.

KEYWORDS

γδ TCR, Vγ9Vδ2$^+$ T cells, TCR distance, TCR sequencing, *TRD* rearrangement

Introduction

γδ T cells are unconventional T cells which have T cell receptors (TCR) consisting of both rearranged γ (TRG gene) and δ (TRD gene) chains. Like αβ T cells, γδ T cells use the recombination of variable, diversity, and joining gene segments (V(D)J recombination) to generate the complementarity-determining region 3 (CDR3) of the *TRG* and *TRD*. The diversity of these CDR3 regions is further amplified by the insertion of palindromic sequences (P nucleotides) and additional non-templated nucleotides (N-insertions) introduced by terminal deoxynucleotidyl transferase (TdT) (1, 2).

However, in contrast to conventional αβ T cells, which use numerous V segments almost randomly, human γδ T cells exclusively use Vδ1, Vδ2, and to a lesser extent also Vδ3 segments to generate delta chains. Further restrictions on diversity are imposed due to Vδ2$^+$ chains mostly pairing with Vγ9-JP chains (2). The resulting Vγ9Vδ2$^+$ T cells are regarded as innate γδ effectors that are quickly activated in anti-tumor, infection, and inflammation within diseases (3). Committed Vγ9Vδ2$^+$ T effector cells are enriched in fetal thymus and blood, where they then persist into adulthood (4–6). The Vγ9Vδ2$^+$ TCRs uniformly recognize phosphoantigens like microbial-derived (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) and host-derived Isopentenyl pyrophosphate (IPP) in a pMHC-unrestricted manner (7–10), leading to fast TCR expansion and cytokine release of Vγ9Vδ2$^+$ γδ T cells (3). The Vγ9Vδ2$^+$ TCRs are featured as "semi-invariant" TCRs whereby the Vγ9 chains always have a *TRGV9-TRGJP* rearrangement. Fetal-derived Vγ9JP chains often express the germline-encoded CDR3 sequence CALWEVQELGKKIKVF due to the lack of TdT in the fetal thymus (5, 6). The Vδ2$^+$ repertoire, on the other hand, that evolves during human development remains both highly diverse and individual (4, 5). In the early stages of life, the *TRDV2* gene segments preferentially rearrange with *TRDJ3* and *TRDJ2*, and gradually switch to *TRDJ1* after birth (11). Meanwhile, more N-insertions and longer CDR3 length are introduced into Vδ2$^+$ TCRs after birth due to the increasing activity of the TdT (6). Public Vδ2$^+$ TCRs are frequent among Vδ2$^+$ repertoires from both the fetus and cord blood (6, 12–14). Public Vδ2$^+$ TCRs have a higher overall diversity than the public Vγ9-JP; they occupy a substantial portion of Vδ2$^+$ repertoires from adult peripheral blood (4). However, the properties and ontogeny of public Vγ9Vδ2$^+$ TCRs are not completely solved. It is also unclear whether public Vγ9Vδ2$^+$ TCRs have any advantage in target recognition, amplification over private TCRs or whether the thymus after birth still preserves the ability to produce public Vδ2$^+$ TCRs.

TCR-sequencing data is high-dimensional data. The CDR3 sequences are typically composed of 10-30 diverse amino acids and factors such as V(D)J recombination, frequency, and MHC restriction need to be considered in the analysis of this. Recently, different computational tools were developed to discover TCR clusters based on the sequence patterns (15–17). For example, TCRdist3 is an open-source python package which transforms TCR repertoires into biochemically informed distance metrics based on the similarity of the TCR amino acid sequences, especially on the CDR3 sequence regions. The calculated distance metrics enabled clustering or meta-clonotype analysis to be carried out on the TCR sequences (18, 19). However, MHC restriction of αβ TCRs and lack of HLA genotyping data for most of the available data impeded these tools from being applied to public TCR datasets on a larger scale. In contrast, the MHC-unrestricted nature of γδ TCR makes it possible to apply TCRdist3 on γδ TCR repertoires across a large number of individuals.

To investigate the heterogeneity and ontogeny of public Vδ2$^+$ TCRs, we determined the publicity of TCRs from Vδ2$^+$ TCR repertoires of 89 individuals from cord blood (CB), infant peripheral blood, and adult peripheral blood. Vδ2$^+$ CDR3 amino acid (CDR3aa) sequences were embedded into the distance matrix by TCRdist3 and visualized by Uniform Manifold Approximation and Projection (UMAP). We found that both the J-usage and length together defined the heterogeneity of Vδ2$^+$ CDR3aa sequences. Both germline-encoded and age-dependent features were preserved among public Vδ2$^+$ TCRs, indicating that they are produced in the fetal and adult thymus. Interestingly, we additionally revealed a higher expansion status of public Vδ2$^+$ TCRs than private Vδ2$^+$ TCRs.

Results

Public Vδ2$^+$ clones prevail in all age groups

To investigate the occurrence of public Vδ2$^+$ clones, we collected TCR repertoires containing 213,391 Vδ2$^+$ CDR3aa sequences from 11 cord blood (CB), 55 infant peripheral blood, and 23 adult peripheral blood samples. Eighty-one samples were collected from our published studies (4, 13, 20, 21), and eight of these samples (five CB and three adult) were included from an unpublished databank to increase further the sample size (Figure 1A and Table S1). The lengths of CDR3s ranged from 4 to 39 amino acids with a median of 18 amino acids (Figure S1A). The *TRDJ3* segment dominated in CB samples and rapidly decreased after birth. Similarly, 15.9% of the *TRDV2* rearranged with *TRDJ2* in CB, but this number decreased to around 2.1% in adults. In contrast, the *TRDJ1* segment increased to a large majority in adult samples compared to the small frequency that was found in CB. The proportions of the *TRDJ4* segment were marginal in all three groups (Figure S1B). "Public" Vδ2$^+$ TCR clones were defined by the proportion of individuals sharing the same CDR3aa sequence Private TCR CD3R regions were found in only one individual. As well as this low and high TCR's appeared in less than or equal to 10% of individuals respectively. In CB samples, 26.8% of TCR sequences were low public and 15.1% were high. Interestingly, although the publicity of adult Vδ2$^+$ TCRs significantly decreased,14.5% of low public and 4.6% of high public TCRs were still found on average (Figure S1C).

Before applying the TCRdist3 tool to Vδ2$^+$ TCR repertoires, data pre-processing and down-sampling were performed (Figure 1A). To reduce the noise caused by rare sequences, we only selected CDR3aa sequences with a length between 14 to 22 amino acids, and all *TRDJ4* rearrangements were also excluded (Figures S1A, 1B, C). Subsequently, this led to 52,199 CDR3aa sequences being obtained after down-sampling. This data cleansing and down-sampling method did not significantly affect the J-usage and publicity of post-procession TCRs in this study (Figures 1C, D).

Highly diverse Vδ2$^+$ TCRs cluster according to CDR3aa length and *TRDJ* segment usage

The distance between every two TCRs was calculated based on CDR3aa sequences by the TCRdist3 which generated a

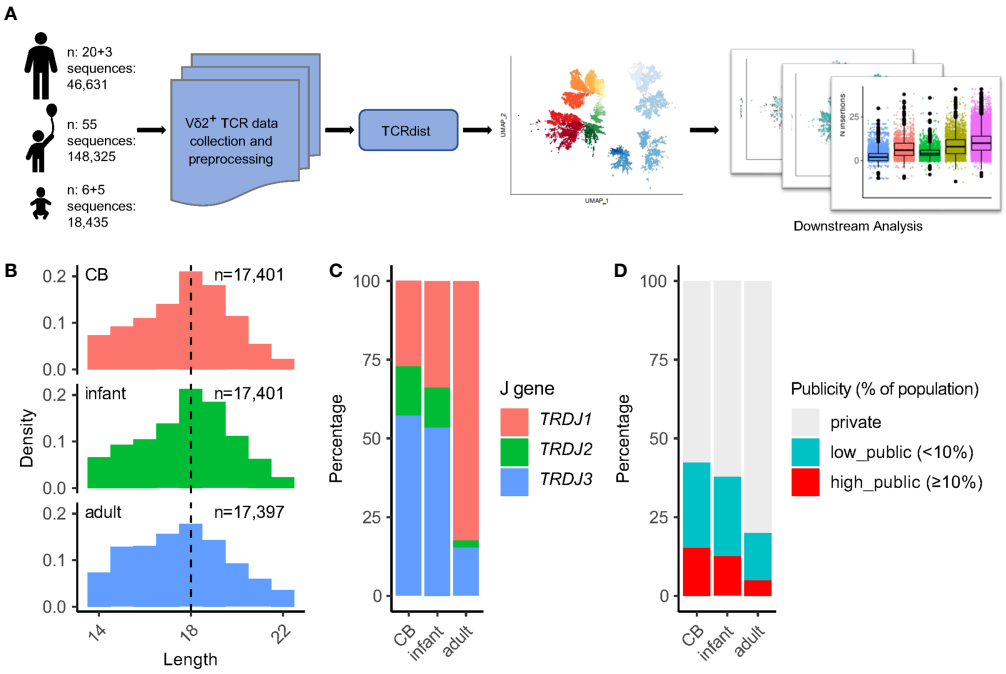

FIGURE 1
Experimental design and data pre-processing. **(A)** Illustration of the data analysis workflow. Our datasets were collected from 11 CB, 55 infants, 23 adults; among them, datasets from five CB and three adults were unpublished data. **(B)** Sequence length distribution of 52,199 CDR3aa sequences from CB, infant, and adult groups after down-sampling and pre-processing. The numbers indicate the number of CDR3aa sequences after down-sampling. Dashed lines indicate the median value of CDR3 length. **(C)** J gene usage among different age groups. **(D)** CDR3aa publicity composition among age groups. The publicity of a CDR3aa sequence is defined by the proportion of individuals that share this sequence.

distance matrix (18, 19) Following this a UMAP was generated to allow data embedding and visualization (Figures 1A, 2A). At first sight, Vδ2$^+$ TCRs were clearly stratified on the UMAP by both the J-usage and CDR3aa length (Figures S2A, B). The J-usage skewed from *TRDJ3*- and *TRDJ2*-dominant in the CB group to *TRDJ1*-dominant in the adult group (Figures 2B, S2C). Longer CDR3s on the other hand were more frequently found in *TRDJ1* and *TRDJ2* adult group. The CDR3aa length distribution between the age groups did however remain similar (Figures 2B, S2C, D). Infant-derived Vδ2$^+$ TCRs showed intermediate features between CB and adult TCRs in terms of both J-usage and CDR3aa length (Figures 2B, S2C, D).

