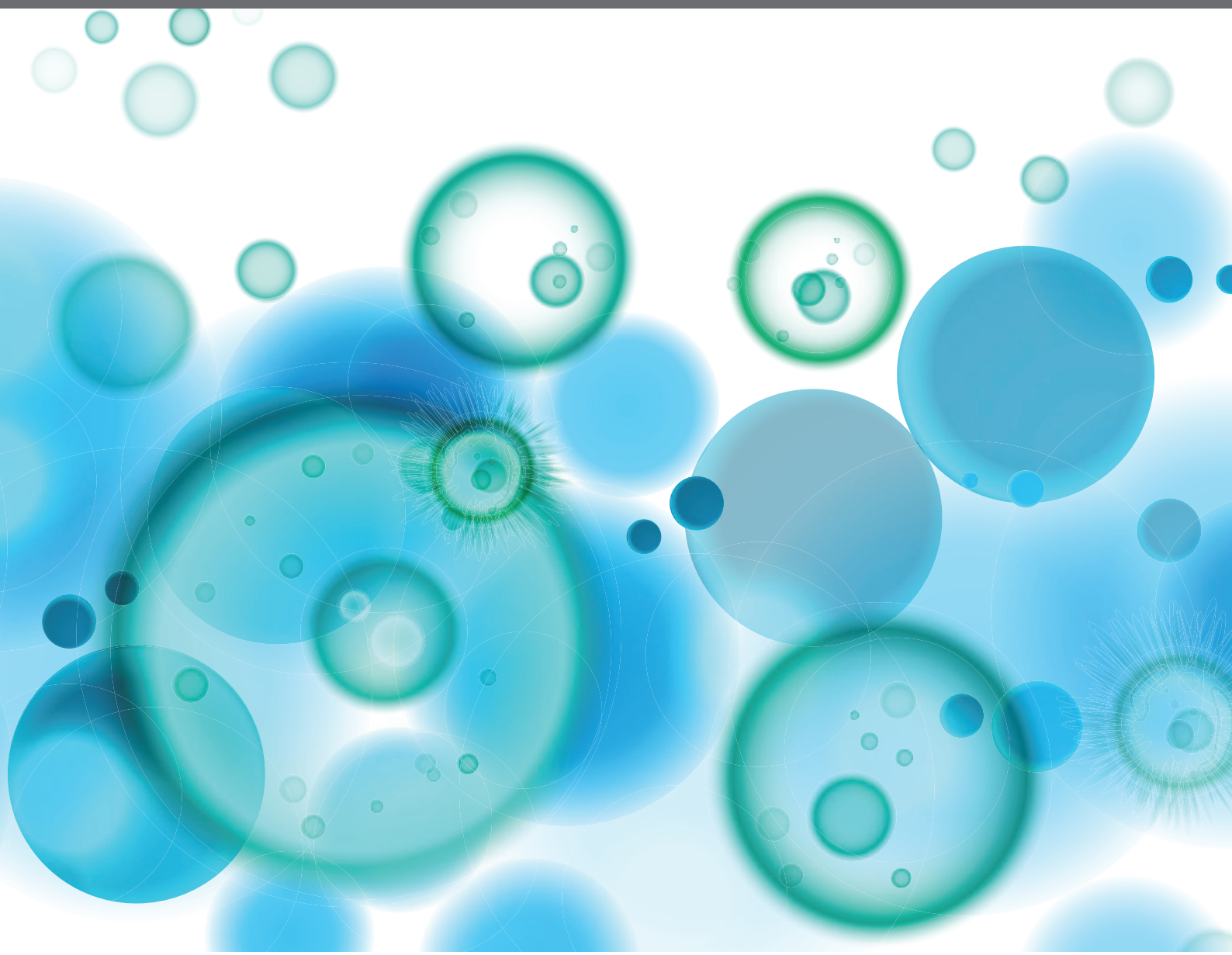
NEXT GENERATION γδ T CELL-BASED TUMOR IMMUNOTHERAPY

EDITED BY: Andy Hee-Meng Tan, Alice Cheung and Dieter Kabelitz
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-502-1
DOI 10.3389/978-2-83250-502-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

NEXT GENERATION γδ T CELL-BASED TUMOR IMMUNOTHERAPY

Topic Editors:
Andy Hee-Meng Tan, Bioprocessing Technology Institute (A*STAR), Singapore
Alice Cheung, Singapore General Hospital, Singapore
Dieter Kabelitz, University of Kiel, Germany

Citation: Tan, A. H.-M., Cheung, A., Kabelitz, D., eds. (2022). Next Generation γδ T Cell-Based Tumor Immunotherapy. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-502-1

Table of Contents

frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 30 September 2022
DOI 10.3389/fimmu.2022.1041362

OPEN ACCESS

EDITED AND REVIEWED BY
Katy Rezvani,
University of Texas MD Anderson Cancer Center, United States

*CORRESPONDENCE
Andy Hee-Meng Tan
andy_tan@bti.a-star.edu.sg
Alice Man Sze Cheung
alice.cheung@duke-nus.edu.sg
Dieter Kabelitz
Dietrich.Kabelitz@uksh.de

SPECIALTY SECTION
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

RECEIVED 10 September 2022
ACCEPTED 20 September 2022
PUBLISHED 30 September 2022

CITATION
Tan AH-M, Cheung AMS and Kabelitz D (2022) Editorial: Next generation γδ T cell-based tumor immunotherapy. *Front. Immunol.* 13:1041362. doi: 10.3389/fimmu.2022.1041362

Editorial: Next generation γδ T cell-based tumor immunotherapy

Andy Hee-Meng Tan[1]*, Alice Man Sze Cheung[2,3]* and Dieter Kabelitz[4]*

[1]Immune Cell Manufacturing, Bioprocessing Technology Institute Agency for Science, Technology and Research (ASTAR), Singapore, Singapore, [2]Department of Hematology, Singapore General Hospital, Singapore, Singapore, [3]SingHealth Duke-National University of Singapore (NUS) Medicine Academic Clinical Programme, Singapore, Singapore, [4]Institute of Immunology, University of Kiel, Kiel, Germany

KEYWORDS

gamma delta (γδ) T cells, tumor immunotherapy, butyrophilins, cytokine programming, gamma delta TCR repertoire, cost-effective manufacturing of gamma delta t cell therapies, chimeric antigen receptor-engineered gamma delta T cells, phosphoantigens

Editorial on the Research Topic
Next generation γδ T cell-based tumor immunotherapy

The discovery of the γδ T-cell receptor (TCR) and its ability to confer potent cytotoxic activity in CD3+ cells some 35 years ago sparked the initial proliferation in research that garnered widespread interest in the biology and function of γδ T cells. The identification of major human γδ TCR clonotypes, their tissue distributions as well as dynamic changes throughout ontogeny and in disease states have contributed to the appreciation of human γδ T cell diversity. Despite this, incomplete understanding of mechanisms underlying the complexity of various γδ T cell subsets in homeostasis, inflammation and malignancy restricted the focus of most early studies to blood circulating Vγ9Vδ2 T cells which could be robustly activated and expanded *ex vivo* using aminobisphosphonates such as zoledronate compared with other γδ T cell subsets for which activating ligands were then largely unknown. This galvanized attempts to harness the tumoricidal potential of Vγ9Vδ2 T cells in clinical trials. Although these cells exhibited highly promising safety profiles in patients, early trial data revealed suboptimal anti-tumor efficacy. Despite these setbacks, recent breakthroughs in deciphering the unique antigen (Ag) binding modes of γδ TCR coupled with high dimensional analyses of tissue- and disease-specific γδ T cell subsets at single cell resolution led to renewed excitement in the development of γδ T cell-based therapeutics. This Research Topic has compiled a series of nine articles of which five review our hitherto understanding of the multifaceted nature of γδ T cells and four report original research providing new insights into the molecular and cellular regulation of a diverse repertoire of γδ T cells, paving the way for next generation γδ T cell-based tumor immunotherapies.

Despite extensive use of phosphoantigens (pAgs) to activate and expand Vγ9+Vδ2+ T cells, the role of butyrophilins (BTNs) in mediating Vγ9Vδ2 TCR-dependent activation is only gaining recent appreciation. Expression of BTN3A1 and BTN2A1 heterodimers, in complex with pAgs, on the surface of accessory innate immune, infected or tumor cells is an absolute requirement for interaction with the Vγ9Vδ2 TCR. Other BTN and BTN-like (BTNL) molecules are involved in the ontogeny and homeostasis of non-Vγ9+Vδ2+ T cell subtypes as noted by Herrmann and Karunakaran. Their review also emphasized dissection of BTN-associated mechanisms that enhance the effector function to broaden the therapeutic applications of γδ T cells in disease settings. Extending the role of TCRδ complementarity-determining region 3 (CDR3δ) in Vγ9Vδ2 TCR-mediated responses, Vyborova et al. adduced evidence for CDR3δ determinants in pAg sensing that are significantly correlated with Vγ9Vδ2 TCR affinity and signal strength, contributing to Vγ9Vδ2 T cell repertoire focusing. Furthermore, surface expression of the inhibitory natural killer receptor (NKR) CD94/NKG2A is biased toward while that of activating NKR NKG2D is independent of these CDR3δ traits, findings which may impact design of high-affinity Vγ9Vδ2 TCR-based therapies.

γδ T cells manifest substantial plasticity and can be polarised to different cell states in response to environmental stimuli. It is thus not uncommon to identify γδ T cells with opposing functional properties in different biological contexts. The mini review by Bhat et al. provides a comprehensive summary of studies highlighting the dichotomous nature of γδ T cells influenced by diverse pathological milieux, leading to beneficial or detrimental outcomes in the host. For example, IL-17 production by γδ T cells has been shown to aggravate autoimmune conditions (1, 2) and also be associated with immunosuppression that exacerbate tumour growth (3, 4). Such phenomena attributable to the varied cellular interactions of γδ T cells can be addressed by appropriate considerations of systems immunology and personalized approaches. In this regard, Chan et al. provided an updated account of crosstalk between γδ T cells and a variety of immune cells which collectively coordinates anti-tumor responses. Additionally, the cytokine milieu plays a major role in shaping the fate commitment of γδ T cells. Consistent with this, Song et al. discussed how cytokines and their combinations differentially direct the polarization of tissue-resident and tumour-infiltrating γδ T cells. Such experimental insights will inform strategies to manipulate the cytokine dependencies of γδ T cells to improve the cytotoxic function and *in vivo* persistence of administered T cells against tumors. Pei et al. elegantly showed that CD137 costimulation of Vγ9+Vδ2+ T cells using CD137L agonist diminished IL-10 receptor expression and alleviated exhaustion in these cells by mitigating the immunosuppressive tumor microenvironment (TME) mediated by IL-10. This increased Vγ9+Vδ2+ T cell efficacy against Epstein Barr virus (EBV)-transformed B lymphoma in a humanized mouse model. Hu et al. examined the immunosuppressive TME of hepatocellular carcinoma (HCC) which the authors showed close correlation with high expression of inhibitory checkpoint molecules, presence of tumor-promoting immune cells and various types of programmed cell death. This is associated with γδ T cell subtype imbalance characterized by selective depletion of cytotoxic Vδ2+ T cells and enrichment of Treg-like Vδ1+ T cells as well as poorer patient prognosis. These studies underscore the importance of performing greater in-depth analyses of the cellular networks and interplay of cytokines in the TME to accelerate the clinical translation of γδ T cell therapies.

Over the years, translating γδ T cell therapies from bench to bedside has encountered tremendous challenges. Saura-Esteller et al. expertly summarized the evolution of γδ T cell therapeutic strategies assessed in clinical trials that signifies past progress in γδ T cell research. With a growing list of companies developing γδ T cell-based or engaging therapies, the authors noted the high cost of manufacturing γδ T cell-based products due in part to requirement for multiple cytokines and prolonged *ex vivo* expansion process. Exemplifying continual efforts to reduce γδ T cell production cost, Ferry et al. described a one-step protocol utilizing a single cytokine to expand Vδ1+ T cells. In addition, the authors demonstrated that these cells were amenable to high efficiency transduction of chimeric antigen receptors (CARs). Going forward, development of other ways that harness the anti-tumor potency of γδ T cells, namely bispecific γδ T cell engagers and advanced γδ T cell genome-editing, is expected to widen the immuno-oncological applications of these cells.

Author contributions

AT and AC discussed findings of various articles of the Research Topic in broader context of tumor immunotherapy. AT collated contributions and prepared final version of editorial by taking into account suggestions from AC and DK.

Funding

This work is supported by the core funds of BTI, A*STAR and Industry Alignment Fund-Industry Collaboration Projects (IAF-ICP) grant ID I1801E0037 awarded to AH-MT. AMSC is supported by National Medical Research Council (NMRC) of Singapore-Centre Grant NMRC/CG21APR2002.

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

References

1. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KHG. Interleukin-1 and IL-23 induce innate IL-17 production from γδ T cells, amplifying Th17 responses and autoimmunity. *Immunity* (2009) 31(2):331–41. doi: 10.1016/j.immuni.2009.08.001

2. Petermann F, Rothhammer V, Claussen MC, Haas JD, Blanco LR, Heink S, et al. γδ T cells enhance autoimmunity by restraining regulatory T cell responses *via* an interleukin-23-Dependent mechanism. *Immunity* (2010) 33(3):351–63. doi: 10.1016/j.immuni.2010.08.013

3. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity* (2014) 40(5):785–800. doi: 10.1016/j.immuni.2014.03.013

4. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang R-F. Tumor-infiltrating γδ T cells suppress T and dendritic cell function *via* mechanisms controlled by a unique toll-like receptor signaling pathway. *Immunity* (2007) 27 (2):334–48. doi: 10.1016/j.immuni.2007.05.020

frontiers in Immunology

MINI REVIEW
published: 17 March 2022
doi: 10.3389/fimmu.2022.876493

Butyrophilins: γδ T Cell Receptor Ligands, Immunomodulators and More

Thomas Herrmann * *and Mohindar M. Karunakaran*

Institute for Virology and Immunobiology, Julius Maximilians Universität Würzburg, Würzburg, Germany

Butyrophilins (BTN) are relatives of the B7 family (e.g., CD80, PD-L1). They fulfill a wide range of functions including immunomodulation and bind to various receptors such as the γδ T cell receptor (γδTCR) and small molecules. One intensively studied molecule is BTN3A1, which binds *via* its cytoplasmic B30.2 domain, metabolites of isoprenoid synthesis, designated as phosphoantigen (PAg), The enrichment of PAgs in tumors or infected cells is sensed by Vγ9Vδ2 T cells, leading to the proliferation and execution of effector functions to remove these cells. This article discusses the contribution of BTNs, the related BTNL molecules and SKINT1 to the development, activation, and homeostasis of γδ T cells and their immunomodulatory potential, which makes them interesting targets for therapeutic intervention.

Keywords: butyrophilin, immune therapy, T cell receptor, γδ T cell, BTN3A1, BTN2A1, phosphoantigen, tumor

OPEN ACCESS

Edited by:
Dieter Kabelitz,
University of Kiel, Germany

Reviewed by:
Jean Jacques Fournie,
INSERM U1037 Centre de Recherche en Cancérologie de Toulouse, France
David Vermijlen,
Université libre de Bruxelles, Belgium

***Correspondence:**
Thomas Herrmann
herrmann-t@vim.uni-wuerzburg.de

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *15 February 2022*
Accepted: *23 February 2022*
Published: *17 March 2022*

Citation:
Herrmann T and Karunakaran MM (2022) Butyrophilins: γδ T Cell Receptor Ligands, Immunomodulators and More. Front. Immunol. 13:876493. doi: 10.3389/fimmu.2022.876493

WHAT ARE BUTYROPHILINS?

Eponymous for the butyrophilin family is butyrophilin 1A1 (BTN1A1 in humans, Btn1a1 in mice). It controls the fat content of milk and is found in membranes of milk-secreting mammary gland epithelial cells and fat droplets (1). Interestingly, it is also expressed in the thymic epithelium (2). The extracellular domains of BTN1A1 and other members of the BTN-family share structural features with the B7 family (**Figure 1**) (5). Its cytoplasmic region contains a juxtamembrane coiled-coiled domain and a B30.2 domain. B30.2 domains are also found in many members of the "tripartite-motif" family (TRIM), known to be involved in innate immune responses and serve as platforms for interaction with other proteins (6). The human *BTN* genes are all part of the gene cluster located at the telomeric end of the *MHC* complex on chromosome 6. It contains protein-encoding genes (7). Four of them; BTN2A1, BTN3A1 (CD277), BTN3A2 and BTN3A3, contribute to γδ T cell activation (8–13). While BTN3 genes first emerged in placental mammals (14) BTN1 and BTN2 genes co-emerged with the evolvement of vertebrates (7).

BUTYROPHILINS AND γδ T CELL DEVELOPMENT

Some BTN-, Butyrophilin like (BTNL) and the BTN-related Skint (Selection and upkeep of intraepithelial T cells) molecules control the development and function of γδ T cells (5). γδ T cells are "non-conventional" T-lymphocytes, which are defined by expression of the γδ T cell

antigen receptor (γδTCR). They can be found in nearly all jawed vertebrates. γδ T cells differ profoundly from MHC restricted αβ T cells in antigen-recognition, thymic development and selecting ligand. In contrast to MHC restricted αβ T cells, whose TCR bind with the CDRs (complementarity determining region) 1, 2 and 3 of both the TCRα and β chains to MHC-peptide complexes, γδTCRs do not share specificity for a common class of ligands. The known γδTCR antigens are soluble molecules such Phycoerythrin, various bacterial proteins and stress-induced MHC- and MHC-like molecules (15). γδ T cells differ from MHC restricted T cells in thymic development, notably that for some populations of γδ T cells the strength of TCR-ligand interaction during thymic development does not result in classical positive or negative selection, but in programming for differentiation to IFNγ or IL-17-producing cells (16, 17). Furthermore, γδTCR rearrangements are typical for certain phases of thymic development and lead to highly specialized γδ T-cell populations. The most prominent example for such a development is the exclusive homing and functional specialization of the DETC (Dendritic Epidermal T cells) which help to maintain the barrier function of the skin (18). They are the first T cell population to develop in a body and are characterized by their dendritic morphology and a uniform TCR. The discovery of a mouse strain variant devoid of these cells led to the discovery of the butyrophilin-related gene *Skint1* gene (4) which together with Skint2 is essential for DETC development (19). Classical DETC has been found so far only in rodents, but interestingly DETC-resembling cells with variable γδ TCR exist in the crab-eating macaques (*Macaca fascicularis*) which, in contrast to the DETC negative hominids, carry a functional *SKINT1L* (Skint1 like) gene (18).

In humans, most of the circulating γδ T cells contain a Vδ2-bearing δ chain, while TCR of resident human γδ T cells are dominated by other Vδ (20). Some BTNs and BTNL molecules control γδ T cell subset homeostasis and activation and bind to their TCR (21).

BTN and BTNL proteins are structurally similar, but the composition and chromosomal location of the gene families varies between species. In humans and mice, activation of γδ T cells and homeostasis of populations of intestinal epithelial γδ T cells are under the control of BTN and BTNL molecules. Murine Btnl1-Btnl6 heterodimers interact with Vγ7 positive γδ T cells, and human BTNL3-BTNL8 heterodimers bind the TCR of Vγ4 positive cells. In the latter case, direct binding to germline-encoded parts of the γ-chain, especially to its hypervariable region 4 (HV4/CDR4), has been demonstrated, showing similarities to the interaction of some superantigens with Vβ-gene-encoded regions of αβTCRs (22–24). It is likely that differential topologies of TCR-ligands prompt unique modes of signaling, and it has been hypothesized that this superantigen-like type of binding maintains local cell homeostasis, while binding to the CDR3s supports an antigen-specific immune

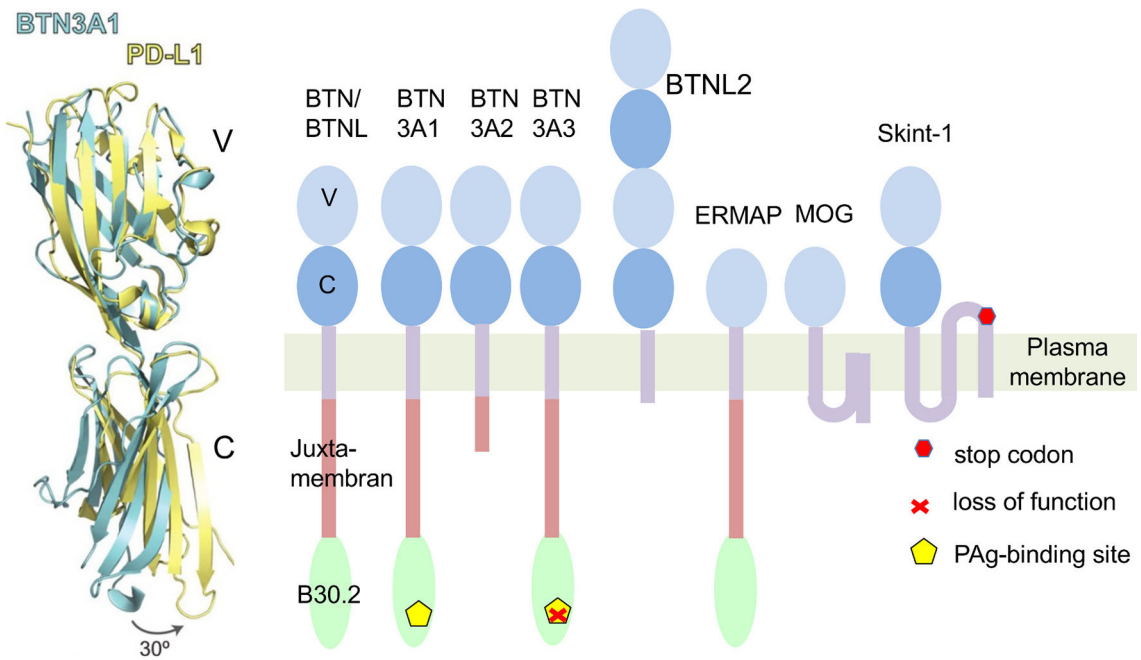


FIGURE 1 | Butyrophilins as members of the B7 family. Left: A comparison of structures of extracellular domains of programmed death ligand 1 (PD-L1: yellow) and Butyrophilin 3A1 (BTN3A1: blue). Shown is an overlay of the tertiary structure of both proteins. Differences of the C-domains of both molecules result mainly from a twist in the orientation of V- and C-Ig domains. In an exclusive overlay of V domain alone, similarities in the Ig-V domains would be even more pronounced. Picture is modified from (3). Right: Schematic representation of human BTN/BTNL family members and mouse Skint1. The protein domains are exemplified for BTN3A1. ERMAP (Erythroblast membrane-associated protein), MOG (Myelin-Oligodendrocyte glycoprotein). Skint 1 (Selection and upkeep of intraepithelial T-cells 1). The indicated Stop codon inactivates the Skint1 function in the DETC deficient FVB/N-Tac mouse strain (4).

response (21). Insights to the physiological significance of BTNL-TCRγ interaction can be expected from a rather frequent copy number variation, which generates a BTNL3*8 fusion product not expected to bind to Vγ4TCRs (25).

PHOSPHOANTIGEN-REACTIVE Vγ9Vδ2 T CELLS AND BUTYROPHILINS

BTN2A1 and BTN3A1 are mandatory for the activation of T cells carrying the eponymous Vγ9Vδ2TCR (10) with a Vγ9JP rearrangement and Vδ2-containing δ-chains. 1-5% of all blood T cells are Vγ9Vδ2 T cells. Usually, Vγ9Vδ2 T cells are cytotoxic cells with a type I cytokine profile, but they also show a remarkable functional plasticity and are activated by pyrophosphorylated metabolites of isoprenoid synthesis, the so-called phosphoantigens (PAgs). One such PAg is isopentenyl diphosphate (IPP), which is found in all organisms. Another well-studied PAg named (E)-4 Hydroxy-3-Methy-but-2-enyl diphosphate (HMBPP) stimulates 10000-fold better than IPP. It is the precursor of IPP in the DOXP metabolic pathway for isoprenoid synthesis, common to many Eubacteria (e.g., Mycobacteria), Apicomplexa such *Plasmodium* spp. or *Toxoplasma gondii* and chloroplasts. HMBPP initiates the massive expansion of the Vγ9Vδ2 T cell population during infections with HMBPP-producers, which can end up with up to 50% of human blood T cells becoming Vγ9Vδ2 T cells. In some tumor cells IPP levels are increased and trigger their elimination *via* Vγ9Vδ2 T cells. Increased IPP levels and concomitant Vγ9Vδ2 T cell activation are also induced by amino-bisphosphonates such as zoledronate, drugs commonly used in the treatment of bone metastasis or osteoporosis (26).

A breakthrough for the understanding of PAg-induced Vγ9Vδ2 T cell activation, was the finding that in cultures with peripheral blood mononuclear cells, monoclonal antibodies against BTN3A stimulated Vγ9Vδ2 T cells (agonist) or inhibited their PAg-response (antagonists) (**Figure 1**). This effect was dependent on the monoclonal antibodies binding to BTN3A molecules expressed by antigen-presenting or tumor cells, and not to the BTN3A molecules on the Vγ9Vδ2 T cells (8). PAg does not bind to the Vγ9Vδ2TCR but needs "presentation" by other cells that is initiated by binding to the intracellular B30.2 domain of BTN3A1 (9). The PAg-binding induces a conformational change which leads to the formation of a BTN3A1-BTN2A1 complex and to a not yet understood change at the cell surface, which is recognized by the Vγ9Vδ2 T cells (27). Whether recognition involves direct binding of BTN3A1 to the TCR or a hypothetical counter receptor on the T cells, or both, is unclear, but the key role of both proteins in PAg-stimulation is undisputed. The function of the PAg-non-binding BTN3A2 and BTN3A3 molecules is to increase the efficacy of the BTN3A1 action in PAg-mediated stimulation (23).

Insights into the mechanism of PAg mediated Vγ9Vδ2 T cell activation came from the comparison of species. The alpaca, beside humans and primates, is one of the few mammals with functional *BTN3*, *TCRVγ9* (*TRGV9*) and *TCRVδ2* (*TRDV2*) genes (14, 28, 29), and possesses PAg reactive Vγ9Vδ2 T cells (14). Replacing the intracellular region of human BTN3A1 with that of an alpaca BTN3 results in a chimeric BTN3 molecule which stimulates as efficiently as the complex of the different human BTN3A molecules (14). Sequence comparison and phylogenetic considerations led to the postulation of an Alpaca-like primordial BTN3 which amalgamates the function of the human BTN3A family and can be imagined as a BTN3A3-like molecule with an intact PAg-binding site (15, 29).

Another example of how a comparison of species' differences helps for a better understanding of PAg stimulation, is the comparison of rodent cell lines expressing human BTN3A1 with cell lines carrying single human chromosomes. The comparison showed that, together with BTN3A1, other genes on human chromosome 6 are mandatory for PAg presentation (30). Our strategy to identify the gene(s) was the generation of "radiation hybrids" by fusing irradiated human chromosome 6-bearing rodent cells with other rodent cells (11). The chromosomes of the non-irradiated fusion partner randomly integrate pieces of the chromosomes of the irradiated cells including those of chromosome 6. The hybrids were tested, and it was assumed that only cells carrying the missing (human) genes can present PAgs. Comparison of human genome fragments of the PAg-presenting hybrids led to the identification of a 150 kB fragment at the telomeric end of the HLA complex, which contained the entire *BTN* cluster including *BTN2A1*. The significance of BTN2A1 for PAg presentation was demonstrated by:

1. Co-expression of BTN2A1 and BTN3A1 in rodent cells which rendered the cells PAg-presenters.
2. BTN2A1 inactivation by CRISPR-Cas9-mutagenesis of human 293T cells abolished their PAg-presentation capacity.

The Wilcox group in Birmingham, UK, demonstrated that the Vγ9 part of the TCR binds to BTN2A1 with a similar topology as BTNL3 to Vγ4 (24), which does not involve the TCRδ chain (11). Independently and with another screening system, the groups of Godfrey and Uldrich, at the University of Melbourne, also identified BTN2A1 as a key compound of PAg presentation. Both groups came to very similar conclusions on the interaction of BTN3A1, BTN2A1 and Vγ9Vδ2TCR (11, 12). However, the ligand(s) of the Vγ9Vδ2TCR in PAg-induced activation is still not known, it is speculated that it could be a particular conformation of BTN2A1 and BTN3A1. Our working hypothesis is that apart from BTN2A1 and BTN3A1, other molecules might be involved and that interaction of the TCR with these molecules implies also additional CDR3-binding ligand(s) which leads to different signals than exclusive binding of BTN(L) molecules to the Vγ part of the TCR (11) (**Figure 2**) 2). In summary, BTN and BTN-like molecules are essential for the development and TCR-mediated activation of many, if not all, γδ T cells.

OTHER FUNCTION OF BUTYROPHILINS AND IMMUNE THERAPY

BTN2A1 and BTN3A1 fulfill various functions. One function of BTN2A1 is its capacity to bind to DC-SIGN, which depends on the expressing cell type and the degree of its glycosylation state

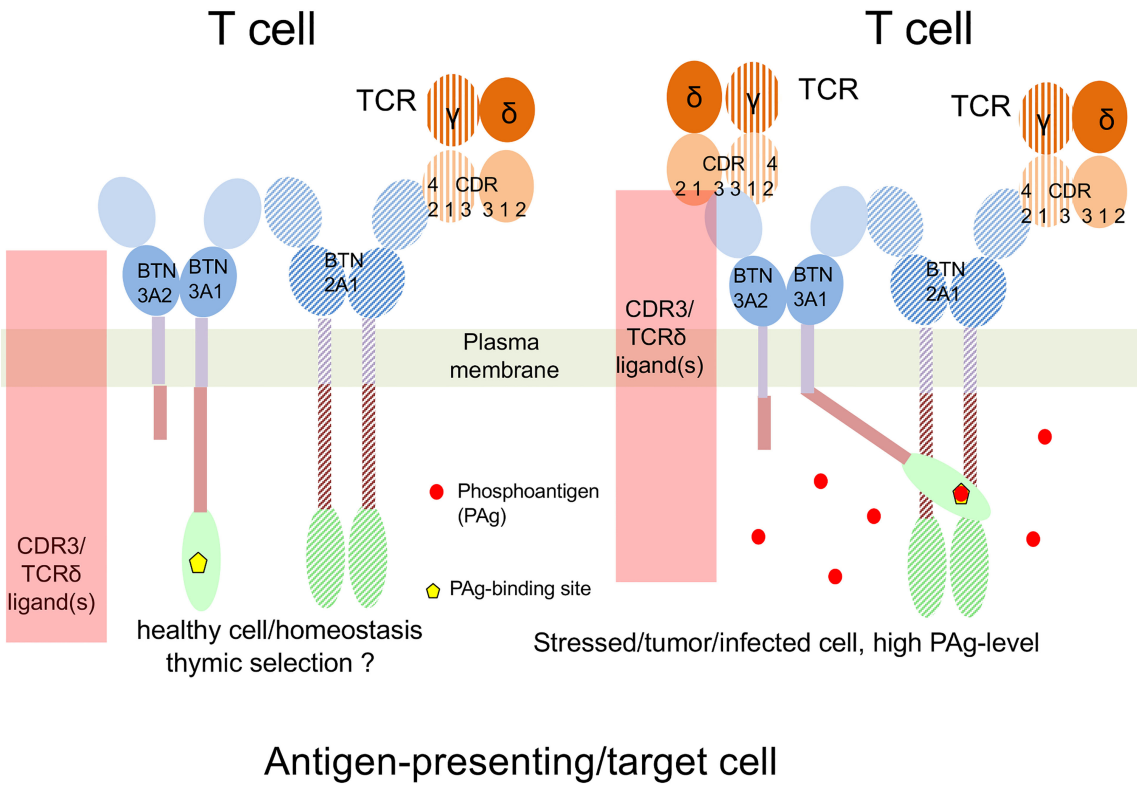


FIGURE 2 | Working hypothesis on PAg-recognition by Vγ9Vδ2 T cells. Left: Under physiological conditions, BTN2A1 binds to the hypervariable region 4 (CDR4 or HV4) of the Vγ9 region of the Vγ9Vδ2TCR. This controls thymic development and homeostasis of Vγ9Vδ2 T cells. Right: Infection or cell transformation increases PAg-levels in the presenting or tumor cell. This leads to PAg-binding to the B30.2 domain of BTN3A1, and subsequent binding of the B30.2-PAg complex to the intracellular domain of BTN2A1. The resulting complex might include additional proteins, which finally bind all CDRs regions of γ and δ chain and trigger Vγ9Vδ2 T cell activation. Please note that so far neither direct binding of the TCR to BTN3A nor existence of an additional ligand recruited by the PAg-binding BTN3A has been demonstrated.

(31). An example of BTN3A1 function is its involvement in the induction of IFNβ production by cytoplasmic- or viral nucleic acids (32).

The immunomodulatory function of BTN(L) and using it as a target of immunotherapy is becoming of greater interest. For some time it has been known that BTN(L)-specific monoclonal antibodies can amplify activation of T cells and NK cells (5, 33), while BTN(L) overexpression, soluble BTN(L)-molecules or BTN(L)-Fc constructs often inhibit T-cell activation [e.g (2, 5)]. However, the involved counter receptors have not been identified and physiological relevance of this suppression is not clear. One of the better-understood examples about the physiological role of BTN(L)s comes from the analysis of BTN2a2-deficient mice. These show an increased αβT cell response, and shown by cell transfer experiments, this results from missing Btn2a2 expression of antigen-presenting cells (34). More recently, involvement was also reported in the regulation of ILC2-T cell crosstalk (35) and bone resorption (36) and reduced levels of soluble Btn2a2 in arthritis of in mouse or BTN2A2 in human arthritis (36).

A study which attracted much attention was the analysis of the suppression of tumor-specific T cells by BTN3A1 (37), which was postulated to be a consequence of BTN3A1-binding to glycosylated CD45 and concomitant disruption of the immunological synapse and TCR-mediated signaling. This immune suppression could be abolished by BTN3A specific monoclonal antibodies *in vitro* and in mouse models. Immunodeficient NGS mice were inoculated with a human ovarian cancer cell line and treated with combinations of human tumor-target specific TCR transductants. γδ T cells cells with and without BTN3 antibodies showed αβ and γδ T cell specific effects, by combining inhibition of BTN3-mediated suppression of the T cell response and agonistic action of agonist on the γδ T cells. The other model used was BTN3A1-transgenic mice inoculated with an immune suppression inducing ovarian tumor cell line (ID8-*Defb29-Vegf-a)*, where the therapeutic effect of the BTN3 specific mAb was even superior to PD1 specific mAb. Interestingly, even in absence of Vγ9Vδ2 T cells, administration of zoledronate had also a beneficial effect (37) which indicates that (partial) reversal of BTN3A1 immunosuppression might also involve PAgs. Another aspect is the interpretation of an ongoing clinical trial with the agonistic BTN3A specific monoclonal antibody ICT01 (ClinicalTrials.gov-Identifier: NCT04243499) where a positive clinical outcome

might not only result from activation of Vγ9Vδ2 T cells (38) but also by reconstituting a BTN3-suppressed αβ T cell.

Very recently a mechanism of immune evasion by expression of BTNL2 on cancer cells was described, where BTNL2 promotes IL-17 production by a local γδ T cell population which enhances tumor resistance by recruitment of myeloid suppressor cells T cells (39). Blockade of BTNL2 by a monoclonal antibody had a significant therapeutic effect for several mouse tumors and acts synergistically with PD1 blockade. Furthermore, BTNL2 is expressed in multiple human solid cancers and its expression level correlates negatively with patients′ survival. Thus, BTNL2 may be a target for therapeutic intervention similar as BTN3 although the mechanism of immune evasion is a different one.

To conclude, BTN(L) molecules fulfill immunological and non-immunological functions of which some affect γδ T cells and bear therapeutic potential by targeting them with monoclonal antibodies similar to those successfully applied for more conventional B7 family members (40).

AUTHOR CONTRIBUTIONS

TH wrote the first version of the manuscript which was finalized together with MK. All authors contributed to the article and approved the submitted version.

FUNDING

This study is funded by the DFG He 2346-8/2 which is part of the FOR 2799. This publication was supported by the Open Access Publication Fund of the University of Wuerzburg.