Adjacent to this, in order to test if other factors contributed to the heterogeneity of Vδ2$^+$ TCRs, we selected the TCRs with the most prevalent lengths for the *TRDJ1* (length 17aa) and *TRDJ3* (length 19aa) regions for a more in-depth re-analysis. This showed that publicity (Figure 2C) and age groups (Figure 2D) were not distinguishable on the re-analyzed UMAP. More evidently, after restricting to the same J-usage and length, the CDR3aa sequence logomap showed almost identical motifs between the different publicity and age groups (Figures S2E, F). This suggests that the heterogeneity of Vδ2$^+$ TCRs is primarily determined by a combination of *TRDJ* usage and CDR3aa length.

Public Vδ2$^+$ repertoire preservers both germline and age-related characteristics

In previous studies by Ravens et al. and Papadopoulou et al., public Vδ2$^+$ TCRs were described as germline-encoded CDR3 with either no or few N-insertions and short CDR3 lengths (12, 13). In our dataset, publicity was also reversely associated with the number of N-insertions and length of CDR3aa (Figures S3A, B). Interestingly, public Vδ2$^+$ TCRs previously have shown age-dependent wave-like dynamics: enriching in fetal blood, then decreasing in cord blood before rising again in 5 to10-week-old infants and then finally dropping in adulthood (12, 13). This then therefore led us to determine whether or not the public clones generated in different time windows would also show similar age-dependent features. Indeed, although public Vδ2$^+$ TCRs were enriched in TCR clusters with shorter lengths, they still demonstrated to preserve the J-usage and length-determined heterogeneity as private Vδ2$^+$ TCRs also displayed (Figures 2E, F).

Following this, to investigate how public Vδ2$^+$ TCRs' features changed during development, we took advantage of the whole dataset before down-sampling. Overlapping of all unique public CDR3aa clones for different age groups showed that only a minor portion of clones were shared between the CB and adult groups (CB&AD shared) (1,175 out of 4,641 in CB and 1,175 out of 5,262 in adult). In contrast, both CB and adult groups largely shared their public Vδ2$^+$ repertoire with the infant group (4,428 out of 4,641 in CB and 4,258 out of 5,262 in adult) (Figure S3C). From combining the transitional features of infant TCRs in the J-usage and length, we considered that age-related differences of public Vδ2$^+$ TCRs mainly exist between CB and adult groups (Figure 3A), while a transitional infant group shared the commonalities from both sides. As TdT activity increases along with human development, we hypothesized that adult-derived TCRs would have more N-insertions than CB-derived ones. Indeed, the private Vδ2$^+$ TCRs from the adult group had the most N-insertions and longest CDR3aa length, whereas the CB&AD shared group Vδ2$^+$ TCRs had the fewest N-insertions (Figures 3B, S3D). The N-insertions of adult-derived *TRDJ2* and *TRDJ3* public Vδ2$^+$ TCRs were slightly more than that of CB-derived public TCRs (Figure 3B). Intriguingly, for *TRDJ1*, we observed more TCRs with higher N-insertions in the CB public group than in the adult public group (Figure 3B). Here 25.0% of CB-derived public Vδ2$^+$ *TRDJ1* TCRs had more than 10 N-insertions. Whereas for adult-derived and CB&AD shared public clones, the number was merely 5.91% and 2.91%, respectively (Figure 3C). Finally, although the CB-derived public Vδ2$^+$ *TRDJ1* TCRs had more residues in the high-variable region, the motifs of the three groups were similar, i.e. polar amino acids were mainly used (Figure 3D).

Synonymous codons in CDR3 nucleotide sequences result from different TRDD-gene usages and N-insertions that contribute to the generation of public Vd2$^+$ CDR3

Since the generation of public Vδ2$^+$ clones did not entirely result from simple germline rearrangements without N-insertions (Figures 3B, C), we explored in more detail how the public CDR3aa sequences were rearranged. The publicity of CDR3aa sequence positively correlated with the number of its corresponding unique encoding transcripts (Figure 4A). The same CDR3aa sequences could be generated by the exceedingly high numbers of different CDR3 nucleotide (CDR3nt) sequences. For example, the public CDR3aa sequence 'CACDTLGDTDKLIF' (2) was detected in 76 different individuals as well as also being transcribed from 80 different transcripts (Figure 4A). Additionally, public Vδ2$^+$ CDR3aa sequences were more likely to have a variable *TRDD*-segment usage. 31.3% and 10.6% of 'high public' and 'low public' CDR3's, respectively, could be rearranged from more than one *TRDD*-segment, whereas a much lower frequency of only 0.16% was observed in private CDR3's (Figure 4B). More surprisingly, public CDR3aa sequences could be generated from multiple CDR3nt sequences even within one individual. For example, in donor SA62, the public CDR3 "CACDTLGDTDKLIF" could be produced by eight different CDR3nt transcripts, either rearranged with *TRDD3* and 0 – 1 N-insertion, *TRDD2* with 2

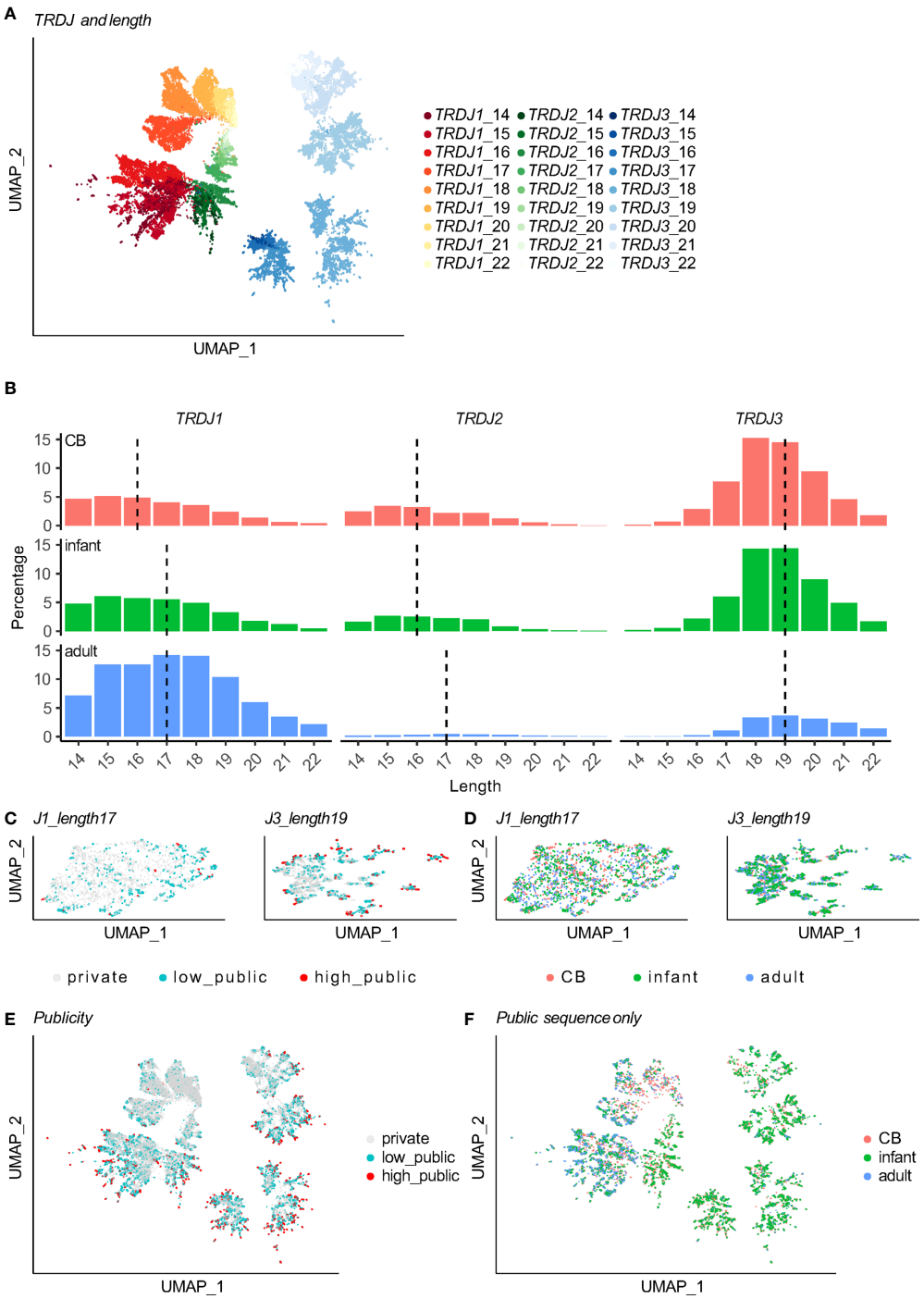

FIGURE 2
The heterogeneity of Vδ2 TCRs is determined by CDR3 lengths and *TRDJ* segments. **(A)** Each point stands for a Vδ2$^+$ CDR3aa sequence. UMAP for 52,199 Vδ2$^+$ CDR3 (same data as in Figure 1B) colored by the combination of J gene usage and CDR3aa length. **(B)** CDR3aa length distribution with different J segments and age groups. The dashed line indicates the median length. **(C)** UMAP of length = 17 *TRDJ1* Vδ2$^+$ CDR3aa sequences (left), UMAP of length = 19 *TRDJ3* Vδ2$^+$ CDR3aa sequences (right) colored by sequence publicity. **(D)** The same UMAPs in **(C)** are colored by age group. **(E)** Same UMAP in **(A)** colored by the publicity label of the sequence. **(F)** UMAP in **(A)** after filtering out private TCRs, colored by age group.