ACKNOWLEDGMENTS

This manuscript is based on the publication: T. Herrmann. Butyrophiline : Verwandte der B7-Familie, γδ-T-Zellrezeptorliganden und vieles mehr. Trillium Immunologie 2021-07-21. DOI: 10.47184/ti.2021.02.04

REFERENCES

1. Ogg SL, Weldon AK, Dobbie L, Smith AJ, Mather IH. Expression of Butyrophilin (Btn1a1) in Lactating Mammary Gland is Essential for the Regulated Secretion of Milk-Lipid Droplets. *Proc Natl Acad Sci U S A* (2004) 101(27):10084–9. doi: 10.1073/pnas.0402930101
2. Smith IA, Knezevic BR, Ammann JU, Rhodes DA, Aw D, Palmer DB, et al. BTN1A1, the Mammary Gland Butyrophilin, and BTN2A2 are Both Inhibitors of T Cell Activation. *J Immunol* (2010) 184(7):3514–25. doi: 10.4049/jimmunol.0900416
3. Palakodeti A, Sandstrom A, Sundaresan L, Harly C, Nedellec S, Olive D, et al. The Molecular Basis for Modulation of Human Vgamma9Vdelta2 T Cell Responses by CD277/butyrophilin-3 (BTN3A)-Specific Antibodies. *J Biol Chem* (2012) 287(39):32780–90. doi: 10.1074/jbc.M112.384354
4. Boyden LM, Lewis JM, Barbee SD, Bas A, Girardi M, Hayday AC, et al. Skint1, the Prototype of a Newly Identified Immunoglobulin Superfamily Gene Cluster, Positively Selects Epidermal Gammadelta T Cells. *Nat Genet* (2008) 40(5):656–62. doi: 10.1038/ng.108
5. Rhodes DA, Reith W, Trowsdale J. Regulation of Immunity by Butyrophilins. *Annu Rev Immunol* (2016) 34:151–72. doi: 10.1146/annurev-immunol-041015-055435
6. D'Cruz AA, Babon JJ, Norton RS, Nicola NA, Nicholson SE. Structure and Function of the SPRY/B30.2 Domain Proteins Involved in Innate Immunity. *Protein Sci* (2013) 22(1):1–10. doi: 10.1002/pro.2185
7. Rhodes DA, Chen HC, Williamson JC, Hill A, Yuan J, Smith S, et al. Regulation of Human Gammadelta T Cells by BTN3A1 Protein Stability and ATP-Binding Cassette Transporters. *Front Immunol* (2018) 9:662. doi: 10.3389/fimmu.2018.00662
8. Harly C, Guillaume Y, Nedellec S, Peigne CM, Monkkonen H, Monkkonen J, et al. Key Implication of CD277/butyrophilin-3 (BTN3A) in Cellular Stress Sensing by a Major Human Gammadelta T-Cell Subset. *Blood* (2012) 120 (11):2269–79. doi: 10.1182/blood-2012-05-430470
9. Sandstrom A, Peigne CM, Leger A, Crooks JE, Konczak F, Gesnel MC, et al. The Intracellular B30.2 Domain of Butyrophilin 3A1 Binds Phosphoantigens to Mediate Activation of Human Vgamma9Vdelta2 T Cells. *Immunity* (2014) 40(4):490–500. doi: 10.1016/j.immuni.2014.03.003
10. Herrmann T, Fichtner AS, Karunakaran MM. An Update on the Molecular Basis of Phosphoantigen Recognition by Vgamma9Vdelta2 T Cells. *Cells* (2020) 9(6):1–26. doi: 10.3390/cells9061433
11. Karunakaran MM, Willcox CR, Salim M, Paletta D, Fichtner AS, Noll A, et al. Butyrophilin-2a1 Directly Binds Germline-Encoded Regions of the Vgamma9Vdelta2 TCR and Is Essential for Phosphoantigen Sensing. *Immunity* (2020) 52(3):487–98.e6. doi: 10.1016/j.immuni.2020.02.014
12. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 is Essential for Phosphoantigen Reactivity by Gammadelta T Cells. *Science* (2020) 367(6478):1–13. doi: 10.1126/science.aay5516
13. Cano CE, Pasero C, De Gassart A, Kerneur C, Gabriac M, Fullana M, et al. BTN2A1, an Immune Checkpoint Targeting Vgamma9Vdelta2 T Cell Cytotoxicity Against Malignant Cells. *Cell Rep* (2021) 36(2):109359. doi: 10.1016/j.celrep.2021.109359
14. Fichtner AS, Karunakaran MM, Gu S, Boughter CT, Borowska MT, Starick L, et al. Alpaca (Vicugna Pacos), the First Nonprimate Species With a Phosphoantigen-Reactive Vgamma9Vdelta2 T Cell Subset. *Proc Natl Acad Sci U S A* (2020) 117(12):6697–707. doi: 10.1073/pnas.1909474117
15. Herrmann T, Karunakaran MM, Fichtner AS. A Glance Over the Fence: Using Phylogeny and Species Comparison for a Better Understanding of Antigen Recognition by Human Gammadelta T-Cells. *Immunol Rev* (2020) 298 (1):218–36. doi: 10.1111/imr.12919
16. Munoz-Ruiz M, Sumaria N, Pennington DJ, Silva-Santos B. Thymic Determinants of Gammadelta T Cell Differentiation. *Trends Immunol* (2017) 38(5):336–44. doi: 10.1016/j.it.2017.01.007
17. Tieppo P, Papadopoulou M, Gatti D, McGovern N, Chan JKY, Gosselin F, et al. The Human Fetal Thymus Generates Invariant Effector Gammadelta T Cells. *J Exp Med* (2020) 217(3):1–17. doi: 10.1084/jem.20190580
18. Sutoh Y, Mohamed RH, Kasahara M. Origin and Evolution of Dendritic Epidermal T Cells. *Front Immunol* (2018) 9:1059:1059. doi: 10.3389/fimmu.2018.01059
19. Jandke A, Melandri D, Monin L, Ushakov DS, Laing AG, Vantourout P, et al. Butyrophilin-Like Proteins Display Combinatorial Diversity in Selecting and Maintaining Signature Intraepithelial Gammadelta T Cell Compartments. *Nat Commun* (2020) 11(1):3769. doi: 10.1038/s41467-020-17557-y
20. Hayday AC. [Gamma][Delta] Cells: A Right Time and a Right Place for a Conserved Third Way of Protection. *Annu Rev Immunol* (2000) 18:975–1026. doi: 10.1146/annurev.immunol.18.1.975
21. Hayday AC, Vantourout P. The Innate Biologies of Adaptive Antigen Receptors. *Annu Rev Immunol* (2020) 38:487–510. doi: 10.1146/annurev-immunol-102819-023144
22. Di Marco Barros R, Roberts NA, Dart RJ, Vantourout P, Jandke A, Nussbaumer O, et al. Epithelia Use Butyrophilin-Like Molecules to Shape Organ-Specific Gammadelta T Cell Compartments. *Cell* (2016) 167(1):203–18.e17. doi: 10.1016/j.cell.2016.08.030
23. Vantourout P, Laing A, Woodward MJ, Zlatareva I, Apolonia L, Jones AW, et al. Heteromeric Interactions Regulate Butyrophilin (BTN) and BTN-Like Molecules Governing γδ T Cell Biology. *Proc Natl Acad Sci U S A* (2018) 5:1039–44. doi: 10.1073/pnas.1701237115
24. Willcox CR, Vantourout P, Salim M, Zlatareva I, Melandri D, Zanardo L, et al. Butyrophilin-Like 3 Directly Binds a Human Vgamma4(+) T Cell Receptor Using a Modality Distinct From Clonally-Restricted Antigen. *Immunity* (2019) 51(5):813–25.e4. doi: 10.1016/j.immuni.2019.09.006
25. Aigner J, Villatoro S, Rabionet R, Roquer J, Jimenez-Conde J, Marti E, et al. A Common 56-Kilobase Deletion in a Primate-Specific Segmental Duplication

Creates a Novel Butyrophilin-Like Protein. *BMC Genet* (2013) 14:61. doi: 10.1186/1471-2156-14-61
26. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide Antigens, Presentation Mechanisms, and Immunological Memory of Human Vgamma2Vdelta2 T Cells: Discriminating Friend From Foe Through the Recognition of Prenyl Pyrophosphate Antigens. *Immunol Rev* (2007) 215:59–76. doi: 10.1111/j.1600-065X.2006.00479.x
27. Hsiao CC, Nguyen K, Jin Y, Vinogradova O, Wiemer AJ. Ligand-Induced Interactions Between Butyrophilin 2A1 and 3A1 Internal Domains in the HMBPP Receptor Complex. *Cell Chem Biol* (2022). doi: 10.1016/j.chembiol.2022.01.004
28. Karunakaran MM, Goebel TW, Starick L, Walter L, Herrmann T. V Gamma 9 and V Delta 2 T Cell Antigen Receptor Genes and Butyrophilin 3 (BTN3) Emerged With Placental Mammals and are Concomitantly Preserved in Selected Species Like Alpaca (Vicugna Pacos). *Immunogenetics* (2014) 66 (4):243–54. doi: 10.1007/s00251-014-0763-8
29. Fichtner AS, Karunakaran MM, Starick L, Truman RW, Herrmann T. The Armadillo (Dasypus Novemcinctus): A Witness But Not a Functional Example for the Emergence of the Butyrophilin 3/Vγ9vδ2 System in Placental Mammals. *Front Immunol* (2018) 9:265. doi: 10.3389/fimmu.2018.00265
30. Riano F, Karunakaran MM, Starick L, Li JQ, Scholz CJ, Kunzmann V, et al. V Gamma 9V Delta 2 TCR-Activation by Phosphorylated Antigens Requires Butyrophilin 3 A1 (BTN3A1) and Additional Genes on Human Chromosome 6. *Eur J Immunol* (2014) 44(9):2571–6. doi: 10.1002/eji.201444712
31. Malcherek G, Mayr L, Roda-Navarro P, Rhodes D, Miller N, Trowsdale J. The B7 Homolog Butyrophilin BTN2A1 is a Novel Ligand for DC-SIGN. *J Immunol* (2007) 179(6):3804–11. doi: 10.4049/jimmunol.179.6.3804
32. Seo M, Lee SO, Kim JH, Hong Y, Kim S, Kim Y, et al. MAP4-Regulated Dynein-Dependent Trafficking of BTN3A1 Controls the TBK1-IRF3 Signaling Axis. *Proc Natl Acad Sci U S A* (2016) 113(50):14390–5. doi: 10.1073/pnas.1615287113
33. Compte E, Pontarotti P, Collette Y, Lopez M, Olive D. Frontline: Characterization of BT3 Molecules Belonging to the B7 Family Expressed on Immune Cells. *Eur J Immunol* (2004) 34(8):2089–99. doi: 10.1002/eji.200425227
34. Sarter K, Leimgruber E, Gobet F, Agrawal V, Dunand-Sauthier I, Barras E, et al. Btn2a2, a T Cell Immunomodulatory Molecule Coregulated With MHC Class II Genes. *J Exp Med* (2016) 213(2):177–87. doi: 10.1084/jem.20150435
35. Frech M, Omata Y, Schmalzl A, Wirtz S, Taher L, Schettl G, et al. Btn2a2 Regulates Ilc2–T Cell Cross Talk in Type 2 Immune Responses. *Front Immun* (2022) 12. doi: 10.3389/fimmu.2022.757436
36. Frech M, Schuster G, Andes FT, Schett G, Zaiss MM, Sarter K. RANKL-Induced Btn2a2 - A T Cell Immunomodulatory Molecule - During Osteoclast Differentiation Fine-Tunes Bone Resorption. *Front Endocrinol (Lausanne)* (2021) 12:685060. doi: 10.3389/fendo.2021.685060
37. Payne KK, Mine JA, Biswas S, Chaurio RA, Perales-Puchalt A, Anadon CM, et al. BTN3A1 Governs Antitumor Responses by Coordinating Alphabeta and Gammadelta T Cells. *Science* (2020) 369(6506):942–9. doi: 10.1126/science.aay2767
38. De Gassart A, Le KS, Brune P, Agaugue S, Sims J, Goubard A, et al. Development of ICT01, a First-in-Class, Anti-BTN3A Antibody for Activating Vgamma9Vdelta2 T Cell-Mediated Antitumor Immune Response. *Sci Transl Med* (2021) 13(616):eabj0835. doi: 10.1126/scitranslmed.abj0835
39. Du Y, Peng Q, Cheng D, Pan T, Sun W, Wang H, et al. Cancer Cell-Expressed BTNL2 Facilitates Tumour Immune Escape *via* Engagement With IL-17A-Producing Gammadelta T Cells. *Nat Commun* (2022) 13(1):231. doi: 10.1038/s41467-021-27936-8
40. Fritz JM, Lenardo MJ. Development of Immune Checkpoint Therapy for Cancer. *J Exp Med* (2019) 216(6):1244–54. doi: 10.1084/jem.20182395

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 04 April 2022
doi: 10.3389/fimmu.2022.845974

Apoptosis, Pyroptosis, and Ferroptosis Conspiringly Induce Immunosuppressive Hepatocellular Carcinoma Microenvironment and γδ T-Cell Imbalance

Yi Hu[1,2†], Dan Chen[1†], Minjing Hong[1†], Jing Liu[1,3†], Yijia Li[1,3], Jianlei Hao[1,3], Ligong Lu[1], Zhinan Yin[1,3] and Yangzhe Wu[1*]*

[1] Guangdong Provincial Key Laboratory of Tumour Interventional Diagnosis and Treatment, Zhuhai Institute of Translational Medicine, Zhuhai People's Hospital Affiliated with Jinan University, Jinan University, Zhuhai, China, [2] Microbiology and Immunology Department, School of Medicine, Faculty of Medical Science, Jinan University, Guangzhou, China, [3] The Biomedical Translational Research Institute, Faculty of Medical Science, Jinan University, Guangzhou, China

OPEN ACCESS

Edited by:
Dieter Kabelitz,
University of Kiel, Germany

Reviewed by:
Jiacheng Bi,
Chinese Academy of Sciences (CAS), China
Shubhada Vivek Chiplunkar,
Advanced Centre for Treatment, Research and Education in Cancer, India

***Correspondence:**
Yangzhe Wu
tyzwu@jnu.edu.cn
Yi Hu
yihu2020@jnu.edu.cn

†These authors share first authorship

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 30 December 2021
Accepted: 07 March 2022
Published: 04 April 2022

Citation:
Hu Y, Chen D, Hong M, Liu J, Li Y, Hao J, Lu L, Yin Z and Wu Y (2022) Apoptosis, Pyroptosis, and Ferroptosis Conspiringly Induce Immunosuppressive Hepatocellular Carcinoma Microenvironment and γδ T-Cell Imbalance. Front. Immunol. 13:845974. doi: 10.3389/fimmu.2022.845974

Hepatocellular carcinoma (HCC) is highly malignant and prone to metastasize due to the heterogeneous and immunosuppressive tumor microenvironment (TME). Programmed cell deaths (PCDs) including apoptosis, ferroptosis, and pyroptosis routinely occur in the HCC TME and participate in tumorigenesis. However, how apoptosis, ferroptosis, and pyroptosis are involved in constructions of the immunosuppressive TME and their underlying cross-talk remains to be further unveiled. In this work, we deciphered the immunosuppressive landscape of HCC TME, which demonstrated high expressions of inhibitory checkpoint molecules and infiltration of protumor immune cells but low infiltration of antitumor effector immune cells. Further investigations unequivocally revealed that marker genes of apoptosis, ferroptosis, and pyroptosis are closely correlated with expressions and infiltrations of inhibitory checkpoint molecules and immune cells and that higher "-optosis" links to poorer patient prognosis. Notably, such three types of "-optosis" interact with each other at both the gene and protein levels, suggesting that they conspiringly induce the establishment of the immunosuppressive HCC TME. Interestingly, examinations of circulating γδ T cells in HCC patients revealed a noticeable dysfunction phenotype. The strikingly elevated ratio of the $V\delta1^+$ versus the $V\delta2^+$ subset suggested that the $V\delta1^+/V\delta2^+$ ratio would be a potential biomarker for the diagnosis and prognosis in HCC patients. Altogether, this work thoroughly decrypted the underlying correlations between apoptosis, ferroptosis, and pyroptosis and the formation of immunosuppressive HCC TME and, meanwhile, indicated that allogeneic $V\delta2^+$ γδ T-cell transfer would be a promising adjuvant strategy for renormalizing circulating γδ T cell and thus achieving sound clinical efficacy against HCC.

Keywords: apoptosis, pyroptosis, ferroptosis, immunosuppressive HCC TME, γδ T-cell imbalance

INTRODUCTION

The liver is a major metabolic organ and plays a crucial role in all metabolism processes in the body. In certain circumstances, such as alcohol abuse, virus infection, and drugs, liver inflammation will occur and can deteriorate into hepatic fibrosis, hepatic cirrhosis, and eventually liver cancer. Generally, tumorigenesis is a result induced by complicated events, and one determinant factor is the long-term suppressive or dysfunctional immunity of hosts. Hepatocellular carcinoma (HCC) is well-known for its high relapse rate and poor long-term prognosis. Current routine treatments of HCC rely heavily on chemotherapeutic approaches (e.g., sorafenib) but have unsatisfactory results. Fortunately, immunotherapy strategies (1) including immune checkpoint blockade (ICB) (2) and immune cell therapy (3–5) provide new opportunities for HCC patients. For example, PD1 blockade therapy is now covered for free by the National Healthcare Security System of China and benefits the increasing HCC population. Nevertheless, further decoding of the HCC tumor microenvironment (TME), especially the immune landscape, will benefit the development of immunotherapies. For HCC, how the immunosuppressive microenvironment is triggered and eventually established remains to be further illustrated.

The excessive proliferation nature of cancer cells and the antitumor immunity of the host unavoidably lead to numerous types of cell death, mainly including programmed (e.g., apoptosis) and non-regulated (e.g., necrosis) cell deaths. Currently, intensively investigated programmed cell deaths (PCDs) in HCC TME include apoptosis, ferroptosis, and pyroptosis. Apoptosis belongs to the non-immunogenic cell death and shall not induce detectable immune responses under normal circumstances. On the other hand, immunogenic deaths, ferroptosis, and pyroptosis can result in immune responses due to the release of various intracellular contents, most acting as pro-inflammatory signals. Though the role of ferroptosis and pyroptosis in HCC prognosis had been previously discussed (6, 7), how non-immunogenic apoptosis as well as immunogenic ferroptosis and pyroptosis cross-talk at the gene and protein levels and collaboratively contribute to the development of the immunosuppressive HCC TME remain to be further addressed.

In the HCC TME, there are various types of infiltrated immune cells, including lymphoid and myeloid linage cells. Among them, γδ T cell occupies 6.8%~34% $CD3^+$ T cell in the liver (8, 9) and plays crucial roles in liver protection from virus infection (10) and tumorigenesis (11, 12). Intra-tumoral γδ T cell was proposed to be one of the best positive prognosis markers for pan-cancers (13). However, evidence indicated that the two major subsets of γδ T cell, namely, $V\delta1^+$ and $V\delta2^+$ subsets, possess contradictory roles in antitumor immunity. The $V\delta1^+$ subset tends to perform a Treg-like function in the context of TME, which expresses high levels of inhibitory surface markers like CD73 (14) and CD39 (15), as well as cytokines such as IL10 (15) and TGFβ (16). The $V\delta2^+$ subset, however, is the early source of IFNγ (17, 18) and performs cytotoxic functions against transformed cells (19). For years, we have mainly focused on the $V\delta2^+$ subset; most importantly, we innovatively proved the safety and efficacy of the allogeneic $V\delta2^+$ γδ T cells in a series of clinical studies (4, 5, 20). Therefore, the primary scientific question we want to illustrate is the performance of the $V\delta2^+$ γδ T cell in the context of HCC TME. Here we not only applied bioinformatics to evaluate the correlations between γδ T-cell infiltration and three types of cell death (apoptosis, ferroptosis, and pyroptosis) but also statistically evaluated the imbalance of circulating γδ T-cell subsets in HCC patients.

Together, our present work revealed that the HCC TME is globally immunosuppressive, and three types of "-optosis" (apoptosis, ferroptosis, and pyroptosis) play crucial roles in creating the immunosuppressive TME and lead to the adverse prognosis of HCC patients. Moreover, we found that the proportion of the Vδ1/Vδ2 subsets of circulating γδ T cell is functionally imbalanced in HCC patients, suggesting that the adoptive transfer of allogeneic $V\delta2^+$ γδ T cells from healthy donors might benefit patients. We further hypothesize that by taking advantage of apoptosis, ferroptosis, and pyroptosis, immune cell-based adjuvant therapy (e.g., $V\delta2^+$ γδT) might lead to a better prognosis than chemotherapy alone or chemotherapy combined with checkpoint blockade.

METHODS AND MATERIALS

γδ T-Cell Subset Proportioning in the Healthy Population and Hepatocellular Carcinoma Patients

To reveal the proportional difference in the two subsets of γδ T cells from peripheral blood mononuclear cells (PBMCs) between the healthy population and HCC patients, the raw immune-phenotyping data of these two populations provided by the Shuangzhi Purui Medical Laboratory Co., Ltd. (Wuhan, China) were obtained and analyzed. The healthy population consisted of 170 disease-free adults (age range, 26–72; gender includes both female and male). The HCC population consisted of 42 patients (age range, 30–71; diagnosis, HCC I to IV including both primary and recurrence; gender includes both female and male). The antibodies used included anti-human CD3, anti-human TCR Vδ1, and anti-human TCR Vδ2 antibodies (BioLegend, San Diego, CA, USA). All examinations were conducted using BD FACSCanto cytometry (BD Biosciences, San Jose, CA, USA).

Online Databases and Bioinformatics Analysis

Tumoral mRNA-sequencing data (level 3) and corresponding clinical information of 371 HCC patients were obtained from The Cancer Genome Atlas (TCGA) dataset (https://portal.gdc.cancer.gov/) by following the related guidelines and policies. The mRNA-seq data of 50 paired peri-tumor tissue samples, together with 226 healthy liver tissue samples from the GTEx database (GTEx V8, https://gtexportal.org/home/datasets), were used as the control group, which described donors' clinical information including gender, race, age, clinical stages, therapy history, and biospecimen collection. Protein–protein interaction was

analyzed using the online database (https://string-db.org/). The R Project for Statistical Computing version 4.0.3 was used for graph plotting and analysis unless specified otherwise. In our analysis, HCC patients were divided into three groups (I, II, and III–IV) according to the clinical stage (pTNM) due to the limited amount of stage IV patients. To analyze the multi-gene correlation, the mRNA-seq dataset from NIH National Cancer Institute (https://tcga-data.nci.nih.gov/tcga/) was also included. The R software packages ggstatsplot and pheatmap were respectively used to produce two-gene and multi-gene correlation maps. Spearman's analysis was used to describe the correlation.

As for ferroptosis analysis, the related marker genes were derived from previously published data (21). The marker genes for apoptosis were adopted from http://biocc.hrbmu.edu.cn/CancerSEA/home.jsp. The marker genes for pyroptosis were from published work (22). Then R packages ggplot2 and pheatmap were used for data visualization. Based on marker genes of apoptosis, ferroptosis, and pyroptosis, we wanted to know whether samples could be clustered into subgroups. The R packages ConsensusClusterPlus and pheatmap were used to generate clustering heatmaps. Moreover, the Kaplan–Meier survival analysis with log-rank test was used to plot survival curves among groups; p-values and hazard ratio (HR) with 95% CI were generated by log-rank tests and univariate Cox proportional hazards regression. For correlation between single gene and survival, R packages ggrisk, survival, survminer, glmnet, and timeROC were applied for analysis.

To evaluate immune cell infiltration in the context of apoptosis, ferroptosis, and pyroptosis, the R package immunedeconv was applied, which integrates six classes of algorithms, including TIMER, xCell, MCP-counter, CIBERSORT, EPIC, and quanTIseq. Meanwhile, the gene expression of inhibitory checkpoint molecules PDCD1(PD1), CD274(PDL1), PDCD1LG2(PDL2), CTLA4, LAG3, TIGIT, HAVCR2(TIM3), and SIGLEC15 in HCC tissues were intensively inspected. To analyze the correlation between "-optosis" related marker genes and 15 checkpoint molecules, both stimulatory checkpoint molecules (CD27, CD28, CD40, DNAM1, ICOS, and TNFRSF9) and inhibitory checkpoint molecules (CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, TIGIT, SIGLEC15, and BTLA) were included. To assay ICB among HCC subgroup samples, the potential ICB response was predicted with the Tumor Immune Dysfunction and Exclusion (TIDE) algorithm (23). p-Value <0.05 was considered statistically significant.

RESULTS

Hepatocellular Carcinoma Tumor Microenvironment Is Highly Immune Suppressed

To comprehensively inspect the immune microenvironment of HCC TME, we firstly compared gene expression of immune inhibitory checkpoint molecules between tumor tissues and normal (peri-tumor) tissues, including CD274, CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, TIGIT, and SIGLEC15. It showed that all inhibitory checkpoint molecules except for CD274 are significantly upregulated in the HCC tumor group (**Figures 1A, B**). Then, 371 HCC samples were divided into 3 groups based on clinical stage (pTNM), and the checkpoint molecules were further compared among the 3 subgroups. It turned out that all checkpoint molecules are not statistically different among subgroups (**Figure 1C**). Further analysis revealed that HCC patients with different clinical stages respond to ICB therapy similarly (**Figure 1D**). We further checked the difference of immune cell infiltration between the HCC and normal groups by CIBERSORT score comparisons. We found that in the HCC group, scores of regulatory T cells (Tregs), resting macrophages M0, resting myeloid dendritic cells (DCs), and activated mast cells are strikingly increased. On the contrary, CIBERSORT scores of infiltrated γδ T cells, monocytes, macrophages M2, and resting mast cells are all depressed (**Figure 1E**). Furthermore, we inspected tissue infiltration of these immune cells among subgroups according to clinical stages (I, II, III–IV, and normal) and observed a similar pattern (**Figure 1F**) to **Figure 1E**.

Apoptosis Contributes to Establishing the Immunosuppressive Hepatocellular Carcinoma Tumor Microenvironment

Due to the excessive proliferation of HCC tumor cells and corresponding antitumor immunity and because cancer cell apoptosis routinely occurs in the TME, we first checked the expression differences of 63 apoptosis marker genes between HCC and normal tissues. We can see that 48 apoptosis-related marker genes are significantly upregulated in the HCC group (9 downregulated genes highlighted by blue arrows and 6 genes with no statistical difference) (**Figure 2A**). We then analyzed the correlation between each of the 63 marker genes and the overall survival (OS) rate and found that only 22 apoptotic genes are significantly correlated with the OS ($p < 0.05$). Specifically, 21 genes have negative correlations, while only one gene (PPP2R1B) has a positive correlation (**Supplementary Figure 2A**). Afterward, the multi-gene correlation among these 22 genes was calculated, as shown in **Figure 2B**. Moreover, we intensively analyzed those genes that have a better correlation with OS ($p \leq 0.01$), including BCL10, E2F2, BAK1, BCAP31, CASP2, SNW1, and STK4. It shows that higher expressions of these 7 genes lead to poorer prognoses (**Figure 2C**). Further analysis revealed that expressions of these 7 genes among different clinical stages (I, II, and III–IV) are consistently and greatly upregulated as compared with the normal group, and the expression level dramatically increases from stage I to stage III–IV (**Figure 2D**). We then found that six of these seven genes (except BCAP31) are positively correlated with the expression level of checkpoint molecules and immune cell infiltration (**Figures 2E, F**; **Supplementary Figure 2B**).

Based on the expressions of those 22 genes that are correlated with OS, 371 HCC samples were further divided into 3 subgroups using the R package ConsensusClusterPlus (**Supplementary Figure 2C**), and the clinical information of these 3 groups is

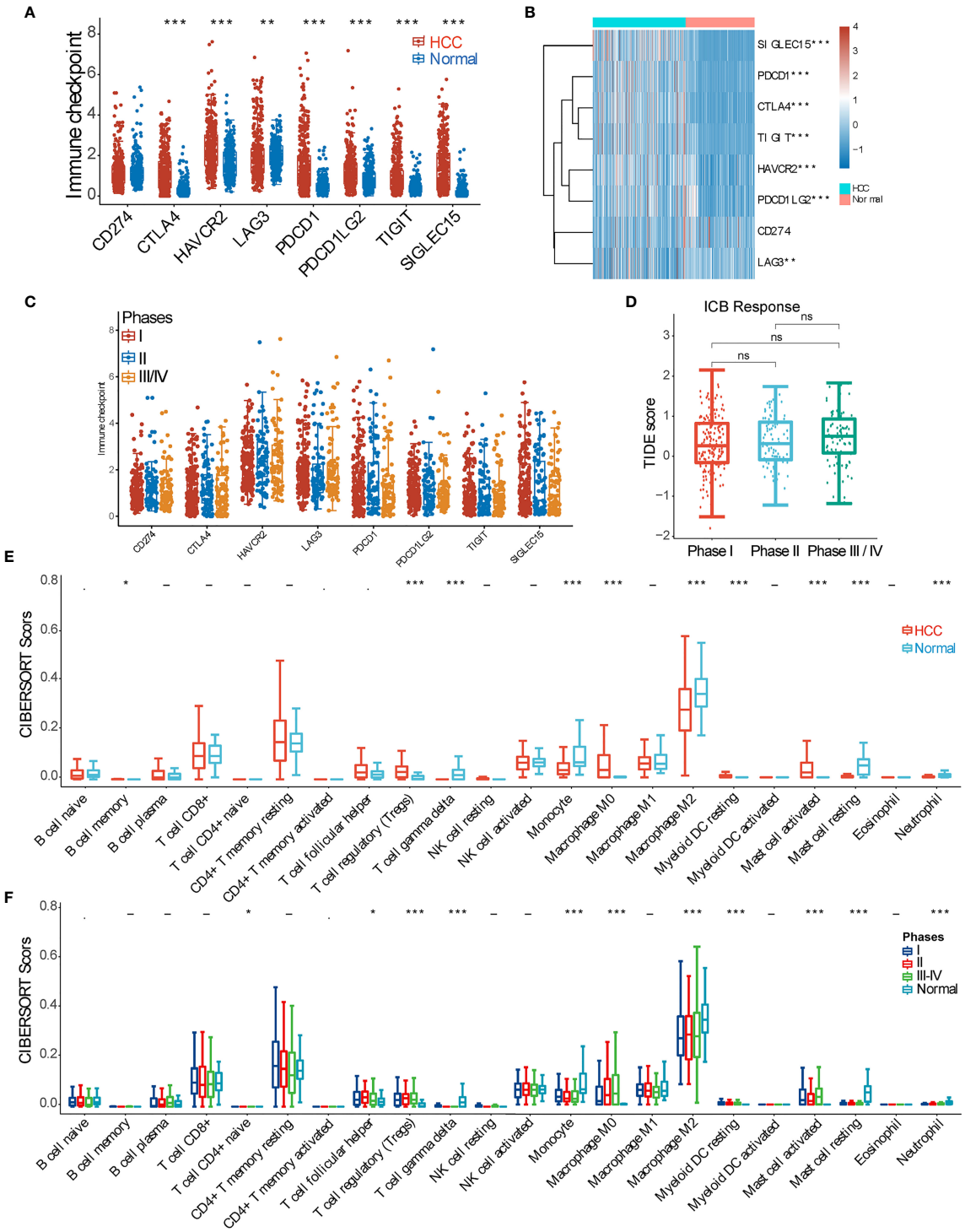


FIGURE 1 | Overview of the immune microenvironment of hepatocellular carcinoma (HCC) tissue. **(A)** Expression difference of inhibitory checkpoint molecules (CD274(PDL1), CTLA4, HAVCR2(TIM3), LAG3, PDCD1(PD1), PDCD1LG2(PDL2), TIGIT, and SIGLEC15) between HCC and normal tissue samples. **(B)** Heatmap of inhibitory checkpoint molecule expression. **(C)** Expression of inhibitory checkpoint molecules in HCC samples of pTNM stages I, II, and III–IV. **(D)** Tumor Immune Dysfunction and Exclusion (TIDE) score analysis was used to predict the responses to immune checkpoint blockade (ICB) of HCC patients at pTNM stages I, II, and III–IV. **(E)** CIBERSORT score of various immune cell infiltrations in HCC and normal tissue samples. **(F)** CIBERSORT score of various immune cell infiltrations among HCC samples of pTNM stages I, II, and III–IV. *p < 0.05; **p < 0.01; ***p < 0.001; '-', ns, no statistical significance.

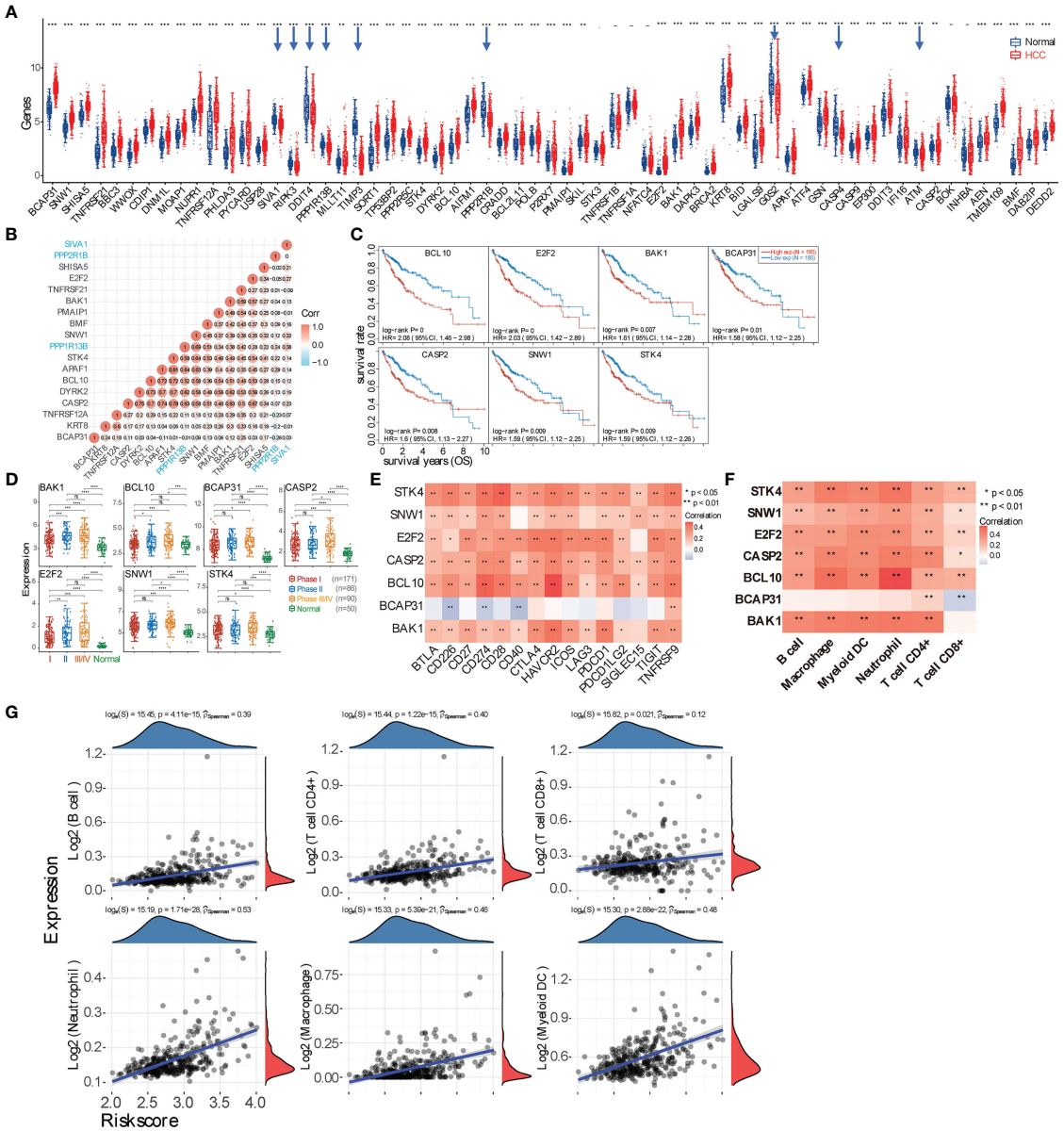


FIGURE 2 | Evaluating the underlying correlation between apoptosis and immune microenvironment. **(A)** Expression spectrum of apoptosis marker genes in hepatocellular carcinoma (HCC) tumor tissue. Downregulated genes are highlighted by blue arrows. **(B)** Multi-gene correlation map generated using the genes with significant ($p < 0.05$) correlation with the overall survival (OS) rate. A positive value represents a positive correlation; otherwise, a negative correlation. The larger correlation value means the better correlation between two genes. The three genes labeled with blue color are downregulated genes in panel **(A, C)** Survival curves of 7 genes (BCL10, E2F2, BAK1, BCAP31, CASP2, SNW1, and STK4) that negatively correlate with prognosis ($p < 0.01$). **(D)** Expression difference of those 7 genes in HCC tumor tissues among patients with stage I, II, or III–IV. **(E)** Correlation analysis between 7 marker genes and checkpoint molecules. Only BCAP31 negatively correlates with checkpoint molecules. **(F)** Correlation between 7 marker genes and major types of immune cell infiltration. **(G)** Spearman's correlation analysis between the risk score of the prognostic model (calculated from the 7 marker genes) and immune score (based on TIMER). The horizontal axis represents the risk score of the prognostic model; the vertical axis represents the immune score. The density curve (red) on the right represents the distribution trend of the immune score, and the dark-blue density curve on the top represents the distribution trend of the risk score. The p-value and correlation coefficient ($\hat{\rho}$ Spearman) are shown at the top of the respective graph. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; '-', ns, no statistical significance.

shown in the table. It shows that the expressions of most checkpoint molecules (except SIGLEC15) are significantly elevated in "G2" and "G3" compared with "G1." CIBERSORT scores indicated that infiltrations of naïve B cells, $CD8^+$ T cells, naïve $CD4^+$ T cells, resting NK cells, monocytes, macrophage M1, and activated mast cells are statistically different among the three groups ($p < 0.01$). Further TIDE score analysis revealed that "G2" and "G3" have better ICB responses. However, OS probability

shows no statistical difference among the three subgroups. Furthermore, 7 genes with a $p \leq 0.01$ correlation with OS were used to construct a prognostic signature model using the least absolute shrinkage and selection operator (LASSO) Cox regression model. The obtained Riskscore equals (0.072) * *BAK1* + (0.1257) * *BCAP31* + (0.0895) * *BCL10* + (0.2596) * *E2F2* + (0.1424) * *SNW1* (**Supplementary Figure 2D**). Based on this prognostic model, the correlation between the Riskscore and immune cell infiltration was further visualized (**Figure 2G**).

Ferroptosis Is Involved in Constructing the Immunosuppressive Hepatocellular Carcinoma Tumor Microenvironment

Given that ferroptosis can be conditionally triggered by chemotherapy or can spontaneously occur under oxidative stress in the HCC microenvironment, we then checked the overall expressions of ferroptosis marker genes between HCC and normal samples. It shows 19 upregulated and 3 downregulated genes in HCC (**Figure 3A**), and the gene correlation in HCC and normal tissues is shown in **Figure 3B** and **Supplementary Figure 3A**. We then calculated how expressions of these genes would affect patient prognosis, and we found that higher expressions of genes SLC1A5, CARS1, SLC7A11, RPL8, and TFRC lead to poorer OS ($p < 0.01$) (**Figure 3C** and **Supplementary Figure 3B**). Moreover, the expressions of these 5 genes are gradually enhanced from tumor stage (pTNM) I to III/IV (**Figure 3D**). Subsequently, the correlation between these 5 genes and checkpoint molecules or immune cells was analyzed, as shown in **Figures 3E, F**. Moreover, HCC samples were also sub-clustered into 4 groups based on the expression of 22 ferroptosis marker genes, showing the difference in immune cell infiltration as well as expressions of inhibitory checkpoint molecules among subgroups (**Supplementary Figure 3C**). TIDE score revealed that these 4 subgroups respond to ICB differently, and the OS probability among groups is statistically different ($p < 0.001$). Furthermore, a prognostic signature model was constructed, and the obtained Riskscore equals (0.1218) * *CARS1* + (0.0359) * *RPL8* + (0.0095) * *TFRC* + (0.1585) * *SLC7A11* + (0.1652) * *SLC1A5* (**Supplementary Figure 3D**), which was used to calculate the correlation between the Riskscore and immune cell infiltration (**Figure 3G**).

Pyroptosis Conspires in Creating the Immunosuppressive Hepatocellular Carcinoma Tumor Microenvironment

In addition to ferroptosis, pyroptosis is another type of inflammatory cell death. It is thus interesting to unveil the relationship between pyroptosis and the immune response in the HCC microenvironment. We found that, among 33 marker genes, 21 genes are upregulated, 6 genes are downregulated, and 6 genes have no statistical difference (**Figure 4A**). Further analysis revealed that only 6 genes (CASP3, GSDME, NLRC4, NLRP6, NOD1, and PLCG1) are statistically correlated with the OS rate of HCC patients ($p < 0.05$) (**Figure 4B** and **Supplementary Figure 4A**), and only GSDME, NLRP6, and NOD1 are significantly correlated with the OS ($p < 0.01$). Moreover, genes GSDME and NOD1 expressed higher in pTNM stage III–IV compared with stages I and II (**Figure 4C**). Further evaluation revealed that these 6 genes except NLRP6 are all positively correlated with checkpoint molecule expression and immune cell infiltration (**Figures 4D, E**). Additionally, all HCC samples can be grouped into two subclusters based on pyroptosis marker genes (**Supplementary Figure 4B**), and then expressions of checkpoint molecules, immune cell infiltration (TIMER and CIBERSORT scores), ICB, and the OS probability between two subgroups were all deciphered (**Supplementary Figures 4C, D**). The prognostic signature model was also generated by using the genes with $p < 0.01$, and the Riskscore equals (0.2413) * GSDME + (−0.2142) * NLRP6 + (0.0892) * NOD1, and the correlation between the Riskscore and immune cell infiltration was obtained (**Figure 4F** and **Supplementary Figure 4E**).

Cross-Talk of Apoptosis, Ferroptosis, and Pyroptosis in Hepatocellular Carcinoma Tumor Microenvironment

In the context of HCC TME, apoptosis, ferroptosis, and pyroptosis occur in an interconnecting manner, and their cross-talk may exist at both the gene and protein levels. We thus evaluated those marker genes that have a $p < 0.01$ correlation with the OS rate of HCC patients and found that these three types of "-optosis" genes do show cross-talk at the genomic level (**Figure 5A** and **Supplementary Figure 5A**). For example, NOD1 (pyroptosis gene) associates with both apoptosis genes (BCL10, CASP2, STK4, E2F2, and BAK1) and ferroptosis genes (TFRC, SLC1A5, and CARS1), and apoptosis gene BCL10 connects with pyroptosis genes (NOD1 and GSDME) and ferroptosis genes (SLC1A5, TFRC, CARS1, and SLC7A11). The protein–protein correlation analysis (STRING database, https://string-db.org/) further supports the analysis at the mRNA level, clearly showing the existing interactions among apoptosis, ferroptosis, and pyroptosis at the protein levels (**Figure 5B** and **Supplementary Figure 5B**). Next, we examined the protein–protein interactions between 15 checkpoint molecules and the "-optosis" genes that significantly correlate ($p < 0.01$ and <0.05, respectively) with the OS rate (**Supplementary Figures 5C, D**); the result indicated that only 5 checkpoint molecules (CD27, CD28, CD40, CTLA4, and PDL1) correlate with "-optosis" proteins. Afterward, 15 "-optosis" genes (significantly correlating with the OS rate, $p < 0.01$) were used to construct the prognostic signature model, and the obtained Riskscore (**Supplementary Figure 5E**) was correlated with immune cell infiltration (**Figure 5C**), indicating positive correlations with 6 main types of immune cells and specifically relative higher correlations with neutrophils, macrophages, and myeloid DCs ($p_{Spearman} > 0.4$).

Apoptosis–Ferroptosis–Pyroptosis Collaboratively Induces Immunosuppressive Hepatocellular Carcinoma Tumor Microenvironment

Since cross-talk of apoptosis, ferroptosis, and pyroptosis was demonstrated at both the gene and protein levels, it is interesting to decode whether they conspiringly participate in inducing the

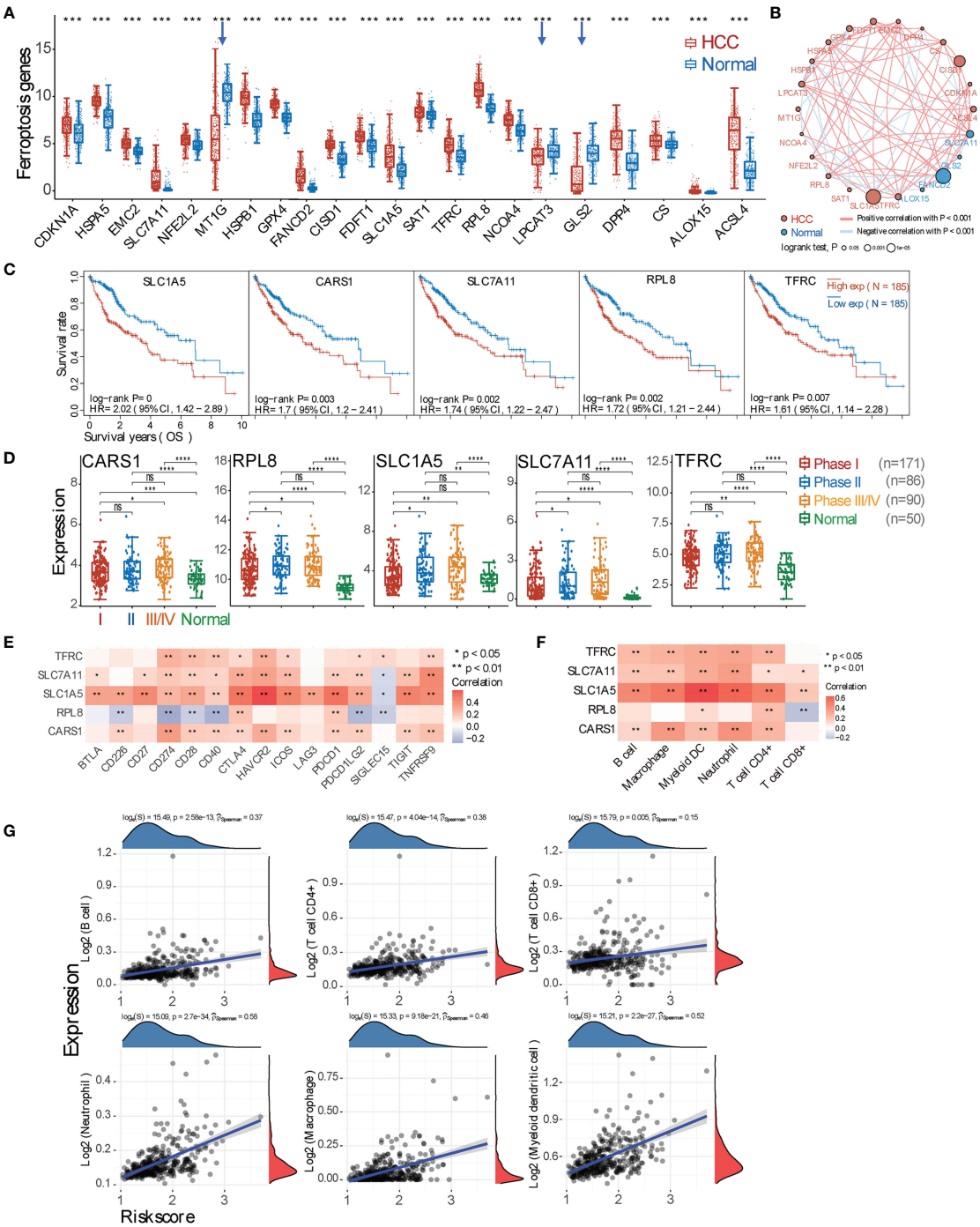


FIGURE 3 | Marker gene expression of ferroptosis and its correlation with tumor microenvironment (TME) immune profile. **(A)** Expression comparison of ferroptosis marker genes between hepatocellular carcinoma (HCC) and normal tissues. The downregulated genes in HCC tissues are highlighted by blue arrows. **(B)** Gene relationship. Each circle represents a gene: the red one means positive, while the blue one represents negative correlation. The different sizes of the circle mean the different log-rank p-value. **(C)** Representative overall survival curves of 5 genes (SLC1A5, CARS1, SLC7A11, RPL8, and TFRC) that are negatively and significantly correlated with prognosis ($p < 0.01$). **(D)** Expression of these 5 marker genes in tumor tissues from pTNM stage I, II, or III–IV patients. **(E)** Correlation analysis between 5 marker genes and checkpoint molecules. **(F)** Correlation between 5 marker genes and major types of immune cell infiltration. **(G)** Spearman's correlation analysis between the risk score of the prognostic model (calculated from the 5 marker genes) and the immune score. The horizontal axis represents the risk score of the prognostic model; the vertical axis represents the immune score. The density curve (red) on the right represents the distribution trend of the immune score, and the dark-blue density curve on the top represents the distribution trend of the risk score. The p-value and correlation coefficient ($\hat{\rho}$ Spearman) are shown at the top of the respective graph. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; '-', ns, no statistical significance.

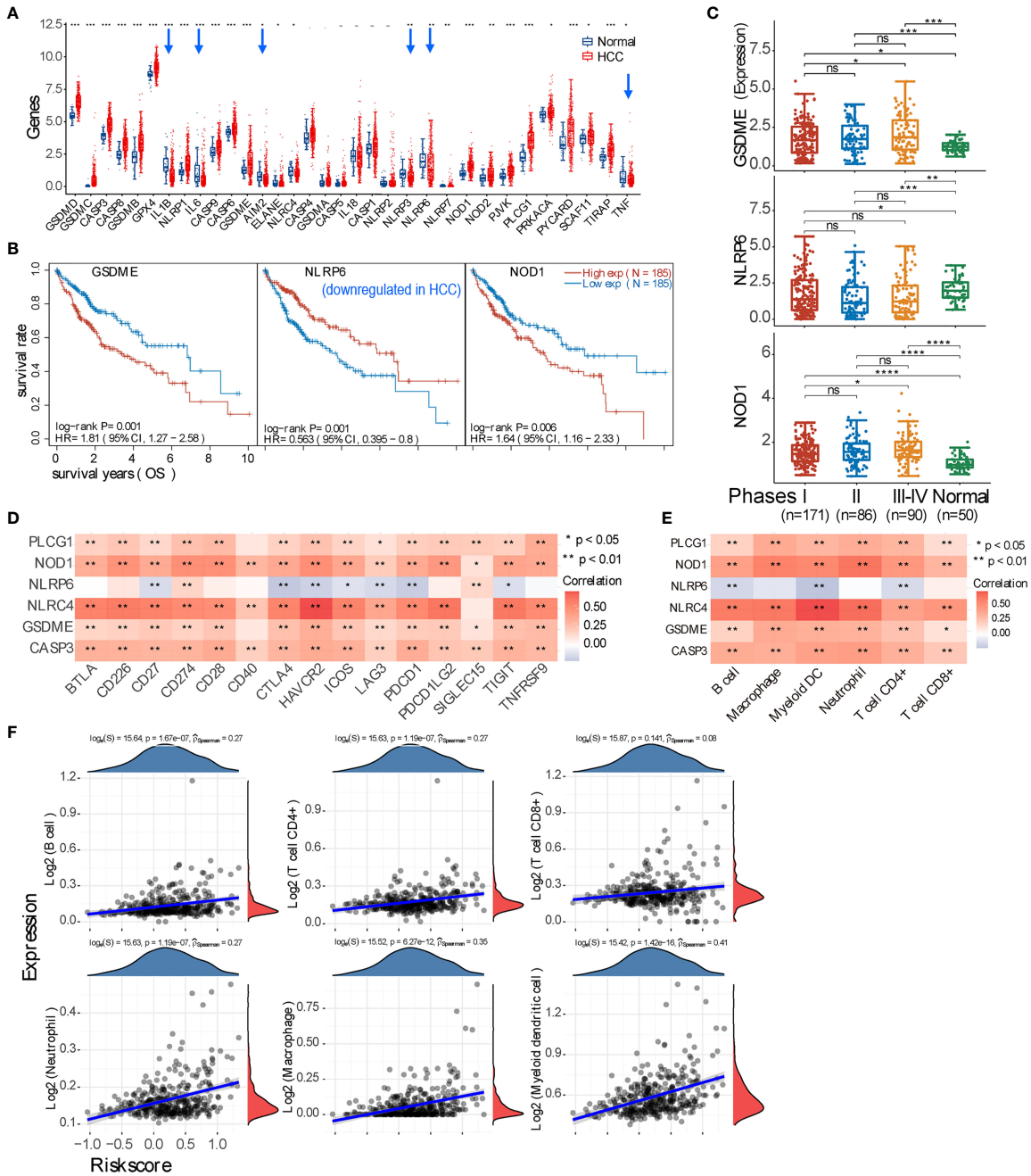


FIGURE 4 | Expression of pyroptosis marker genes and their immune correlation in the context of hepatocellular carcinoma (HCC). **(A)** Expression profile of pyroptosis marker gene in HCC and normal tissue. The downregulated genes in HCC are pointed by blue arrows. **(B)** Three marker genes are significantly correlated with prognosis ($p < 0.01$); specifically, NLRP6 positively correlates with the overall survival of HCC patients. **(C)** Expression comparison of these 3 genes (GSDME, NLRP6, and NOD1) in HCC patients with pTNM stage I, II, or III–IV. **(D, E)** Correlation analysis between all genes that have $p < 0.05$ correlation with prognosis and checkpoint molecules, or immune cell infiltration. **(F)** Spearman's correlation analysis between the risk score of the prognostic model (calculated from the 3 marker genes) and immune score. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; '-', ns, no statistical significance.

immune-suppressiveness of HCC TME. We thus analyzed how each marker gene ($p < 0.01$ correlation with the OS) correlated with immune cell infiltration (**Figure 5D**). The result unequivocally indicates that tumor-infiltrating Tregs, macrophage M0, resting myeloid DCs, and activated mast cells are all significantly enriched in the context of expressions of the listed marker genes. Meanwhile, γδ T cell, monocytes, macrophage M2, resting mast cells, and neutrophils are all

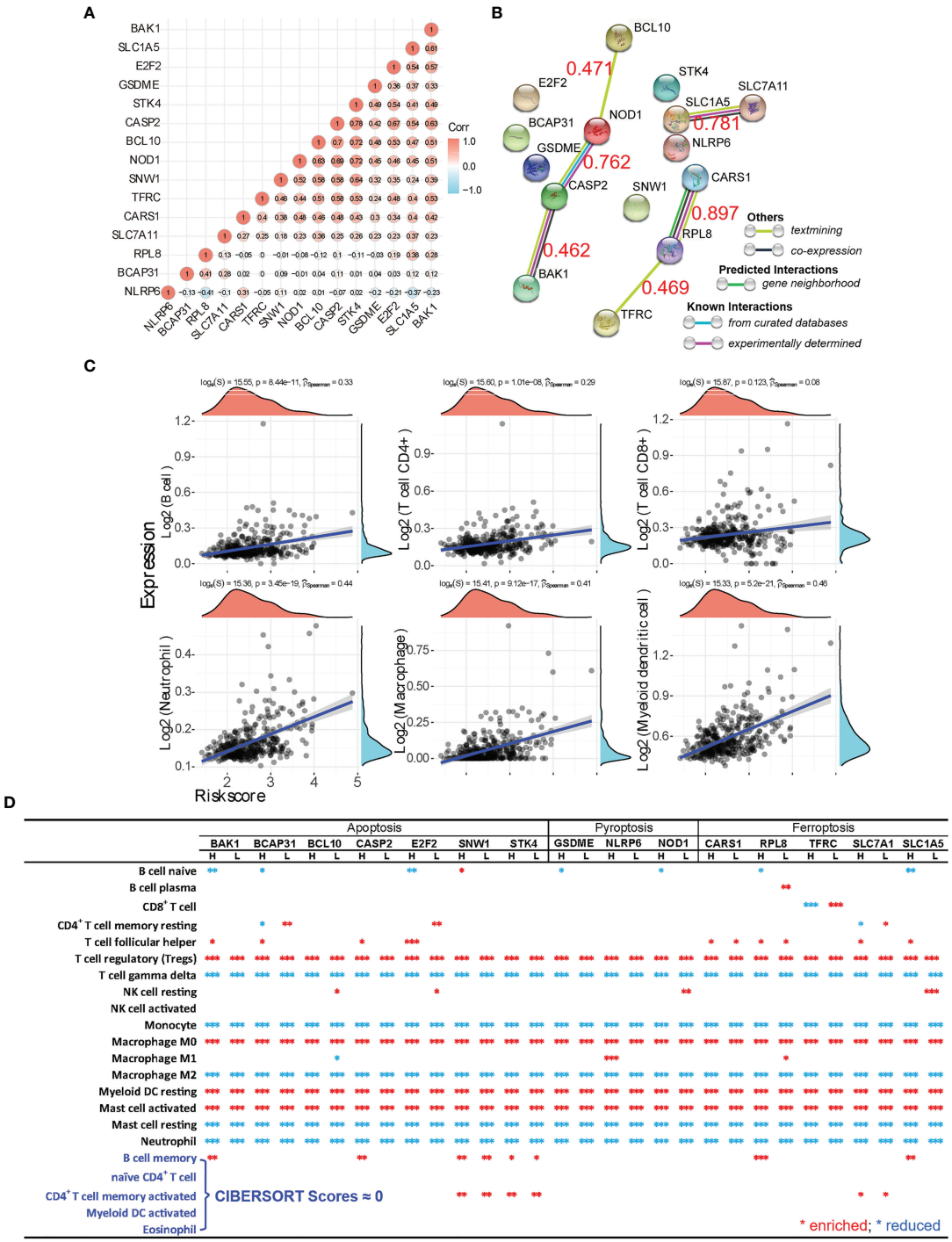


FIGURE 5 | Marker gene cross-talking analysis of apoptosis, ferroptosis, and pyroptosis. **(A)** The gene-gene correlation of all "-optosis" marker genes that have significant correlations with the overall survival (OS) ($p < 0.01$) is analyzed. The correlation coefficient is shown on each circle, and the red circle refers to a positive correlation, while the blue one means a negative correlation. **(B)** Using the analysis tool provided by the STRING database, the interaction of protein corresponding to each "-optosis" marker gene was calculated as well, and the correlation coefficient between genes is marked as red numeric. **(C)** Spearman's correlation analysis between the risk score of the prognostic model (calculated from the 15 "-optosis" marker genes, BCL10, E2F2, BAK1, BCAP31, CASP2, SNW1, STK4, SLC1A5, CARS1, SLC7A11, RPL8, TFRC, GSDME, NLRP6, and NOD1) and immune score. **(D)** Using the CIBERSORT analysis, the comprehensive relationship between the expression of each "-optosis" marker gene ($p < 0.01$ correlating with the OS) and immune cell infiltration is revealed. The red asterisk means enriched infiltration, and the blue one means reduced infiltration of immune cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

significantly suppressed. Importantly, both enriched and reduced immune cells significantly ($p < 0.001$) correlate with the "-optosis" marker genes regardless of their high or low expression in HCC tissue compared with normal tissue. Additionally, OS analysis generated by the prognostic signature model shows that higher expressions of marker genes ($p < 0.01$ correlation with the OS) of apoptosis, ferroptosis, and pyroptosis lead to poorer prognosis both individually (**Figures 6A–C**) and collaboratively (**Figure 6D**) (**Supplementary Figures 2D, 3D, 4E,** and **5E**). These results again suggest that apoptosis, ferroptosis, and pyroptosis altogether participate in creating the immunosuppressive HCC TME.

Immunosuppressive Hepatocellular Carcinoma Tumor Microenvironment Induces γδ T-Cell Imbalance

Since the liver is one of the richest sources of tissue-resident γδ T cells, and our previous works unequivocally proved that adoptive transfer therapy of allogeneic $V\delta2^+$ γδ T cells could safely and efficiently help control liver cancer progression or even achieve complete remission (4, 5), we thus focused on exploring how γδ T cell is repressed in the HCC TME (**Figure 5D**). We tried to inspect the underlying difference of circulating γδ T cells between the healthy population and HCC patients. We found that even though the global proportion of γδ T cells in $CD3^+$ T cells is not statistically different between healthy and HCC populations, the $V\delta1^+$ subset dramatically elevated, while the $V\delta2^+$ subset strikingly reduced in the HCC population ($p < 0.0001$) (**Figure 6E**). This leads to a striking augmentation of the $V\delta1^+/V\delta2^+$ ratio in the HCC population. Further analysis revealed the significant reduction of NKG2D expression in the $V\delta1^+$ subset, but not expressions of PD1, NKP30, and NKP46. For the $V\delta2^+$ subset, expression of PD1 is greatly increased in the HCC group, implying depressed antitumor immunity of $V\delta2^+$ γδ T cells. For other makers, NKG2D, NKP30, and NKP46 show no statistical difference between the healthy and HCC groups (**Figures 6F, G**).

DISCUSSION

It is commonly recognized that the TME is immunosuppressive, which results from many regulatory factors, including low pH value, hypoxia, nutritional deficiency, metabolic pathways remodeling, inflammation, and others (24, 25). Our focus in this work is to decipher the immune landscape of the HCC TME, facilitating clinical efficacy predictions of immunotherapies. Firstly, our global evaluation of HCC transcriptomic data unequivocally reveals that compared with normal tissue, HCC tissue expresses significantly higher inhibitory checkpoint molecules, including CTLA4, HAVCR2, LAG3, PDCD1, PDCD1LG2, TIGIT, and SIGLEC15. Notably, expressions of these checkpoint molecules are independent of the pTNM stages of patients, therefore clearly suggesting that HCC patients with various pTNM stages will have similar responses to ICB therapy. Then, CIBERSORT evaluation revealed that HCC TME is indeed immunosuppressive, evident by significantly more infiltration of Tregs, activated mast cells, and M0 macrophages. Meanwhile, HCC TME strikingly suppresses γδ T-cell infiltration, inhibits the differentiation of macrophages from M0 to M2, dampens the activation of myeloid DCs, and reduces neutrophil infiltration. Importantly, the infiltration of these immune cells shows no statistical difference among samples of different pTNM stages I–IV. Together, we can conclude that HCC TME is highly immunosuppressive, and the patients with different pTNM stages I–IV probably respond to ICB and immune cell therapy similarly.

Inflammation, which causes various types of diseases, is one of the major inducers that closely coordinates with the establishment of the immunosuppressive TME. In HCC TME, inflammation mainly originates from continuous cell death in the context of excessive proliferation of cancer cells. Cancer cell death could be classified into two types, immunogenic and non-immunogenic deaths. Ferroptosis and pyroptosis belong to immunogenic cell death, while apoptosis is a type of non-immunogenic cell death. We thus tried to understand how these three types of PCD (apoptosis, ferroptosis, and pyroptosis) are involved in constructing the immunosuppressive HCC TME. We found that in HCC, most marker genes of the three types of "-optosis" are significantly upregulated, implicating that apoptosis, ferroptosis, and pyroptosis indeed occur routinely in HCC TME. Notably, further inspections indicated that only 7 apoptosis marker genes, 5 ferroptosis marker genes, and 3 pyroptosis marker genes have strong correlations ($p < 0.01$) with the OS rate. This implies that the occurrence of "-optosis" in HCC TME can directly impact the clinical prognosis. Moreover, most of these 15 marker genes are upregulated gradually from pTNM stage I to IV, implying an increasing tendency of "-optosis" from the early to the late stage of liver cancer.

Previously, the gene–gene correlations between checkpoint molecules (both stimulatory and inhibitory) and "-optosis" related marker genes remain largely unknown. After thorough analysis, we found that nearly all "-optosis" marker genes ($p < 0.01$ correlation with the OS) are positively and significantly correlated with expressions of checkpoint molecules except BCAP31, RPL8, and NLRP6 (**Figures 2E**, **3E**, and **4D**). This partially explains why the higher expression of "-optosis" marker genes leads to poor prognoses. Even though these marker genes positively correlated with infiltrations of immune cells (B cells, macrophages, myeloid DCs, neutrophils, $CD4^+$ T, and $CD8^+$ T) as well (**Figures 2F**, **3F**, and **4E**), further immune cell subsets explorations revealed that the enriched immune cells mainly consist of inhibitory cells (Tregs and activated mast cells) and resting immune cells (resting macrophages and DCs) (**Figure 5D**). Meanwhile, antitumor effector immune cells are greatly suppressed, including γδ T cells, monocytes, and neutrophils. Such immune phenotype of HCC TME probably links with checkpoint molecules CD27, CD40, CD28, and CTLA4, which leads to enriched inhibitory cells while depleting effector immune cells, although this hypothesis needs to be validated experimentally. These pieces of evidence altogether indicated that the three types of "-optosis" collaboratively orchestrated the immunosuppressive nature of HCC TME, thus

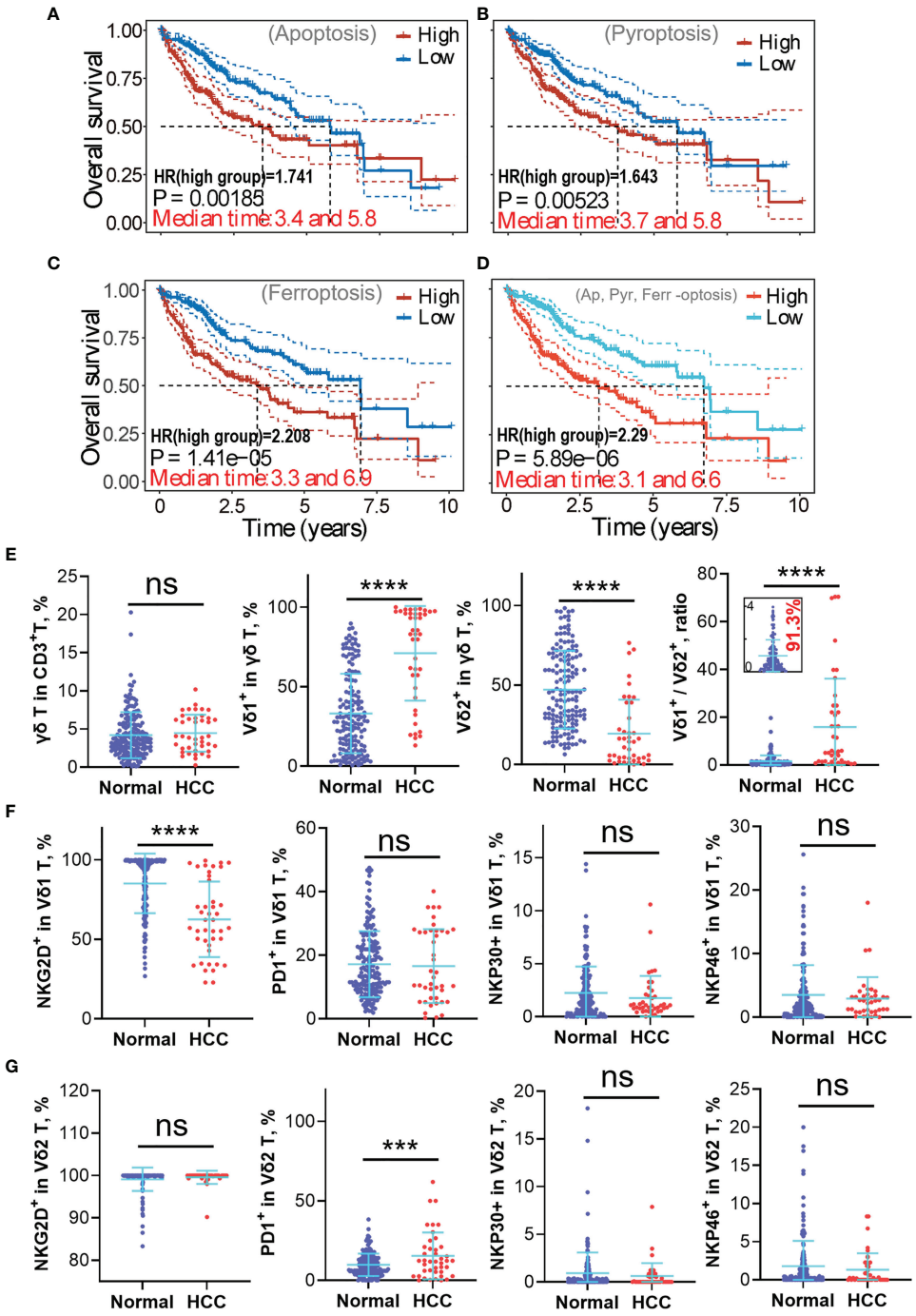


FIGURE 6 | High expressions of apoptosis, ferroptosis, and pyroptosis-related marker genes in hepatocellular carcinoma (HCC) lead to poorer prognosis and implicate γδ T-cell depletion, particularly Vδ2$^+$ γδ T-cell depletion. **(A–C)** The "-optosis" marker genes were used to create the prognostic signature model using the least absolute shrinkage and selection operator (LASSO) Cox regression model (cited from **Supplementary Figures 2D, 3D, 4E**), the correlations between the Riskscore and overall survival (OS) are graphically shown here. It shows higher expression of marker genes positively correlates with higher risk coefficient (HR > 1), leading to poorer clinical outcome. **(D)** Correlation between the Riskscore (generated using 15 "-optosis" marker genes) and the OS (cited from **Supplementary Figure 5E**). Higher Riskscore leads to a poorer prognosis. **(E)** Circulating γδ T-cell phenotypes from the healthy population and HCC patients were analyzed using flow cytometry, including the proportion of γδ T cell in CD3$^+$ T cells, Vδ1$^+$ subset in γδ T cell, Vδ2$^+$ subset in γδ T cell, and the Vδ1$^+$/Vδ2$^+$ ratio. **(F)** Functional phenotypes of circulating Vδ1$^+$ γδ T cell, including expressions of NKG2D, PD1, NKP30, and NKP46. **(G)** Functional phenotypes of circulating Vδ2$^+$ γδ T cell, including expressions of NKG2D, PD1, NKP30, and NKP46. ns, no statistical significance; ***p < 0.001; ****p < 0.0001.

addressing why HCC patients with higher expression of "-optosis" marker genes had poorer responses to ICB therapy.

Since γδ T cells reside preferentially in the liver and play a key role in preventing liver tumorigenesis, we thus analyzed phenotypic profiles of circulating γδ T cells in HCC and healthy populations. Universally, $V\delta2^+$ is dominant over $V\delta1^+$ subset in the peripheral blood of the healthy population and accounts for over 50% of total γδ T cells. Under immune-suppressed conditions, the $V\delta2^+$ subset is slowly depleted in the context of long-term stimulation of phosphoantigens presented by cancer cells and myeloid cells; however, the $V\delta1^+$ subset can survive, as it is more Treg-like. This interpretation can be supported by results that the $V\delta1^+$ subset was enriched in HCC patient blood, while the percentage of the $V\delta2^+$ subset was strikingly decreased. Because the data show that more than 90% of the healthy population has the $V\delta1^+/V\delta2^+$ ratio ≤ 4 and more than 63.4% of HCC patients have the $V\delta1^+/V\delta2^+$ ratio > 4, we thus propose that 4 can be used as the threshold value of the $V\delta1^+/V\delta2^+$ ratio since higher $V\delta1^+/V\delta2^+$ ratio implies more suppressive immunity of the host. Whether or not the proposed $V\delta1^+/V\delta2^+$ ratio can be a predictive factor for discriminating healthy (≤4) or sub-healthy conditions (>4 with no medical abnormalities) and for predicting HCC prognosis (≤4 links with better prognosis) needs further validation with a larger scale of clinical samples. Nevertheless, the imbalanced $V\delta1^+/V\delta2^+$ ratio has the potential to be used as an indicator in health checkups and clinical prognosis. Additionally, reduced NKG2D expression of the $V\delta1^+$ population suggests depressed cell activation and probably enhanced survival ability of the $V\delta1^+$ subset in HCC. In contrast, a significantly higher PD1 expression in the $V\delta2^+$ population indicated the depressed cytotoxicity of the $V\delta2^+$ γδ T cell in the HCC group (**Figures 6F, G**). Together, we can conclude that in the context of HCC, the $V\delta1^+$ subset survives and consists of the main γδ T-cell population, whereas the $V\delta2^+$ subset is more vulnerable to long-term stimulation of phosphoantigens and is easily depleted or suppressed.

In conclusion, the HCC TME is highly immunosuppressive, leading to high expressions of inhibitory checkpoint molecules and limited infiltrations of antitumor immune cells. Our study revealed that apoptosis, ferroptosis, and pyroptosis conspiringly

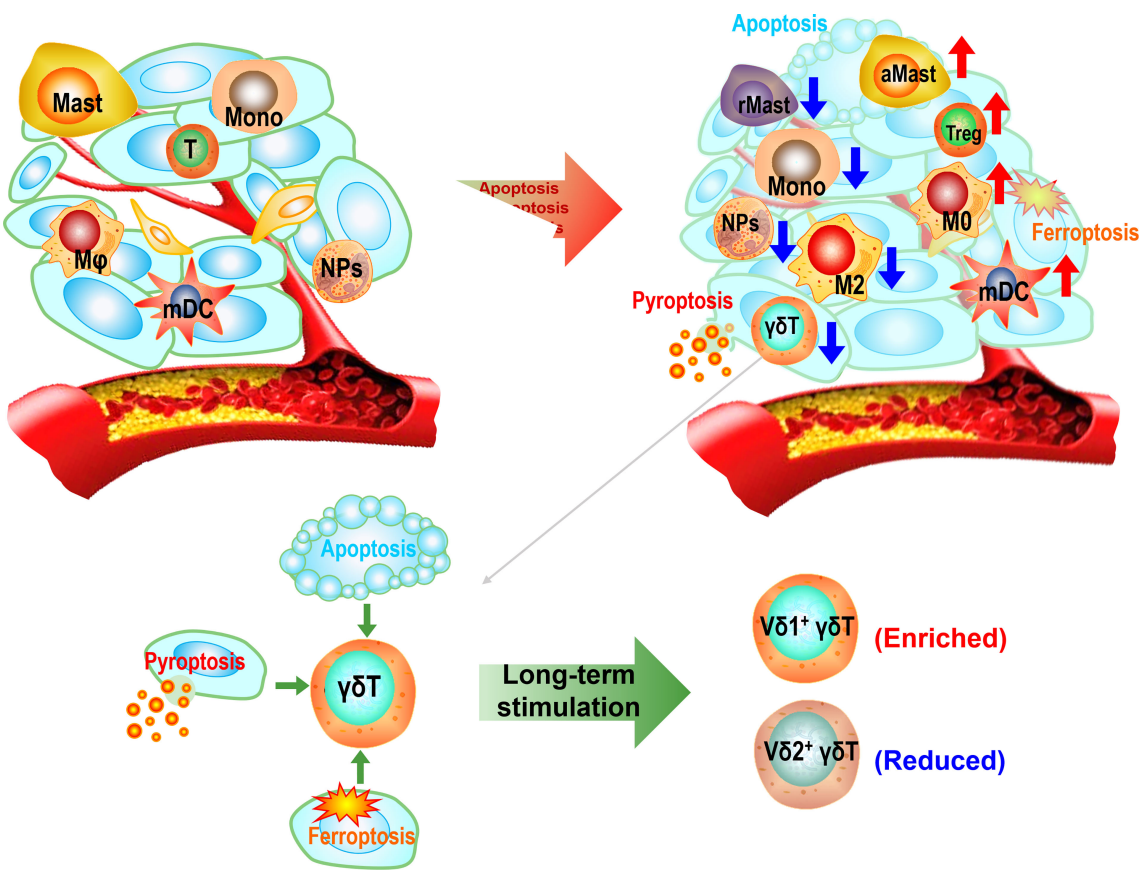


FIGURE 7 | The sketch diagram depicts the establishment of immunosuppressive hepatocellular carcinoma (HCC) tumor microenvironment (TME). The excessive growth of cancer cells accompanies by apoptotic, ferroptotic, and pyroptotic deaths, leading to the construction of the inflammatory microenvironment and subsequently immunosuppressive TME. The immune profile of the TME appears as enrichments of Tregs, activated mast cells, M0, and resting myeloid dendritic cells (DCs) but reductions of γδ T cells, M2 cells, monocytes, resting mast cells, and neutrophils. Long-term stimulation in the TME leads to a predominant $V\delta1^+$ population in total γδ T cells.

induce the establishment of the immunosuppressive HCC TME. The immunosuppressive landscape of the HCC TME was shaped by the high expression of inhibitory checkpoint molecules and enrichments of Tregs, activated mast cells, M0, and non-activated myeloid DCs but low enrichments of antitumor effector cells such as γδ T cells, M2 cells, monocytes, and neutrophils (**Figure 7**). Our bioinformatics evidence also showed that the inactivation of the TME infiltrating immune cells might be mediated by checkpoint molecules. Finally, since cytotoxic $V\delta2^+$ γδ T cell is selectively depleted whereas Treg-like $V\delta1^+$ γδ T cell is upregulated in HCC, the adoptive transfer of allogeneic $V\delta2^+$ γδ T cells could be a promising immunotherapeutic strategy for this malignant cancer (4, 5).

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

Work supervision and project design: YH and YW. Experiments: DC, MH, JL, and YJL. Data analysis and bioinformatics: YW and YH. Data discussion: YW, YH, JH, LL, and ZY. Manuscript drafting, proofreading, and revision: YH and YW. All authors approved manuscript submission.