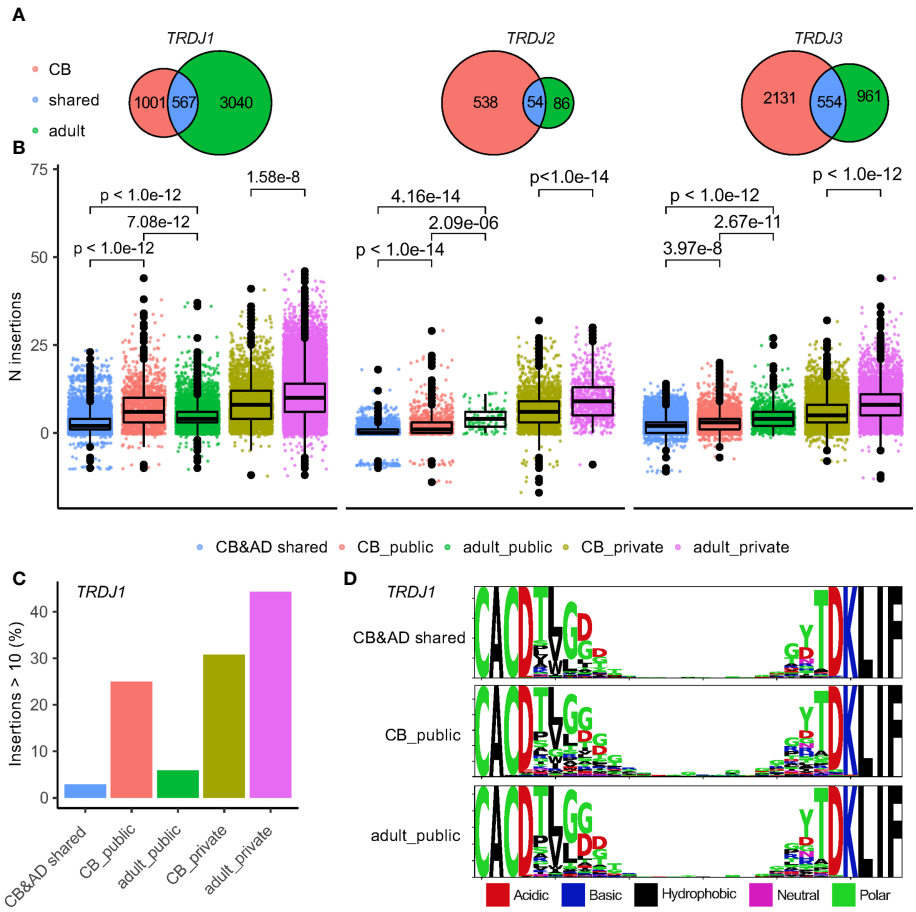

FIGURE 3
Patterns of public clones alter between age groups. **(A)** Venn plots show the overlap of public Vδ2$^+$ clones between CB and adult groups. The sizes of ellipses correlate to number of unique clones. **(B)** N-insertion of Vδ2$^+$ TCRs in corresponding groups. Each point stands for a CDR3 nucleotide sequence. The distribution is summarized by box plot; the three horizontal lines of the box-whisker plot represent the higher quartile, median, and lower quartile, respectively. Games-Howell test was used for P-value calculation. Vδ2$^+$ TCRs were grouped by J-usage and the publicity between CB and adult. **(C)** barplot shows the ratio of sequences with N insertions ≥ 10 in publicity groups with *TRDJ1* gene usage. **(D)** Logomap for sequences with public *TRDJ1* gene usage.

N-insertions, or 9 N-insertions without *TRDD* segment (Table 1). 19.8% (median value, ranging from 4% to 62.6%) of high public Vδ2$^+$ CDR3 in each individual were generated by at least five unique transcripts. In contrast, the number of private CDR3s was much lower at 1.28% (median value, ranging from 0.26% to 5.56%) (Figure 4C).

The publicity of Vδ2$^+$ clones positively associated with expansion status

To determine whether the publicity of Vδ2$^+$ TCRs correlated to the expansion ability, we assigned the top 25% of most expanded TCRs in each sample as high frequency (high-freq) TCRs and then labelled the remaining as low frequency (low-freq) TCR's (Figure S4A). The high-freq and low-freq TCRs were not distinguishable on the UMAP (Figure 5A). In order to understand which groups of Vδ2$^+$ TCRs are more likely to be high-freq TCR's, we calculated the "expansion status score" based on high-freq to low-freq TCRs (Methods section). For a group of TCRs in one individual, the expansion status is calculated by dividing the number of high-freq TCRs in the group by the number of low-freq TCRs followed by a log-transformation. Hence, the higher the expansion status score, the more high-freq TCR's in that group. An expansion status score of > 0 means the group has more high-freq TCRs than low-freq ones. Interestingly, the median expansion status score of "high public" TCRs was 0.37, and that of the "low public" TCR's remained significantly higher than the private TCR values (-0.50 vs -1.28, median value) (Figure 5B). We further examined the expansion status score for TCRs with different J-usages, and similar results were observed (Figure S4B). Given that the

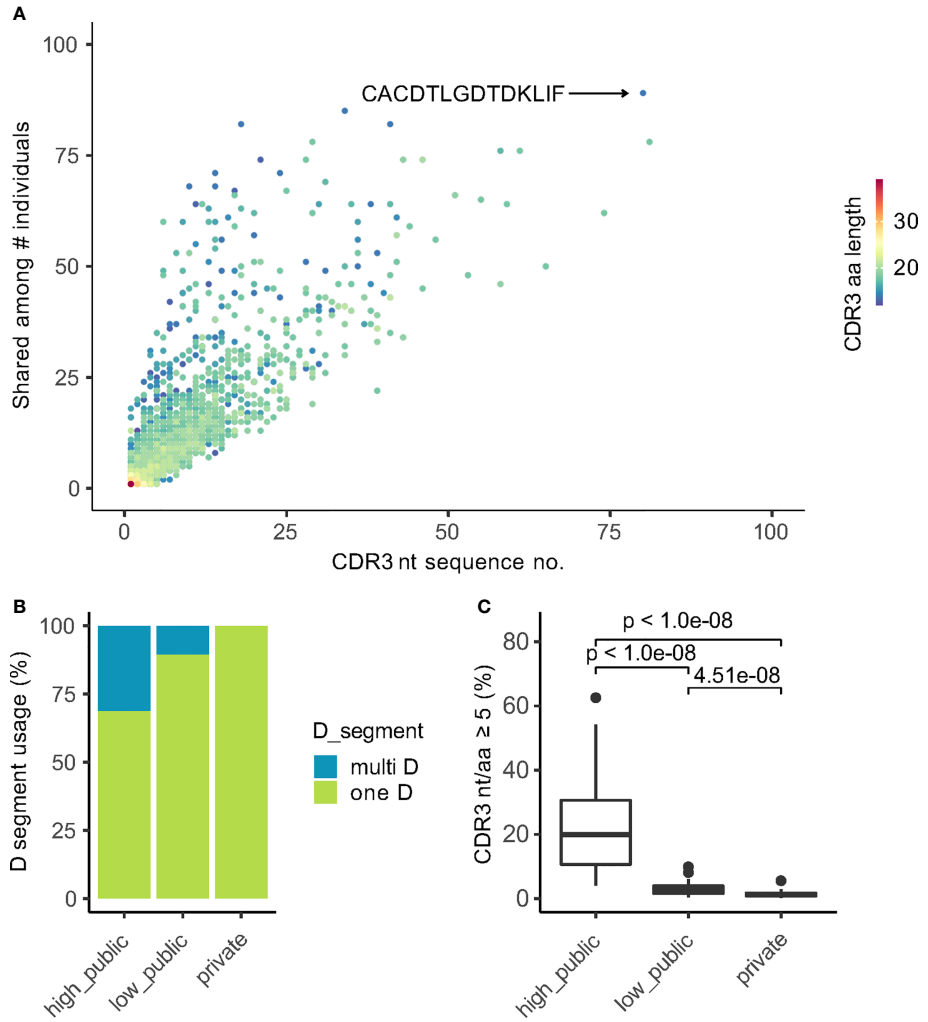

FIGURE 4
Public CDR3aa clones have more corresponding CDR3nt transcripts. **(A)** Scatter plot of the publicity of CDR3aa (number of individuals sharing the CDR3aa) vs the number of corresponding CDR3nt sequences. Each point indicates one unique CDR3aa sequence colored by the CDR3aa length. The most public CDR3aa sequence are indicated on the plot. **(B)** TRDD-segment usage of CDR3 sequences in different publicity groups. **(C)** Box plot shows the ratio of CDR3aa sequences translated from 5 or more different nucleotide transcripts in each individual. Games-Howell *Post-Hoc* Test was used to test the mean difference between groups. Adjusted P-values are shown between groups.

TABLE 1 CDR3aa "CACDTLGDTDKLIF" corresponding CDR3nt sequences for individual SA62.

CDR3nt sequence	*TRDD*	*TRDJ*	N-insertion
TGTGCCTGTGACAC**C**CT**A**GG**A**GA**C**ACCGATAAACTCATCTTT	*TRDD2*	*TRDJ1*	2
TGTGCCTGTGACAC**C**CTGGGGGGATACCGATAAACTCATCTTT	*TRDD3*	*TRDJ1*	0
TGTGCCTGTGACAC**A**CTGGGGGATACCGATAAACTCATCTTT	*TRDD3*	*TRDJ1*	0
TGTGCCTGTGACAC**G**CTGGGGGATACCGATAAACTCATCTTT	*TRDD3*	*TRDJ1*	1
TGTGCCTGTGACAC**T**CTGGGGGATACCGATAAACTCATCTTT	*TRDD3*	*TRDJ1*	1
TGTGCCTGTGACAC**T**CTGGGGGATAC**T**GATAAACTCATCTTT	*TRDD3*	*TRDJ1*	1
TGTGCCTGTGACAC**A**CTGGGGGA**C**ACCGATAAACTCATCTTT	*TRDD3*	*TRDJ1*	0
TGTGCCTGTGACAC**C**CT**A**GG**C**GATACCGATAAACTCATCTTT	.	*TRDJ1*	9

Under scores indicate variable nucleotide residues.

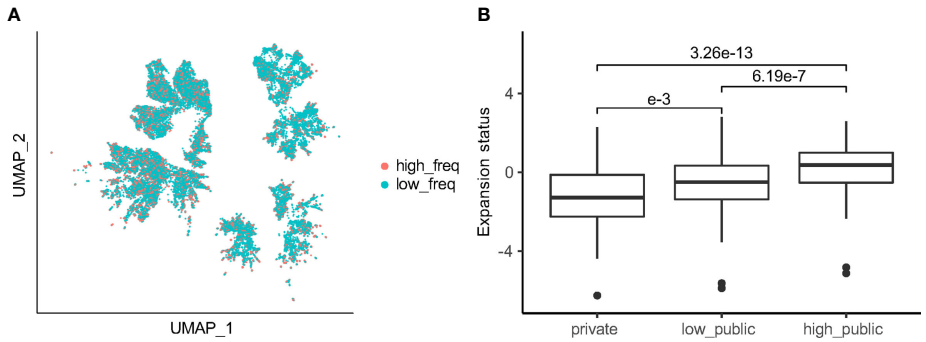

FIGURE 5
Public clones have a greater expansion status compared to private clones. **(A)** UMAP of Vδ2$^+$ TCR colored by high-freq/low-freq category. **(B)** Expansion status score for each publicity group. Games-Howell *Post-Hoc* Test was used to test the mean difference between groups. Adjusted P-values are shown between groups.

publicity is reversely associated with CDR3 length (Figure 4A), expansion status could also be associated with CDR3 length. However, CDR3aa lengths only demonstrated to have a minimal impact on expansion status, and the median expansion status scores of all lengths and *TRDJ*s remained below 0 (Figures S4C, D).