ACKNOWLEDGMENTS

This work was partially supported by the Key Program of the National Natural Science Foundation of China (32030036) (ZNY), the National Natural Science Foundation of China (82002787) (YH), the Startup Foundation of the Zhuhai People's Hospital (YNXM20210305) (YW), and the Natural Science Foundation of Guangdong Province (2020A1515010132) (YW). We also thank Ms. Qian Jin from the Shuangzhi Purui Medical Laboratory Co., Ltd. (Wuhan, China) for helping to compile the immunophenotype data of the healthy and HCC population, and we thank Mr. Yuncong Zhang from the Zhuhai Institute of Translational Medicine for helping with the data analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.845974/full#supplementary-material

REFERENCES

1. Johnston MP, Khakoo SI. Immunotherapy for Hepatocellular Carcinoma: Current and Future. *World J Gastroenterol* (2019) 25(24):2977–89. doi: 10.3748/wjg.v25.i24.2977
2. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in Patients With Advanced Hepatocellular Carcinoma (CheckMate 040): An Open-Label, non-Comparative, Phase 1/2 Dose Escalation and Expansion Trial. *Lancet* (2017) 389(10088):2492–502. doi: 10.1016/S0140-6736(17)31046-2
3. Mizukoshi E, Kaneko S. Immune Cell Therapy for Hepatocellular Carcinoma. *J Hematol Oncol* (2019) 12(1):52. doi: 10.1186/s13045-019-0742-5
4. Xu Y, Xiang Z, Alnaggar M, Kouakanou L, Li J, He J, et al. Allogeneic Vgamma9Vdelta2 T-Cell Immunotherapy Exhibits Promising Clinical Safety and Prolongs the Survival of Patients With Late-Stage Lung or Liver Cancer. *Cell Mol Immunol* (2021) 18(2):427–39. doi: 10.1038/s41423-020-0515-7
5. Alnaggar M, Xu Y, Li J, He J, Chen J, Li M, et al. Allogenic Vgamma9Vdelta2 T Cell as New Potential Immunotherapy Drug for Solid Tumor: A Case Study for Cholangiocarcinoma. *J Immunother Cancer* (2019) 7(1):36. doi: 10.1186/s40425-019-0501-8
6. Huo J, Cai J, Guan G, Liu H, Wu L. A Ferroptosis and Pyroptosis Molecular Subtype-Related Signature Applicable for Prognosis and Immune Microenvironment Estimation in Hepatocellular Carcinoma. *Front Cell Dev Biol* (2021) 9:761839. doi: 10.3389/fcell.2021.761839
7. Liao H, Shi J, Wen K, Lin J, Liu Q, Shi B, et al. Molecular Targets of Ferroptosis in Hepatocellular Carcinoma. *J Hepatocell Carcinoma* (2021) 8:985–96. doi: 10.2147/JHC.S325593
8. Racanelli V, Rehermann B. The Liver as an Immunological Organ. *Hepatology* (2006) 43(2 Suppl 1):S54–62. doi: 10.1002/hep.21060
9. Norris S, Collins C, Doherty DG, Smith F, McEntee G, Traynor O, et al. Resident Human Hepatic Lymphocytes are Phenotypically Different From Circulating Lymphocytes. *J Hepatol* (1998) 28(1):84–90. doi: 10.1016/S0168-8278(98)80206-7
10. Wu X, Zhang JY, Huang A, Li YY, Zhang S, Wei J, et al. Decreased Vdelta2 Gammadelta T Cells Associated With Liver Damage by Regulation of Th17 Response in Patients With Chronic Hepatitis B. *J Infect Dis* (2013) 208 (8):1294–304. doi: 10.1093/infdis/jit312
11. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-Infiltrating Gammadelta T Cells Suppress T and Dendritic Cell Function via Mechanisms Controlled by a Unique Toll-Like Receptor Signaling Pathway. *Immunity* (2007) 27(2):334–48. doi: 10.1016/j.immuni.2007.05.020
12. Yi Y, He HW, Wang JX, Cai XY, Li YW, Zhou J, et al. The Functional Impairment of HCC-Infiltrating Gammadelta T Cells, Partially Mediated by Regulatory T Cells in a TGFbeta- and IL-10-Dependent Manner. *J Hepatol* (2013) 58(5):977–83. doi: 10.1016/j.jhep.2012.12.015
13. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. *Nat Med* (2015) 21(8):938–45. doi: 10.1038/nm.3909
14. Ni C, Fang QQ, Chen WZ, Jiang JX, Jiang Z, Ye J, et al. Breast Cancer-Derived Exosomes Transmit lncRNA SNHG16 to Induce CD73+gammadelta1 Treg Cells. *Signal Transduct Target Ther* (2020) 5(1):41. doi: 10.1038/s41392-020-0129-7
15. Chabab G, Barjon C, Bonnefoy N, Lafont V. Pro-Tumor Gammadelta T Cells in Human Cancer: Polarization, Mechanisms of Action, and Implications for Therapy. *Front Immunol* (2020) 11:2186. doi: 10.3389/fimmu.2020.02186
16. Hua F, Kang N, Gao YA, Cui LX, Ba DN, He W. Potential Regulatory Role of In Vitro-Expanded Vdelta1 T Cells From Human Peripheral Blood. *Immunol Res* (2013) 56(1):172–80. doi: 10.1007/s12026-013-8390-2
17. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, et al. Gamma Delta T Cells Provide an Early Source of Interferon Gamma in Tumor Immunity. *J Exp Med* (2003) 198(3):433–42. doi: 10.1084/jem.20030584
18. Sun G, Yang S, Cao G, Wang Q, Hao J, Wen Q, et al. Gammadelta T Cells Provide the Early Source of IFN-Gamma to Aggravate Lesions in Spinal Cord Injury. *J Exp Med* (2018) 215(2):521–35. doi: 10.1084/jem.20170686
19. Silva-Santos B, Mensurado S, Coffelt SB. Gammadelta T Cells: Pleiotropic Immune Effectors With Therapeutic Potential in Cancer. *Nat Rev Cancer* (2019) 19(7):392–404. doi: 10.1038/s41568-019-0153-5

20. Liang J, Fu L, Li M, Chen Y, Wang Y, Lin Y, et al. Allogeneic Vg9Vd2 T-Cell Therapy Promotes Pulmonary Lesion Repair: An Open-Label, Single-Arm Pilot Study in Patients With Multidrug-Resistant Tuberculosis. *Front Immunol* (2021) 12. doi: 10.3389/fimmu.2021.756495
21. Liu Z, Zhao Q, Zhu ZX, Yuan SQ, Yu K, Zhang Q, et al. Systematic Analysis of the Aberrances and Functional Implications of Ferroptosis in Cancer. *iScience* (2020) 23(7):101302. doi: 10.1016/j.isci.2020.101302
22. Lin W, Chen Y, Wu B, Chen Y, Li Z. Identification of the Pyroptosis-Related Prognostic Gene Signature and the Associated Regulation Axis in Lung Adenocarcinoma. *Cell Death Discov* (2021) 7(1):161. doi: 10.1038/s41420-021-00557-2
23. Jiang P, Gu S, Pan D, Fu J, Sahu A, Hu X, et al. Signatures of T Cell Dysfunction and Exclusion Predict Cancer Immunotherapy Response. *Nat Med* (2018) 24(10):1550–8. doi: 10.1038/s41591-018-0136-1
24. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
25. Li Y, Wu Y, Hu Y. Metabolites in the Tumor Microenvironment Reprogram Functions of Immune Effector Cells Through Epigenetic Modifications. *Front Immunol* (2021) 12:641883. doi: 10.3389/fimmu.2021.641883

Conflict of Interest: ZY is the founder of the Shuangzhi Purui Medical Laboratory Co., Ltd. (Wuhan, 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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

MINI REVIEW
published: 20 May 2022
doi: 10.3389/fimmu.2022.894580

Contemplating Dichotomous Nature of Gamma Delta T Cells for Immunotherapy

Jaydeep Bhat[1*†], *Katarzyna Placek*[2†] *and Simon Faissner*[3]

[1] Department of Molecular Immunology, Ruhr-University Bochum, Bochum, Germany, [2] Department of Molecular Immunology and Cell Biology, Life and Medical Sciences Institute, University of Bonn, Bonn, Germany, [3] Department of Neurology, Ruhr-University Bochum, St. Josef-Hospital, Bochum, Germany

γδ T cells are unconventional T cells, distinguished from αβ T cells in a number of functional properties. Being small in number compared to αβ T cells, γδ T cells have surprised us with their pleiotropic roles in various diseases. γδ T cells are ambiguous in nature as they can produce a number of cytokines depending on the (micro) environmental cues and engage different immune response mechanisms, mainly due to their epigenetic plasticity. Depending on the disease condition, γδ T cells contribute to beneficial or detrimental response. In this review, we thus discuss the dichotomous nature of γδ T cells in cancer, neuroimmunology and infectious diseases. We shed light on the importance of equal consideration for systems immunology and personalized approaches, as exemplified by changes in metabolic requirements. While providing the status of immunotherapy, we will assess the metabolic (and other) considerations for better outcome of γδ T cell-based treatments.

Keywords: gamma delta T cells, immunotherapy, cancers, infection, neuroimmunology, metabolism, multi-omics

OPEN ACCESS

Edited by:
Alice Cheung, Singapore General Hospital, Singapore

Reviewed by:
Ilan Bank, Sheba Medical Center, Israel

****Correspondence:***
Jaydeep Bhat
jaydeep.bhat@rub.de

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *11 March 2022*
Accepted: *22 April 2022*
Published: *20 May 2022*

Citation:
Bhat J, Placek K and Faissner S (2022) Contemplating Dichotomous Nature of Gamma Delta T Cells for Immunotherapy. Front. Immunol. 13:894580. doi: 10.3389/fimmu.2022.894580

INTRODUCTION

T cells and B cells have emerged as primary lymphocytes lineages throughout 500 million years of evolutionary conservation, mainly generating antigen receptor diversity through somatic recombination (1, 2). The broad range of diversity is achieved by the recombination events occurring on human chromosome 7 for TCR γ and β chain genes, and on human chromosome 14 for TCR α and δ chain genes. TCR γ and δ genes in mice are located on chromosomes 13 and 14, respectively (3, 4). The variable regions of TCR chains comprising of variable (V), diversity (D), and joining (J) elements give rise to the broad range of diversity which enables recognition of foreign molecular patterns (3). Conventionally, TCR α and β chains are rearranged and expressed on the surface to become αβ T cells (~95% of $CD3^+$ T cells in human peripheral blood), while TCR γ and δ chain-expressing cells become γδ T cells (~5% of $CD3^+$ T cells in human peripheral blood). TCR γ and δ chain genes are further classified into subfamilies, consequently, the multiple combinations of these TCR family genes generate many functional γδ T-cell subsets such as $Vγ9^+Vδ2$, $Vγ9^-Vδ2$, Vδ1 and Vδ3 which can be paired with various Vγ chains. Depending on the ontogeny of the subset, the phenotypic distribution and ligand recognition change dramatically (5). The Vδ1 subset is abundant in the intestine and gut, but it is a minor population in the peripheral blood. This is in contrast to the Vδ2 subset which is a major population in circulation and a minor subset in the mucosa.

The features and functions of both αβ and γδ T cells differ remarkably well depending on the thymic and extrathymic origin, as extrathymic T cells are more functionally "innate" immune cells (6, 7). Mouse γδ T-cell subsets develop through successive but coordinated waves and reside in most of the peripheral tissues. These murine γδ T cells, classified based on TCR γ chains, are mainly found in two functional states depending on interferon-γ (IFN-γ) or interleukin-17 (IL-17) production (8).

γδ T cells possess a unique potential of functional plasticity. Within the tumor milieu, γδ T cells produce cytokines (e.g. IFN-γ or IL-17), which are associated with the prognosis of different kinds of cancers. This dual roles of human γδ T cells in cancer has been recently reviewed (9). Since γδ T cells possess a dichotomous nature in cancer, autoimmunity and infections, this review will focus on mechanisms of γδ T cells in those areas; however, being aware about the fact there are important advances in other fields such as modes of antigen recognition (5, 10), fetal ontology (11, 12), and involvement in hepatic or gastro-intestinal diseases (13–16), which is out of the scope of this review. Apart from the basic understanding of γδ T-cell subsets and their function in health and diseases, the use of γδ T cells for immunotherapeutic applications is of great interest. Additionally, other recent technological advances such as single-cell omics, 3D organoid models or humanized mice will facilitate the progress in harnessing the therapeutic potential of human γδ T cells. In this review, we discuss the pivotal features of γδ T cells and their potential for therapeutic approaches.

DICHOTOMY OF γδ T CELLS

γδ T cells in Cancers – a Double-Edged Sword

Due to their potential of immunoserveillance and anti-tumor response, γδ T cells are found to be involved in several types of cancer including hematological malignancies (17), glioblastoma (18), gastric (19), colorectal (20) and breast cancer (21). Dysregulated mevalonate metabolism in cancer cells often leads to the accumulation of phosphoantigens (pAg) such as Isopentenyl Pyrophosphate (IPP), which potentiates Vγ9Vδ2 T-cell cytotoxicity (22). IPP can be released to the extracellular space where it is recognized by Vδ2 T cells *via* ATP-binding cassette transporter A1 (ABCA1) and apoliprotein A-I (apoA-1) (23). Recent studies have shown that phosphoantigens are bound by butyrophilins (BTN), specifically BTN3A1 and BTN2A1, which then interact with the TCR of Vδ2 T cells. Formation of such a signaling complex results in Vδ2 T-cell activation and in the anti-tumor activity (24, 25). Though the molecular details of butyrophilins- γδ TCR signaling complex is largely unknown, a landmark study showed that BTN3A1 (an isoform of CD277) and its intracellular B30.2 domain are absolutely essential for inside-out signaling to activate Vδ2 T cells (26), which is further modulated by Rho-GTPase (27). Dissecting this molecular complexity further, it was revealed that Vγ9Vδ2 TCR is required initially for T-cell activation and formation of immune synapse (IS) with CD277 (recruiting BTN3A1 and BTN2A1, independent of pAg), upon which latter provides mandatory coactivation signal and stabilizes IS in a pAg-dependent manner (28).

In addition to the butyrophilins (as mentioned above) and B7 superfamily-like proteins, major histocompatibility class (MHC) - like antigens and immunoglobulin (Ig) -like antigens have also been identified as antigens for γδ T-cell subsets [extensively reviewed in (5)]. For example, Vδ1 T cells recognize self-derived or foreign lipids bound by the CD1d molecule on the surface of target cells (29, 30). Other interesting examples of MHC-like antigens are MHC-related protein 1 (MR1), ephrin type-A receptor 2 (EphA2) and endothelial protein C receptor (EPCR) (31–33). MR1 is a Vitamin B precursor and known antigen for mucosal associated invariant T cells, but recently shown to be recognized by Vδ1 T cells from healthy individuals and in some diseases (31). EphA2 and EPCR are well known stress-ligands. EPCR serves as a ligand for human Vγ4Vδ5 subset-specific recognition of endothelial cells infected by cytomegalovirus and epithelial tumors (33). Not only EPCR, annexin A2 (an Ig-like antigen) is also recognized by Vγ8Vδ3 subset during cellular stress surveillance (34). Interestingly, non-physiological molecules like red algal protein phycoerythrin have also been reported as antigens for human and murine IL-17-producing γδ T cells (35). Furthermore, the functional plasticity of γδ T cells includes a response mediated by CD16 and thus participates in the antibody-dependent cellular cytotoxicity (ADCC). It has been shown to enhance Vδ2 T cell function towards lymphoma cells with the use of anti-CD20 (36, 37). Vγ9Vδ2 T-cell cytotoxicity can also be mediated by the production of cytokines (e.g. IFN-γ and TNF-α), cytotoxic (e.g. granzymes) and apoptotic molecules (e.g. TRAIL), and/or *via* NKG2D receptor-ligand axis (22).

γδ T cells are highly pleiotropic in function as they possess both anti-tumor and pro-tumor activities in the tumor microenvironment (TME). γδ T cells are the early producers of IFN-γ during tumorigenesis (38), while IL-2 and IL-15 are the potent inducers of cytotoxic potential (39, 40), which provide an important cancer immunomodulating factor to promote other cytotoxic T lymphocyte responses. Tumor-infiltrating γδ T cells preferentially produce IFN-γ and are positively associated with better patient outcome in case of colon cancer (20). Also, intracellular IFN-γ expression only after phorbol ester and ionomycin (PMA/Iono) stimulation was remarkable in γδ T cells from TME of ovarian cancer (41). IFN-γ producing γδ T cells exert their anti-tumor functions by upregulating MHC class I molecules and CD54, thus further enhancing CD8 T-cell-mediated killing (42). Conversely, γδ T cells producing IL-17 have been suggested to negatively impact the progression of colon (43), gallbladder (44), and breast cancer (45), either by suppressing immune cell functions, promoting immune cell pro-tumor activity, or by inducing angiogenesis. Hypoxia, which is commonly found in solid tumors, was attributed to reduce cytotoxic activity of γδ T cells in oral cancer patients (46) and enhance IL-17 production. Furthermore, γδ T cells provide pro-tumor inflammatory conditions and thus favor tumor

progression, participating in most of the hallmarks of cancer (47, 48).

γδ T Cells in Autoimmune Disease of the Central Nervous System

Classically, γδ T cells are known to possess the properties of innate immune cells such as rapid expression of IFN-γ or IL-17 in response to cytokine supplementation without TCR engagement. TME drives IL-17 production in γδ T cells and hence provide evidence for the γδ T-cell function as a consequence of (micro) environmental signals. The capacity to produce IL-17 is attributed to epigenetic regulation (49). IL-17 producing γδ T cells are implicated in autoimmunity and inflammatory conditions. Results from experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), provide evidence that γδ T cells serve as important source of cytokines IL-17 and IL-23 and consequently amplify IL-17 production by Th17 cells (50). Vice versa, IL-17A is also important for the recruitment of IL-1β secreting myeloid cells that prime pathogenic γδT17 and Th17 cells in EAE (51), suggesting a regulatory loop. One effect of IL-17 producing γδ T cells is to interfere with regulatory T cells (Treg) development by preventing the conversion of conventional T cells into Foxp3$^+$ Treg cells as elicited using IL23R reporter mice (52). This observation was additionally supported by enhanced antigen-specific T cell responses by γδ T cells. In EAE, Vγ4$^+$IL-17 producing γδ T cells differentiate in the draining lymph nodes, mediated by IL23R and through activation of *Il17* locus, but not *via* IL-1R1 (53).

Those alterations hold also true for MS. Using single-cell RNA-seq and spatial transcriptomics Th17/Tfh cells have been identified as cellular marker of MS disease progression (54). Though EAE is skewed towards IL-17 producing γδ T cells, studies in human have shown a more remarkable association of MS with IFN-γ producing γδ T cells. Vδ1 T cells were shown to produce a high amount of IFN-γ in newly diagnosed, untreated MS patients, which was decreased by treatment with natalizumab (55). Contrarily, single or dual expression of IFN-γ and IL-17 by Vδ2 T cells is lower in MS patients compared to healthy controls (56). There is also evidence of direct cytotoxicity towards oligodendrocytes by γδ T cells (57). γδ T cells could therefore serve as marker of disease activity. Circulating CCR5$^+$ γδ T cells are decreased during MS relapse in line with higher frequency of IFN-γ$^+$ γδ T cells, assuming a Th1 profile (58). Hence, beside other nonconventional immune cells, single-cell resolution identified specific γδ T cell subsets as contributor of MS disease activity with potential as therapeutic target.

γδ T Cells in Infection

IL-17 production by Th17 cells is usually associated with protection against bacterial and fungal infection through their effector function (59). In 2009, the pivotal role of CCR6$^+$ γδ T cells characterized by IL-17 production, innate receptor expression and recruitment of neutrophils was identified for the first time as first line response to mycobacteria and *Candida albicans* (60). Unlike αβ T cells, IL-17 producing γδ T cells are not associated with the engagement of TCR (50, 60, 61). These observations are highly intriguing, since transcriptionally distinct αβ-γδ co-expressing T cells have been discovered, which produce IL-17 upon stimulation by IL-1β and IL-23 and play a pathogenic role in the CNS autoimmunity in EAE. The characterization of TCR revealed that these hybrid αβ-γδ T cells are mainly Vγ4$^+$ and TCRβ$^+$ and importantly, provide protection against *Staphylococcus aureus* infection (62). This is consistent with findings showing that Vγ6$^+$Vδ4$^+$ T cells are clonally expanded in skin-draining lymph nodes after *S. aureus* infection in mice. RNA-seq analysis of TRG and TRD sequences revealed the clonal expansion of TRGV5, TRGV6, and TRDV4 (63). In contrast to murine γδ T cells, human γδ T cells play a diverse role in infection immunity. Human Vδ2 T cells have been known to respond strongly to phosphoantigens such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) which is a metabolite produced by microbes *via* the 2-c-methyl-D-erythritol 4-phosphate (MEP) pathway (64). Vδ2 cells are protective in *Plasmodium falciparum* infection (65). Recently, it was shown that γδ T cells kill infected red blood cells by phagocytosis and opsonization *via* CD16, in addition to the BTN3A1-TCR mediated degranulation process (66). This host defense mechanism adds a new aspect to the γδ T-cell function and engagement during immune response. Whether the phagocytotic machinery of γδ T cells is also involved during a response to other pathogens remains to be determined.

For a long time, the role of γδ T cells in mycobacterial infection has been studied in human and animal models. *Mycobacterium tuberculosis* (Mtb) was one of the first bacteria described to induce γδ T cell immune responses (67) by recognizing Mtb antigens. Bacillus Calmette-Guérin (BCG) vaccine, the only vaccine protecting against tuberculosis, has been broadly administered worldwide and it has been shown to generate a protective Vδ2 T cell memory response against Mtb infection (68). BCG has been also suggested to provide heterologous protection against infections that are not related to Mtb (69–72). In fact, in the early 90s, it was shown that *in vitro* pre-expanded γδ T cells with *M. tuberculosis* were able to proliferate in response to re-challenge with unrelated pathogens such as *Listeria monocytogenes*, group A streptococci or *S. aureus* (73). These results indicate that human γδ T cell responses are not pathogen-specific therefore raising the question, whether BCG-induced γδ T cells contribute to their cross-protective effect and whether they can develop innate immune cell memory referred to as "trained immunity". Trained immunity was first described in monocytes and macrophages (which have a shorter half-life) (74, 75). Therefore, those might be less suitable vaccination targets to provide long-term protection. The characterization of trained immunity in long-lived γδ T cells could potentially open new avenues in designing effective vaccines with cross-protective effects. Whether immune memory responses of γδ T cells contribute to the protection induced by other vaccines still needs to be explored.

Altogether, it is crucial to broaden the mechanistic knowledge about the role of γδ T cells in infections using systems immunology approaches such as single-cell multi-omics to provide better therapeutic interventions. This is especially vital

for the development of new generation vaccines, which would not only trigger αβ T cell memory responses but also harness the therapeutic potential of γδ T cells.

γδ T CELLS IN SYSTEMS IMMUNOLOGY – A HOLISTIC APPROACH

The systems approach is defined by the use of a broad strategy for understanding the outcome of a complex set of components (76). Multi-omics methods and systems immunology measures help to investigate changes in the proteome, phenome, transcriptome, epigenome, metabolome, microbiome as well as cell-to-cell communication, all of which shape immune cell responses. A decade ago, these measurements were performed on a bulk cell population. Nowadays, it is possible to use these methods at a single-cell resolution. Such datasets are made publicly available by international consortia such as ImmGen (https://www.immgen.org) for mouse immune cells and the cell atlas for humans (https://www.humancellatlas.org). Additionally, due to increasing efforts to combine interdisciplinary approaches such as computational biology together with data science and machine learning (77), a comprehensive study of immune cells and their responses is feasible in defining disease severity/progression, therapeutic response, or even vaccine effects. Indeed, a new field of "systems vaccinology" has emerged with the aim to comprehensively analyze the immune response to vaccination and understand potential new mechanisms of protection (78). This will allow immunologists to consider the individual human variation, possibly identifying the reasons driving differential immune responses. This holds specifically true for γδ T cell responses, which are largely shaped by environmental factors and not genetic control (79). A milestone study for chimeric antigen receptor (CAR) T cells by Melenhorst et al. (2022) shows an impeccable use of single-cell multi-omics and systems immunology methods (80). In this longitudinal study, authors analyzed CD19 chimeric antigen receptor (CAR) T cells in two chronic lymphocytic leukemia patients over 10 years after successful CAR T cell transfer. In the earlier time-points, $CD4^-$ $CD8^-Helios^{hi}$ CAR T cells in one of these patients were found using cytometry-by-flight (CyTOF) method. Further characterization using 5'cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) with TCR-seq found that these cells were γδ CAR T cells with specific TRDV1 and TRGV4 gene expression. However, long-term surviving CAR T cells were predominated by the $CD4^+$ T cell population with cytotoxic properties especially at later time points. Yet, the origins and contribution of these cells to the remission remains to be determined. Despite a limited number of patients analyzed, this study is the first to show the potential of systems biology and single cell omics to understand the efficacy of CAR T-cell immunotherapy.

Another good example emphasizing the importance of the systems immunology approach is to evaluate the prognostic significance of immune cells in various cancers using CIBERSORT (a machine learning-based algorithm), where γδ T cells have emerged as the most favorable leukocyte with global prognostic association across 25 human cancers (81). Optimizing this computational identification approach further, tumor-infiltrating Vγ9Vδ2 T cells were variably associated with disease outcome due to considerable high inter-individual variation in its abundance (82). A combination of flow cytometry and sequencing results with the help of single sample gene set enrichment analysis (ssGSEA) method has inferred abundance of 24 immune cell types in cancer including γδ T cells. This algorithm called "Immune Cell Abundance Identifier (ImmuneCellAI)" could accurately predict response to anti-PD1 immunotherapy (83). To a limited extent, we have previously used a comprehensive approach to assess the disease progression and therapeutic response in patients with γδ T cells malignancies (84, 85). Though the transcriptome and epigenome of γδ T cells are already available, the focus is now shifted to single-cell studies (**Table 1**) as it allows to create a compendium of cell types as exemplified in mice (104) and humans (105, 106).

An emerging area in system immunology and single-cell methodology is to decipher the metabolic changes in γδ T cells during the development and differentiation process. Mechanistic target of rapamycin complex 1 (mTORC1) regulates a distinct metabolic requirement for thymic development of αβ and γδ T cells. Interestingly, mTORC1 signaling further coordinates developmental signals with TCR and NOTCH pathways (107). Diving into the details of metabolic requirements at a single-cell level, Single Cell ENergetIc metabolism (SCENITH) has been recently developed and used to assess γδ T cell energy metabolism (108, 109). Consequently, this study found that the metabolic requirements of IL-17^+ γδ T cells are imprinted during early thymic development and are maintained in the periphery and tumor of obese mice (109). A distinct metabolic usage by IFN-γ^+ versus IL-17^+ γδ T cells shows a need for glycolysis versus oxidative metabolism, respectively. Interestingly, glucose supplementation elevated the anti-tumor function of IFN-γ^+ γδ T cells (109). Similarly, altered tumor metabolism also needs to be studied as it is sensed by γδ T cells (32), which may ultimately implicates γδ T cell responses in TME causing hypoxia (46, 110) or tumor resistance (111). Moreover, recent reports highlight a crucial role of γδ T cells in thermogenesis and sympathetic innervation (112, 113). Furthermore, the ketogenic diet has been shown to expand protective γδ T cells during an infection with influenza virus in the lungs (114) and which restrain inflammation in adipose tissues (97). However, prolonged intake of the ketogenic diet causes obesity and significantly reduces the adipose tissue-resident γδ T cells (97). Thus, targeting metabolic changes together with transcriptional changes will help to understand the γδ T-cell differentiation process and its implications in diseases.

γδ T CELL-BASED IMMUNOTHERAPY: MISSING LINKS AND UNEXPLORED AVENUES

Due to their unique characteristics distinct from conventional αβ T cells, γδ T cells are an attractive cellular target for allogeneic transfer as exemplified by the recent phase I clinical trial in 132

TABLE 1 | Summary of single-cell multi-omics datasets from healthy individuals or diseases.

Study	Year	Disease	model organism	Biological source	sc-omics method
Watkin et al. (86)	2020	Allergy	Human	PBMCs from peanut allergic (PA) patients and healthy controls	scRNA-seq
Boufea et al. (87)	2020	Breast cancer	Human	peripheral blood γδ T cells from healthy adult donors and from fresh tumor biopsies of breast cancer patients	scRNA-seq
Pizzolato et al. (88)	2019	CMV	Human	PBMC and purified γδ T cells from CMV^+ and CMV^- healthy donors	scRNA-seq
Jaeger et al. (89)	2021	Crohn's disease	Human	IEL T cells sorted from two Crohn's disease patients and two controls	scRNA-seq
10xgenomics	–	Healthy	Human	10k PBMC from a healthy donor (v3 chemistry)	scRNA-seq
Park et al. (90)	2020	Healthy	Human	dissociated cells from human thymus during development, childhood, and adult life	scRNA-seq
Tan et al. (91)	2021	Healthy	Human	γδ T cells sorted from neonatal and adult blood	scRNA-seq and paired TCR sequencing
Reitermaier et al. (92)	2021	Healthy	Human	$CD3^+$ T cells FACS-sorted from single-cell suspensions of three fetal skin donors	scRNA-seq
Tan et al. (93)	2019	Healthy	Mouse	FACS-sorted $Vγ6^+$ T cell, $CD4^+$ and/or $CD8^+$ thymocytes	scRNA-seq
Sagar et al. (94)	2020	Healthy	Mouse	Healthy fetal and adult thymus	scRNA-seq
Lee et al. (95)	2020	Healthy	Mouse	total iNKT, MAIT, and γδ T cells from the pooled thymi of BALB/c mice	scRNA-seq and paired V(D)J sequencing
Alves de Lima et al. (96)	2020	Healthy	Mouse	sorted γδ T cells from the dural meninges and spleen of 7-d-old (P7) or 8-week-old adult mice	scRNA-seq
Goldberg et al. (97)	2020	Healthy	Mouse	pan-CD45 FACS-sorted tissue-resident haematopoietic cells from white adipose tissue	scRNA-seq
Hu et al. (98)	2021	Healthy	Mouse	sorted hepatic and thymic γδ T cells	scRNA-seq
Li et al. (99)	2022	Healthy	Mouse	mouse γδ T cells from peripheral lymph nodes, spleen, and thymus	scRNA-seq and scATAC-seq
Wang et al. (100)	2021	Leukemia	Human	$CD45^+CD3^+$ cell populations from B cell-acute lymphoblastic leukemia and healthy controls	scRNA-seq and paired TCR sequencing
Melenhorst et al. (80)	2022	Leukemia	Human	sorted single $CD3^+$ CAR^+ nuclei from patient PBMC	scRNA-seq, CITE-seq and paired TCR sequencing
Gherardin et al. (101)	2021	Merkel Cell Carcinoma	Human	sorted $CD3^+$ and $γδ^+$ T cells from dissociated Merkel Cell Carcinomas tumor	scRNA-seq and paired TCR sequencing
Schafflick et al. (102)	2020	MS	Human	CSF and blood from MS and healthy donors	scRNA-seq
Kaufmann et al. (54)	2021	MS	Human	PBMC from MS and healthy donors	scRNA-seq
Cerapio et al. (103)	2021	Ovarian cancer	Human	γδ T-cell infiltrating lymphocytes from ovarian carcinoma	scRNA-seq

The listed datasets are generated either directly using γδ T cells or have identified γδ T cells in their computational approaches. PBMC, peripheral blood mononuclear cells; CMV, cytomegalovirus; FACS, fluorescence activated cell sorting; iNKT, invariant natural killer T cells; MAIT, mucosal associated invariant T cells; MS, multiple sclerosis; CAR, chimeric antigen receptor.

late-stage cancer patients (115) and ongoing clinical trial on patients with solid tumors (https://clinicaltrials.gov/ct2/show/NCT04765462). The concept of γδ T cell-based immunotherapy has been under development for more than a decade. Earlier, the immunotherapy with γδ T cells for cancer was mainly based on two approaches: *in vivo* activation of γδ T cells using aminobisphonates (e.g. zoledronate) and adoptive transfer of *in vitro* expanded γδ T cells (116). The approach of *in vivo* activation of γδ T cells has extended its toolbox. Because of the basic research on γδ T-cell activation (26), the molecular mediators of activation (e.g. butyrophilins) can be targeted to improve immunotherapy outcome. In an ongoing clinical trial, monoclonal anti-BTN3A1 antibodies (ICT01) are administered alone or in combination with the checkpoint-inhibitor pembrolizumab for hematological cancers (https://www.clinicaltrials.gov/ct2/show/NCT04243499). Besides checkpoint inhibitors (117), other immunomodulatory agents that could be used in combination with γδ T cell-based immunotherapy include epigenetic drugs (118, 119), toll-like receptor ligands (120), or even bispecific antibodies targeting Vγ9 chains of Vγ9Vδ2 T cells (121). Since most of these modulators have been proposed or shown clinical relevance *in vitro*, many are being tested in a clinical trial. Likewise, the approach of using adoptive transfer of *in vitro* expanded γδ T cells is now suggested to be supplemented with biomaterials such as cytokines [e.g. TGF-β (122)] or nutrients [e.g. Vitamin C (123)]. Furthermore, engineered cytokines have emerged as an attractive tool to improve T cell immunotherapy by modulating cell expansion, persistence, tumor homing and adaptation to TME (124). As engineered IL-2 and IL-15 are already in clinical trials, their use in γδ T cell-based therapies might also be beneficial. The use of naturally occurring nutrient supplementation such as Vitamin C could potentially be beneficial too. However, the metabolic requirement and effect of these nutrients need to be considered given the inter-individual variation in γδ T cell frequency and their subset distribution due to age, gender, and race (125). These needs can be complemented for a more personalized approach to immunotherapy to provide benefit to patients.

γδ T cell-based immunotherapy has been progressing over the last decades along with the development of T cell-based therapies (**Figure 1**). The novel first-in-class approach is being exploited to apply genetically modified γδ T cells in human (138). Previously, the Vδ1 subset of γδ T cells were designed and applied as a new cellular product called "DOT cells" for adoptive immunotherapy of leukemia patients (17, 139). Further advancing the approach of genetic modifications, CAR γδ T cell therapy is under investigation (114, 140, 141). The patient-derived xenograft model showed anti-leukemic activity and IL-15-mediated long-term persistence of CD123-CAR-DOTs in acute myeloid leukemia (142). Following the United States Food and Drug Administration (US FDA) approval for ADI-01 in the year 2020, an allogenic CAR γδ T cell therapy targeting CD20 protein was manufactured at clinical scale. As observed in a preclinical study with B-cell malignancies, CAR γδ T cell therapy generated an innate and adaptive anti-tumor immune response but no xenogeneic graft-versus-host disease (143). This CD20 CAR γδ T cell therapy is now in a phase I clinical trial (https://www.clinicaltrials.gov/ct2/show/NCT04735471). The interim analysis of the ADI-01 phase I clinical trial showed that 50% patients achieved complete response (CR) while 75% patients achieved objective response rate (ORR) without any ADI-01-related serious adverse events using CD20-CAR γδ T cell therapy (press release dated on December 6, 2021; https://www.adicetbio.com). Furthermore, the ADI-01 therapy has been just granted the Fast Track Designation by the US FDA raising hopes for even faster implementation of γδ T cell-based therapies into daily clinical practice (press release dated on April 19, 2022; https://www.adicetbio.com). CAR T cell therapy is being broadened towards T cells engineered with γδTCR (TEG) (138, 144). With approval by US FDA, genetically modified γδ T cells might benefit a broad spectrum of cancer patients. In the future, targeting γδ T cells with tailored immunotherapies might also be a potential new avenue for the treatment of other diseases such as MS and infections.

CONCLUSION

Though neglected for a long time, γδ T cells have emerged as a key immune cell type, especially in cancer biology and are already investigated in clinical trials. Its pleiotropic role is further being investigated in other disorders including immune diseases such as MS, infections or transplantation. Use of "big data" and integrative multi-omics approaches enable us to more specifically unravel molecular mechanisms. This is complemented by the implementation of new methods such as 3D organoids combined with state-of-the-art technologies such as spatial transcriptomics. However, γδ T cells still require a clinical testing model for development of immunotherapy. Altogether, targeting γδ T cells will allow us to more precisely address a broad range of conditions, eventually allowing a γδ T cell targeted personalized immunotherapy.

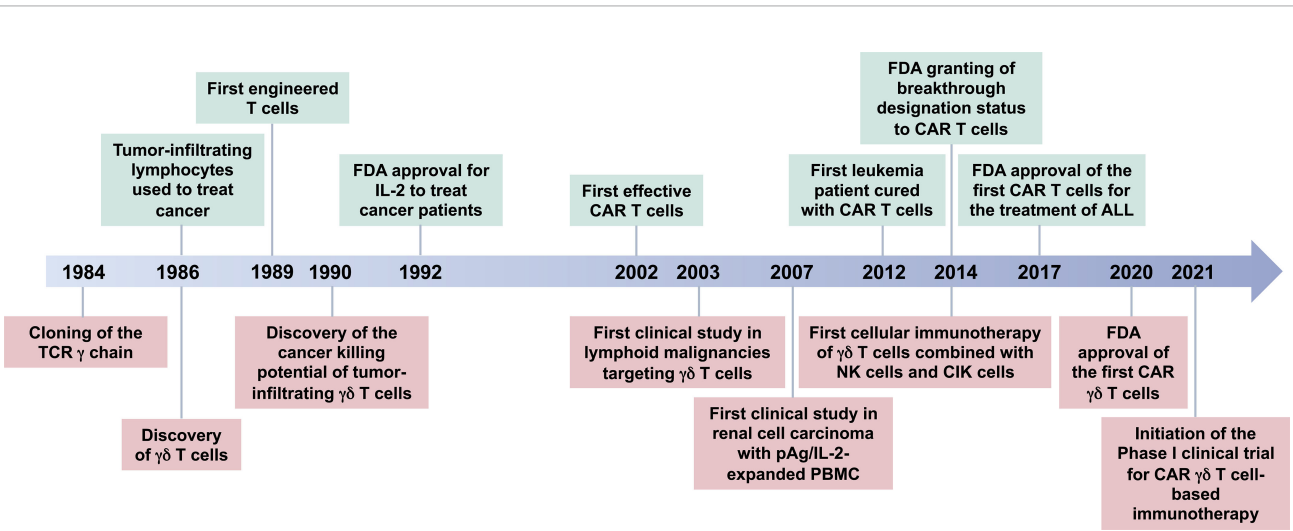


FIGURE 1 | Timeline for γδ T-cell-based immunotherapy. A brief history of the breakthrough findings that led to the development of γδ T cell-based immunotherapies. γδ T cells were discovered in 1986 (126, 127), after accidental cloning of the gamma chain of the T cell receptor (TCR) in 1984 (128). At the same time, Rosenberg's group started to treat cancer patients with their own tumor-infiltrating lymphocytes leading to the first patient to be cured from cancer using this method (129, 130). Fast forward from 1989 to year 2017 (131–137), the FDA approved the first CAR T cells for the treatment of B-cell lymphomas, Kymriah® and Yescarta® developed by Novartis Pharmaceuticals Corp. (https://www.hcp.novartis.com/home/) and Kite Pharma, Inc. (https://www.kitepharma.com/), respectively. The first big success came in 2020 for CAR therapy with γδ T cells, when the FDA cleared an investigational new drug (IND) application and orphan drug designation for GDX012 (an allogenic Vδ1 T-cell-based therapy) developed by Lymphact and later GammaDelta Therapeutics (https://gammadeltatx.com/). Also, at the same time, the Adicet Bio (https://www.adicetbio.com/) received the FDA approval for an IND application ADI-01, an allogenic CAR γδ T cell therapy targeting CD20 protein in non-Hodgkin lymphomas. In 2021, the first Phase I Clinical Trials of γδ T-cell-based immunotherapies were initiated. TCR, T-cell receptor; FDA, The United States Food and Drug Administration; IL-2, interleukin-2; CAR, chimeric antigen receptor; pAg, phosphoantigen; PBMC, peripheral blood mononuclear cells; ALL, acute lymphoblastic leukemia; NK cells, natural killer cells; CIK cells, cytokine-induced killer cells.

AUTHOR CONTRIBUTIONS

JB and KP conceptualized and wrote the first draft of manuscript. JB, KP, and SF contributed to the discussion and made final corrections. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Ruhr University Bochum, Germany to JB. KP has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 798582 and from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation under Germany's Excellence Strategy - EXC2151 - 390873048.

ACKNOWLEDGMENTS

We thank Prof. Ingo Schmitz for reading and support to the manuscript. We apologize to all the colleagues whose studies cannot be cited due to space constraint. We acknowledge support by the Open Access Publication Funds of the Ruhr-Universität Bochum.

REFERENCES

1. Hirano M, Guo P, McCurley N, Schorpp M, Das S, Boehm T, et al. Evolutionary Implications of a Third Lymphocyte Lineage in Lampreys. *Nature* (2013) 501(7467):435–8. doi: 10.1038/nature12467
2. Willcox BE, Willcox CR. γδ TCR Ligands: The Quest to Solve a 500-Million-Year-Old Mystery. *Nat Immunol* (2019) 20(2):121–8. doi: 10.1038/s41590-018-0304-y
3. Glusman G, Rowen L, Lee I, Boysen C, Roach JC, Smit AFA, et al. Comparative Genomics of the Human and Mouse T Cell Receptor Loci. *Immunity* (2001) 15(3):337–49. doi: 10.1016/S1074-7613(01)00200-X
4. Baum T-P, Hierle V, Pasqual N, Bellahcene F, Chaume D, Lefranc M-P, et al. IMGT/GeneInfo: T Cell Receptor Gamma TRG and Delta TRD Genes in Database Give Access to All TR Potential V(D)J Recombinations. *BMC Bioinf* (2006) 7(1):224. doi: 10.1186/1471-2105-7-224
5. Deseke M, Prinz I. Ligand Recognition by the γδ TCR and Discrimination Between Homeostasis and Stress Conditions. *Cell Mol Immunol* (2020) 17 (9):914–24. doi: 10.1038/s41423-020-0503-y
6. Blais M-È, Brochu S, Giroux M, Bélanger M-P, Dulude G, Sékaly R-P, et al. Why T Cells of Thymic Versus Extrathymic Origin Are Functionally Different. *J Immunol* (2008) 180(4):2299–312. doi: 10.4049/jimmunol.180.4.2299
7. Melandri D, Zlatareva I, Chaleil RAG, Dart RJ, Chancellor A, Nussbaumer O, et al. The γδtcr Combines Innate Immunity With Adaptive Immunity by Utilizing Spatially Distinct Regions for Agonist Selection and Antigen Responsiveness. *Nat Immunol* (2018) 19(12):1352–65. doi: 10.1038/s41590-018-0253-5
8. Ribot JC, Lopes N, Silva-Santos B. γδ T Cells in Tissue Physiology and Surveillance. *Nat Rev Immunol* (2021) 21(4):221–32. doi: 10.1038/s41577-020-00452-4
9. Li Y, Li G, Zhang J, Wu X, Chen X. The Dual Roles of Human γδ T Cells: Anti-Tumor or Tumor-Promoting. *Front Immunol* (2021) 11. doi: 10.3389/fimmu.2020.619954
10. Herrmann T, Karunakaran MM. Butyrophilins: γδ T Cell Receptor Ligands, Immunomodulators and More. *Front Immunol* (2022) 13. doi: 10.3389/fimmu.2022.876493
11. Clark BL, Thomas PG. A Cell for the Ages: Human γδ T Cells Across the Lifespan. *Int J Mol Sci* (2020) 21(23):8903. doi: 10.3390/ijms21238903
12. Xu W, Lau ZW, Fulop T, Larbi A. The Aging of γδ T Cells. *Cells* (2020) 9 (5):1181. doi: 10.3390/cells9051181
13. Wang X, Tian Z. γδ T Cells in Liver Diseases. *Front Med* (2018) 12(3):262–8. doi: 10.1007/s11684-017-0584-x
14. Rajoriya N, Fergusson JR, Leithead JA, Klenerman P. Gamma Delta T-Lymphocytes in Hepatitis C and Chronic Liver Disease. *Front Immunol* (2014) 5. doi: 10.3389/fimmu.2014.00400
15. Catalan-Serra I, Sandvik AK, Bruland T, Andreu-Ballester JC. Gammadelta T Cells in Crohn's Disease: A New Player in the Disease Pathogenesis? *J Crohn's Colitis* (2017) 11(9):1135–45. doi: 10.1093/ecco-jcc/jjx039
16. Suzuki T, Hayman L, Kilbey A, Edwards J, Coffelt SB. Gut Gammadelta T Cells as Guardians, Disruptors, and Instigators of Cancer. *Immunol Rev* (2020) 298(1):198–217. doi: 10.1111/imr.12916
17. Di Lorenzo B, Simões AE, Caiado F, Tieppo P, Correia DV, Carvalho T, et al. Broad Cytotoxic Targeting of Acute Myeloid Leukemia by Polyclonal Delta One T Cells. *Cancer Immunol Res* (2019) 7(4):552–8. doi: 10.1158/2326-6066.CIR-18-0647
18. Chauvin C, Joalland N, Perroteau J, Jarry U, Lafrance L, Willem C, et al. NKG2D Controls Natural Reactivity of Vγ9vδ2 T Lymphocytes Against Mesenchymal Glioblastoma Cells. *Clin Cancer Res* (2019) 25(23):7218–28. doi: 10.1158/1078-0432.CCR-19-0375
19. Wang J, Lin C, Li H, Li R, Wu Y, Liu H, et al. Tumor-Infiltrating γδt Cells Predict Prognosis and Adjuvant Chemotherapeutic Benefit in Patients With Gastric Cancer. *Oncoimmunology* (2017) 6(11):e1353858. doi: 10.1080/2162402X.2017.1353858
20. Meraviglia S, Lo Presti E, Tosolini M, La Mendola C, Orlando V, Todaro M, et al. Distinctive Features of Tumor-Infiltrating γδ T Lymphocytes in Human Colorectal Cancer. *Oncoimmunology* (2017) 6(10):e1347742. doi: 10.1080/2162402X.2017.1347742
21. Boissière-Michot F, Chabab G, Mollevi C, Guiu S, Lopez-Crapez E, Ramos J, et al. Clinicopathological Correlates of γδ T Cell Infiltration in Triple-Negative Breast Cancer. *Cancers* (2021) 13(4):765. doi: 10.3390/cancers13040765
22. Todaro M, D'Asaro M, Caccamo N, Iovino F, Francipane MG, Meraviglia S, et al. Efficient Killing of Human Colon Cancer Stem Cells by γδ T Lymphocytes. *J Immunol* (2009) 182(11):7287–96. doi: 10.4049/jimmunol.0804288
23. Castella B, Kopecka J, Sciancalepore P, Mandili G, Foglietta M, Mitro N, et al. The ATP-Binding Cassette Transporter A1 Regulates Phosphoantigen Release and Vγ9vδ2 T Cell Activation by Dendritic Cells. *Nat Commun* (2017) 8(1):15663. doi: 10.1038/ncomms15663
24. Sandstrom A, Peigne CM, Leger A, Crooks JE, Konczak F, Gesnel MC, et al. The Intracellular B30.2 Domain of Butyrophilin 3A1 Binds Phosphoantigens to Mediate Activation of Human Vgamma9Vdelta2 T Cells. *Immunity* (2014) 40(4):490–500. doi: 10.1016/j.immuni.2014.03.003
25. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 is Essential for Phosphoantigen Reactivity by γδ T Cells. *Science* (2020) 367:eaay5516. doi: 10.1126/science.aay5516
26. Harly C, Guillaume Y, Nedellec S, Peigne CM, Monkkonen H, Monkkonen J, et al. Key Implication of CD277/butyrophilin-3 (BTN3A) in Cellular Stress Sensing by a Major Human Gammadelta T-Cell Subset. *Blood* (2012) 120 (11):2269–79. doi: 10.1182/blood-2012-05-430470
27. Sebestyen Z, Scheper W, Vyborova A, Gu S, Rychnavska Z, Schiffler M, et al. RhoB Mediates Phosphoantigen Recognition by Vgamma9Vdelta2 T Cell Receptor. *Cell Rep* (2016) 15(9):1973–85. doi: 10.1016/j.celrep.2016.04.081
28. Vyborova A, Beringer DX, Fasci D, Karaiskaki F, van Diest E, Kramer L, et al. γ9δ2t Cell Diversity and the Receptor Interface With Tumor Cells. *J Clin Invest* (2020) 130(9):4637–51. doi: 10.1172/JCI132489
29. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, McPherson KG, Lim RT, et al. CD1d-Lipid Antigen Recognition by the Gammadelta TCR. *Nat Immunol* (2013) 14(11):1137–45. doi: 10.1038/ni.2713
30. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The Majority of CD1d-Sulfatide-Specific T Cells in Human Blood Use a

Semiinvariant Vdelta1 TCR. *Eur J Immunol* (2012) 42(9):2505–10. doi: 10.1002/eji.201242531
31. Le Nours J, Gherardin Nicholas A, Ramarathinam HSri, Awad W, Wiede F, Gully Benjamin S, et al. A Class of γδ T Cell Receptors Recognize the Underside of the Antigen-Presenting Molecule MR1. *Science* (2019) 366 (6472):1522–7. doi: 10.1126/science.aav3900
32. Harly C, Joyce Stephen P, Domblides C, Bachelet T, Pitard V, Mannat C, et al. Human γδ T Cell Sensing of AMPK-Dependent Metabolic Tumor Reprogramming Through TCR Recognition of Epha2. *Sci Immunol* (2021) 6 (61):eaba9010. doi: 10.1126/sciimmunol.aba9010
33. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and Tumor Stress Surveillance by Binding of a Human γδ T Cell Antigen Receptor to Endothelial Protein C Receptor. *Nat Immunol* (2012) 13(9):872–9. doi: 10.1038/ni.2394
34. Marlin R, Pappalardo A, Kaminski H, Willcox Carrie R, Pitard V, Netzer S, et al. Sensing of Cell Stress by Human γδ TCR-Dependent Recognition of Annexin A2. *Proc Natl Acad Sci* (2017) 114(12):3163–8. doi: 10.1073/pnas.1621052114
35. Zeng X, Wei Y-L, Huang J, Newell Evan W, Yu H, Kidd Brian A, et al. γδ T Cells Recognize a Microbial Encoded B Cell Antigen to Initiate a Rapid Antigen-Specific Interleukin-17 Response. *Immunity* (2012) 37(3):524–34. doi: 10.1016/j.immuni.2012.06.011
36. Mounia Sabrina B, Bernard K, Geneviève F, Jean-François R. γδ T-Cell Killing of Primary Follicular Lymphoma Cells is Dramatically Potentiated by GA101, a Type II Glycoengineered Anti-CD20 Monoclonal Antibody. *Haematologica* (2011) 96(3):400–7. doi: 10.3324/haematol.2010.029520
37. Tokuyama H, Hagi T, Mattarollo SR, Morley J, Wang Q, So HF, et al. V Gamma 9 V Delta 2 T Cell Cytotoxicity Against Tumor Cells is Enhanced by Monoclonal Antibody Drugs–Rituximab and Trastuzumab. *Int J Cancer* (2008) 122(11):2526–34. doi: 10.1002/ijc.23365
38. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, et al. γδ T Cells Provide an Early Source of Interferon γ in Tumor Immunity. *J Exp Med* (2003) 198(3):433–42. doi: 10.1084/jem.20030584
39. Ribot JC, Ribeiro ST, Correia DV, Sousa AE, Silva-Santos B. Human γδ Thymocytes Are Functionally Immature and Differentiate Into Cytotoxic Type 1 Effector T Cells Upon IL-2/IL-15 Signaling. *J Immunol* (2014) 192 (5):2237–43. doi: 10.4049/jimmunol.1303119
40. Silva-Santos B, Mensurado S, Coffelt SB. γδ T Cells: Pleiotropic Immune Effectors With Therapeutic Potential in Cancer. *Nat Rev Cancer* (2019) 19 (7):392–404. doi: 10.1038/s41568-019-0153-5
41. Oberg HH, Janitschke L, Sulaj V, Weimer J, Gonnermann D, Hedemann N, et al. Bispecific Antibodies Enhance Tumor-Infiltrating T Cell Cytotoxicity Against Autologous HER-2-Expressing High-Grade Ovarian Tumors. *J Leukocyte Biol* (2020) 107(6):1081–95. doi: 10.1002/JLB.5MA1119-265R
42. Chen HC, Joalland N, Bridgeman JS, Alchami FS, Jarry U, Khan MWA, et al. Synergistic Targeting of Breast Cancer Stem-Like Cells by Human γδ T Cells and CD8(+) T Cells. *Immunol Cell Biol* (2017) 95(7):620–9. doi: 10.1038/icb.2017.21
43. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. γδt17 Cells Promote the Accumulation and Expansion of Myeloid-Derived Suppressor Cells in Human Colorectal Cancer. *Immunity* (2014) 40(5):785–800. doi: 10.1016/j.immuni.2014.03.013
44. Patil RS, Shah SU, Shrikhande SV, Goel M, Dikshit RP, Chiplunkar SV. IL17 Producing γδt Cells Induce Angiogenesis and Are Associated With Poor Survival in Gallbladder Cancer Patients. *Int J Cancer* (2016) 139(4):869–81. doi: 10.1002/ijc.30134
45. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau C-S, et al. IL-17-Producing γδ T Cells and Neutrophils Conspire to Promote Breast Cancer Metastasis. *Nature* (2015) 522(7556):345–8. doi: 10.1038/nature14282
46. Sureshbabu SK, Chaukar D, Chiplunkar SV. Hypoxia Regulates the Differentiation and Anti-Tumor Effector Functions of γδt Cells in Oral Cancer. *Clin Exp Immunol* (2020) 201(1):40–57. doi: 10.1111/cei.13436
47. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov* (2022) 12(1):31–46. doi: 10.1158/2159-8290.CD-21-1059
48. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
49. Schmolka N, Serre K, Grosso AR, Rei M, Pennington DJ, Gomes AQ, et al. Epigenetic and Transcriptional Signatures of Stable Versus Plastic Differentiation of Proinflammatory Gammadelta T Cell Subsets. *Nat Immunol* (2013) 14(10):1093–100. doi: 10.1038/ni.2702
50. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KHG. Interleukin-1 and IL-23 Induce Innate IL-17 Production From γδ T Cells, Amplifying Th17 Responses and Autoimmunity. *Immunity* (2009) 31 (2):331–41. doi: 10.1016/j.immuni.2009.08.001
51. McGinley AM, Sutton CE, Edwards SC, Leane CM, DeCourcey J, Teijeiro A, et al. Interleukin-17a Serves a Priming Role in Autoimmunity by Recruiting IL-1beta-Producing Myeloid Cells That Promote Pathogenic T Cells. *Immunity* (2020) 52(2):342–56.e6. doi: 10.1016/j.immuni.2020.01.002
52. Petermann F, Rothhammer V, Claussen MC, Haas JD, Blanco LR, Heink S, et al. γδ T Cells Enhance Autoimmunity by Restraining Regulatory T Cell Responses *via* an Interleukin-23-Dependent Mechanism. *Immunity* (2010) 33(3):351–63. doi: 10.1016/j.immuni.2010.08.013
53. Papotto PH, Gonçalves-Sousa N, Schmolka N, Iseppon A, Mensurado S, Stockinger B, et al. IL-23 Drives Differentiation of Peripheral γδ17 T Cells From Adult Bone Marrow-Derived Precursors. *EMBO Rep* (2017) 18 (11):1957–67. doi: 10.15252/embr.201744200
54. Kaufmann M, Evans H, Schaupp A-L, Engler JB, Kaur G, Willing A, et al. Identifying CNS-Colonizing T Cells as Potential Therapeutic Targets to Prevent Progression of Multiple Sclerosis. *Med* (2021) 2(3):296–312.e8. doi: 10.1016/j.medj.2021.01.006
55. Singh AK, Novakova L, Axelsson M, Malmeström C, Zetterberg H, Lycke J, et al. High Interferon-γ Uniquely in Vδ1 T Cells Correlates With Markers of Inflammation and Axonal Damage in Early Multiple Sclerosis. *Front Immunol* (2017) 8. doi: 10.3389/fimmu.2017.00260
56. Maimaitijiang G, Shinoda K, Nakamura Y, Masaki K, Matsushita T, Isobe N, et al. Association of Decreased Percentage of Vδ2+Vγ9+ γδ T Cells With Disease Severity in Multiple Sclerosis. *Front Immunol* (2018) 9. doi: 10.3389/fimmu.2018.00748
57. Zeine R, Pon R, Ladiwala U, Antel JP, Filion LG, Freedman MS. Mechanism of γδ T Cell-Induced Human Oligodendrocyte Cytotoxicity: Relevance to Multiple Sclerosis. *J Neuroimmunology* (1998) 87(1):49–61. doi: 10.1016/S0165-5728(98)00047-2
58. Monteiro A, Cruto C, Rosado P, Martinho A, Rosado L, Fonseca M, et al. Characterization of Circulating Gamma-Delta T Cells in Relapsing vs Remission Multiple Sclerosis. *J Neuroimmunol* (2018) 318:65–71. doi: 10.1016/j.jneuroim.2018.02.009
59. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol* (2009) 27(1):485–517. doi: 10.1146/annurev.immunol.021908.132710
60. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-Producing γδ T Cells Selectively Expand in Response to Pathogen Products and Environmental Signals. *Immunity* (2009) 31(2):321–30. doi: 10.1016/j.immuni.2009.06.020
61. Kapsenberg ML. γδ T Cell Receptors Without a Job. *Immunity* (2009) 31 (2):181–3. doi: 10.1016/j.immuni.2009.08.004
62. Edwards SC, Sutton CE, Ladell K, Grant EJ, McLaren JE, Roche F, et al. A Population of Proinflammatory T Cells Coexpresses αβ and γδ T Cell Receptors in Mice and Humans. *J Exp Med* (2020) 217(5):e20190834. doi: 10.1084/jem.20190834
63. Marchitto MC, Dillen CA, Liu H, Miller RJ, Archer NK, Ortines RV, et al. Clonal Vγ6(+)Vδ4(+) T Cells Promote IL-17-Mediated Immunity Against Staphylococcus Aureus Skin Infection. *Proc Natl Acad Sci USA* (2019) 116 (22):10917–26. doi: 10.1073/pnas.1818256116
64. Puan K-J, Jin C, Wang H, Sarikonda G, Raker AM, Lee HK, et al. Preferential Recognition of a Microbial Metabolite by Human Vγ2vδ2 T Cells. *Int Immunol* (2007) 19(5):657–73. doi: 10.1093/intimm/dxm031
65. Howard J, Zaidi I, Loizon S, Mercereau-Puijalon O, Déchanet-Merville J, Mamani-Matsuda M. Human Vγ9vδ2 T Lymphocytes in the Immune Response to P. Falciparum Infection. *Front Immunol* (2018) 9. doi: 10.3389/fimmu.2018.02760
66. Junqueira C, Polidoro RB, Castro G, Absalon S, Liang Z, Sen Santara S, et al. γδ T Cells Suppress Plasmodium Falciparum Blood-Stage Infection by Direct Killing and Phagocytosis. *Nat Immunol* (2021) 22(3):347–57. doi: 10.1038/s41590-020-00847-4

67. Ladel CH, Blum C, Dreher A, Reifenberg K, Kaufmann SH. Protective Role of Gamma/Delta T Cells and Alpha/Beta T Cells in Tuberculosis. *Eur J Immunol* (1995) 25(10):2877–81. doi: 10.1002/eji.1830251025
68. Hoft DF, Brown RM, Roodman ST. Bacille Calmette-Guerin Vaccination Enhances Human Gamma Delta T Cell Responsiveness to Mycobacteria Suggestive of a Memory-Like Phenotype. *J Immunol* (1998) 161(2):1045–54.
69. Garly ML, Martins CL, Balé C, Baldé MA, Hedegaard KL, Gustafson P, et al. BCG Scar and Positive Tuberculin Reaction Associated With Reduced Child Mortality in West Africa. A Non-Specific Beneficial Effect of BCG? *Vaccine* (2003) 21(21-22):2782–90. doi: 10.1016/S0264-410X(03)00181-6
70. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized Trial of BCG Vaccination at Birth to Low-Birth-Weight Children: Beneficial Nonspecific Effects in the Neonatal Period? *J Infect Dis* (2011) 204(2):245–52. doi: 10.1093/infdis/jir240
71. Benn CS, Netea MG, Selin LK, Aaby P. A Small Jab - A Big Effect: Nonspecific Immunomodulation by Vaccines. *Trends Immunol* (2013) 34 (9):431–9. doi: 10.1016/j.it.2013.04.004
72. Higgins JP, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and Measles Containing Vaccines With Childhood Mortality: Systematic Review. *Bmj* (2016) 355: i5170. doi: 10.1136/bmj.i5170
73. Munk ME, Gatrill AJ, Kaufmann SH. Target Cell Lysis and IL-2 Secretion by Gamma/Delta T Lymphocytes After Activation With Bacteria. *J Immunol* (1990) 145(8):2434–9.
74. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida Albicans Infection Affords Protection Against Reinfection *via* Functional Reprogramming of Monocytes. *Cell Host Microbe* (2012) 12(2):223–32. doi: 10.1016/j.chom.2012.06.006
75. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic Programming of Monocyte-to-Macrophage Differentiation and Trained Innate Immunity. *Science* (2014) 345(6204):1251086. doi: 10.1126/science.1251086
76. Davis MM, Tato CM, Furman D. Systems Immunology: Just Getting Started. *Nat Immunol* (2017) 18(7):725–32. doi: 10.1038/ni.3768
77. Villani A-C, Sarkizova S, Hacohen N. Systems Immunology: Learning the Rules of the Immune System. *Annu Rev Immunol* (2018) 36(1):813–42. doi: 10.1146/annurev-immunol-042617-053035
78. Pulendran B, Davis Mark M. The Science and Medicine of Human Immunology. *Science* (2020) 369(6511):eaay4014. doi: 10.1126/science.aay4014
79. Mangino M, Roederer M, Beddall MH, Nestle FO, Spector TD. Innate and Adaptive Immune Traits are Differentially Affected by Genetic and Environmental Factors. *Nat Commun* (2017) 8:13850. doi: 10.1038/ncomms13850
80. Melenhorst JJ, Chen GM, Wang M, Porter DL, Chen C, Collins MA, et al. Decade-Long Leukaemia Remissions With Persistence of CD4(+) CAR T Cells. *Nature* (2022) 602(7897):503–9. doi: 10.1038/s41586-021-04390-6
81. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. *Nat Med* (2015) 21(8):938–45. doi: 10.1038/nm.3909
82. Tosolini M, Pont F, Poupot M, Vergez F, Nicolau-Travers M-L, Vermijlen D, et al. Assessment of Tumor-Infiltrating Tcrvγ9vδ2 γδ Lymphocyte Abundance by Deconvolution of Human Cancers Microarrays. *Oncoimmunology* (2017) 6(3):e1284723. doi: 10.1080/2162402X.2017.1284723
83. Miao Y-R, Zhang Q, Lei Q, Luo M, Xie G-Y, Wang H, et al. ImmuCellAI: A Unique Method for Comprehensive T-Cell Subsets Abundance Prediction and Its Application in Cancer Immunotherapy. *Advanced Sci* (2020) 7 (7):1902880. doi: 10.1002/advs.201902880
84. Bhat J, Bergmann AK, Waschina S, Nerl C, Kaleta C, Siebert R, et al. DNA Methylation Profile of a Hepatosplenic Gamma/Delta T-Cell Lymphoma Patient Associated With Response to Interferon-α Therapy. *Cell Mol Immunol* (2021) 18(5):1332–5. doi: 10.1038/s41423-020-0518-4
85. Mirji G, Bhat J, Kode J, Banavali S, Sengar M, Khadke P, et al. Risk Stratification of T-Cell Acute Lymphoblastic Leukemia Patients Based on Gene Expression, Mutations and Copy Number Variation. *Leukemia Res* (2016) 45:33–9. doi: 10.1016/j.leukres.2016.03.002
86. Watkin L, Anvari S, Hassan O, Schuster K, Davis C. Increased Costimulatory Molecule Expression in γδ-T Cells of Peanut Allergic Individuals. *J Allergy Clin Immunol* (2020) 145(2, Supplement):AB244. doi: 10.1016/j.jaci.2019.12.151
87. Boufea K, González-Huici V, Lindberg M, Symeonides S, Oikonomidou O, Batada NN. Single-Cell RNA Sequencing of Human Breast Tumour-Infiltrating Immune Cells Reveals a γδ T-Cell Subtype Associated With Good Clinical Outcome. *Life Sci Alliance* (2021) 4(1):e202000680. doi: 10.26508/lsa.202000680
88. Pizzolato G, Kaminski H, Tosolini M, Franchini D-M, Pont F, Martins F, et al. Single-Cell RNA Sequencing Unveils the Shared and the Distinct Cytotoxic Hallmarks of Human Tcrvδ1 and Tcrvδ2 γδ T Lymphocytes. *Proc Natl Acad Sci* (2019) 116(24):11906–15. doi: 10.1073/pnas.1818488116
89. Jaeger N, Gamini R, Cella M, Schettini JL, Bugatti M, Zhao S, et al. Single-Cell Analyses of Crohn's Disease Tissues Reveal Intestinal Intraepithelial T Cells Heterogeneity and Altered Subset Distributions. *Nat Commun* (2021) 12(1):1921. doi: 10.1038/s41467-021-22164-6
90. Park J-E, Botting Rachel A, Domínguez Conde C, Popescu D-M, Lavaert M, Kunz Daniel J, et al. A Cell Atlas of Human Thymic Development Defines T Cell Repertoire Formation. *Science* (2020) 367(6480):eaay3224. doi: 10.1126/science.aay3224
91. Tan L, Fichtner Alina S, Bruni E, Odak I, Sandrock I, Bubke A, et al. A Fetal Wave of Human Type 3 Effector γδ Cells With Restricted TCR Diversity Persists Into Adulthood. *Sci Immunol* (2021) 6(58):eabf0125. doi: 10.1126/sciimmunol.abf0125
92. Reitermaier R, Krausgruber T, Fortelny N, Ayub T, Vieyra-Garcia PA, Kienzl P, et al. αβγδ T Cells Play a Vital Role in Fetal Human Skin Development and Immunity. *J Exp Med* (2021) 218(4):e20201189. doi: 10.1084/jem.20201189
93. Tan L, Sandrock I, Odak I, Aizenbud Y, Wilharm A, Barros-Martins J, et al. Single-Cell Transcriptomics Identifies the Adaptation of Scart1+ Vγ6+ T Cells to Skin Residency as Activated Effector Cells. *Cell Rep* (2019) 27 (12):3657–71.e4. doi: 10.1016/j.celrep.2019.05.064
94. Sagar, Pokrovskii M, Herman JS, Naik S, Sock E, Zeis P, et al. Deciphering the Regulatory Landscape of Fetal and Adult γδ T-Cell Development at Single-Cell Resolution. *EMBO J* (2020) 39(13):e104159. doi: 10.15252/embj.2019104159
95. Lee M, Lee E, Han SK, Choi YH, D-i K, Choi H, et al. Single-Cell RNA Sequencing Identifies Shared Differentiation Paths of Mouse Thymic Innate T Cells. *Nat Commun* (2020) 11(1):4367. doi: 10.1038/s41467-020-18155-8
96. Alves de Lima K, Rustenhoven J, Da Mesquita S, Wall M, Salvador AF, Smirnov I, et al. Meningeal γδ T Cells Regulate Anxiety-Like Behavior *via* IL-17a Signaling in Neurons. *Nat Immunol* (2020) 21(11):1421–9. doi: 10.1038/s41590-020-0776-4
97. Goldberg EL, Shchukina I, Asher JL, Sidorov S, Artyomov MN, Dixit VD. Ketogenesis Activates Metabolically Protective γδ T Cells in Visceral Adipose Tissue. *Nat Metab* (2020) 2(1):50–61. doi: 10.1038/s42255-019-0160-6
98. Hu Y, Fang K, Wang Y, Lu N, Sun H, Zhang C. Single-Cell Analysis Reveals the Origins and Intrahepatic Development of Liver-Resident IFN-γ-Producing γδ T Cells. *Cell Mol Immunol* (2021) 18(4):954–68. doi: 10.1038/s41423-021-00656-1
99. Li Z, Yang Q, Tang X, Chen Y, Wang S, Qi X, et al. Single-Cell RNA-Seq and Chromatin Accessibility Profiling Decipher the Heterogeneity of Mouse γδ T Cells. *Sci Bull* (2022) 67(4):408–26. doi: 10.1016/j.scib.2021.11.013
100. Wang X, Chen Y, Li Z, Huang B, Xu L, Lai J, et al. Single-Cell RNA-Seq of T Cells in B-ALL Patients Reveals an Exhausted Subset With Remarkable Heterogeneity. *Adv Sci (Weinh)* (2021) 8(19):e2101447. doi: 10.1002/advs.202101447
101. Gherardin NA, Waldeck K, Caneborg A, Martelotto LG, Balachander S, Zethoven M, et al. γδ T Cells in Merkel Cell Carcinomas Have a Proinflammatory Profile Prognostic of Patient Survival. *Cancer Immunol Res* (2021) 9(6):612–23. doi: 10.1158/2326-6066.CIR-20-0817
102. Schafflick D, Xu CA, Hartlehnert M, Cole M, Schulte-Mecklenbeck A, Lautwein T, et al. Integrated Single Cell Analysis of Blood and Cerebrospinal Fluid Leukocytes in Multiple Sclerosis. *Nat Commun* (2020) 11(1):247. doi: 10.1038/s41467-019-14118-w

103. Cerapio J-P, Perrier M, Balança C-C, Gravelle P, Pont F, Devaud C, et al. Phased Differentiation of γδ T and T CD8 Tumor-Infiltrating Lymphocytes Revealed by Single-Cell Transcriptomics of Human Cancers. *Oncoimmunology* (2021) 10(1):1939518. doi: 10.1080/2162402X.2021.1939518
104. Schaum N, Karkanias J, Neff NF, May AP, Quake SR, Wyss-Coray T, et al. Single-Cell Transcriptomics of 20 Mouse Organs Creates a Tabula Muris. *Nature* (2018) 562(7727):367–72. doi: 10.1038/s41586-018-0590-4
105. Han X, Zhou Z, Fei L, Sun H, Wang R, Chen Y, et al. Construction of a Human Cell Landscape at Single-Cell Level. *Nature* (2020) 581(7808):303–9. doi: 10.1038/s41586-020-2157-4
106. Karlsson M, Zhang C, Méar L, Zhong W, Digre A, Katona B, et al. A Single–Cell Type Transcriptomics Map of Human Tissues. *Sci Adv* (2020) 7(31): eabh2169. doi: 10.1126/sciadv.abh2169
107. Yang K, Blanco DB, Chen X, Dash P, Neale G, Rosencrance C, et al. Metabolic Signaling Directs the Reciprocal Lineage Decisions of αβ and γδ T Cells. *Sci Immunol* (2018) 3(25):eaas9818. doi: 10.1126/sciimmunol.aas9818
108. Argüello RJ, Combes AJ, Char R, Gigan J-P, Baaziz AI, Bousiquot E, et al. SCENITH: A Flow Cytometry-Based Method to Functionally Profile Energy Metabolism With Single-Cell Resolution. *Cell Metab* (2020) 32(6):1063–75.e7. doi: 10.1016/j.cmet.2020.11.007
109. Lopes N, McIntyre C, Martin S, Raverdeau M, Sumaria N, Kohlgruber AC, et al. Distinct Metabolic Programs Established in the Thymus Control Effector Functions of γδ T Cell Subsets in Tumor Microenvironments. *Nat Immunol* (2021) 22(2):179–92. doi: 10.1038/s41590-020-00848-3
110. Li L, Cao B, Liang X, Lu S, Luo H, Wang Z, et al. Microenvironmental Oxygen Pressure Orchestrates an Anti- and Pro-Tumoral γδ T Cell Equilibrium *via* Tumor-Derived Exosomes. *Oncogene* (2019) 38(15):2830–43. doi: 10.1038/s41388-018-0627-z
111. Gonnermann D, Oberg HH, Kellner C, Peipp M, Sebens S, Kabelitz D, et al. Resistance of Cyclooxygenase-2 Expressing Pancreatic Ductal Adenocarcinoma Cells Against γδ T Cell Cytotoxicity. *Oncoimmunology* (2015) 4(3):e988460. doi: 10.4161/2162402X.2014.988460
112. Kohlgruber AC, Gal-Oz ST, LaMarche NM, Shimazaki M, Duquette D, Koay H-F, et al. γδ T Cells Producing Interleukin-17A Regulate Adipose Regulatory T Cell Homeostasis and Thermogenesis. *Nat Immunol* (2018) 19(5):464–74. doi: 10.1038/s41590-018-0094-2
113. Hu B, Jin C, Zeng X, Resch JM, Jedrychowski MP, Yang Z, et al. γδ T Cells and Adipocyte IL-17RC Control Fat Innervation and Thermogenesis. *Nature* (2020) 578(7796):610–4. doi: 10.1038/s41586-020-2028-z
114. Makkouk A, Yang X, Barca T, Lucas A, Turkoz M, Wong JTS, et al. Off-The-Shelf Vδ1 Gamma Delta T Cells Engineered With Glypican-3 (GPC-3)-Specific Chimeric Antigen Receptor (CAR) and Soluble IL-15 Display Robust Antitumor Efficacy Against Hepatocellular Carcinoma. *J ImmunoTher Cancer* (2021) 9(12):e003441. doi: 10.1136/jitc-2021-003441
115. Xu Y, Xiang Z, Alnaggar M, Kouakanou L, Li J, He J, et al. Allogeneic Vγ9vδ2 T-Cell Immunotherapy Exhibits Promising Clinical Safety and Prolongs the Survival of Patients With Late-Stage Lung or Liver Cancer. *Cell Mol Immunol* (2021) 18(2):427–39. doi: 10.1038/s41423-020-0515-7
116. Kabelitz D, Serrano R, Kouakanou L, Peters C, Kalyan S. Cancer Immunotherapy With γδ T Cells: Many Paths Ahead of Us. *Cell Mol Immunol* (2020) 17(9):925–39. doi: 10.1038/s41423-020-0504-x
117. Bhat SA, Vedpathak DM, Chiplunkar SV. Checkpoint Blockade Rescues the Repressive Effect of Histone Deacetylases Inhibitors on Gammadelta T Cell Function. *Front Immunol* (2018) 9:1615. doi: 10.3389/fimmu.2018.01615
118. Bhat J, Kabelitz D. Gammadelta T Cells and Epigenetic Drugs: A Useful Merger in Cancer Immunotherapy? *Oncoimmunology* (2015) 4(6):e1006088. doi: 10.1080/2162402X.2015.1006088
119. Bhat J, Kouakanou L, Peters C, Yin Z, Kabelitz D. Immunotherapy With Human Gamma Delta T Cells—Synergistic Potential of Epigenetic Drugs? *Front Immunol* (2018) 9(512). doi: 10.3389/fimmu.2018.00512
120. Wang H, Chen H, Liu S, Zhang J, Lu H, Somasundaram R, et al. Costimulation of γδtcr and TLR7/8 Promotes Vδ2 T-Cell Antitumor Activity by Modulating mTOR Pathway and APC Function. *J Immunother Cancer* (2021) 9(12):e003339. doi: 10.1136/jitc-2021-003339
121. Ganesan R, Chennupati V, Ramachandran B, Hansen MR, Singh S, Grewal IS. Selective Recruitment of γδ T Cells by a Bispecific Antibody for the Treatment of Acute Myeloid Leukemia. *Leukemia* (2021) 35(8):2274–84. doi: 10.1038/s41375-021-01122-7
122. Peters C, Meyer A, Kouakanou L, Feder J, Schricker T, Lettau M, et al. TGF-β Enhances the Cytotoxic Activity of Vδ2 T Cells. *Oncoimmunology* (2019) 8 (1):e1522471. doi: 10.1080/2162402X.2018.1522471
123. Kouakanou L, Peters C, Sun Q, Floess S, Bhat J, Huehn J, et al. Vitamin C Supports Conversion of Human γδ T Cells Into FOXP3-Expressing Regulatory Cells by Epigenetic Regulation. *Sci Rep* (2020) 10(1):6550. doi: 10.1038/s41598-020-63572-w
124. Yang Y, Lundqvist A. Immunomodulatory Effects of IL-2 and IL-15; Implications for Cancer Immunotherapy. *Cancers (Basel)* (2020) 12 (12):3586. doi: 10.3390/cancers12123586
125. Cairo C, Armstrong CL, Cummings JS, Deetz CO, Tan M, Lu C, et al. Impact of Age, Gender, and Race on Circulating γδ T Cells. *Hum Immunol* (2010) 71 (10):968–75. doi: 10.1016/j.humimm.2010.06.014
126. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, et al. Identification of a Putative Second T-Cell Receptor. *Nature* (1986) 322 (6075):145–9. doi: 10.1038/322145a0
127. Bank I, DePinho RA, Brenner MB, Cassimeris J, Alt FW, Chess L. A Functional T3 Molecule Associated With a Novel Heterodimer on the Surface of Immature Human Thymocytes. *Nature* (1986) 322(6075):179–81. doi: 10.1038/322179a0
128. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. A Third Rearranged and Expressed Gene in a Clone of Cytotoxic T Lymphocytes. *Nature* (1984) 312(5989):36–40. doi: 10.1038/312036a0
129. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, et al. Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients With Metastatic Cancer. *N Engl J Med* (1985) 313(23):1485–92. doi: 10.1056/NEJM198512053132327
130. Rosenberg SA, Spiess P, Lafreniere R. A New Approach to the Adoptive Immunotherapy of Cancer With Tumor-Infiltrating Lymphocytes. *Science* (1986) 233(4770):1318–21. doi: 10.1126/science.3489291
131. Gross G, Waks T, Eshhar Z. Expression of Immunoglobulin-T-Cell Receptor Chimeric Molecules as Functional Receptors With Antibody-Type Specificity. *Proc Natl Acad Sci USA* (1989) 86(24):10024–8. doi: 10.1073/pnas.86.24.10024
132. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-Lymphocyte Cytotoxicity and Proliferation Directed by a Single Chimeric TCRzeta /CD28 Receptor. *Nat Biotechnol* (2002) 20(1):70–5. doi: 10.1038/nbt0102-70
133. Rosenbaum L. Tragedy, Perseverance, and Chance - The Story of CAR-T Therapy. *N Engl J Med* (2017) 377(14):1313–5. doi: 10.1056/NEJMp1711886
134. Zocchi MR, Ferrarini M, Rugarli C. Selective Lysis of the Autologous Tumor by Delta TCS1+ Gamma/Delta+ Tumor-Infiltrating Lymphocytes From Human Lung Carcinomas. *Eur J Immunol* (1990) 20(12):2685–9. doi: 10.1002/eji.1830201224
135. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, et al. Gammadelta T Cells for Immune Therapy of Patients With Lymphoid Malignancies. *Blood* (2003) 102(1):200–6. doi: 10.1182/blood-2002-12-3665
136. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety Profile and Anti-Tumor Effects of Adoptive Immunotherapy Using Gamma-Delta T Cells Against Advanced Renal Cell Carcinoma: A Pilot Study. *Cancer Immunol Immunother CII* (2007) 56(4):469–76. doi: 10.1007/s00262-006-0199-6
137. Cui J, Wang N, Zhao H, Jin H, Wang G, Niu C, et al. Combination of Radiofrequency Ablation and Sequential Cellular Immunotherapy Improves Progression-Free Survival for Patients With Hepatocellular Carcinoma. *Int J Cancer* (2014) 134(2):342–51. doi: 10.1002/ijc.28372
138. Sebestyen Z, Prinz I, Déchanet-Merville J, Silva-Santos B, Kuball J. Translating Gammadelta (γδ) T Cells and Their Receptors Into Cancer Cell Therapies. *Nat Rev Drug Discov* (2020) 19(3):169–84. doi: 10.1038/s41573-019-0038-z
139. Almeida AR, Correia DV, Fernandes-Platzgummer A, da Silva CL, da Silva MG, Anjos DR, et al. Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept. *Clin Cancer Res* (2016) 22(23):5795–804. doi: 10.1158/1078-0432.CCR-16-0597