Discussion

In this study, we applied TCRdist3 to systematically investigate the Vδ2$^+$ TCR repertoire and revealed that Vδ2$^+$ TCRs retain a high heterogeneity that is primarily determined by the J-usage and CDR3aa length. It was observed that public Vδ2$^+$ TCRs were as diverse as private TCRs. In previous studies, TCR's with high publicity or shared between cord blood (CB) and adult age groups were characterized to show only a few or no N-insertions and shorter CDR3length (6, 13). Unexpectedly, our study also demonstrated that the *TRDJ1* of public (but not of private) γδ TCRs in CB displayed a relatively high number of N-insertions and longer CDR3 lengths. Moreover, it was additionally revealed that, compared to private Vδ2$^+$ CDR3aa sequences, the public Vδ2$^+$ CDR3aa sequences were prone to be generated from multiple CDR3nt transcripts even within one individual. Thus, it could be concluded that germline-like rearrangement and synonymous codons used by CDR3nt sequences contribute to the generation of public CDR3aa. Finally, public Vδ2$^+$ TCRs displayed a higher expansion status than private Vδ2$^+$ TCRs.

By using TCRdist3 and various other tools for investigating CDR3 motif or amino acid properties 'clustering' of TCR's can be carried out. This strategy was particularly useful in linking αβ TCR sequences to antigen-specificity based on similarity (18, 22–24). In contrast to highly rearranged αβ TCRs, which have the ability to recognize any possible antigen, most of the rearranged Vγ9Vδ2$^+$ TCRs are instead generated from relatively fixed options and are thought to uniformly recognize phosphoantigens (2, 7). Complex TCR repertoire data can be extracted to generate a single UMAP by applying the TCRdist3 method to conveniently analyze the heterogeneity of γδ TCR's. Hence, it is useful when investigating the shift of the Vδ2$^+$ TCR repertoire under different physiological and pathological conditions. For example, in our study, the repertoire shift from CB-derived to adult-derived repertoire was notably highlighted. Moreover, from this, it would be interesting to see if TCRdist3 could be applied to the more adaptive Vδ1$^+$ or Vδ3$^+$ γδ TCRs to possibly determine their function and antigen-specificity.

Vδ2$^+$ TCRs derived after birth displayed more N-insertions and longer CDR3 length than those from CB, considering the increasing TdT activity. However, in contrast to this, the public clones among CB-derived *TRDJ1* Vδ2$^+$ TCRs showed more N-insertions and longer CDR3 than their adult public TCR counterparts. This property was not seen among public Vδ2$^+$ TCRs with other J-usage meaning it is difficult to fully explain and understand this complex feature as yet. One possibility for this could be that it may associate with the intrathymic differentiation of Vγ9Vδ2$^+$ T effectors. Mouse and human innate γδ T effectors are committed in waves within the fetal thymus, and have shown to acquire phenotypes that are closely related with certain TCR usages (3, 4, 6, 25). While the development of human γδ T cells is not fully elucidated, it could be hypothesized that a number of underappreciated Vγ9Vδ2$^+$ T effectors develop later in the fetus when the TdT becomes much more active. These specialized effector cells do not remain in peripheral blood after birth. By comparing specific γδ T cells from mice relevant information can be obtained. Mouse Vγ6$^+$ and Vγ4$^+$ IL-17-producing γδ T cells are a rare population of cells which reside in mucosal tissues like the skin or lungs (26, 27). These specialized cells exclusively develop at embryonic days of E15 to E18 after

gestation in the fetal thymus where they will then home to specific tissues (28). Thus, there is only a narrow window in which these cells can easily be observed whilst they travel within the circulation. This therefore means that the existence of previously unknown tissue-resident γδ T cell populations which are generated shortly after birth cannot be excluded.

We demonstrated that the publicity of Vδ2$^+$ TCRs positively associates with a higher expansion status. This remains in line with previous studies which also suggest that higher abundance was found on high public clones (12, 13). One of the most debatable questions regarding public Vδ2$^+$ TCRs continues to determine if the generation and expansion of public Vδ2$^+$ TCRs are driven by interactions with BTN2A1 and BTN3A1 butyrophilin molecules. It is also yet to be discovered if the recognition of specific antigens may additionally alter the expansion process within these Vδ2$^+$ TCRs. Although the CDR3 is essential for recognition, previous studies failed to find evidence that the CDR3 of Vδ9Vδ2$^+$ TCRs specifically recognize phosphoantigens (7–10). We cannot exclude the possibility that even the family of Vγ9Vδ2$^+$ TCRs recognizes antigens in an "adaptive-like" way *via* the CDR3 until a complete structure of interacting Vγ9Vδ2$^+$ TCR, phosphoantigen, and butyrophilins BTN2A1 and BTN3A1 is revealed. However, based on the current understanding of Vγ9Vδ2$^+$ T cells, it is unlikely that public or expanded Vδ2$^+$ TCR clones result from antigen-specific clonal expansion. First of all, previous *ex vivo* experiments suggested that phosphoantigen stimulation induced both polyclonal and unbiased expansion of Vδ9Vδ2$^+$ T cells (6, 20). Moreover, in our study, by calculating the geometric distance between Vδ2$^+$ CDR3 based on sequence patterns, it was found that there is no significant difference between public and private Vδ2$^+$ CDR3 patterns or between high-freq and low-freq Vδ2$^+$ CDR3s. The results suggest that the binding between Vδ2$^+$ CDR3 and phosphoantigen-activated butyrophilins BTN2A1 and BTN3A1 does not favor specific CDR3 variants or motifs.

Taking this all into account it can be determined why public Vδ2$^+$ TCRs appear to have a survival advantage? Based on the rearrangement bias and development ontogeny, various speculations can be made as follows: 1). There is a rearrangement bias. where the publicity of Vδ2$^+$ TCR CDR3aa positively associates with the number of corresponding CDR3nt sequences. Therefore, the γδ T cells with a public Vδ2$^+$ TCR may have multiple sources from different TCR rearrangements, resulting in a higher copy number. 2). Most public Vδ2$^+$ TCRs, especially those shared between many individuals, are rearranged early in life and persist into adulthood (4, 12–14). They may simply have more time to accumulate. A similar situation was observed in human αβ T cells, where it was found that T cells carrying public αβ TCRs were generated before birth and then continued to maintain high abundances for a long time throughout adulthood (29).

One of the major limitations to this current study was that it was only viable to investigate the Vδ2$^+$ chains, meaning information on the corresponding pairing of Vγ9 chains was lost. Within our study it was also difficult to prove or disapprove the possibility that public Vγ9Vδ2$^+$ TCRs may interact with antigens in a different way compared to antigen interaction by private TCRs. However, recent advancements in single-cell TCR sequencing do make it possible to sequence paired γδ TCR and relate it to phenotypes of other cells (4). From this it can be expected that more such data will soon become available. Another limitation to this study was the fact that undersampling could possibly impair accuracy. As the library protocol only enabled a survey of up to tens of thousands of γδ T cells from a portion of a PBMC sample, this underrepresented the huge vast number of γδ T cells that are actually living within our body. This undersampling may make it difficult to accurately identify moderately expanded clones. However, considering the relatively low diversity of Vδ2$^+$ TCRs, undersampling may compromise some details, but the major findings are unlikely to be greatly affected.

The TCRdist3 method has proven to be a very useful tool for analyzing human αβ T cells, and the software is able to support γδ TCR analysis (19). However, as mentioned above, the αβ TCRs have a much higher heterogeneity than Vδ2$^+$ TCRs provided by the V(D)J rearrangement. Thus, detecting the different patterns between αβ TCRs is considerably easier. In our case, the TCRdist3 detected the heterogeneity of Vδ2$^+$ TCRs generated by length and J-usage, but not by publicity or age. Our sequence pattern analysis also failed to find heterogeneity between public and private TCRs. Furthermore, the existing possibility that more subtle and essential substitutions hiding in public Vδ2$^+$ TCRs cannot be excluded. Currently, methods for TCR clustering are all based on the CDR3aa sequences, which is sufficient to study antigen-specificity. However, the ontogeny of TCRs can be better determined if CDR3nt sequences are included to provide crucial information about VDJ rearrangement and N-insertions.

Our study established that TCR sequence analysis tools such as the TCRdist3 are very useful for investigating the γδ TCR repertoire. By using TCRdist3 and downstream analysis, it could be demonstrated that public Vδ2$^+$ TCRs are a heterogeneous population with both germline and age-related features that confer expansion advantages over private TCRs. Given that expressing γδ TCRs on αβ T cells is a promising immunotherapy strategy against tumors (30, 31), those "more successful" public Vγ9Vδ2 TCR might improve the performance of immunotherapy using Vγ9Vδ2$^+$ T cell clones or engineered αβ T cells carrying Vγ9Vδ2 TCR.

Materials and methods

Human sample isolation and preparation

Data from 8 healthy donors in this study were newly generated. Blood samples from adult donors (n = 3) and cord

blood (CB) donors (n = 5) were collected at Hannover Medical School (Hannover, Germany) after written informed consent. This study was performed in accordance with the Declaration of Helsinki and approved by the institutional ethics review board at Hannover Medical School under study numbers 1303-2012 (CB individuals) and 7901-2018 (healthy adult individuals). PBMCs and CBMCs were purified from the blood samples by Ficoll-Paque density gradient media separation. These cells were then stored at -80°C in 90% fetal bovine serum and 10% DMSO freezing medium before use.