140. Wawrzyniecka PA, Ibrahim L, Gritti G, Pule MA, Maciocia PM. Chimeric Antigen Receptor T Cells for Gamma–Delta T Cell Malignancies. *Leukemia* (2022) 36(2):577–9. doi: 10.1038/s41375-021-01385-0
141. Rozenbaum M, Meir A, Aharony Y, Itzhaki O, Schachter J, Bank I, et al. Gamma-Delta CAR-T Cells Show CAR-Directed and Independent Activity Against Leukemia. *Front Immunol* (2020) 11. doi: 10.3389/fimmu.2020.01347
142. Sánchez-Martínez D, Tirado N, Mensurado S, Martínez-Moreno A, Romecin P, Gutiérrez-Agüera F, et al. Generation and Proof-of-Concept for Allogeneic CD123 CAR-Delta One T (DOT) Cells in Acute Myeloid Leukemia. *bioRxiv* (2022) 2022:03.15.484289. doi: 10.1101/2022.03.15.484289
143. Nishimoto KP, Barca T, Azameera A, Makkouk A, Romero JM, Bai L, et al. Allogeneic CD20-Targeted Gammadelta T Cells Exhibit Innate and Adaptive Antitumor Activities in Preclinical B-Cell Lymphoma Models. *Clin Transl Immunol* (2022) 11(2):e1373. doi: 10.1002/cti2.1373
144. Straetemans T, Kierkels GJJ, Doorn R, Jansen K, Heijhuurs S, dos Santos JM, et al. GMP-Grade Manufacturing of T Cells Engineered to Express a Defined γδtcr. *Front Immunol* (2018) 9. doi: 10.3389/fimmu.2018.01062

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 31 May 2022
doi: 10.3389/fimmu.2022.863155

A Simple and Robust Single-Step Method for CAR-Vδ1 γδT Cell Expansion and Transduction for Cancer Immunotherapy

Gabrielle M. Ferry[1†], Charles Agbuduwe[1†], Megan Forrester[2], Sophie Dunlop[2], Kerry Chester[3], Jonathan Fisher[1], John Anderson[1‡] and Marta Barisa[1*‡]*

[1] Developmental Biology and Cancer Section, University Colloge of London (UCL) Great Ormond Street Institute of Child Health, London, United Kingdom, [2] TC-BioPharm, Holytown, United Kingdom, [3] Research Department of Oncology, Unicersity College of London (UCL) Cancer Institute, London, United Kingdom

OPEN ACCESS

Edited by:
Dieter Kabelitz,
University of Kiel, Germany

Reviewed by:
Jurgen Kuball,
Utrecht University, Netherlands
Jean Jacques Fournie,
INSERM U1037 Centre de Recherche en Cancérologie de Toulouse, France

***Correspondence:**
John Anderson
j.anderson@ucl.ac.uk
Marta Barisa
m.barisa@ucl.ac.uk

[†]These authors share first authorship

[‡]These authors share senior authorship

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 26 January 2022
Accepted: 25 April 2022
Published: 31 May 2022

Citation:
Ferry GM, Agbuduwe C, Forrester M, Dunlop S, Chester K, Fisher J, Anderson J and Barisa M (2022) A Simple and Robust Single-Step Method for CAR-Vδ1 γδT Cell Expansion and Transduction for Cancer Immunotherapy. Front. Immunol. 13:863155. doi: 10.3389/fimmu.2022.863155

The γδT cell subset of peripheral lymphocytes exhibits potent cancer antigen recognition independent of classical peptide MHC complexes, making it an attractive candidate for allogeneic cancer adoptive immunotherapy. The Vδ1-T cell receptor (TCR)-expressing subset of peripheral γδT cells has remained enigmatic compared to its more prevalent Vγ9Vδ2-TCR and αβ-TCR-expressing counterparts. It took until 2021 before a first patient was dosed with an allogeneic adoptive Vδ1 cell product despite pre-clinical promise for oncology indications stretching back to the 1980s. A contributing factor to the paucity of clinical progress with Vδ1 cells is the lack of robust, consistent and GMP-compatible expansion protocols. Herein we describe a reproducible one-step, clinically translatable protocol for Vδ1-γδT cell expansion from peripheral blood mononuclear cells (PBMCs), that is further compatible with high-efficiency gene engineering for immunotherapy purposes. Briefly, αβTCR- and CD56-depleted PBMC stimulation with known-in-the-art T cell stimulators, anti-CD3 mAb (clone: OKT-3) and IL-15, leads to robust Vδ1 cell expansion of high purity and innate-like anti-tumor efficacy. These Vδ1 cells can be virally transduced to express chimeric antigen receptors (CARs) using standard techniques, and the CAR-Vδ1 exhibit antigen-specific persistence, cytotoxicity and produce IFN-γ. Practicable, GMP-compatible engineered Vδ1 cell expansion methods will be crucial to the wide-spread clinical testing of these cells for oncology indications.

Keywords: gamma delta (gammadelta) T cells, γδT cells, γδ T cells, V delta-1 (Vδ1) cells, CAR-gamma delta T cells, cancer immunotherapy, CAR-Vδ1 cells

INTRODUCTION

Characterized by expression of a T cell receptor (TCR) composed of gamma and delta chains (γδTCR), γδT cells are an innate-like subset of human T cells representing up to 15% of peripheral CD3-positive cells and up to 60% of intraepithelial lymphocytes in healthy donors. Whilst their physiological role in humans remains an area of active study and debate (1–3), γδT cells are a major

area of interest in adoptive cell therapy for oncology indications (4–7). Exome characterisation of >16,000 patient tumors identified infiltrating γδT cells as the immune cell species most positively associated with patient survival across all cancers (8). A recent examination of patient brain tumor samples further defined γδT cell infiltration as most predictive of patient survival, in unexpected contrast to αβT cells, which correlated negatively with survival (9).

Two subsets composed of MHC-unrestricted Vγ9Vδ2-TCR and Vγ*x*Vδ1-TCR-expressing cells (where x denotes one of 6 functional gamma chain genes) dominate the peripheral γδT cell compartment. In contrast to the oligoclonal and phosphoantigen-reactive Vγ9Vδ2-TCR population, Vγ*x*Vδ1 cells (referred to hereafter as 'Vδ1 cells') express a Vδ1-TCR chain paired with one of various Vγ-chains. The peripheral human Vδ1 population has a polyclonal TCR repertoire that is reactive to a range of antigen types including peptides, lipids and various CD1 proteins of self and non-self origin (3, 10).

Adoptive transfer of the Vγ9Vδ2 cell subset has been clinically tested for anti-cancer efficacy for nearly 20 years (11). Several groups have also previously demonstrated methods to expand Vδ1 cells (12–18). 2021 saw publication of data from two first-in-man Vδ1 cell adoptive transfer clinical trials. GammaDelta Therapeutics Ltd are exploring unengineered, allogeneic 'delta one T' cell ('DOT'; 'GDX012' product) safety, tolerability, and preliminary antileukemic activity in patients with minimal residual disease-positive acute myeloid leukemia (trial ID: NCT05001451). Adicet Bio Inc are testing the safety and efficacy of 'ADI-001' product anti-CD20 CAR-engineered allogeneic Vδ1 cells in adults with B cell malignancies, as a monotherapy or in combination with IL-2 (trial ID: NCT04735471). The discrepancy in the numbers of clinical investigations between Vδ1 and Vγ9Vδ2 γδT cell subsets does not stem from a lack of pre-clinical promise of the Vδ1 subset. Indeed, there is literature stretching back decades describing potent Vδ1 cell responses against tumor targets *in vitro* and graft-*versus*-leukemic effects following bone marrow transplantation, hypothesized to be mediated by atypical T cell subsets (19–22).

The discovery that Vγ9Vδ2 cells can be expanded to high numbers and purity using phosphoantigens (*e.g.* IPP or BrHPP) or phosphoantigen-inducing aminobisphophonates (*e.g.* zoledronic acid) enabled high-throughput Vγ9Vδ2 cell pre-clinical exploration, and consequently accelerated clinical translation. A promising two-step multi-cytokine clinical-grade protocol for Vδ1 cell expansion was published and patented by Almeida and colleagues in 2016 (23) (referred to hereafter as the 'DOT protocol') and is set for clinical translation in trial NCT05001451. Herein we describe a one-step, single-cytokine gene-engineered Vδ1 cell product manufacturing protocol that utilizes processes and reagents already employed to generate genetically modified αβT and Vγ9Vδ2 cell biotherapeutics. We show that Vδ1 cells are readily expandable to high numbers and purity by stimulation of αβTCR- and CD56-depleted PBMC with OKT-3 anti-CD3 mAb in the presence of IL-15-supplemented media. Thus-stimulated Vδ1 cells are efficiently and stably transduced with a chimeric antigen receptor (CAR) using standard viral transduction protocols. The resulting Vδ1-CAR-T cells exhibit innate recognition of targets in addition to antigen-specific boosting of function, and do not exhibit alloreactivity to allogeneic PBMC.

MATERIALS AND METHODS

Ethical Approval

Expansion of T cells from healthy donors was performed under the governance of the following UCL UK research ethics committee approvals: "Establishing cell cultures for pediatric cancers", IRAS project ID-154668. This ethical approval allows for expansion cell lines from tissue samples following written informed consent or from anonymized blood samples from healthy volunteers. For this study, only anonymized commercially available blood samples or anonymized small samples from healthy volunteers were used.

γδT Cell Expansion

PBMC were isolated from purchased whole blood leucocyte cones *via* density gradient centrifugation using Lymphoprep (Stemcell) according to manufacturer's instruction. PBMC were either cryopreserved in 90% FBS 10% DMSO or re-suspended in complete T cell culture media for further processing. Complete T cell culture media consisted of xeno- and serum-free CTS-OpTmizer (Thermo Fisher) with 10% synthetic serum replacement (Thermo Fisher) and GlutaMAX (Thermo Fisher), all of which are available to research as well as GMP-grade from Thermo Fisher with the following product catalogue numbers: research-grade CTS-OpTmizer (A1048501) and GMP-compatible alternative GMP-grade OpTmizer-CTS (A3705003), synthetic immune cell serum replacement that is compatible with both manufacturing standards (A2596101) and GlutaMAX also compatible with both standards (35050061). If starting with cryopreserved material, PBMC were thawed and rested at $10x10^6$ cells/mL in complete pre-warmed media overnight before further processing to avoid over-stressing the lymphocytes and to enhance depletion quality. PBMC at $2\text{-}4x10^6$ cells/mL density were then either stimulated in standard cell culture plates right away or first depleted of αβT cells using the TCRα/β Product Line (Miltenyi Biotec) according to manufacturer's instructions concurrently with depletion of CD56-positive cells using CD56 MicroBeads (Miltenyi Biotec) according to manufacturer's instructions. Briefly, cells were first labelled with anti-TCRα/β-biotin, then a mix of anti-biotin microbeads and anti-CD56 beads, and then depleted using MACS Cell Separation LD Columns (Miltenyi Biotec). If cultured in G-Rex vessels (Wilson Wolf), depleted PBMC were initiated at $2\text{-}4x10^6$ cells/cm^2. Thus-prepared PBMC were stimulated with either 1μg/mL OKT-3 (Miltenyi Biotec Cat# 130-093-387, RRID : AB_1036144) or 1μg/mL PHA (Merck) and various cytokine combinations: (i) 100 IU/mL IL-2 aldesleukin (Proleukin; Novartis), (ii) 70 ng/mL IL-15 (Peprotech), (iii) 20 ng/mL rhIL-7 (Peprotech), or the (iv) 'DOT protocol' cytokine

cocktail, which consisted of a first culture in 100 ng/mL rIL-4, 70 ng/mL rIFN-γ, 7 ng/mL rIL-21 and 15 ng/mL rIL-1β followed by a second culture in 70 ng/mL rIL-15 and 30 ng/mL IFN-γ (all from Peprotech). When comparing the full 'DOT protocol' to test expansion protocols, the methodology described by Almeida and colleagues was used (23), albeit with the omission of a positive selection step using OKT-3 following the alpha beta TCR depletion. Briefly, depleted PBMC were stimulated for a first cytokine culture with 70ng/mL OKT-3, and then a second cytokine culture with 1μg/mL OKT-3. Live cells before and during expansion were counted using Trypan Blue exclusion, an automatic cell counter (Invitrogen) and flow cytometry-based Precision Count Beads (Biolegend).

Vδ2 γδT Cell Depletion

Vδ2 γδT cells were depleted from PBMC at one of three stages of expansion: pre-initiation, at midway split or at harvest. All depletions were done using anti-TCR/Vδ2 mAb clone B6 (BioLegend Cat# 331404, RRID : AB_1089228) at a concentration of 0.5μg/10^6 PBMC. When depleting at initiation Vδ2 cell initiation was incorporated into the αβTCR/CD56 depletion process. This was done as follows: PBMC were co-incubated with αβTCR-biotin mAb and Vδ2 (clone: B6)-biotin mAb, washed, and then co-incubated with anti-biotin and anti-CD56 microbeads according to manufacturer's protocol, then washed and depleted using Miltenyi LD magnetic column separation, as above and according to manufacturer's protocol. If depleting at midway split or final harvest, expanding cells were harvested, washed and labelled with 0.5μg clone B6/10^6 PBMC, incubated for 20min, washed and incubated and depleted using Miltenyi anti-biotin microbeads and LD columns as above.

Flow Cytometry

The following fluorochrome-antibody conjugates and dyes were used according to manufacturer's instruction in Biolegend Cell Staining Buffer to detect different lymphocyte subpopulations in culture: Zombie Green Viability Dye (BioLegend), Zombie Yellow Viability Dye (BioLegend), LIVE/DEAD Fixable Near IR kit (Thermo Fisher), anti-CD3 PE/Dazzle594 (BioLegend Cat# 980006, RRID : AB_2715768), anti-αβTCR APC (BioLegend Cat# 306717, RRID : AB_10612747), anti-TCRVδ1 APC-Vio770 (Miltenyi Biotec Cat# 130-120-440, RRID : AB_2752099), anti-TCRVδ2 VioBlue and PE (Miltenyi Biotec Cat# 130-101-152, RRID : AB_2660779), anti-CD69 FITC (BioLegend Cat# 310903, RRID : AB_314838), anti-NKG2D PercP/Cy5.5 (BioLegend Cat# 320817, RRID : AB_2562791) anti-CD56 Alexa Fluor 488 (BioLegend Cat# 318311, RRID : AB_604094), anti-PD-1 APC/Fire750 (BioLegend Cat# 329953, RRID : AB_2616720) and BUV737 (BD Biosciences Cat# 612791, RRID : AB_2870118), anti-LAG-3 PE/Cy7 (BioLegend Cat# 369309, RRID : AB_2629752), anti-TIM-3 BV711 (BioLegend Cat# 345023, RRID : AB_2564045), anti-CD34 QBend10 Alexa Fluor700 (BioTechne), anti-CD34 QBend10 Alexa Fluor488 (Novus Biologicals), anti-NKp44 PerCP/Cy5.5 (BioLegend Cat# 325114, RRID : AB_2616752), anti-NKp30 DyLight 650 (NovusBio, Cat # FAB1849W, clone 210845). When detecting intracellular and cell surface accumulation of IFN-γ and CD107a, respectively, PBMC were challenged with relevant targets overnight, and incubated at 37°C and 5% CO_2 with anti-CD107a FITC (BioLegend Cat# 328605, RRID : AB_1186058), then 1x monensin (Biolegend) was added followed by incubation for another 4h, stained for cell surface markers, and then permeabilized using Biolegend Intracellular Staining Permeabilization Wash Buffer and stained with anti-IFN-γ Brilliant Violet 605 (BioLegend Cat# 502535, RRID : AB_11125368), anti-IL17a PerCPCy5.5 (BioLegend Cat# 512313, RRID : AB_961397) or anti-Granzyme B Pacific Blue (BioLegend Cat# 372217, RRID : AB_2728384) according to manufacturer's instructions. All expansion and activation samples were analyzed on a BD LSR II flow cytometer using FACSDiva software (BD FACSDiva Software, RRID : SCR_001456), while CAR-Vδ1 proliferation was analyzed on a Beckman Coulter CytoFlex using CytExpert software (CytExpert Software, RRID : SCR_017217). For setting of gates in analysis of panels we employed fluorescence minus one (FMO) controls. Post-acquisition data processing was carried out using FlowJo software (FlowJo, RRID : SCR_008520). T-SNE analysis on flow cytometry data was performed using FlowJo software and concatenated using R language Statistical Computing (RRID : SCR_001905).

Retroviral Production and T Cell Transduction

293T cells (ATCC Cat# CRL-3216, RRID : CVCL_0063) were plated at 1.5x10^6 cells per 10cm^2 plate (Corning) in 10mL 10% fetal bovine serum (FBS)-supplemented Gibco IMDM (Thermo Fisher). At 70% confluence, 293T cells were transfected using GeneJuice (Merck) according to manufacturer's protocol. Triple plasmid transient transfection was carried out using SFG-gammaretroviral vectors (RRID : Addgene_22493). The anti B7H3 CAR-T was synthesized within SFG and contains the following components: IL-2 signal peptide, TE9 ScFv, CD8 hinge and transmembrane, CD28 endodomain, CD3zeta. The CAR was co-expressed with the RQR8 sort suicide gene allowing detection with anti-CD34 antibody.

The B7H3-CAR (second generation with CD28 and 325 CD3-zeta endomains synthesized in our laboratory), gag+pol 326 (RRID : Addgene_8449) and RD114 envelope (RRID : 327 Addgene_17576) plasmids were added at an equimolar ratio. Retroviral supernatant was harvested at 48 and 72 hours following transfection and used immediately for T cell transduction. Briefly, non-tissue culture treated 24 well plates (Costar) were coated with RetroNectin (Takara) in PBS (final concentration of 1mg/mL) and incubated at 4°C for 24 hours. The retronectin was removed and 1.5 mL of retroviral supernatant was added to each retronectin coated well. Following this, 3x10^5 stimulated T cells in 500 μL was added and plates were centrifuged at 1000 *x* g for 40 minutes, at room temperature before incubation in complete T cell culture media at 37°C, supplemented with IL-15 to a final concentration of 70ng/mL (~140 IU/mL). Transduced T cells were harvested after three days, washed and re-suspended for expansion in specified cytokine-supplemented complete T cell culture medium.

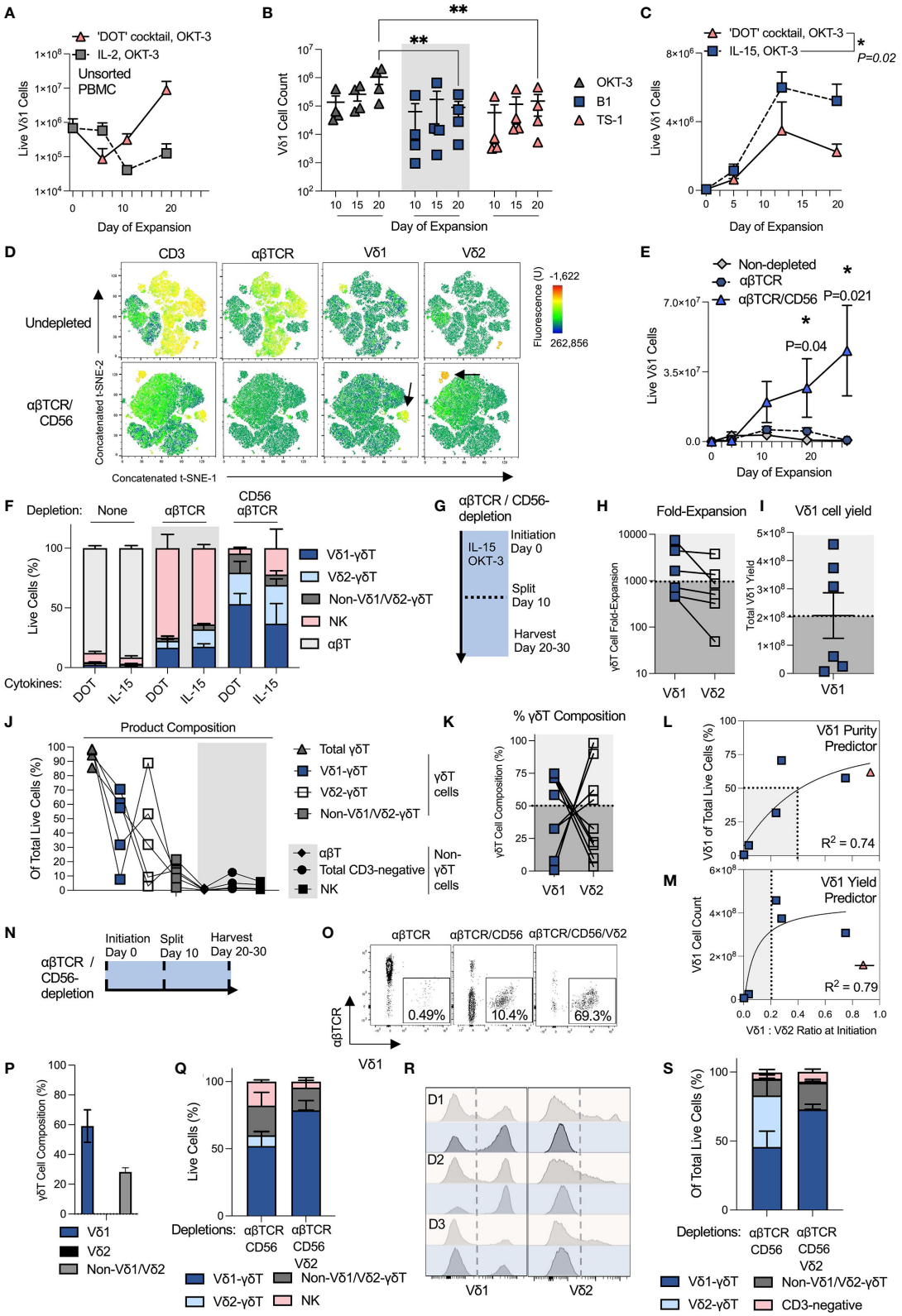


FIGURE 1 | Continued

FIGURE 1 | OKT-3 and IL-15 stimulation of αβTCR- and CD56-depleted PBMC leads to robust and reproducible Vδ1 cell expansion. All experiments used separate and independent donors except where indicated they were from the same donors. **(A)** Vδ1 cell yield was compared when expanding PBMC with either the 'DOT' cocktail of cytokines or IL-2 (N=2; mean +/- standard error mean (SEM). **(B)** αβT-depleted PBMC were expanded with the 'DOT' cocktail of cytokines and a single stimulation with 1ug/mL of either anti-CD3 mAb clone OKT-3, anti-γδTCR mAb clone B1 or Vδ1-TCR clone TS-1. (N=4; mean +/- SEM; statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test). **(C)** αβT-depleted PBMC were expanded with either the 'DOT' cocktail of cytokines or IL-15. Expansion was measured over a period of 20 days, and compared at day 20 post-stimulation (N=3; mean +/- SEM; statistical significance was determined by two-way ANOVA with Sidak's multiple comparisons test). Vδ1 cell numbers in the starting material ranged from 40e3 – 80e3/well. **(D)** Three separate donor PBMC were stained for flow cytometry analysis of population composition before and after αβTCR/CD56 depletion and visualized using a t-SNE algorithm. The three donor t-SNE data was concatenated using 'R'. The black arrows on the bottom row indicate Vδ1 and Vδ2 cells among the depleted PBMC. **(E)** Vδ1 cells were expanded using OKT-3 and IL-15 from PBMC either unmanipulated, depleted only of αβT cells, or depleted of αβT cells and CD56-positive cells (N=3; mean +/- SEM; statistical significance was determined by ordinary two-way ANOVA with Sidak's multiple comparisons test). Vδ1 cell numbers in the starting material ranged from 7e3 – 80e3/well. **(F)** Day 20-harvested expansate composition was compared after culture in either the 'DOT' cocktail of cytokines or IL-15 alone, from either undepleted, αβTCR single-depleted or αβTCR/CD56-double depleted PBMC at initiation (N=3). **(G)** To achieve optimal 20 day expansion, expanding T cell cultures were split 1:4 at day 10 of expansion to avoid overconfluence. **(H)** Matched Vδ1 cell expansions using OKT-3 and IL-15 from αβTCR/CD56-depleted PBMC in 6-well G-Rex vessels were compiled from six different donors and two separate experimental repeats and compared in terms of fold-expansion and **(I)** absolute cell yield. **(J)** A further five donors were initiated for G-Rex culture in a third αβTCR/CD56-depleted OKT-3/IL-15 manufacturing run. Day 20 expansates were analyzed for product composition (N=5). **(K)** G-Rex expansion donor data was pooled to examine the Vδ1/Vδ2 cell composition of total product γδT cells (N=11). **(L)** The effect on αβTCR/CD56-depleted IL-15/OKT-3-expanded Vδ1 cell purity of pre-depleted, pre-stimulation PBMC Vδ1:Vδ2 ratio was determined (N=6; statistical significance was determined by a non-linear least squares model). Purity in this context denotes Vδ1 cells of total live cells in the product at day 20. **(M)** The effect on αβTCR/CD56-depleted IL-15/OKT-3-expanded Vδ1 cell yield of pre-depleted, pre-stimulation PBMC Vδ1:Vδ2 ratio was determined (N=6; statistical significance was determined by a non-linear least squares model; one data point indicated in red and crossed out was not included in the statistical analysis of the data). **(N)** Three potential timepoints (shown with black, dotted lines) were identified for testing Vδ2 cell depletion from Vδ1 cell product: at initiation, at midway split or at harvest. **(O)** Initiation Vδ2 depletion: Vδ1 cell purity of total T cells (CD3-positive cells) were compared in single (αβTCR), double (αβTCR/CD56) or triple (αβTCR/CD56/Vδ2)-depleted freshly-isolated PBMC at initiation. Shown are representative dot plots from one donor. **(P)** Initiation Vδ2 depletion: Three donor triple αβTCR/CD56/Vδ2-depleted PBMC OKT-3/IL-15 expansates were harvested at day 10 of expansion to examine γδT cell subset composition (N=3). **(Q)** Midway Vδ2 depletion: αβTCR/CD56-depleted PBMC were expanded with OKT-3/IL-15, then depleted of Vδ2 cells at day 10 and plated for another 10 days' expansion until day 20. Resulting day 20 PBMC composition was analyzed and compared in Vδ2-depleted (αβTCR/CD56/Vδ2) or undepleted (αβTCR/CD56) expansates (N=3). **(R)** Harvest Vδ2 depletion: Expansate depletion of Vδ2 cells was tested at day 20 harvest of cell cultures. Shown are three donor (D1, D2, D3) PBMC pre- (red) and post- (blue) depletion, in which Vδ1 and Vδ2 cells was measured (N=3). **(S)** Harvest Vδ2 depletion: Expansate product composition was characterized following day 20 product Vδ2-depletion (N=3). * means P <0.05; ** means P ≤0.01.

Transduction efficiency was assessed by flow cytometric detection of the CD34 marker gene (26).

Cytotoxicity Assays

Cytotoxicity was determined either by staining for cell surface accumulation of CD107a as above where indicated, or by four-hour chromium (^{51}Cr)-release assay. Briefly, 1x10^6 target cells were labelled with 20 µL ^{51}Cr amounting to 3.7 MBq (PerkinElmer) for 60 minutes at 37°C. Following this, target cells were co-cultured with effector CAR T cells at range of effector: target (E:T) ratios (10:1, 5:1, 2.5:1 and 1.25:1) for four hours at 37°C in 96 well U bottom plates (Grenier). After incubation, the plates were centrifuged at 1500RPM for 5 minutes and 50 µL of the supernatant was transferred to 96 well OptiPlate-96 HB (PerkinElmer). 150 µL of scintillation fluid was added per well and the plates were sealed and incubated at room temperature overnight. ^{51}Cr release from lysed target cells was counted on 1450 MicroBeta Trilux Scintillation Counter (PerkinElmer). The scintillation counts from wells with only targets (without effectors) were used as spontaneous release controls and target cells lysed with 1% Triton X-100 (Thermofisher) were used as a maximum ^{51}Cr release control.

Proliferation Assay

Proliferation of expanded and harvested Vδ1 cells following repeated stimulation was evaluated to determine CAR-Vδ1 persistence in the presence of an antigen-expressing target cell line. Briefly, CAR-Vδ1 cells were labelled with CellTrace Violet proliferation dye (ThermoFisher) according to manufacturer's instructions for 20 minutes at 37°C. Once labelled, CAR-Vδ1 cells were plated at 5x10^5 per well of a 48-well plate (Corning) and co-cultured at a 1:1 E:T ratio with irradiated tumor targets, either B7H3-negative Jurkat wild type cells (Jurkat-WT) or isogenic Jurkat cells transduced to express high levels of B7H3 (Jurkat-B7H3). Plates were incubated at 37°C and 5% CO_2 for 6 days, without exogenous cytokine supplementation. Freshly irradiated target cells were fed every two days following co-culture and proliferation was evaluated by flow cytometry.

Cell Lines

Jurkat (ATCC Cat# TIB-152, RRID : CVCL_0367), HeLa (ATCC Cat# CCL-2.2, RRID : CVCL_0058), NOMO-1 (DSMZ Cat# ACC-542, RRID : CVCL_1609), K562 (ATCC Cat# CCL-243, RRID : CVCL_0004) and U87 (ATCC Cat# HTB-14, RRID : CVCL_0022) cell lines were all acquired from ATCC and cultured as recommended by the supplier. LAN-1 cell line (DSMZ Cat# ACC-655, RRID : CVCL_1827) was acquired from DSMZ and cultured as recommended by the supplier. Cell lines were screened monthly for mycoplasma contamination Briefly, Jurkat, K562 and NOMO-1 cells were grown in 10% FBS-supplemented RPMI1640 (Sigma Aldrich) suspension culture and kept at <1x10^6/mL density. LAN-1, HeLa and U87 cell lines were grown in 10% FBS-supplemented DMEM (Thermo Fisher) adherent culture and split regularly at around 80-90% confluency using trypsin (Thermo Fisher)-based disaggregation, to avoid overgrowth.

Production of B7-H3 Positive Jurkat Cells

A truncated B7-H3 (T-B7-H3) in an SFG γ-retroviral expression cassette was a gift from Karin Straathof (UCL). A 4Ig-B7-H3

isoform of B7-H3 was purchased (Sinobiological) and cloned into a γ-retroviral expression cassette. Retroviral transduction was used to stably transduce Jurkat cells with 4Ig-B7-H3.

Statistical Design

Data was analyzed with GraphPad Prism (GraphPad Prism, RRID : SCR_002798). Data are displayed at mean ± SEM unless otherwise stated. For normally distributed numerical data, parametric tests were used to determine significance of difference between groups. Analysis of variance (ANOVA) was used, unless otherwise stated. Significance is represented by: *p<0.5, **P<0.01, ***p<0.001, ****p<0.0001.

Calculation of Earth Mover's Distance (EMD)

EMD describes change in signal strength based on difference in probability distribution, with a higher EMD denoting a larger change. The use of EMD to describe changes in protein accumulation allows multiple biological replicates to be characterized with a high degree of consistency, without collapsing the data to mean or median values at the expense of interpretability (24). EMD was computed between bulk CAR-transduced T cell culture versus non-transduced culture. Samples were time- and donor-matched. EMD was calculated between T cell populations that had undergone the same processing. The Python (Python Programming Language, RRID : SCR_008394) module 'wasserstein_distance', which is a component of 'scipy.stats', was used to calculate EMD between samples.

RESULTS

OKT-3 and IL-15 Stimulation of αβTCR- and CD56-Depleted PBMC Leads to Robust and Reproducible Vδ1 Cell Expansion

Benchmarking our efforts to the two-step and multi-cytokine 'DOT protocol' [described in detail by Almeida and co-workers (23)], we compared its ability to expand Vδ1 cells with canonical *ex vivo* T cell expansion methodology, consisting of a single step PBMC stimulation with clone OKT-3 anti-CD3 mAb and IL-2 at 100 IU/mL. Briefly, the 'DOT protocol' entails a first 10 day culture in OKT-3 with IL-4, IFN-γ, IL-21 and IL-1β, followed by a second culture in OKT-3 with IL-15 and IFN-γ. Over 20 days of expansion, the 'DOT' cocktail of cytokines yielded a mean 100-times more Vδ1 cells than culture in IL-2 following activation of unsorted PBMC with a single dose of OKT-3 at initiation (**Figure 1A**). The inferiority of IL-2 monoculture to the 'DOT' cocktail of cytokines is consistent with what was reported in the original 'DOT' protocol publication (23). In pursuit of an allogeneically-applicable expansion protocol that generates a product without potentially alloreactive αβT cells, we then depleted the starting PBMC of αβT cells using a standard and GMP-compatible αβTCR-biotin and anti-biotin bead-based protocol from Miltenyi. Aside from removing contaminating αβT cells, αβTCR-depletion further enhanced the yield of Vδ1 cells following 20 day culture in the 'DOT' cocktail of cytokines with a single dose of OKT-3 at initiation (**Supplementary Figures 1A, B**). This may be at least in part be due to the removal of αβT cell competition for cytokines.

We then investigated, in the context of the DOT cocktail of cytokines, whether we could expand Vδ1 cells more efficiently by using a more specific γδTCR stimulus, such as an anti-γδTCR mAb (clone: B1) or specific anti-Vδ1-TCR mAb (clone: TS-1). A single stimulating 1 μg/mL mAb dose at initiation has been previously reported to be effective for TS-1/B1 mAb-driven Vδ1 cell expansion (25). Anti-CD3 OKT-3 stimulation led to an order of magnitude higher Vδ1 cell expansion from non-depleted PBMC than either γδT cell-specific clone (**Figure 1B**). Moreover, specific anti-γδTCR stimulation applied to non-depleted PBMC failed to prevent expansion of contaminating αβT cells in culture (**Supplementary Figure 1C**). Vδ1 cell numbers in DOT cytokine cocktail culture were evaluated and found equivalent between single stimulation with OKT-3 at initiation or repeated OKT-3 stimulation at 5 day intervals over the course of expansion, suggesting that a single OKT-3 administration is sufficient for optimal T cell expansion (**Supplementary Figure 1D**). As a result, αβTCR-depletion and a single stimulation with OKT-3 anti-CD3 mAb were progressed for further study.

We next evaluated Vδ1 cell expansion in this culture setup using either the two-step DOT cocktail of cytokines or continuous culture in 70ng/mL (corresponding to ~140 IU/mL) of IL-15 alone, it also being a component of the latter half of the DOT protocol regimen. Having discarded IL-2 alone as an optimal milieu, IL-15 was chosen as the second most commonly-employed GMP-compatible T cell manufacturing mitogen. Unexpectedly, IL-15 monoculture yielded at least equivalent or higher Vδ1 cell numbers to the DOT cocktail of cytokines (**Figure 1C**). We next examined whether IL-15-driven Vδ1 cell expansion could be improved by further depleting competition for cytokine from NK cells. We first confirmed that freshly-isolated PBMC Vδ1 cells do not express canonical NK cell-marker, CD56, while CD3-negative freshly-isolated PBMC and some Vδ2 cells do (**Supplementary Figure 1E**). We then combined the αβTCR-biotin and anti-biotin depletion step with GMP-compatible Miltenyi anti-CD56 magnetic beads according to manufacturer's protocol. Three donor concatenated t-SNE analysis of culture initiation material demonstrates the difference between undepleted and αβTCR/CD56-depleted freshly-isolated PBMC (**Figure 1D**). The double-depleted material is predominantly CD3-negative, though with enriched Vδ1 and Vδ2 composition relative to undepleted PBMC.

Undepleted, αβTCR- and αβTCR/CD56-depleted PBMC starting material was then compared for its ability to expand Vδ1 cells when stimulated with IL-15 and OKT-3. Double-depleted PBMC yielded not only substantially greater Vδ1 cell numbers, but also purity (**Figures 1E, F**). Of note, no substantive differences in product composition from any of the starting materials could be found when comparing IL-15 monoculture with culture in the DOT cocktail of cytokines (**Figure 1F**). We, therefore, progressed a single-step OKT-3 + IL-15-based

αβTCR/CD56-depleted Vδ1 expansion protocol for further optimization. We note that in this setup, a majority of donor αβTCR/CD56-depleted PBMC initially plated at $1x10^6$ cells/cm2 approached over-confluence by day 10 of culture (**Supplementary Figure 1F**). We, therefore, opted for a 1:4 culture split midway through the protocol (**Figure 1G**). While feasibly the cells can be cultured for shorter or longer periods as per desired product specification, we progressed a 20-day expansion period with a midway split for further analysis.

We benchmarked our OKT-3 and IL-15-based single step protocol against other published single step Vδ1 expansion methods that utilize phytohaemagglutinin (PHA) instead of anti-CD3 mAb (16–18). We stimulated αβTCR- and CD56-depleted PBMC with OKT-3 and IL-15, or PHA with either IL-2 (16, 17) or IL-7 (18). OKT-3 with IL-15 outperformed both PHA-based protocols in terms of Vδ1 yield in all donors tested (**Supplementary Figures 2A-D**). The choice of anti-CD3 stimulation was further re-enforced by data indicating that, at harvest, OKT-3-stimulated Vδ1 cells expressed higher activation marker levels with concurrently lower exhaustion markers than PHA-stimulated Vδ1 cells, all the while expressing more NKG2D and CD56 receptors, indicative of favorable functional phenotype (**Supplementary Figure 2E**). Indeed, PHA-stimulated Vδ1 cells expressed higher apoptotic markers than CD3-stimulated Vδ1 cells at harvest (**Supplementary Figure 2F**).