Vγ9Vδ2$^+$ T cells sorting

Fluorescence-activated cell sorting (FACS) was performed using the FACS Aria Fusion flow cytometer (BD, USA). PBMC and CBMC were incubated with 5% Fc-receptor block before staining. The following antibodies were used: anti-CD3 (clone REA613; Miltenyi Biotec), anti-CD3 (clone SK7; BD Bioscience), anti-γδ TCR (clone 11F2, BD Bioscience or Miltenyi Biotec), anti-Vγ9 (clone IMMU 360; Beckman Coulter), anti-Vδ2 (clone 123R3; Miltenyi Biotec).

Vδ2$^+$ TCR library construction and sequencing

All the newly generated data was sequenced and pre-processed in the same way as other published data used in this study (4, 13, 20, 21). Briefly, RNA was extracted from sorted DAPI$^-$CD3$^+$γδ$^+$Vγ9$^+$Vδ2$^+$ cells from PBMC or CMBC by an RNAeasy Micro Kit (Qiagen). Reverse transcription was carried out with Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers. As previously described (21), δ chains was amplified *via TRDV2* specific primers hTRDV2: ATTGCAAAGAACCTGGCTGT and hTRDC: GACAAAAACGGATGGTTTGG. The PCR program was set as follows: 1). 95°C for 3 min; 2). 95°C, 63°C, and 72°C for 30s each, for 5 cycles; 3). 95°C for 30s, 72°C for 35s, for 20–25 cycles; 4). 72°C for 4 min.

The amplified cDNA library with Illumina P5 and P7 adaptor was sequenced by Illumina Miseq using 500 cycles of paired-end sequencing.

Raw sequencing data alignment and annotation

Raw reads alignment annotation was performed with MiXCR software v.2.1.12 to international immunogenetics information system (IMGT) reference (32). Unproductive TCRs were filtered out. Annotated TCRs were further counted and summarized by VDJtools (33).

Data integration and processing

VDJtools output files from all the 89 individuals from the published and newly generated datasets were merged together. Since some datasets comprised entire TCRδ repertoires (13, 21), non-Vδ2 TCRs were filtered out. Numbers of N-insertions were calculated *via* VDJtools output as following: For TCRs rearranged with a *TRDD* segment: N-insertion = (Jstart – Dend – 1) + (Dstart – Vend - 1); For TCRs without D-usage: N-insertion = Jstart – Vend – 1.

Vend, Dstart, Dend, Jstart are the start/end position of V, D, J segments on CDR3nt sequence.

Publicity of TCRs were defined based on the CDR3aa sequence by whether a sequence is shared among a certain percentage of the population. "private" CDR3aa is defined as CDR3aa that only appears in only one individual, "high public" TCRs are shared among at least 10% of the population, i.e. shared among 9 or more individuals in our study, the remaining TCRs are defined as "low public", i.e. shared by at least 2 individuals to 10% of the population.

TCR distance calculation and UMAP embedding

Vδ2$^+$ CDR3aa sequences with a length from 14 to 22 aa were preselected and downsampled for TCR distance calculation. CDR3s rearranged with *TRDJ4* segment were excluded. For each age group, the numbers of CDR3s were randomly down-sampled to 17,398 - 17,401 sequences. TCR distances were computed according to the protocol of TCRdist3 (34). Briefly, CDR3aa sequence, V-usage, and J-usage were then included as input for the TCRdist3 in the Python 3.8 environment. CDR1, CDR2, and CDR2.5 sequences were reconstructed from the V-usage. After alignment, penalties were given to each mismatch between two TCRs according to the BLOSUM62 substitution matrix. Finally, distance was calculated as the weighted sum of penalties across all CDRs. The TCR distance matrix was further embedded into latent spaces by UMAP.

Calculation of expansion potential

In each individual, CDR3aa sequences were ranked by the frequencies from high to low. The top 25% of CDR3s were assigned as "high frequency" TCRs, and the rest were labelled as "low frequency" TCRs. (Figure S4A). The expansion status score is calculated for a pre-defined group of TCRs within an individual (*i.e.* the high public *TRDJ1* Vδ2$^+$ TCR in the donor CB2) as follows:

$$Expansion\ status\ score = \ln\left(\frac{n_{\text{highfreq}}+1}{n_{\text{lowfreq}}+1}\right)$$

$n_{highfreq}$ and $n_{lowfreq}$ are the number of high-freq and low-freq CDR3aa sequences in the group.

Sequence alignment and logomap

Sequences of selected groups were aligned using Clustal Omega (35–37), logomap was generated from the aligned sequences using Logomaker (38).

Statistics

Statistical analyses were performed under R v4.1.2. The statistical methods are described in the figure legends, in all cases, considering the sample size, variance and number of comparisons. Either a one-way ANOVA or a Tukey's HSD test after a one-way ANOVA or Games-Howell *Post-Hoc* Test was used and *P*-values were then calculated.

Data availability statement

The previously unpublished raw data presented in this study are deposited in the GEO repository, accession number GSE213280. All codes and processed data are available from Github repository https://github.com/isihh-uke/gdTCR_analysis.git.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional ethics review board at Hannover Medical School. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

LD and LT conducted and interpreted bioinformatics analysis. AH and SR organized and performed TCR sequencing. LD, IP, and LT designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

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SUPPLEMENTARY FIGURE 1
CDR3 delta chain sequence distribution among different age groups. **(A)** Sequence length distribution of 213,391 CDR3aa sequences from 89 individuals, including 11 CB, 55 infants, and 23 adults. The numbers on the plot indicate the number of CDR3s in each group. Black dashed lines indicate the median value of CDR3 length. Grey dash lines indicate the range of length of CDR3aa sequences that are used for TCRdist3 computation. **(B)** J gene composition among different age groups. **(C)** CDR3 publicity composition among age groups. The publicity of a CDR3aa sequence is defined by the proportion of individuals that share this sequence.

SUPPLEMENTARY FIGURE 2
The heterogeneity of Vδ2 TCRs is determined by CDR3 lengths and *TRDJ* segments. UMAP in colored by **(A)** J-usage and **(B)** CDR3aa length. **(C)** Contour plot visualization on the density distribution of TCRs on the UMAP, split by age groups. **(D)** Boxplot of CDR3aa sequence length for different age groups by different J gene usage. Adjusted *P*-values calculated from Tukey's HSD test after one-way ANOVA are shown

between groups. **(E,F)** Logomap for length = 17 *TRDJ1* (left) length = 19 *TRDJ3* (right) Vδ2$^+$ TCRs in **(E)** different age groups and **(F)** different publicity groups.

SUPPLEMENTARY FIGURE 3

Sequence length and N insertion exploration for CDR3 sequences in different publicity groups. **(A)** N-insertion of Vδ2$^+$ TCRs. Each point stands for a CDR3 nucleotide sequence. The distribution is summarized by a box plot. (**B**) CDR3 aa length of Vδ2$^+$ TCRs with different J gene usages and different publicity groups. (**A,B**) P-values calculated from one-way ANOVA are shown. (**C**) Venn plots show the overlap of public Vδ2$^+$ clones between CB, infant, and adult groups. The sizes of ellipses correlate to the number of unique clones. (**D**) CDR3 aa length of Vδ2$^+$ TCRs from CB and adults with different J gene usages and publicity sharing groups. Each point stands for a CDR3aa sequence.

SUPPLEMENTARY FIGURE 4

Public clones have greater expansion status compared to private clones. **(A)** The frequency distribution for the CDR3aa sequences in different individuals, from left to right, shows three representative individuals. The low/high frequency label was defined within each individual using the corresponding 75th percentile number of the frequency as a threshold. **(B)** Expansion potential for each publicity group according to different J gene usage. Games-Howell *Post-Hoc* Test was used to test the mean difference between groups. Adjusted P-values are shown between groups. **(C)** Expansion potential for *TRDJ1* sequences with different length. **(D)** Expansion potential for *TRDJ3* sequences with different length.

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COVID-19 vaccination influences subtypes of γδ-T cells during pregnancy

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Up to now, there has been insufficient clinical data to support the safety and effects of vaccination on pregnancy post COVID-19 vaccination. The γδ-T cells are considered an important component in the immune system to fight against viral infection and exhibit critical roles throughout the pregnancy period. However, the immunological roles of γδ-T cells in pregnant women with the COVID-19 vaccination remain unclear. Therefore, the objective of this study is to investigate the alteration of frequency and expression pattern of activation receptors and inhibitory receptors in γδ-T cell and its subsets in peripheral blood samples collected from non-pregnant vaccinated women, vaccinated pregnant women, and unvaccinated pregnant women. Our findings indicated that the frequency of $CD3^+γδ\text{-}T^+$ cells is lower in vaccinated pregnant women than in unvaccinated pregnant women. But no significant difference was found in the frequency of $CD3^+γδ\text{-}T^+$ cells between non-pregnant vaccinated women and vaccinated pregnant women. In addition, there were no significant differences in the frequencies of $CD3^+γδ\text{-}T^+Vδ1^+T$ cells, $CD3^+γδ\text{-}T^+Vδ2^+T$ cells, $CD3^+γδ\text{-}T^+Vδ1^-Vδ2^-T$ cells, and $Vδ1^+T$ cell/$Vδ2^+T$ cell ratio between the pregnant women with or without COVID-19 vaccination. Similar results were found after comparing non-pregnant and pregnant women who received the COVID-19 vaccine. However, there was a significant difference in the fraction of $Vδ1^-Vδ2^-T$ cells in $CD3^+γδ\text{-}T^+$ cells between non-pregnant vaccinated women and vaccinated pregnant women. The frequency of $NKG2D^+$ cells in $Vδ2^+T$ cells was not significantly different in the vaccinated pregnant women when compared to that in unvaccinated pregnant women or non-pregnant vaccinated women. But the percentage of $NKG2D^+$ cells in $Vδ1^+T$ cells was the lowest in pregnant women after COVID-19 vaccination. Furthermore, down-regulation of NKP46 and NKP30 were found in $Vδ2^+T$ and

Vδ1$^+$T cells in the vaccinated pregnant women, respectively. After the vaccination, up-regulation of PD-1 expression in Vδ1$^+$T cells and Vδ2$^+$T cells indicated γδ-T cells could respond to COVID-19 vaccination and display an exhausted phenotype following activation. In conclusion, COVID-19 vaccination influences subtypes of γδ-T cells during pregnancy, but the side effects might be limited. The phenotypical changes of Vδ1$^+$T cells and Vδ2$^+$T cells will be a promising predictor for evaluating the clinical outcome of the COVID-19 vaccine.