Encouragingly for clinical practicality, OKT-3 with IL-15 expanded not only freshly-isolated PBMC, but also Vδ1 cells from thawed cryopreserved PBMC that were αβTCR-/CD56-depleted following an overnight rest upon resuscitation (**Supplementary Figures 3A, B**). We note that an overnight PBMC 'rest and recovery' step in complete media at standard culture conditions enabled retention of a pre-cryopreservation Vδ1/Vδ2 cell ratio (**Supplementary Figure 3C**), and substantially increased the quality of αβTCR-/CD56-depletions as well as Vδ1 cell expansion. Resting was carried out at a high cell density (10^6 PBMC/mL) in complete expansion media, without cytokine supplementation.

To simulate a potential manufacturing process, we compiled expansion data of six arbitrarily chosen healthy donor cryopreserved leukapheresate-derived PBMC from two experimental runs, each of which consisted of PBMC thaw and overnight rest in complete media, followed by αβTCR/CD56-depletion and OKT-3/IL-15 stimulation the following day, as described above, except that in this iteration Vδ1 cells were cultured in 6-well G-Rex (as opposed to standard cell culture) vessels. Expansions were split into new wells at a 1:4 culture surface area ratio on day 10 of expansion and harvested at day 20 for analysis. Out of six donors tested, three achieved >1,000-fold Vδ1 cell expansion, and all achieved >400-fold expansion (**Figure 1H**). While in every donor examined Vδ1 cell expansion rate was greater than that of Vδ2 cells, in five out of six donors the difference was minimal suggesting a relatively unbiased γδT subset expansion by OKT-3/IL-15 (**Figure 1H**). The total Vδ1 cell yield per harvested 6-well G-Rex well was $>2x10^8$ Vδ1 cells per $4x10^6$ PBMC initiated in three out of six donors tested, delineating apparent 'good' and 'poor' expanders (**Figure 1I**). These donors further clustered by product composition. While all yielded 80-100% pure γδT cells, γδT cell composition varied greatly (**Figure 1J**). αβT cell contamination was negligible, though some CD3-negative cells (mostly NK cells) persisted (**Figure 1J**). We investigated γδT cell product composition further and found that in all G-Rex-expanded donor products, an apparently inverse relationship existed between high purity Vδ1-donors and Vδ2-donors (**Figure 1K**). Given the relatively unbiased subset expansion by OKT-3/IL-15 we observed, we interrogated whether a high-purity (or 'good') Vδ1-donor could be predicted by examining the undepleted leukapheresates of donors entered for expansion.

The pre-depletion donor PBMC Vδ1:Vδ2 ratios were compared in six donors and correlated to Vδ1 cell purity and total Vδ1 cell count after 20 day stimulation of αβTCR/CD56-depleted PBMC with OKT-3 and IL-15. In these donors, a pre-initiation Vδ1:Vδ2 ratio of greater than 0.4:1 was associated with at least 50% Vδ1 cell purity at harvest ($R^2 = 0.74$) (**Figure 1L**). The relationship between Vδ1:Vδ2 ratio and absolute Vδ1 cell yield was also investigated. We observed, however, that with excluding one donor from analysis for yield (indicated in red in **Figures 1L, M**), high Vδ1:Vδ2 ratio at initiation correlated with high Vδ1 cell yield at harvest in this small sampling of independent donors. A minimum pre-initiation Vδ1:Vδ2 ratio of 0.2:1 was associated with harvests of $>2x10^8$ Vδ1 cells per $4x10^6$ PBMC initiated ($R^2 = 0.79$) (**Figure 1M**). We hypothesize, therefore, that a high Vδ1:Vδ2 ratio at initiation of expansion may serve as a biomarker for high ultimate Vδ1 cell yield and purity at harvest, though more donor material screening is required to substantiate this observation.

We next examined whether αβTCR/CD56-depleted OKT-3/IL-15-stimulated product can be further enriched for Vδ1 cells by depleting contaminating Vδ2 cells. To this end, we identified three potential depletion points during the manufacture: at initiation concurrently with αβTCR/CD56-depletion, midway at the split, or at harvest (**Figure 1N**). First examining the initiation depletion strategy, we compared the following depletions for Vδ1 cell purity among freshly-isolated PBMS, (i) αβTCR, (ii) αβTCR/CD56, and (iii) αβTCR/CD56/Vδ2. The triple depletion was performed as follows: PBMC were co-incubated with αβTCR-biotin mAb and Vδ2 (clone: B6)-biotin mAb, washed, and then co-incubated with anti-biotin and anti-CD56 microbeads according to manufacturer's protocol. Each depletion step further increased Vδ1 cell purity among the initiation T cell compartment (**Figure 1O**). Not only was the clone B6 Vδ2-depletion highly effective at the outset, it also prevented re-growth of Vδ2 cells during expansion (**Figure 1P**). Though, it also encouraged non-Vδ1/Vδ2 γδT cell expansion, resulting in a Vδ1: non-Vδ1/Vδ2 γδT cell ratio of ~ 2:1. Product Vδ2 depletion midway was highly efficacious; at harvest it yielded a ~77% pure Vδ1 cell product, with a ~17% non-Vδ1/Vδ2 γδT cell presence (**Figure 1Q**). Product depletion at harvest yielded a similar purity of ~72% Vδ1 cells and ~20% non-Vδ1/Vδ2 γδT cells (**Figures 1R, S**). Non-γδT cell content was ~10% in all methods tested and was largely CD3-negative. A majority of these were NK cells (CD3-negative/CD56-positive PBMC)

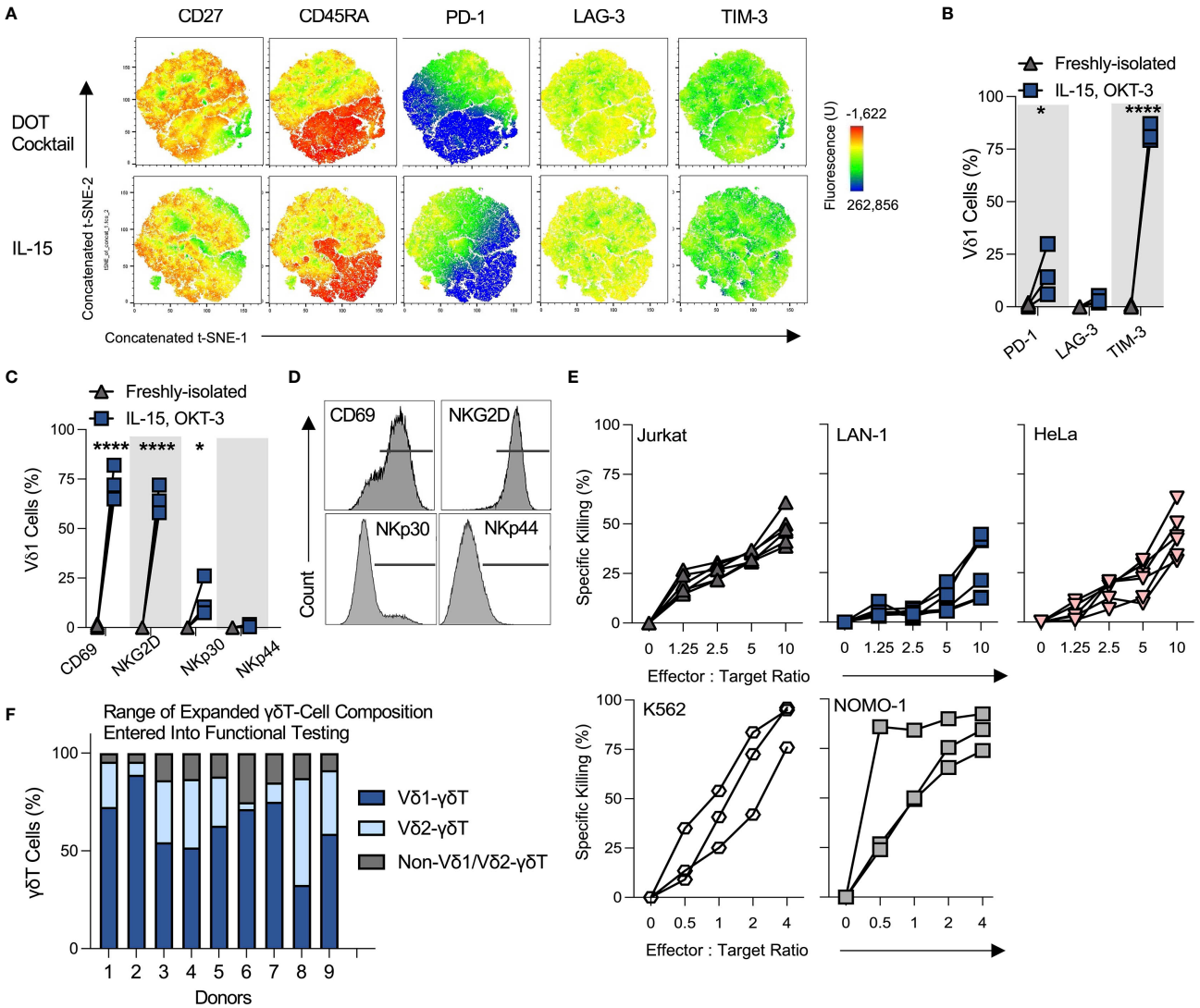


FIGURE 2 | OKT-3/IL-15-expanded Vδ1 cells are innately cytotoxic against a range of tumor targets. All the following data describes phenotyping and performance of Vδ1 cells expanded using OKT-3 and IL-15 from αβT and CD56-depleted PBMC, unless specified otherwise. **(A)** Three donor day 20-expanded Vδ1 cell expression of a range of memory and exhaustion markers was analyzed using t-SNE and concatenated. Profiles were compared between IL-15 or 'DOT' cytokine cocktail milieus (N=3). **(B)** Vδ1 cell % positivity of 'exhaustion' markers was tallied in three donors (N=3; statistical significance was ascertained using a matched two-way ANOVA with Sidak's multiple comparison test). **(C)** Activation and cytotoxicity markers CD69, NKG2D, NKp30 and NKp44 were analyzed using flow cytometry (N=3; statistical significance ascertained using a matched two-way ANOVA with Sidak's multiple comparison test), **(D)** with histograms of marker expression shown from a representative donor. **(E)** Expanded Vδ1 cell product cytotoxicity was studied by target chromium (Cr^{51}) release upon 4h co-culture at different E:T ratios (N=6; each dot and trajectory represent a single donor) for Jurkat, LAN-1 and HeLa target cell lines, and by use of an overnight flow cytometric cytotoxicity assay for K562 and NOMO-1 target cell lines (N=3). **(F)** Donors for functional assay testing were not selected based on Vδ1 cell purity. Shown is a representative range of 9 donor day 20 OKT-3/IL-15-expanded product γδT cell composition from αβTCR/CD56-depleted PBMC (N=9). * means $P < 0.05$; **** means $P \leq 0.0001$.

(**Supplementary Figure 4B**), that we hypothesize either escaped initial depletion or upregulated CD56 during expansion. We note that, while initially negative, also 50-70% of Vδ1 cells upregulated CD56 upon expansion (**Supplementary Figure 4C**), negating the possibility of a CD56-based contaminant depletion at harvest.

Of the Vδ2 cell depletion strategies tested, we hesitate to recommend the best, nor indeed whether it is required at all - as optimal product specifications in terms of γδT cell subset purity for maximal therapeutic efficacy are yet to be determined. It is not necessarily the case that the purest Vδ1 cell product is the most efficacious against cancer, and it is feasible that other γδT cell subsets in the product will synergize rather than suppress Vδ1 cell anti-cancer functionality. Substantial further study in this area is required. Other factors that will impact the decision on Vδ2 cell depletion include post-harvest processing, such as

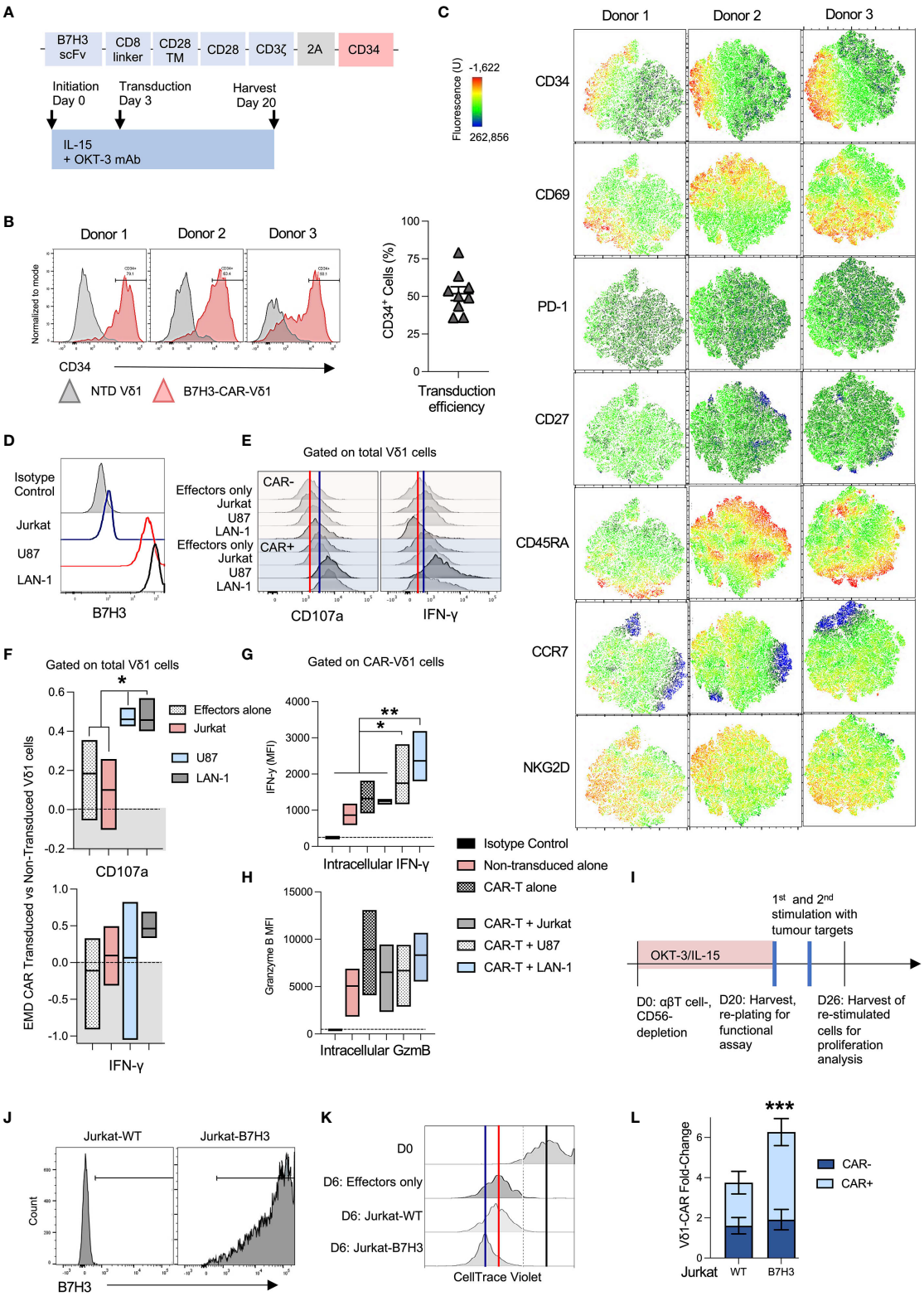


FIGURE 3 | Continued

FIGURE 3 | OKT-3/IL-15-expanded Vδ1 cells are readily transducible with chimeric antigen receptors (CAR). **(A)** Vδ1 cells were transduced with an SFG retroviral vector encoding a B7H3-28ζ-CAR and a CD34 marker gene separated with a T2A cleavage sequence at day 3 post-activation. **(B)** Three representative donor Vδ1 cell CD34 marker gene expression is shown (left panel), along with a compilation of 9 different donor transduction efficiencies 5 days post-transduction (right panel). **(C)** Vδ1 cell activation and memory markers were characterized in a whole population of CAR-transduced cells. t-SNE data is shown for three individual donor day-20 harvested PBMC, gated on live Vδ1 cells. **(D)** A panel of B7H3-positive and negative target cell lines was selected. **(E)** Vδ1 cell accumulation of intracellular IFN-γ and cell surface CD107a was measured after overnight co-culture with targets followed by a 4h culture in monensin-supplemented media. Marker accumulation was measured in CAR-transduced and non-transduced Vδ1 cells. Shown are representative histograms of marker expression from a representative donor. The red line indicates median fluorescence intensity (MFI) of CAR-negative effectors alone, while the blue line indicates MFI of CAR-positive effectors alone. **(F)** IFN-γ and CD107a expression histogram data from 3 separate donors was converted to earth mover's distance (EMD) values that compared marker expression between CAR-transduced *versus* non-transduced cells. A score of '0.0' indicates no difference and is indicated by the dotted line (N=3; mean and distribution indicated). **(G)** The same data was analyzed using MFI measurements, compared in stratified CAR-positive (CD34+) and non-transduced (CD34-) Vδ1 cells in the same culture (N=3; mean and distribution indicated; statistical significance ascertained using one-way ANOVA). **(H)** Vδ1 cells in the same culture were similarly analyzed for intracellular granzyme B levels (N=3; mean and distribution indicated). **(I)** To test expanded CAR-Vδ1 persistence and proliferation, expanded cells were harvested and challenged twice at a 1:1 E:T ratio with **(J)** irradiated B7H3 antigen-positive and negative Jurkat targets. **(K)** CAR-Vδ1 CellTrace Violet dye dilution was measured after 6 day co-culture. One representative donor matched data is shown. The black line indicates the edge of undiluted dye at day 0 of the assay, the red line indicates dye MFI of CAR-Vδ1 only at day 6 indicative of background proliferation, while the blue line indicates dye MFI of CAR-Vδ1 in co-culture with antigen-positive targets. **(L)** To account for ongoing background proliferation, Vδ1 cells were counted pre and post-co-culture using flow cytometric counting beads, and Vδ1 fold-change was normalized to effectors alone (N=3; mean +/- SEM; statistical significance was ascertained using a two-way ANOVA with Sidak's multiple comparison). * means $P < 0.05$; ** means $P \leq 0.01$; *** means $P \leq 0.001$.

intention to cryopreserve, *etc*. The remainder of the functional data in this study is presented on Vδ1 cell products derived from double (αβTCR/CD56)-depleted PBMC.

We note that the above depletions could be reproduced to GMP-standard by replacing research-grade αβTCR and CD56 depletion reagents with GMP-grade alternatives from Miltenyi Biotec (CliniMACS TCRα/β Product Line cat nr: 200-070-407; CliniMACS CD56 Product Line cat nr: 170-076-713) and carried out on either CliniMACS *Plus* or CliniMACS *Prodigy* hardware. We note the lack of commercially-available GMP-compatible clone B6 Vδ2-biotin products, though anticipate that those could be obtained from suppliers through custom manufacture.

OKT-3/IL-15-Expanded Vδ1 Cells Are Innately Cytotoxic Against a Range of Tumor Targets

20-day-expanded OKT-3/IL-15 Vδ1 cells exhibited a similar memory and exhaustion profile to DOT cytokine cocktail counterparts. As indicated in concatenated t-SNE plots of various markers, Vδ1 cells were broadly positive for CD27, with a subpopulation brightly expressing CD45RA. While a proportion of CD27+/CD45RA- cells expressed PD-1, few Vδ1 cells bound anti-LAG-3 antibody above isotype control (**Figures 2A, B**). Nearly all Vδ1 cells were dimly but universally TIM-3-positive. OKT-3/IL-15-expanded Vδ1 cells further upregulated activation marker CD69 as well as cytotoxic differentiation marker NKG2D, but not NKp44 (**Figures 2C, D**). A small subpopulation of expanded Vδ1 cells consistently upregulated NKp30.

Functionally, OKT-3/IL-15-expanded Vδ1 cells exhibited highly consistent innate cytotoxicity against a range of hematological and solid tumor targets, including T cell leukemia Jurkat cells, cervical cancer HeLa cells, neuroblastoma LAN-1 cells, chronic myelogenous leukemia K562 cells and acute myeloid leukemia NOMO-1 cells (**Figure 2E**). This is of note, as donors were not specifically selected for only high Vδ1 cell purity, but rather represented a range of γδT-subset compositions (a range of harvested αβTCR/CD56-depleted PBMC-derived products is illustrated in **Figure 2F**). This suggests that maximal Vδ1 cell purity does not uniquely determine the cytotoxic potential of the OKT-3/IL-15-expanded product.

OKT-3/IL-15-Expanded Vδ1 Cells Are Readily Transducible With Chimeric Antigen Receptors (CAR)

To assess the suitability of this expansion protocol for generating genetically-modified immunotherapeutics, we evaluated Vδ1 cell retroviral transduction with an anti-B7H3 2nd generation 28ζ chimeric antigen receptor (CAR) (**Figure 3A**). A consistent ~50% transduction efficiency (ranging from 35.8% - 79.1%) was achieved transducing nine different donors in three experimental runs (**Figure 3B**).

We queried the impact of viral transduction with an ITAM-containing CAR on OKT-3/IL-15 Vδ1 cell product by comparing expression of a range of memory, exhaustion and functional markers within the transduced cell population. Cells were transduced on day 3 following initiation, and thereafter expanded for an additional 17 days until harvest at day 20. Anti-CD34 staining was used to detect expression of the RQR8 CAR marker gene (26) in the transduced cell product. Unexpectedly, none of the activation, memory or exhaustion markers we tested mapped neatly onto CAR(CD34+)-Vδ1 cells (**Figure 3C**). The closest matches were increased expression of NKG2D and a dim but consistent association of CD34 with PD-1 expression in CAR-Vδ1 compared to unmodified Vδ1 cells. Curiously, there was little association between CD69 and CD34 in any of the donors tested, suggesting that the Vδ1 cell product was highly activated regardless of CAR expression. The most notable difference between CAR-transduced Vδ1 cells as a whole compared to OKT-3/IL-15 Vδ1 cells that were never exposed to retrovirus (**Figure 2A**) was the downregulation of CD27 in virus-exposed compared to non-exposed cells. Most other queried markers were similar between both populations.

CAR-Vδ1 were then tested against a range of antigen-positive and negative hematological and solid tumor targets: B7H3-negative Jurkat cells, and B7H3-positive cell lines U87 (originating from glioblastoma) and LAN-1 cells (**Figure 3D**). Intracellular IFN-γ and cytotoxic degranulation cell surface

marker CD107a accumulation was compared in CAR-transduced *versus* unmodified Vδ1 cells using flow cytometry (**Figure 3E**). The red line in **Figure 3E** indicates marker median fluorescence intensity (MFI) of unmodified target-free Vδ1 cells, while the blue line indicates the MFI of target-free CAR-Vδ1 cells. These measures are included to account for the innate, B7H3-independent reactivity of Vδ1 cells, as well as potential baseline activation mediated by CAR-transduction. Differences between histograms were quantified using the statistical analysis tool Earth Mover's Distance (EMD), which quantifies the dissimilarity between two dimensional distributions whilst respecting the single-cell nature of the dataset; a greater value of EMD indicates greater difference (see methods). EMD scores were generated measuring the difference between bulk Vδ1 cell IFN-γ or CD107a accumulation when either unmodified or transduced with a B7H3-28ζ-CAR and challenged with different tumor targets. An EMD score of 0 indicates no relative change between transduced and non-transduced Vδ1 cells. Interestingly, while CAR-Vδ1 CD107a-mediated cytotoxic degranulation was significantly higher upon challenge with antigen-positive U87 and LAN-1 targets than without challenge or challenge with antigen-negative Jurkat cells, IFN-γ production was less consistently impacted by the presence of target antigen and highly variable on a donor-donor basis (**Figure 3F**). This inconsistency was caused not by the inability of CAR engagement to mediate IFN-γ production, but rather high innate and non-CAR-dependent IFN-γ production in some of the donors. All donor Vδ1 cells demonstrated intracellular IFN-γ with and without CAR transduction. A significant, antigen-dependent upregulation of intracellular IFN-γ could be observed when gating on specifically CAR-positive Vδ1 cells, rather than bulk Vδ1 cells in culture (**Figure 3G**). IFN-γ production correlated positively with Vδ1 cell CAR marker gene, CD34, expression when challenged with antigen-positive but not negative targets (**Supplementary Figure 5**). Consistent with a high and sustained cytotoxic potential, granzyme B levels were at least as high or higher in matched unmodified Vδ1 cells compared to CAR-Vδ1 cells before and after challenge with targets (**Figure 3H**).

To test proliferative and persistence capacity, CAR-Vδ1 were harvested post-expansion, plated with no exogenous cytokine and challenged twice at a 1:1 E:T ratio at three day intervals with irradiated B7H3-negative Jurkat wild type cells (Jurkat-WT) or isogenic Jurkat cells transduced to express B7H3 (Jurkat-B7H3) (**Figures 3I, J**). Expansion was monitored *via* dilution of CellTrace Violet proliferation dye, as well as cell counts performed using Precision Count beads and flow cytometry. While all CAR-Vδ1 were highly activated and continued low-grade proliferation after re-plating, more proliferation was seen upon challenge with Jurkat-B7H3 compared to no targets or Jurkat-WT (**Figure 3K**). The black line in **Figure 3K** indicates the CellTrace Violet MFI of Vδ1 cells at plating, the red line of effectors only after 6 days in culture, and the blue line – of effectors co-cultured with antigen-positive targets. Normalized to effectors only, Vδ1 cells expanded more when expressing a CAR but only in response to antigen-positive Jurkat cells (**Figure 3L**). Unmodified Vδ1 cells expanded ~2-fold over target-free matched effectors, likewise CAR-Vδ1 in response to Jurkat-WT. In response to Jurkat-B7H3, meanwhile CAR-Vδ1 cells expanded 4-fold.

DISCUSSION

We set out to develop a single-step, GMP-compatible CAR-Vδ1 cell expansion and transduction protocol that utilizes standard T cell therapy expansion reagents already employed in the CAR-T field. To that end, we focused on pan-T cell stimulating anti-CD3 mAb, clone OKT-3, and the classic T cell cytokine expansion milieu of IL-2 and IL-15. While OKT-3 with IL-2 failed to support sufficient Vδ1 cell expansion, OKT-3 with IL-15 led to substantial Vδ1 cell expansion that was further boosted by depletion of CD56-positive cells. The additive effect of CD56-positive cell depletion was likely at least in part mediated by decreasing competition for IL-15 from CD56-expressing PBMC, such as NK cells. Given the pan-T cell stimulatory nature of both OKT-3 and IL-15, stringent αβT cell depletion prior to initiation was obligate for achieving Vδ1 cell yield and purity. αβTCR/CD56-depleted OKT-3/IL-15-stimulated Vδ1 cells were highly tumor-reactive in their own right and amenable to transduction to high efficiency with a second generation B7H3-28ζ CAR using standard retroviral protocols. CAR-Vδ1 cells retained innate tumor responsiveness while also engaging in CAR-directed reactivity. Upon challenge with targets, B7H3-28ζ-Vδ1 exhibited antigen-specific persistence, cytotoxicity and IFN-γ production. Taken together, we have described a fully GMP-compatible CAR-Vδ1 manufacturing protocol that utilizes reagents and processes well practiced in the CAR-T field.

We further examined the additional purification of Vδ1 cell product with Vδ2 cell depletion. Vδ2 cells were effectively removable using anti-Vδ2TCR mAb clone B6 conjugated to biotion, magnetically removed with anti-biotin microbeads. These depletions could be successfully carried out at initiation of culture with a triple αβTCR/CD56/Vδ2 depletion, midway through depletion at culture split or at harvest. We reserve judgement as to the best approach in this instance, or whether Vδ2 cell depletion is required at all. We hypothesize that an ultra-pure Vδ1 cell product may not exhibit improved efficacy over a product that contains other γδT cell populations. Though, this warrants substantial further investigation with a range of donors. Indeed, it will be difficult to assess optimal product composition until such products are tested clinically. As the debate for "which γδT cell subset is best?" pervades the immunotherapy field, we expect that only clinical testing and conscientious and scientific clinical trial design will shed light on these questions. Ultimately, it may be that no single subset is superior, but rather that a correct balance of the different subsets is optimal for anti-cancer targeting.

In developing the optimized protocol described herein we used the "DOT protocol" cytokine cocktail described by Almeida and colleagues (23) as a comparator, investigating whether we can design a simplified process. Vδ1 cell yield and phenotype

were broadly similar between cells expanded with either OKT3/IL-15 or the "DOT protocol" cocktail of cytokines. There are four main modifications in our process compared to the published "DOT protocol": (1) a simultaneous αβTCR- and CD56-bead depletion step replaces the αβTCR-depletion only, (2) the "DOT protocol" employs a second OKT-3-based CD3 positive selection step while our protocol adds OKT-3 to the depleted product without the need for a second selection step, 3) the multi-cytokine cocktail of the "DOT protocol" is replaced by IL-15 alone, 4) a second OKT-3 stimulation in the "DOT protocol" midway through expansion in omitted in our protocol. Together these changes represent a considerable simplification of the Vδ1 cell expansion process, and a reduction in cost. It was beyond the scope of the current study to perform a detailed side-by-side comparison in terms of *in vitro* and *in vivo* effector function. Further studies are warranted to compare the long-term effector function between these approaches.

We anticipate an increase of pre-clinical and clinical gene-engineered Vδ1 cell investigations for oncology indications in what is a rapidly evolving immunotherapeutic landscape. With the clinical success of canonical autologous CAR-αβT for a range of B cell malignancies, a role may be carved out for allogeneic non-canonical cell therapies. This includes γδT cells of Vδ1 and Vγ9Vδ2 subsets, as well as NK cells, for the targeting of solid tumor indications and CAR-αβT refractory hematological cancers. Allogeneic approaches of this type may further play an important role in democratizing access to a new generation of gene-engineered cell therapy drugs that can be manufactured in bulk from healthy donor material, with accompanying reductions in price and supply chain complexity, as well as possible improvement in product clinical efficacy.

An important area of ongoing research remains the identification of 'optimal' donors for allogeneic cell therapy products. It remains unclear whether high product yield during manufacture is a sure indicator of maximum therapeutic performance, or as recent data from the CAR-αβT field suggests (5) – that cell 'quality', including memory and exhaustion status, is a more predictive metric than quantity. The elucidation of the factors that govern γδT cell product 'quality' will be crucial to sustained clinical success. Vδ1 cells expanded with this one-step IL-15/OKT-3 process expressed high CD27 and CD45RA, in a pattern that is consistent with naïve and central memory in αβT cells and was diminished upon transduction with B7H3-28ζ-CAR. It is unclear whether this marker expression profile correlates with αβT-like memory phenotypes in Vδ1 cells. Indeed, relatively little is known of γδT cell memory, and less still how such cell surface marker phenotypes correlate with anti-tumor functionality. Expanded and CAR-transduced Vδ1 cells weakly upregulated PD-1 and strongly upregulated TIM-3 'exhaustion' markers, the significance of which on γδT cells is little understood. It is unclear whether their presence is indicative of true T cell exhaustion, activation or something other still.

These properties may further vary between the types of indications targeted and gene engineering applied. Intelligent clinical trial design and study of adoptively transferred γδT cells pre- and post-infusion into patients will be crucial in elucidating the specific qualities of cells that confer the greatest therapeutic benefit.

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/s.

ETHICS STATEMENT

Ethical approval was granted by the UCL UK research ethics committee under IRAS project ID-154668.

AUTHOR CONTRIBUTIONS

MB and JA designed the experiments and wrote the manuscript. GF, CA, MF, and SD performed the data-generating experiments for this paper. JF provided data analysis. KC co-supervised CA. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the following research grants and awards: TC BioPharm Ltd studentship to GF (SR16A33), Cancer Research UK-City of London Centre Clinical Academic Training Programme Award [C355/A28852] to CA, Stand up to Cancer/Cancer Research UK Pediatric Cancer New Discoveries Challenge (RT6188) to MB, Debbie Fund award to KC, Children with Cancer UK (15-502), Great Ormond Street Charity Infrastructure award (VS0118). JA is supported by the NIHR Great Ormond Street Biomedical Research centre award.

ACKNOWLEDGMENTS

We would like to thank Glenn Haggerty, Gabriele Pizzolato, Oddette Pomenya and Tommaso Del Buono D'Ondes from TC BioPharm Ltd who contributed to the preliminary data for the manuscript. Emilio Cosimo from TC BioPharm Ltd supported assembly of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.863155/full#supplementary-material

REFERENCES

1. Lopes N, McIntyre C, Martin S, Raverdeau M, Sumaria N, Kohlgruber AC, et al. Distinct Metabolic Programs Established in the Thymus Control Effector Functions of γδ T Cell Subsets in Tumor Microenvironments. *Nat Immunol* (2021) 22:179–92. doi: 10.1038/s41590-020-00848-3
2. Barisa M, Kramer AM, Majani Y, Moulding D, Saraiva L, Bajaj-Elliott M, et al. Coli Promotes Human Vγ9vδ2 T Cell Transition From Cytokine-Producing Bactericidal Effectors to Professional Phagocytic Killers in a TCR-Dependent Manner. *Sci Rep-uk* (2017) 7:2805. doi: 10.1038/s41598-017-02886-8
3. Willcox BE, Willcox CR. γδ TCR Ligands: The Quest to Solve a 500-Million-Year-Old Mystery. *Nat Immunol* (2019) 20:121–8. doi: 10.1038/s41590-018-0304-y
4. Fisher J, Sharma R, Don D, Barisa M, Hurtado M, Abramowski P, et al. Engineering γδt Cells Limits Tonic Signaling Associated With Chimeric Antigen Receptors. *Sci Signal* (2019) 12:eaax1872. doi: 10.1126/scisignal.aax1872
5. Gavriil A, Barisa M, Halliwell E, Anderson J. Engineering Solutions for Mitigation of Chimeric Antigen Receptor T-Cell Dysfunction. *Cancers* (2020) 12:2326. doi: 10.3390/cancers12082326
6. Fowler D, Nattress C, Navarrete AS, Barisa M, Fisher J. Payload Delivery: Engineering Immune Cells to Disrupt the Tumour Microenvironment. *Cancers* (2021) 13:6000. doi: 10.3390/cancers13236000
7. Barisa M, Fowler D, Fisher J. Interplay Between γδt-Cell Metabolism and Tumour Microenvironment Offers Opportunities for Therapeutic Intervention. *Immunometabolism* (2021) 3:210026. doi: 10.20900/immunometab20210026
8. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. *Nat Med* (2015) 21:938–45. doi: 10.1038/nm.3909
9. Park JH, Kim H-J, Kim CW, Kim HC, Jung Y, Lee H-S, et al. Tumor Hypoxia Represses γδ T Cell-Mediated Antitumor Immunity Against Brain Tumors. *Nat Immunol* (2021) 22:336–46. doi: 10.1038/s41590-020-00860-7
10. Davey MS, Willcox CR, Joyce SP, Ladell K, Kasatskaya SA, McLaren JE, et al. Clonal Selection in the Human Vδ1 T Cell Repertoire Indicates γδ TCR-Dependent Adaptive Immune Surveillance. *Nat Commun* (2017) 8:14760. doi: 10.1038/ncomms14760
11. Fisher JP, Heuijerjans J, Yan M, Gustafsson K, Anderson J. γδ T Cells for Cancer Immunotherapy: A Systematic Review of Clinical Trials. *Oncoimmunology* (2014) 3:e27572. doi: 10.4161/onci.27572
12. Hurtado MO, Wolbert J, Fisher J, Flutter B, Stafford S, Barton J, et al. Tumor Infiltrating Lymphocytes Expanded From Pediatric Neuroblastoma Display Heterogeneity of Phenotype and Function. *PLoS One* (2019) 14:e0216373. doi: 10.1371/journal.pone.0216373
13. Landin AM, Cox C, Yu B, Bejanyan N, Davila M, Kelley L. Expansion and Enrichment of Gamma-Delta (γδ) T Cells From Apheresed Human Product. *J Vis Exp Jove* (2021) 175:e62622. doi: 10.3791/62622
14. Wang S. Expansion of Gamma Delta T Cells - A Short Review on Bisphosphonate and K562-Based Methods. *J Immunol Sci* (2018) 2:6–12. doi: 10.29245/2578-3009/2018/3.1133
15. Siegers GM, Dhamko H, Wang X-H, Mathieson AM, Kosaka Y, Felizardo TC, et al. Human Vδ1 γδ T Cells Expanded From Peripheral Blood Exhibit Specific Cytotoxicity Against B-Cell Chronic Lymphocytic Leukemia-Derived Cells. *Cytotherapy* (2011) 13:753–64. doi: 10.3109/14653249.2011.553595
16. Knight A, Mackinnon S, Lowdell MW. Human Vdelta1 Gamma-Delta T Cells Exert Potent Specific Cytotoxicity Against Primary Multiple Myeloma Cells. *Cytotherapy* (2012) 14:1110–8. doi: 10.3109/14653249.2012.700766
17. Correia DV, Fogli M, Hudspeth K, da SMG, Mavilio D, Silva-Santos B. Differentiation of Human Peripheral Blood Vδ1+ T Cells Expressing the Natural Cytotoxicity Receptor NKp30 for Recognition of Lymphoid Leukemia Cells. *Blood* (2011) 118:992–1001. doi: 10.1182/blood-2011-02-339135
18. Wu D, Wu P, Wu X, Ye J, Wang Z, Zhao S, et al. Ex Vivo Expanded Human Circulating Vδ1 γδt Cells Exhibit Favorable Therapeutic Potential for Colon Cancer. *Oncoimmunology* (2015) 4:e992749. doi: 10.4161/2162402x.2014.992749
19. Chien Y, Konigshofer Y. Antigen Recognition by Gammadelta T Cells. *Immunol Rev* (2007) 215:46–58. doi: 10.1111/j.1600-065X.2006.00470.x
20. Butturini A, Bortin MM, Seeger RC, Gale RP. Graft-Vs-Leukemia Following Bone Marrow Transplantation: A Model of Immunotherapy in Man. *Prog Clin Biol Res* (1987) 244:371–90.
21. Lamb LS, Gee AP, Hazlett LJ, Musk P, Parrish RS, O'Hanlon TP, et al. Influence of T Cell Depletion Method on Circulating γδ T Cell Reconstitution and Potential Role in the Graft-Versus-Leukemia Effect. *Cytotherapy* (1999) 1:7–19. doi: 10.1080/0032472031000141295
22. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang K-Y, Abhyankar S, et al. Long Term Disease-Free Survival in Acute Leukemia Patients Recovering With Increased γδ T Cells After Partially Mismatched Related Donor Bone Marrow Transplantation. *Bone Marrow Transpl* (2007) 39:751–7. doi: 10.1038/sj.bmt.1705650
23. Almeida AR, Correia DV, Fernandes-Platzgummer A, da SCL, da SMG, DR A, et al. Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept. *Am Assoc Cancer Res* (2016) 22:5795–804. doi: 10.1158/1078-0432.ccr-16-0597
24. Orlova DY, Zimmerman N, Meehan S, Meehan C, Waters J, Ghosn EEB, et al. Earth Mover's Distance (EMD): A True Metric for Comparing Biomarker Expression Levels in Cell Populations. *PLoS One* (2016) 11:e0151859. doi: 10.1371/journal.pone.0151859
25. Fisher JPH, Yan M, Heuijerjans J, Carter L, Abolhassani A, Frosch J, et al. Neuroblastoma Killing Properties of Vδ2 and Vδ2-Negative γδt Cells Following Expansion by Artificial Antigen-Presenting Cells. *Clin Cancer Res* (2014) 20:5720–32. doi: 10.1158/1078-0432.ccr-13-3464
26. Philip B, Kokalaki E, Mekkaoui L, Thomas S, Straathof K, Flutter B, et al. A Highly Compact Epitope-Based Marker/Suicide Gene for Easier and Safer T-Cell Therapy. *Blood* (2014) 124:1277–87. doi: 10.1182/blood-2014-01-545020

Conflict of Interest: MF and SD are employed by TC BioPharm Ltd. JA and JF are both inventors on a patent pertaining to CCRs in γδT cells, which was licensed to TC Biopharm (WO/2016/174461). JF has undertaken paid consultancy work for TC BioPharm Ltd. MB was previously employed by TC BioPharm Ltd. JA holds founder stock in Autolus Ltd and share options in TC BioPharm Ltd and holds patents in CAR-T technology. JA received consultancy payments from TC-Biopharm between 2018-2021.