KEYWORDS

COVID-19, vaccination, pregnancy, γδ-T cells, Vδ1$^+$ T cells, Vδ2$^+$ T cells

Introduction

Due to a lack of clinical and scientific knowledge, the challenge of the Coronavirus Disease 2019 (COVID-19) pandemic has exposed the limitations of our understanding about severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as well as the immune response during viral infection and vaccination (1). Pregnant women especially are at increased risk of severe illness from COVID-19. There have been a few studies that demonstrated the efficacy and safety of COVID-19 vaccines in pregnant women, who have been excluded from the clinical trials because of the ethical issues (2). However, we still struggle with a complete understanding about whether vaccination could modulate the immune response of pregnant women (3). During normal human pregnancy, γδ-T cells have been reported to play a role in this process (4).

Human γδ T cells can be divided into three subgroups, Vδ1, Vδ2 and Vδ3, according to the structural differences of γ and δ chains. Although γδ-T cells represent only a small fraction of T lymphocytes (1-10%) (5), they have a variety of immune functions, such as resistance to virus infection (6), inflammatory regulation, tissue homeostasis (6), helping B cells to produce antibodies (7), and even maintenance of successful pregnancy (6). Some studies reported that, during normal human pregnancy, γδ-T cells secrete some anti-inflammatory cytokines to reduce nature killer activity (8, 9).Indeed, γδ-T cells regulate the release of inflammatory factors such as IFN-γ,TNF-α, granzyme A/B, and perforin by regulating the expression of surface-activated receptors such as NKG2D, NKp30, NKP46, and the inhibitory receptor PD-1 to regulate their cytotoxic functions (10–13). In addition, low cytotoxic activity of γδ-T cells is necessary during normal pregnancy (14). Most importantly, the imbalance between Vδ1$^+$ T cells and Vδ2$^+$ T cells was observed in adverse pregnancy (15).

Therefore, in this study, we aimed to interrogate the alternation of subtypes of γδ-T cells in pregnant women after COVID-19 vaccination, which could provide a better understanding of the role of γδ-T cells in pregnant women after COVID-19 vaccination, and in turn can further help us to monitor and evaluate the safety and efficacy of COVID-19 vaccines during the pregnancy period.

Materials and methods

Study population

The research objects were selected from pregnant women vaccinated against COVID-19 who went to Tianhe Campus of the Third Affiliated Hospital of Sun Yat-sen University in Guangzhou, China from August 2021 to February 2022. These women did not confirm their pregnancies until after they had received the COVID-19 vaccine. The inclusion criteria included: (1) age of 18-35 years; (2) singleton pregnancy; (3) the onset of pregnancy as calculated by crown-rump length (CRL) on NT ultrasound, with at least one dose of vaccine after pregnancy; and (4) signing an informed consent form, providing vaccination information and confirming their participation in the study. The exclusion criteria included: (1) the gestational age of the documented prenatal examination is not 11-13+6 weeks; (2) other vaccines have been received within 1 year, such as HPV vaccine and hepatitis B vaccine; or (3) complications with basic diseases, requiring long-term medication. The peripheral blood of the patients enrolled in this study was collected to perform immunological assays. The study included 27 vaccinated pregnant women, 11 unvaccinated pregnant women, and 20 non-pregnant vaccinated women. The clinical and demographic characteristics and vaccination scheme of pregnant women were described in Tables 1, 2, respectively. This study was approved

TABLE 1 Basic characteristics of vaccinated and unvaccinated pregnant women.

Groups	Non-pregnant vaccinated group	Un-vaccinated group	Vaccinated group
Included	20	11	27
Age (median and range)	24 (23-35)	29 (26-33)	29 (28-32)
Previous history of SARS-CoV-2 infection	No	No	No
Hypertension	No	No	No
Diabetes	No	No	No
Gestational age of laboratory tests (weeks, median and range)	No	12.4 (12.3-12.7)	12.9 (12.6-13.3)
NT (mm, mean and standard deviation)	No	1.4 (0.4)	1.4 (0.4)
Gestational age of laboratory tests (weeks, median and range)	No	12.4 (12.3-12.7)	12.9 (12.6-13.3)

Gestational age is equal to the number of gestational days divided by 7.

by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University in Guangzhou.

Immune cell phenotype analyzing by flow cytometry

Collected peripheral blood samples were analyzed using flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque centrifugation, then stained by the following antibodies: anti-human CD3-APC-H7 (BD biosciences, clone: SK7), anti-human TCR γδ-BV421 (BD biosciences, clone: 11F2), anti-human PD-1-BB515 (BD biosciences, clone: EH12.1), anti-human NKP46-BV510 (BD biosciences, clone: 9E2/NKP46), anti-human NKP30-Alexa Fluor®647 (BD biosciences, clone: P30-15), anti-human NKG2D-PE-Cy™7 (BD biosciences, clone: 1D11), anti-human TCR Vδ2-PE (BD biosciences, clone: B6), and anti-human TCR Vδ1-PerCP-Vio700 (Miltenyi Biotec, clone: REA173). Data was analyzed using FlowJo 10.1 software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc.). All results are expressed as the mean ± SEM (standard error of the mean). To analyze the difference in γδ T cells and its subsets between vaccinated women and unvaccinated women, Mann-Whitney U tests were performed.

Results

The frequencies of γδ T cell and its subsets in pregnant women after COVID-19 vaccination

The aim of this study was to investigate whether COVID-19 vaccination influences the distribution of total γδ T cells, $V\delta1^+$T cells, $V\delta2^+$T cells, and $V\delta1^-V\delta2^-$T cells in the peripheral blood samples of 58 individuals enrolled in this study. Total γδ T cell was identified among $CD3^+$ lymphocytes using flow cytometry (Figures 1A, B). The results indicated that frequency of total γδ T cells was markedly higher in unvaccinated pregnant women compared to vaccinated pregnant women, but there was no significant difference between non-pregnant vaccinated women and vaccinated pregnant women. Furthermore, to investigate the impact of COVID-19 vaccination on frequencies of γδ T cells subsets, we next compared $V\delta2^+$T cells, $V\delta1^+$T cells, and $V\delta1^-V\delta2^-$ T cells' frequencies as well as Vδ1/Vδ2 ratio in non-pregnant vaccinated women, vaccinated pregnant women, and unvaccinated pregnant women. The results indicated that there were no significant differences in frequencies of $V\delta1^+$T, $V\delta2^+$T cells, and $V\delta1^-V\delta2^-$ T cells in $CD3^+\gamma\delta^+$ T cells between vaccinated pregnant women and unvaccinated pregnant women (Figures 1C, E). However, compared with non-pregnant vaccinated women, the proportion of $V\delta1^-V\delta2^-$ T cells in $CD3^+\gamma\delta^+$ T was significantly reduced in vaccinated pregnant women (Figure 1D). Additionally, Vδ1/Vδ2 ratio was similar in these women with or without vaccination (Figure 1D).

Phenotypical changes of $V\delta2^+$T cells in vaccinated pregnant women

It is well known that the activating and inhibitory receptors define the degree of immune cell maturation and responsiveness to stimuli, so we next investigated the frequencies of $NKG2D^+$, $NKp30^+$, $NKp46^+$, and $PD\text{-}1^+$ cells in $V\delta2^+$T cells. We found no significant difference in the proportions of $NKG2D^+$ $V\delta2^+$T cells among these three groups (Figure 2A). γδ T cell subsets usually express activating natural killer (NK) receptors, such as NKp30 and NKp46, which are involved in regulating immunological functions of γδ T cell and its subsets. There was no significant difference in percentage of $NKp30^+V\delta2^+$T cells between vaccinated pregnant women and unvaccinated pregnant women (Figure 2B). In addition, a similar result was found in comparison of vaccinated pregnant women and non-pregnant vaccinated women (Figure 2B). By contrast, there was no

TABLE 2 Scheme of vaccination of vaccinated pregnant women.

Vaccinated pregnant women	Pregnancy time	Date of laboratory tests	Scheme of vaccination	
			Date of 1st dose	Date of 2nd dose
Vg-1	2021/05/30	2021/08/27	2021/07/27	–
Vg-2	2021/06/03	2021/08/27	2021/06/12	–
Vg-3	2021/05/31	2021/08/27	2021/05/22	2021/06/29
Vg-4	2021/05/25	2021/08/30	2021/05/23	2021/06/22
Vg-5	2021/06/08	2021/08/30	2021/06/13	2021/04/30
Vg-6	2021/05/30	2021/08/30	2021/06/10	2021/07/13
Vg-7	2021/05/29	2021/08/30	2021/06/23	–
Vg-8	2021/05/29	2021/08/30	2021/06/02	2021/05/04
Vg-9	2021/06/04	2021/09/01	2021/07/01	–
Vg-10	2021/05/31	2021/09/01	2021/04/14	2021/06/07
Vg-11	2021/06/04	2021/09/02	2021/05/29	2021/06/26
Vg-12	2021/06/06	2021/09/06	2021/05/10	2021/06/09
Vg-13	2021/06/13	2021/09/10	2021/05/06	2021/06/16
Vg-14	2021/06/06	2021/09/10	2021/05/09	2021/06/12
Vg-15	2021/06/16	2021/09/15	2021/07/16	–
Vg-16	2021/06/25	2021/09/18	2021/05/29	2021/06/25
Vg-17	2021/06/26	2021/09/22	2021/04/22	2021/07/08
Vg-18	2021/06/28	2021/09/26	2021/06/23	2021/07/19
Vg-19	2021/06/26	2021/09/27	2021/06/28	2021/07/20
Vg-20	2021/06/26	2021/09/27	2021/05/30	2021/06/26
Vg-21	2021/06/24	2021/09/27	2021/05/29	2021/06/29
Vg-22	2021/07/15	2021/10/12	2021/07/11	2021/08/07
Vg-23	2021/07/24	2021/10/12	2021/07/25	2021/08/24
Vg-24	2021/07/06	2021/10/11	2021/07/19	–
Vg-25	2021/07/23	2021/10/14	2021/06/29	2021/07/21
Vg-26	2021/07/20	2021/10/15	2021/06/20	2021/07/28
Vg-27	2021/07/17	2021/10/19	2021/07/20	–
Np-1	–	–	2021/04/22	2021/05/27
Np-2	–	–	2021/04/22	2021/05/25
Np-3	–	–	2021/04/01	2021/05/25
Np-4	–	–	2021/04/08	2021/05/07
Np-5	–	–	2021/04/24	2021/05/11
Np-6	–	–	2021/04/02	2021/05/31
Np-7	–	–	2021/04/02	2021/04/30
Np-8	–	–	2021/04/02	2021/04/30
Np-9	–	–	2021/04/02	2021/04/30
Np-10	–	–	2021/04/02	2021/04/30
Np-11	–	–	2021/04/02	2021/04/30
Np-12	–	–	2021/04/02	2021/04/30
Np-13	–	–	2021/04/02	2021/04/30
Np-14	–	–	2021/04/02	2021/04/30
Np-15	–	–	2021/04/02	2021/04/30
Np-16	–	–	2021/03/11	2021/04/12
Np-17	–	–	2021/03/11	2021/04/12
Np-18	–	–	2021/03/11	2021/04/12
Np-19	–	–	2021/03/11	2021/04/12
Np-20	–	–	2021/03/11	2021/04/12

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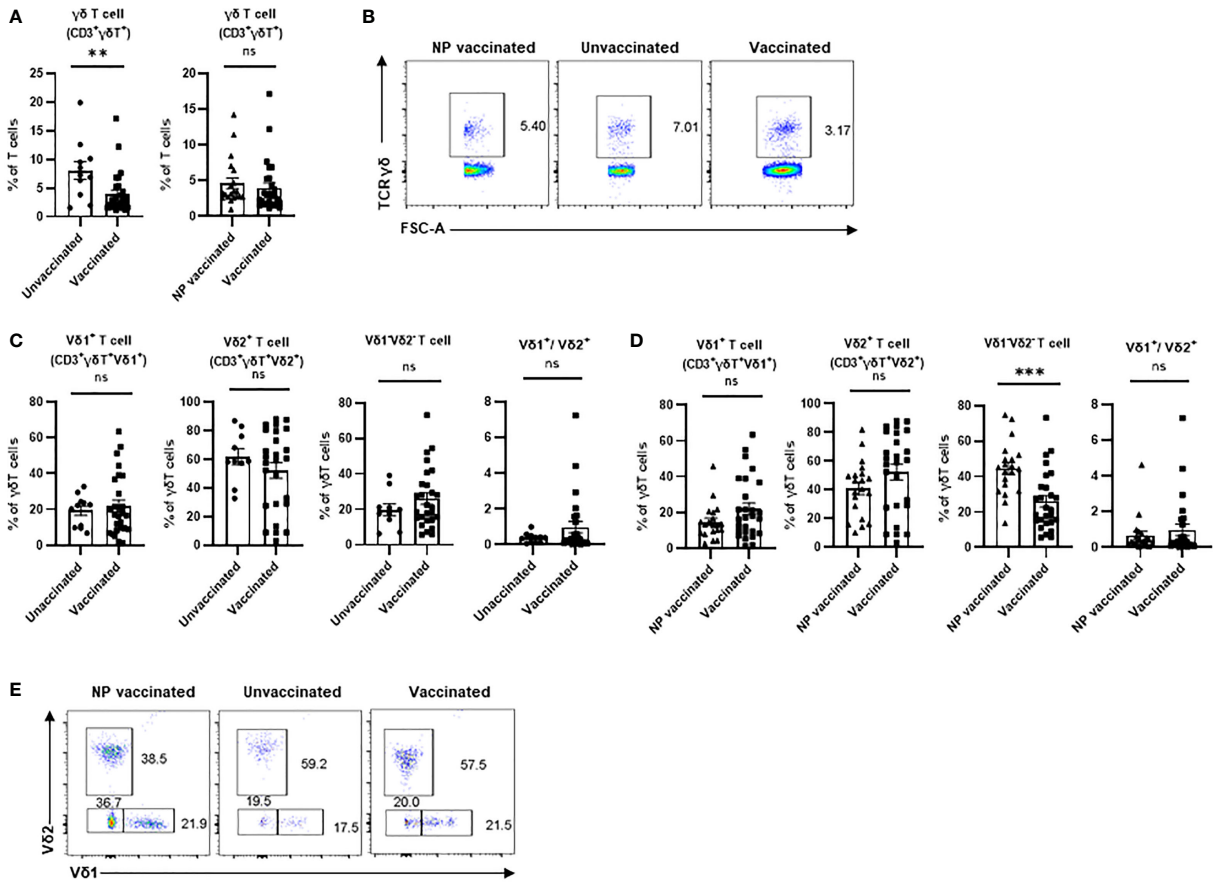

FIGURE 1
Peripheral γδ T cells and their subsets in non-pregnant vaccinated women, vaccinated, and unvaccinated pregnant women. Statistical comparison of γδ T cell proportions in $CD3^+$ T cells **(A, B)** Typical flow cytometry plots and gating for a $CD3^+$TCR $γδ^+$;$Vδ1^+$, $Vδ2^+$, and $Vδ1^-Vδ2^-$ subsets in γδ T cells **(C, D)**, and the $Vδ1^+/Vδ2^+$ ratio between the vaccinated and unvaccinated pregnant women. **(E)** Gate from TCR $γδ^+$ cells,$Vδ1^+$, $Vδ2^+$, and $Vδ1^-Vδ2^-$ subsets are shown as representative flow cytometry plots. ns, no significance; **P < 0.01. ***P < 0.001. (NP vaccinated, non-pregnant vaccinated).

significant difference in the percentage of $NKp46^+Vδ2^+$T cells between vaccinated pregnant women and unvaccinated pregnant women. But compared with non-pregnant vaccinated women, the percentage of $NKp46^+Vδ2^+$T cells was significantly decreased in vaccinated pregnant women (Figure 2C). Furthermore, we supposed that γδ T cell would exhibit the exhausted phenotype after vaccination. Actually, the frequency of PD-1^+ $Vδ2^+$T cells was significantly elevated in vaccinated pregnant women compared to unvaccinated pregnant women. However, there was no difference between non-pregnant vaccinated women and vaccinated pregnant women (Figure 2D).

Phenotypical changes of $Vδ1^+$T cells in vaccinated pregnant women

To investigate the alteration of expression pattern of activating and inhibitory receptors in $Vδ1^+$T cells after COVID-19 vaccination in the pregnant women, the frequencies of $NKG2D^+$, $NKp30^+$, $NKp46^+$, and PD-1^+ cells in $Vδ2^+$T cells were determined. Unsimilar to $Vδ2^+$T cells, the frequencies of $NKG2D^+$ and $NKp30^+$ cells in $Vδ1^+$ cells were much lower in vaccinated pregnant women than that in unvaccinated pregnant women (Figures 3A, B). In addition, a significant difference in the frequency of $NKG2D^+Vδ1^+$ cells was found between non-pregnant vaccinated women and vaccinated pregnant women. Similar to $Vδ2^+$T cells, the frequency of $NKP46^+$ cells were no different in all three groups (Figure 3C). Finally, PD-1^+ $Vδ1^+$T cells were significantly increased in the pregnant women after vaccination (Figure 3D).

Discussion

Our study demonstrated that frequencies of total γδ T cell ($CD3^+γδ^+$ T cells) was significantly decreased in the pregnant

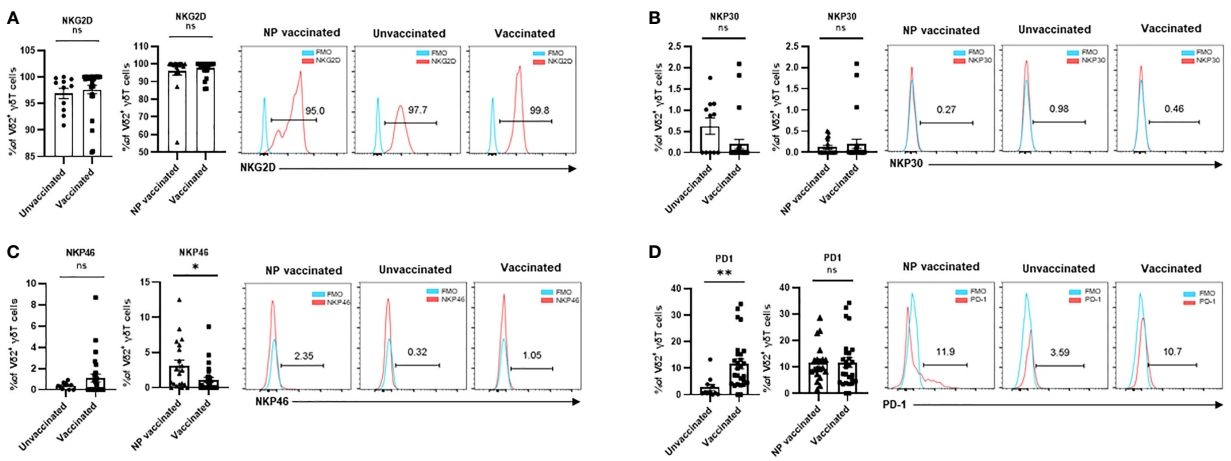

FIGURE 2
Expressions of crucial molecular of Vδ2$^+$ γδ T cells in non-pregnant vaccinated women, vaccinated, and unvaccinated pregnant women. Comparable analysis of expressions of NKG2D **(A)**, NKP30 **(B)**,NKP46 **(C)** and PD-1 **(D)** receptors of Vδ2$^+$ γδ T cells acquired by flow cytometry. ns, no significance; *P < 0.05, **P < 0.01.

women with COVID-19 vaccination compared to that in the pregnant women without COVID-19 vaccination. Furthermore, analysis of the frequencies of Vδ1$^+$T cells and Vδ2$^+$T cells in total γδ T cells indicated that there were no significant differences between vaccinated pregnant women and unvaccinated pregnant women. Importantly, no significant difference was found on the Vδ1/Vδ2 ratio between these two groups of women who were with or without COVID-19 vaccination. For evaluation of activated and exhausted phenotypes in of Vδ1$^+$T cells and Vδ2$^+$T cells after COVID-19 vaccination in the pregnant women, the frequencies of NKG2D$^+$, NKp30$^+$, NKp46$^+$, and PD-1$^+$ cells in these two subsets were analyzed. Our findings suggested that Vδ1$^+$T cells and Vδ2$^+$T cells developed an exhausted phenotype post COVID-19 vaccination. These results demonstrate that COVID-19 vaccination exhibits a certain degree of influence on the frequency of total γδ T cell and alteration of phenotype of Vδ1$^+$T cells and Vδ2$^+$T cells. However, the Vδ1/Vδ2 ratio is similar between vaccinated pregnant women and unvaccinated pregnant women, which indicates that vaccination did not break the important balance between these two main subsets of peripheral γδ T cells. Taken together, the results suggest

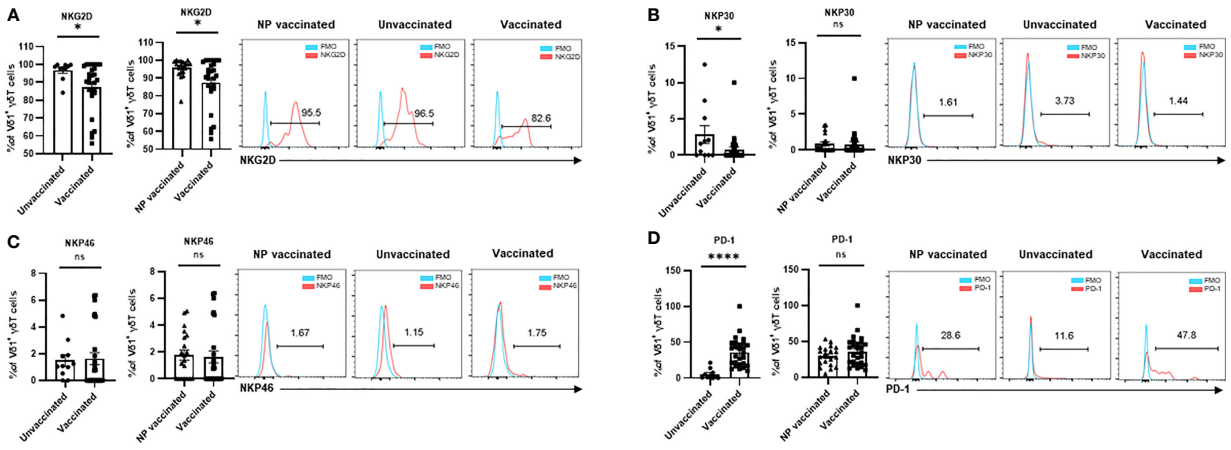

FIGURE 3
Expressions of crucial molecular of Vδ1$^+$ γδ T cells in non-pregnant vaccinated women, vaccinated, and unvaccinated pregnant women. Comparable analysis of expressions of NKG2D **(A)**, NKP30 **(B)**,NKP4 6 **(C)**, and PD-1 **(D)** receptors of Vδ1$^+$ γδ T cells acquired by flow cytometry. ns, no significance; *P < 0.01; ****P < 0.001.

COVID-19 vaccination influences subtypes of γδ-T cells without effects on pregnancy.

In this study, we found that the frequency of total γδ-T cells was similar between the pregnant women and non-pregnant women after COVID-19 vaccination (Figure 1A). In addition, there were no significant differences in frequencies of $V\delta1^+$T cells and $V\delta2^+$T cells between these two groups of individuals. A similar result was also found in the $V\delta1^+$T/$V\delta2^+$T ratio (Figure 1C). However, the frequency of $V\delta1^-V\delta2^-$ T cells was much higher in the non-pregnant vaccinated women compared with that in vaccinated pregnant women (Figure 1D). Furthermore, our data indicated that the proportions of $NKP46^+V\delta2^+$T cells and $NKG2D^+V\delta1^+$T cells were lower in the vaccinated pregnant women than that in non-pregnant vaccinated women (Figures 2C, 3A). By contrast, no significant differences were found in the percentages of $NKG2D^+$, $NKP30^+$, and PD-1^+ cells in $V\delta2^+$T cells and $NKP30^+$ and $NKP46^+$ cells in $V\delta1^+$T cells between the non-pregnant vaccinated women and vaccinated pregnant women (Figures 2A, B, D; Figures 3B-D). These data further demonstrated that pregnancy is not a potential influencing factor for our findings, and this will help us to achieve more confident conclusions.

In humans, $V\delta1^+$T cells and $V\delta2^+$T cells are the two major subsets of γδ T cells which are identified by the Vδ chains. $V\delta1^+$T cells constitute the majority of T cells in the thymus and mucosal tissues, and $V\delta2^+$T cells are predominant in the peripheral blood (16). As the main γδ T cell subsets, $V\delta1^+$T cells and $V\delta2^+$T cells exhibit different immunological functions. $V\delta1^+$T cells display regulatory and effector features, and $V\delta2^+$T cells exert a cytotoxic activity targeting pathogenic characteristics. According to previous studies, the frequency of peripheral γδ T cells is higher in women with a successful pregnancy compared to women with pregnancy failure (9). In addition, one study suggested that $V\delta1^+$T cells could produce IL-10 to down-regulate the cytotoxic NK cells during pregnancy. In healthy pregnant women, the predominant subpopulation of peripheral γδ T cells is $V\delta1^+$T cells, whereas $V\delta2^+$T cells is the most frequent subset in women with recurrent miscarriage (16). Therefore, this evidence demonstrated that an imbalance of Vδ1/Vδ2 ratio leads to adverse pregnancy outcome (15, 16). It has been reported that γδ T cells (mostly expressing Vδ2) are able to destruct influenza A virus-infected cells as efficient as $CD8^+$ T cells or NK cells in a polycytotoxic manner and by releasing IFN-γ against infected cells *in vitro* (17). In several contexts, including infection with Mtb, malaria, influenza, and HIV and vaccination with BCG and live attenuated influenza, there are clear patterns of γδ T-cell expansion, particularly of the $V\delta2^+$ subset, in response to both infection and vaccination (18). However, there is no literature report on the effect of other vaccines after pregnancy on γδ T cells' function. In the future, we could detect the difference in γδ T cells' function between other vaccines and the COVID-19 vaccine during pregnancy, so as to explore whether this result is specific to the COVID-19 vaccine. In our study, COVID-19 vaccination did not change Vδ1/Vδ2 ratio in the pregnant women, which indicates the impact of vaccination might not cause an adverse outcome.

NK cell activating receptors, such as NKG2D, NKp30, and NKp46, are widely involved in regulating NK functions during pregnancy (19). Additionally, one recent study suggested that the frequency of $NKG2D^+V\delta2^+$T cells was negatively correlated with a successful clinical pregnancy (8). Our data demonstrate that no significant difference was found on the percentages of $NKG2D^+$ cells in $V\delta1^+$T cells and $V\delta2^+$T cells, which suggest that COVID-19 vaccination did not induce the highly activated peripheral γδ T cells in the pregnant women. Because of the activated γδ T cells, a high level of inflammation is considered as the major cause of abortion (15). Additionally, frequencies of $NKp30^+$ and $NKp46^+$ cells in $V\delta1^+$T and $V\delta2^+$T were largely similar between vaccinated pregnant women and unvaccinated pregnant women. In summary, these data suggested that the immunological functions of γδ T cells were not altered after COVID-19 vaccination in the pregnant women.

Accordingly, many previous studies suggested that exhaustion of γδ T cells is accompanied by a decrease in the frequency of cells in different types of disease. For instance, in acute myeloid leukemia (AML), the data demonstrated that the proportion of total γδ T cells was decreased in AML patients (20). Subsequently, in these γδ T cells, the authors observed increased PD-1 expression and decreased NKG2D expression, indicating highly activated or even exhausted states in the γδ T cells at diagnosis of AML (20). Additionally, in the acute viral infection, a lower frequency of Vδ2+T cells was also observed (21). Moreover, these $V\delta2^+$T cells highly expressed CD95, which in turn could lead to cell apoptosis that induces the loss of cells (21). Interestingly, this work demonstrated that the expression level of CTLA-4 exhaustion marker was elevated during acute viral infection. In line with these previous results, we also found that stimulation of COVID-19 vaccine induces the exhaustion status within the loss of γδ T cells in pregnant women, suggesting the involvement of γδ T cells in the complex network of protective response induced by COVID-19 vaccination. PD-1 expression is induced on activated T cells and is correlated to exhaustion status in anti-infection and anti-tumor responses (22, 23). In our study, we found that the frequency of PD-1^+ cells in $V\delta1^+$T and $V\delta2^+$T were much higher in vaccinated pregnant women compared to unvaccinated pregnant women. We suspect that γδ T cells developed an exhausted phenotype following activation by COVID-19 vaccination. This switch from activation and exhaustion might be the reason why the frequency of γδ T cells was decreased in the pregnant women with COVID-19 vaccination. Since the objective of this study is to investigate whether COVID-19 vaccination influences subtypes of γδ-T cells during pregnancy, the changes of frequency and immunological phenotypes of γδ-T cells were determined in this study. γδ-T cells are well known as having

multiple functions in innate immune cells, suggesting they play important roles in anti-viral infection and immune response to vaccination. The results of our study could be considered as the clues to address the questions about the potential immunological functions of γδ-T cell and its subsets involved in the immune activity associated with COVID-19 vaccination. This deserves further study in the future.

Despite the positives provided by this study, there are still some limitations. First, a further larger sample size study is warranted to validate these findings. And the effects of age, vaccination scheme, vaccine types, and other factors in impacting the immunological features of γδ T cell should be further evaluated in pregnant women after COVID-19 vaccination. Second, in this study, we only determined the phenotypic changes of γδ T cell subsets in the peripheral blood of individuals. The functional capacity of γδ T cell and its subpopulations are rarely investigated in COVID-19 vaccination and should be determined in the future. Third, we found the phenotypic changes of γδ T cell subsets by comparing the data collected in the pregnant women with or without COVID-19 vaccination. In the future, using samples from each vaccinated individual at different time points in the vaccination scheme will further help us to clarify the significance of functional alterations of γδ T cell and its subsets in establishing protective immunity against COVID-19 infection after vaccination.

Taken together, our study suggests that γδ T cell and its subsets could respond to COVID-19 vaccination and display an exhausted phenotype following activation. In addition, COVID-19 vaccination influences subtypes of γδ-T cells during pregnancy, but the side effects are limited. Last but not least, the contribution of γδ T cell and its subsets to the immunology of COVID-19 vaccination needs to be further investigated.

Data availability statements

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University in Guangzhou, PR China. The participants provided their written informed consent to participate in this study. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CX and ZY designed the study; YX and ZX designed the study, performed the data analysis, and wrote the manuscript. LW and SJ enrolled the subjects and collected the peripheral blood, performed the experiments, and assisted in the preparation of the figures. JL, YL, and RG performed the data analysis and plotted the graphs. YYC, YC, HY, QQ, and MZ collected the peripheral blood and performed the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author JL was employed by the company Guangzhou PuruiBiotechnology Co., Ltd, Guangdong, China.

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

Publisher's note

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