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.

frontiers | Frontiers in Immunology

REVIEW
published: 07 June 2022
doi: 10.3389/fimmu.2022.914839

Targeting Cytokine Signals to Enhance γδT Cell-Based Cancer Immunotherapy

Yuan Song[1,2], *Yonghao Liu*[1,2], *Huey Yee Teo*[1,2] *and Haiyan Liu*[1,2*]

[1] *Immunology Programme, Life Sciences Institute, National University of Singapore, Singapore, Singapore,*
[2] *Immunology Translational Research Program and Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore*

γδT cells represent a small percentage of T cells in circulation but are found in large numbers in certain organs. They are considered to be innate immune cells that can exert cytotoxic functions on target cells without MHC restriction. Moreover, γδT cells contribute to adaptive immune response *via* regulating other immune cells. Under the influence of cytokines, γδT cells can be polarized to different subsets in the tumor microenvironment. In this review, we aimed to summarize the current understanding of antigen recognition by γδT cells, and the immune regulation mediated by γδT cells in the tumor microenvironment. More importantly, we depicted the polarization and plasticity of γδT cells in the presence of different cytokines and their combinations, which provided the basis for γδT cell-based cancer immunotherapy targeting cytokine signals.

Keywords: γδT cell, cytokine, cancer, immunotherapy, cellular therapy

OPEN ACCESS

Edited by:
*Andy Hee-Meng Tan, Bioprocessing Technology Institute (A*STAR), Singapore*

Reviewed by:
Wenwei Tu, The University of Hong Kong, Hong Kong SAR, China
Jaydeep Bhat, Ruhr University Bochum, Germany

***Correspondence:**
Haiyan Liu
micliuh@nus.edu.sg

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *07 April 2022*
Accepted: *09 May 2022*
Published: *07 June 2022*

Citation:
Song Y, Liu Y, Teo HY and Liu H (2022) Targeting Cytokine Signals to Enhance γδT Cell-Based Cancer Immunotherapy. Front. Immunol. 13:914839. doi: 10.3389/fimmu.2022.914839

CHARACTERISTICS AND ANTIGEN RECOGNITION OF γδT CELLS

Although γδT cells share the same progenitors with conventional αβT cells and develop in the thymus, they are considered as innate immune cells due to their major histocompatibility complex (MHC) unrestricted antigen recognition, as well as the expressions of Natural Killer Receptors (NKRs) and Toll-like Receptors (TLRs) along with rapid cytokine production. The majority of γδT cells are negative for CD4 and CD8. In both human and mice, γδT cells account for 5% of total peripheral T cells.

Based on the TCR δ chain usage, human γδT cells can be subtyped to Vδ1, Vδ2, Vδ3 and Vδ5 cells (**Table 1**). Vδ1 and Vδ2 are the major subsets, which are of great interest among human γδT cells. Human Vδ2 cells are generally paired with T cell receptor (TCR) γ9, also named as Vγ9Vδ2 cells. Vγ9Vδ2 cells are the dominant γδT subset in human peripheral blood mononuclear cells (PBMCs). Vγ9Vδ2 TCRs recognize phosphoantigens (PAgs) such as isopentenyl pyrophosphate (IPP), which is accumulated in tumor cells, and (E)-4-hydroxy-3- methyl-but-2-enyl pyrophosphate (HMBPP) that is produced during microbial infections (**Figure 1A**). Interestingly, although γδT cells bind PAgs in the MHC independent manner, PAgs-mediated activation of Vγ9Vδ2 requires butyrophilin (BTN) and BTN-like molecules (1). Recent studies reported that BTN2A1 associated with BTN3A1 to initiate antigen-presentation to Vγ9Vδ2 T cells (2, 3). Besides TCR-associated antigen recognition, Vγ9Vδ2 T cells also express NK receptors including NKG2D and DNAM1, which recognize

TABLE 1 | γδT subsets and distribution in human and mouse.

Species	δ Chain	γ Chain	Distribution
human	Vδ1	Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, and Vγ9	dermis, gut, thymus, liver, and other epithelial tissues, PB
	Vδ2	Vγ9, Vγ8, Vγ4	PB, liver
	Vδ3	various γ chains	liver, gut, PB
	Vδ5	Vγ4	PB
mouse	Vγ1	high diversity	spleen, blood, lymph node, liver, lung, dermis
	Vγ4	high diversity	spleen, blood, lymph node, liver, lung, dermis
	Vγ5	Vδ1	dermis
	Vγ6	Vδ1, Vδ4	reproductive mucosa, skin
	Vγ7	Vδ4, Vδ5, Vδ6	gut

MHC class I chain-related molecules (MICA, MICB), ULBP-binding proteins (ULBPs) and Nectin-like-5 that are broadly expressed on tumor cells (4).

Human Vδ1 cells are mainly distributed in epithelial tissues, such as skin, gut, spleen, and liver. Human Vδ1 cells constitute only up to 15% of human γδT cells in PBMCs (5), but they exhibit fast and marked expansion during CMV infections (6). γδT cell compartment involved in HCMV-specific response is non-Vγ9Vδ2 T cells with the TCRVδ1$^+$ lymphocytes representing the prominent non-Vγ9Vδ2 γδ T cell subset (7–9). Furthermore, these Vδ1 cells display a mixed CD27$^-$/CD45RA$^+$ or CD27$^+$/CD45RA$^+$ phenotype that is identified as cytotoxic effector/memory populations in CMV$^+$ individuals (10). These findings indicated the potential immune surveillance function of Vδ1 cells. Whereas the TCRγ chains paired with Vδ1 display high diversity and the antigens recognized by Vδ1 cells are not well revealed, it has been shown that CD1 molecules with or without loaded lipid antigens can specifically activate Vδ1 cells. The direct interactions between Vδ1 and CD1b, CD1c, or CD1d have been identified by CD1 tetramers, mutagenesis experiments and crystal structures (11–15). In addition to CD1-associated recognition, Vγ4Vδ1 cells have been reported to respond to BTNL3 and BTNL8 expressing cells *via* Vγ4 chain (**Figure 1B**) (16). Annexin A2 and Annexin A6 that are known as stress-induced phospholipid-binding proteins and involved in tumorigenesis also stimulated the proliferation and the production of TNF-α in Vγ4Vδ1 cells (17). Another newly identified stress-induced antigen that is recognized by Vδ1 TCR is ephrin type-A receptor 2 (EphA2) (**Figure 1B**), which is upregulated upon AMP-activated protein kinase (AMPK)-dependent metabolic reprogramming of cancer cells. It can be recognized co-ordinately by ephrin A to govern the activation of Vγ9Vδ1 cells (18). The involvement of EphA2 in Vδ1-mediated tumor cell lysis was demonstrated by reduced susceptibility to killing by EphA2 blocking (19). Human Vδ1 cells from peripheral blood and tissues exhibit autoreactivity to the monomorphic MHC-related protein 1 (MR1) without binding with any ligands, indicating MR1 as a ligand of Vδ1 γδTCR (20). Similar to Vδ2 cells, Vδ1 cells also mediate tumor cell lysis through recognizing ULBP3 and MICA by NKG2D (**Figures 1A, B**) (21–23).

Vδ3 cells account for ~0.2% of lymphocytes in PBMCs from healthy donors but are enriched in the liver and gut and can be expanded in patients with CMV activation and B cell chronic lymphocytic leukemia (24, 25). Human Vδ3 cells were identified as CD1d-restricted T cells and can mediate specific killing against CD1d$^+$ cells (**Figure 1C**). Different from Vδ1 cells, Vδ3 cells can not recognize other CD1 molecules (such as CD1b,CD1c) (26). Annexin A2 was identified as the direct ligand of Vγ8Vδ3 TCR (**Figure 1C**) (17). Recently, human Vδ3 cells have also been shown to bind to MR1 in an antigen-independent manner (**Figure 1C**). Another notable population of human γδT cells is Vδ5 subset. Human

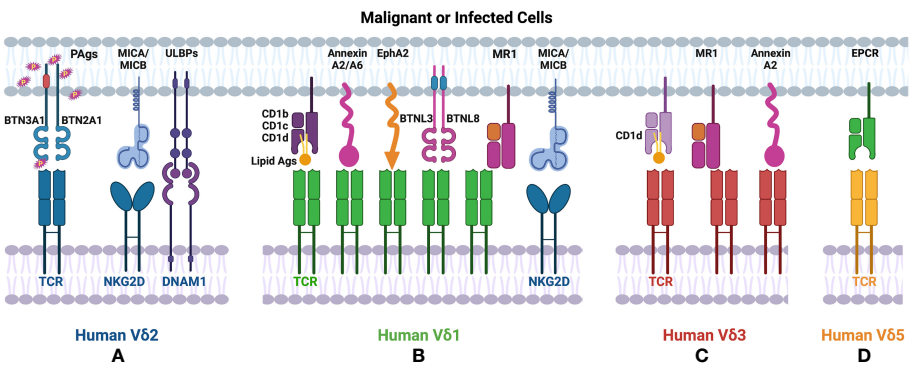


FIGURE 1 | Ligands recognized by human γδT cells. **(A)** Human Vδ2 T cells recognize PAgs via TCR in a BTN molecule dependent manner. **(B)** TCRs of human Vδ1 cells recognize lipid antigens presented by CD1. Human Vδ1 also binds to Annexin A2/A6, EphA2, MR1 in an antigen-independent manner. **(A, B)** Both human Vδ1 and Vδ2 T cells express NKRs (such as NKG2D, DNAM1), which bind to MICA/MICB, ULBPs expressed on tumor cells. **(C)** Human Vδ3 cells interact with CD1d with/without antigen via TCR, also recognize Annexin A2 or MR1 without antigen loading. **(D)** Human Vd5 cells bind to EPCR *via* TCR.

Vγ4Vδ5 T cells were reported to bind directly with endothelial protein C receptor (EPCR) (**Figure 1D**), which is a MHC-like molecule and binds to phospholipid (27). However, the phospholipid binding is not required for the recognition between human Vδ5 cells and EPCR (28).

Taken together, in contrast to αβT cells and other unconventional T cells, such as NKT and MAIT cells, human γδT cells usually recognize specific molecules in an antigen-independent manner except for Vδ2 cells. For example, Vδ1 and Vδ3 TCRs bind to the underside of MR1 and the side of the MR1 antigen-binding groove respectively. Vδ1 cells also respond to CD1 without the loading of lipid antigens. Vδ5 cells recognize EPCR without the involvement of antigens. Other than the recognition of these MHC-like structures in the absence of antigens, Vδ1 TCR can also interact with Annexin A2 and A6 and Vδ3 TCR can recognize Annexin A2 in an Ig-like manner.

With regard to murine γδT cells, they are generally grouped by the usage of TCR γ chains (**Table 1**). Vγ1 and Vγ4 are the predominant subsets in the splenic and circulating γδT cells (29). They are located in many mouse tissues. Vγ5 is invariably paired with Vδ1 and the Vγ5Vδ1 cells are found in dermis and are also named as dendritic epidermal T cells (DETC) (30). Vγ6 cells are mainly paired with Vδ1 or Vδ4 and can home to the mucosa of reproductive tissues and skin (30–32). Vγ7 cells are restricted to intestinal epithelial lymphocytes (33). However, the recognition of PAgs of γδTCR was not found in mouse. Only limited studies reported antigens recognized by murine γδT cells, such as H2–T10, H2–T22, and algae protein phycoerythrin (PE) (34–37). A recent study found that BTNL molecules shape the local Vγ7 and Vγ5 compartments in murine intestinal epithelium and skin (16, 38). The requirement of BTNL during the selection and maintenance of tissue-resident γδT cells indicates the potential interaction between γδTCR and BTNL. However, it is still not clear how the murine and human γδT cell subsets can be matched with each other, and it is difficult to translate some of the findings with murine γδT cells directly to human.

ANTI-TUMOR AND PRO-TUMOR FUNCTIONS OF γδT CELLS MEDIATED BY CYTOKINES AND RECEPTOR-LIGAND INTERACTIONS

After the recognition of antigens or other stress-induced molecules expressed on tumor cells by TCR or NKR, γδT cells can mediate the direct tumor lysis by producing granzyme B, perforin, TNF-α and IFN-γ (**Figure 2**: top right) (39, 40). For example, human Vγ9Vδ2 T cells induced human hepatocellular carcinoma cell lysis in a DNAM-1-dependent manner (4). IL-17 produced by γδT17 cells significantly inhibited tumor

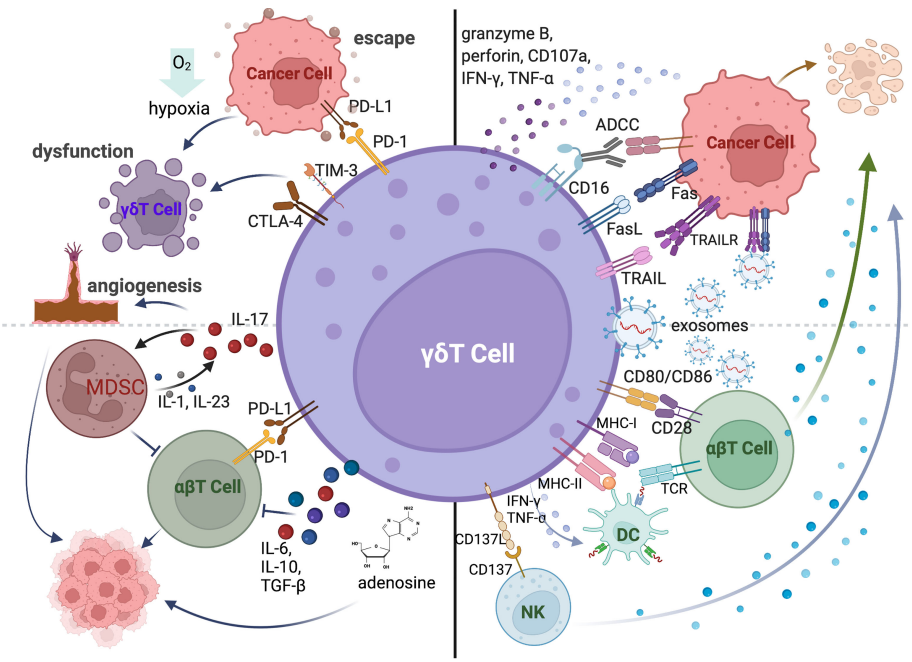


FIGURE 2 | The anti-tumor and pro-tumor functions of γδT cells mediated by cytokines and receptor-ligand interactions. γδT cells can directly kill tumor cells by expressing death receptor ligands (FasL, TRAIL), producing cytotoxic molecules (granzyme B, perforin, CD107a, IFN-γ and TNF-α) and mediating ADCC *via* CD16 expression. The exosomes derived from γδT cells can also directly induce the apoptosis of cancer cells. γδT-APC can activate conventional T cells *via* MHC-I, MHC-II, and co-stimulatory molecules. γδT cells induce the maturation of DCs by secreting IFN-γ and TNF-α and trigger the activation of NK cells *via* CD137L. The pro-tumor function of γδT cells is mediated by the expression of co-inhibitory receptors. The co-inhibitory molecules contribute to tumor cell escape from immune surveillance. Hypoxic tumor microenvironment also induces the dysfunction of γδT cells. γδT cells also promote the tumor growth by recruiting immunosuppressive cells and inhibiting conventional T cells *via* producing IL-17, IL-6, IL-10, TGF-β or adenosine.

development in mice and patients with lung cancer (41, 42). Additionally, activated γδT cells also express death induced ligands CD95L (also known as FasL) and TNF-related apoptosis-inducing ligand (TRAIL), which engage with death receptor CD95 (Fas) and TRAIL receptor, and apoptosis of infected or malignant cells (43–45). Similar to NK cells, the majority of γδT cells in peripheral blood express CD16. CD16 acts as an activation site triggering antibody dependent cellular cytotoxicity (ADCC) (**Figure 2**: top right) (46). A recent study showed that exosomes derived from human Vγ9Vδ2T cells (γδT-Exos) efficiently induced the apoptosis of tumor cells through death receptor ligation (**Figure 2**: top right) (47, 48).

In addition to the direct killing against tumor cells, γδT cells can exert the indirect anti-tumor function by regulating other immune cells in the tumor microenvironment (**Figure 2**: bottom right). Human Vδ2 T cells are described as professional antigen-presenting cells, which can process antigens and provide co-stimulatory signals to induce the proliferation and differentiation of αβT cells (49). It is also reported that human γδT-APCs efficiently cross-present soluble antigens to $CD8^+$T cells *via* MHC-I (50, 51). The high expression levels of APC-associated molecules and tumor antigen presenting capability of *in vitro* expanded human Vγ9Vδ2 T cells were also detected during the early stage of differentiation (52). Activated human γδT cells boost NK cell mediated killing of tumor cells through CD137L (53).

Besides ligand-receptor interactions, cytokine production is the pivotal pathway to regulate other immune cells. Like conventional T cells, γδT cells can be polarized to different subsets based on the secreted cytokines, including IFN-γ-producing γδT cells (γδT-IFN or γδT1), IL-4-producing γδT cells (γδT2), IL-17-producing γδT cells (γδT17) and $Foxp3^+$ regulatory γδT cells (γδTreg). These cytokine-producing γδT cells exist in both human and mouse and can regulate other immune cell functions *via* their signature cytokine productions (**Figure 2**: bottom right). For instance, activated γδT1 cells promoted the maturation of DCs *via* IFN-γ dependent manner in mouse (54). Human freshly isolated γδT1 cells also induced the upregulation of HLA-DR, CD86, CD83 and release of IFN-γ, IL-6, and TNF-α of monocyte-derived DCs through the production of TNF-α and IFN-γ (55, 56). Both human Vδ2 and Vδ3 cells can promote B cell differentiation, antibody maturation and cytokine production (25, 55). IL-4 producing mouse Vγ1Vδ6 T cells can drive the proliferation and IgA secretion of Germinal Centre (GC) B cells (57). In additions, γδT17 cells promoted the infiltration of CTLs within the tumor bed *via* IL-17 production after chemotherapy (58).

Although the anti-tumor functions of γδT cells have been shown in many murine models and in cancer patients, the pro-tumor activities of γδT cells were also reported in numerous studies (**Figure 2**: left). Co-inhibitory molecules can be upregulated on human and murine γδT cells in tumors, which can bind to the co-inhibitory receptors expressed on αβT cells to restrain their activation, infiltration, and anti-tumor efficiency (59). The expressions of PD-1, TIM3 and TIGIT also induced the exhaustion and dysfunction of γδT cells in AML and MM patients (60). Moreover, co-inhibitory receptors on γδT cells contribute to the tumor immune escape by interaction with immunosuppressive molecules (**Figure 2**: top left) (61). Meanwhile, hypoxic tumor microenvironment induced by metabolic status of cancer cells is a critical factor in mediating immunosuppression. The anti-tumor function of γδT cells can be inhibited by hypoxia *via* the downregulation of NKG2D and CD107a expressions (62, 63). Over the past decade, IL-17-producing γδT cells have been found to associate with enhanced tumor growth and metastasis. γδT is one of the major sources of IL-17 in the tumor microenvironment and reduced tumor burden was observed in IL-17-producing Vγ4-depleted and IL-17-deficient mice (64). γδT17 cells recruit myeloid-derived suppressor cells (MDSCs) to the tumor site, which can suppress $CD8^+$T cell responses (64, 65). Consistently, this is also demonstrated in human colorectal cancer (66). In addition, IL-17-produing γδT cells can accelerate tumor progression by promoting angiogenesis and mobilizing pro-tumor macrophages (67, 68). γδTreg cells were found to impair DC maturation and function and $CD8^+$T cell-mediated anti-tumor function in cancer patients *via* TGF-β, IL-6 or IL-10 dependent or independent manner (69, 70). Moreover, $CD39^+$ γδTregs were implicated in the immunosuppressive environment *via* producing adenosine in human colorectal cancer (71). The IL-6-adenosine positive feedback loop between $CD73^+$ γδTregs and cancer-associated fibroblast (CAF) was also involved in tumor progression in breast cancer patients (72).

The role of γδT cells during tumor development is still controversial. Their functions could be cancer type specific. For example, human Vδ1 cells exhibit potent cytotoxicity against colon cancer cells and B-cell chronic lymphocytic leukemia (73, 74), whereas Vδ2 cells are shown to kill a wide variety of tumors including acute myeloid leukemia, multiple myeloma and lung cancer (60, 75). On the other hand, some γδT subsets may exert different functions in the same type of cancer under different treatment conditions/environment. γδT17 cells promoted CTL infiltration into colon cancer after chemotherapy (58), whereas they have been reported to inhibit anti-tumor immune response *via* promoting the recruitment, proliferation, and survival of MDSCs in colorectal cancer and hepatocellular carcinoma (66). Therefore, γδT cell function during tumor development may be greatly influenced by the cytokines present in the tumor microenvironment under specific conditions.

CYTOKINE-MEDIATED REGULATION OF γδT CELL FUNCTION

IL-2 is the commonly used cytokine for expanding human and murine γδT cells. IL-2 is identified as T cell growth factor and is necessary for the proliferation and differentiation of naïve T cells into effector T cells (76). However, γδ T cells produce relatively less IL-2 than αβ T cells (77). Due to the PAgs recognition of human Vγ9Vδ2 T cells, the combination of IL-2 with synthetic PAgs, such as Zoledronate (Zol) and BrHPP, was widely used for the generation of human Vγ9Vδ2 T cells from PBMCs for γδT cell-based immunotherapy (**Figure 3**). Adoptive transfer of pamidronate-expanded Vγ9Vδ2 cells alone effectively

prevented EBV-induced B cell lymphoproliferative disease (EBV-LPD) in mouse and the injection of pamidronate significantly controlled the development through specific activation and expansion of Vγ9Vδ2 cells in humanized mice (78). The adoptive transfer of IL-2/PAgs *ex vivo* expanded Vγ9Vδ2 cells from autologous or allogeneic hosts exhibited potent anti-tumor effects in a variety of cancer patients, such as gastric cancer, osteolytic breast cancer, prostate cancer, and colorectal cancer and so on (79–81). The *in vivo* administration of pamidronate/Zol and low-dose IL-2 also triggered the proliferation of γδT cells in clinical trials and engaged the anti-tumor response without appreciable toxicity in patients (82–84).

IL-15, another proinflammatory cytokine in IL-2 superfamily, has been shown to contribute to the effector functions and maintain the survival of human NK cells *via* IL-15-AKT-XBP1s signalling pathway (**Figure 3**) (85). It is also a promising candidate for enhancing the expansion and cytotoxicity of γδT cells. With the stimulation of IL-2 or IL-15, human Vδ1 cells were selectively induced to express NKp30, NKp44 and NKp46 in a PI3K/AKT dependent manner. The expression of NCRs is associated with increased production of granzyme B and improved cytotoxicity against tumor cells (86, 87). Although low IL-2 and additional IL-15 did not affect NKR expression level on human Vγ9Vδ2 cells, IL-15 significantly increased the expressions of perforin, granzyme B, granulysin and T-bet, which led to enhanced cytotoxic capacity of Vγ9Vδ2 cells. A recent study showed that IL-15 and vitamin C (VC) promoted the proliferation and differentiation and reduced the apoptosis of human Vγ9Vδ2 T cells *in vitro* (88). Moreover, these cells possessed improved cytotoxicity, both *in vitro* and in humanized mouse model. The adoptive transfer of IL-15+VC expanded Vγ9Vδ2 T cells prolonged the survival of patients with late-stage lung cancer or liver cancer (89). IL-15 receptor α signalling limited the development of IL-17-produing γδT cells in a mouse model (90). A global increase of γδT17 cells was found in IL-15Rα-KO mice, but only modest dysregulation of IL-17 production was observed on γδT cells from IL-15-KO mice (90).

Other members of IL-2 cytokine family, including IL-4, IL-7, and IL-21, can also act on γδT cells (**Figure 3**). IL-4 was demonstrated to negatively regulate the anti-tumor function of γδT cells *via* inhibiting the expression of NKG2D and promoting the IL-10 production from Vδ1 cells, which in turn suppressed IFN-γ production and the proliferation of Vδ2 cells (91). IL-7 was used to expand Vδ1 cells from PBMCs in the presence of PHA *in vitro*. These expanded Vδ1 cells exhibited great anti-tumor function and prolonged the survival of human colon carcinoma xenografted mice *via* expressing high levels of cytotoxicity-related molecules, chemokine receptors and NCRs (73). However, IL-7 selectively promoted the IL-17 production of human Vδ1, Vδ2 from cord blood and murine CD27- γδT cells (92). The combination of IL-2 and IL-21 directly enhanced the cytotoxicity of human γδT cells to hepatocellular carcinoma cells *in vitro* (93). In the presence of IL-21, PAgs-expanded Vγ9Vδ2 T cells expressed high level of CXCR5, which enhanced their potential to support antibody production by B cells (94). On the other hand, IL-21-stimulated Vγ9Vδ2 T cells can differentiate to $CD73^+$ γδTreg cells, which exert immunosuppressive function *via* inhibiting T cell responses (95).

The synergistic function of IL-12 and IL-18 in inducing the IFN-γ production of T cells and NK cells has been demonstrated

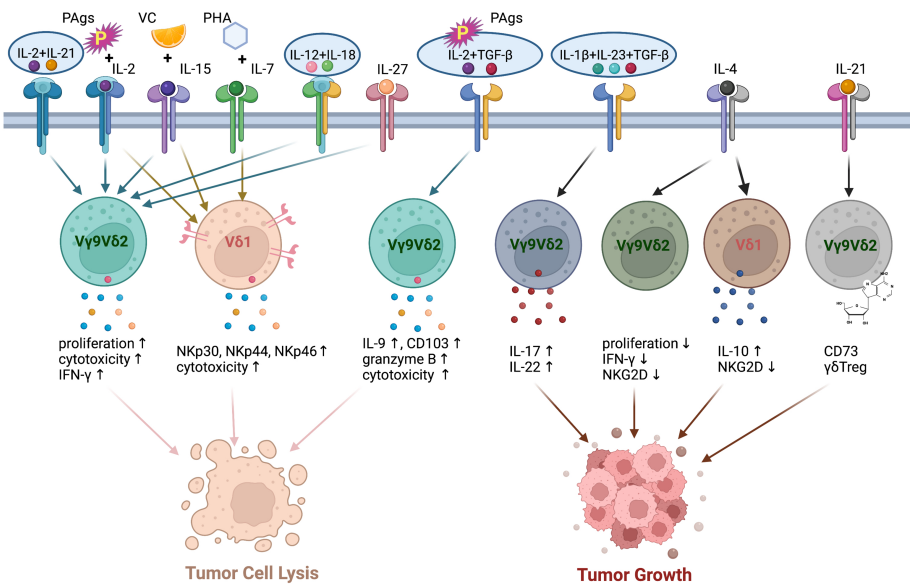


FIGURE 3 | The polarization of human γδT cells induced by different cytokine combination. PAgs and IL-2 with the addition of VC and IL-15, IL-12+IL-18, IL-27, IL-21+IL-2 enhance the cytotoxicity of human Vγ9Vδ2 T cells. IL-2 or IL-15 induces the expressions of NKp30, NKp44, NKp46 on human Vδ1 cells. PHA and IL-7 enhance the cytotoxic capacity of human Vδ1 cells. TGF-β increases the anti-tumor cytotoxicity of human Vγ9Vδ2 T cells in the presence of PAgs and IL-2. The combination of IL-1β, IL-23 and TGF-β promotes the differentiation of Vγ9Vδ2 T cells to IL-17-producing γδT cells. IL-4 reduces the proliferation, NKG2D expression and IFN-γ production of Vγ9Vδ2 T cells *via* promoting IL-10 secretion of Vδ1 cells. IL-21 alone induces Vγ9Vδ2 T cell differentiation to $CD73^+$ γδTreg cells, which promote tumor growth.

(96–99). Similarly, IL-12 and IL-18 also induced the production of IFN-γ and increased cytotoxicity in γδT cells in an antigen-independent manner (**Figure 3**) (100, 101). However, the combination of IL-12 and IL-18 led to the upregulation of TIM3 on γδT cells (102). That might indicate the exhaustion or dysfunction of γδT cell under the treatment of IL-12/18. IL-27 is a heterodimeric cytokine of IL-12 cytokine family. The expression of IL-12R on T cells can be induced by IL-27 (103). The expression of IL-27R was also detected on human Vγ9Vδ2 cells. As expected, IL-27 enhanced the cytotoxicity of human Vγ9Vδ2 T cells by promoting the production of cytotoxic molecules (**Figure 3**) (104).

In addition to cytokines inducing IFN-γ production in γδT cells, IL-17-inducing cytokines are responsible for the polarization of γδT17 cells. It is well known that combination of IL-1β, IL-6, IL-23 and TGF-β induce Th17 differentiation in mouse (105). In human, IL-1 and IL-23 but not TGF-β and IL-6 serve as a rheostat tuning the magnitude of Th17 development (106). The stimulation of IL-1 and IL-23 also promoted RORγt, IL-17, IL-21, and IL-22 expression by γδT cells without the engagement of T cell receptor in mouse (107, 108). TGF-β was found to play a key role in the generation of murine γδT17 in thymus during the postnatal period (109). In adults, IL-1β, TGF-β and IL-23 are required for the commitment of human Vγ9Vδ2 T cells to IL-17-producing γδT cells, which also produce IL-22 (110). The function of IL-6 during the differentiation of γδT17 is uncertain. However, the cocktail of cytokines (IL-1β, TGF-β, IL-6 and IL-23) was used to selectively generate IL-17$^+$ Vγ9Vδ2 T cells *in vitro* (111). These expanded IL-17$^+$ Vγ9Vδ2 T cells produce IL-17 but neither IL-22 nor IFN-γ. The expressions of granzyme B, TRAIL, FasL and CD161 on IL-17$^+$ Vγ9Vδ2 T cells indicated that they contributed to host immune responses against infectious microorganisms. By contrast, TGF-β surprisingly augmented the cytotoxic activity of human Vδ2 T cells when they were stimulated with PAgs and IL-2 or IL-15 in the presence of TGF-β. TGF-β enhanced the migration and anti-tumor function of Vδ2 T cells through upregulating the expressions of CD54, CD103, IFN-γ, IL-9 and granzyme B (112, 113).

In conclusion, γδT cells display high functional plasticity depending on the cytokine environment (**Figure 3**). In view of the cytokine-dependent polarization of γδT cells, it is crucial to understand the roles of various cytokines regulating γδT cell function, which can guide the effective γδT cell-based cancer immunotherapy.

CURRENT γδT CELL-BASED CANCER IMMUNOTHERAPIES

Currently, the majority of the preclinical and clinical studies on γδT cell-based cancer immunotherapy focus on adoptive transfer of expanded γδT cells and its combination with other treatments (**Table 2**, **Figure 4**: top left and bottom left). Due to the feasible expansion of human Vγ9Vδ2 T cells using PAgs or aminobisphosphonates, Zol has been used to expand human γδT cells for adoptive transfer or directly injected to induce the proliferation of human γδT cells *in vivo* for cancer immunotherapy (115, 136).

Due to the successful application of chimeric antigen receptor (CAR) technology in αβT cells, it has also been applied in γδT cell therapy (**Figure 4**: bottom right). The study of allogeneic CAR-Vδ1 T cells targeting CD20 antigen exhibited strong anti-tumor activity and minimum xenogeneic graft-versus-host diseases (GVHD) post transplantation (137). This result further supports the clinical evaluation of ADI-001, an allogeneic CD20-CAR-Vδ1 T cell-associated clinical trial (NCT04735471). CAR-Vδ2 T cells also showed promising results in clearing tumor *in vivo* (138). Mucin 1 (MUC1) with the Tn epitope is a tumor associated antigen that is highly expressed on the surface of a variety of cancer cells. MUC1-Tn CAR-modified Vγ9Vδ2 T cells exhibited similar or stronger anti-tumor effect against breast cancer cell and gastric cancer cell *in vitro* compared with CAR-αβT cells. MUC1-Tn-CAR-Vγ9Vδ2 T cells more effectively suppressed tumor growth than Vγ9Vδ2 T cells in a xenograft murine gastric cancer model (138).

Many recent studies focus on antibody-induced γδ T cell activation (**Figure 4**: top right). Fab fragment of anti-CD3e antibody UCHT1 could bind to γδTCR and enhance the tumor killing of Vγ9Vδ2 T cells (139). Aude De Gassart et al. constructed a humanized antibody, ICT01, that could activate Vγ9Vδ2T cells (140). This antibody activated γδT cells that could kill various tumor cell lines and primary tumor cells but not normal healthy cells. Rajkumar Ganesan et al. designed a bispecific antibody, anti-TRGV9/anti-CD123, that could simultaneously bind to the Vγ9 chain of Vγ9Vδ2 T cells and AML target antigen, CD123, then induce the recruitment and activation of Vγ9Vδ2 T cells to target AML blasts (141). Recently, it is demonstrated that tribody activated γδT cells efficiently. Hans H Oberg et al. reported that tribody [(HER2)2 X CD16] is more effective than anti-HER2 monoclonal antibodies in enhancing γδT cell killing against HER2-expressing cancer cells (142). Similarly, tribody of (Her2)2X Vγ9 targets human Vγ9 T cells and HER2-expressing tumor cells to induce γδT cell-mediated tumor killing (143).

The combination therapy of γδT cells with chemotherapy, monoclonal antibody, immune checkpoint blockade or surgery can exert better anti-tumor efficacy than monotherapy (**Figure 4**: bottom left). The combination of γδT cells with locoregional therapy enhanced clinical efficacy (134). The study using rituximab combined with obinutuzumab and daratumumab activated γδT cells expanded the therapeutic potential of distinctive tumor-antigen-targeting mAbs induced ADCC by γδT cells (144). Targeting the costimulatory signals such as CD137 agonist antibody may promote the anti-tumor functions of Vγ9Vδ2 T cells (145). γδT cell therapy enhanced chemotherapy-induced cytotoxicity to advanced bladder cancer cells (146). Chemotherapeutic agent temozolomide (TMZ) may promote the anti-tumor efficacy of the adoptively transferred *ex vivo* expanded γδT cells for malignant glioblastoma (147). A few studies demonstrated that nanoparticles could also enhance γδ T cells function. In a recent work, it was found that selenium nanoparticles (SeNPs) pre-treatment strengthened the anti-tumor cytotoxicity of Vγ9Vδ2 T cells by increasing the expression of cytotoxicity related molecules, such as NKG2D, CD16, and IFN-γ (148). Chitosan nanoparticles (CSNPs) also exhibited the role of enhancing anti-

TABLE 2 | Clinical trials of γδT cell-based immunotherapy.

Cell types	Cancer type	Phase	Stimulation	Ref
Both Vδ1 and Vδ2 cells	Lymphoma	I	Anti-γδ T-cell receptor (TCR) antibody combine with IL-2 *in vitro* expanded	(114)
Vγ9Vδ2	Renal cell carcinoma	I/II	Zoledronate and IL-2 *in vivo*	(115)
Vγ9Vδ2	Renal cell carcinoma, Colon cancer, Oesophagus carcinoma, Gastric cancer, Ovarian cancer, Breast cancer	I	Bromohydrin pyrophosphate (IPH1101) combine with IL-2 *in vivo*	(116)
Vγ9Vδ2	Metastatic renal cell carcinoma	I	Bromohydrin pyrophosphate (IPH1101) combine with IL-2 *in vivo*	(117)
Vγ9Vδ2	Non-Hodgkin lymphoma (NHL) or Multiple myeloma (MM)	Pilot study	IL-2 combine with pamidronate	(84)
Vγ9Vδ2	Renal cell carcinoma	Pilot study	IL-2 *in vivo*	(118)
Vγ9Vδ2	Breast cancer	II	Neoadjuvant letrozole (LET) plus zoledronic acid	(119)
Vγ9Vδ2	Colorectal cancer	Unknown	Zoledronate and IL-2 *in vitro* expansion	(120)
Vγ9Vδ2	Myeloma	II	Zoledronate and IL-2 *in vivo*	(121)
Vγ9Vδ2	Neuroblastoma	I	Zoledronate and IL-2 *in vivo*	(82)
Vγ9Vδ2	Leukaemia	Pilot study	Zoledronate and IL-2 *in vivo*	(122)
Vγ9Vδ2	Renal cell carcinoma [RCC], Malignant melanoma, and Acute myeloid leukemia	I/II	Zoledronate and IL-2 *in vivo*	(123)
Vγ9Vδ2	Renal cell carcinoma	Pilot study	Zoledronate and IL-2 *in vivo*	(124)
Vγ9Vδ2	Breast cancer	II	zoledronic acid *in vivo*	(125)
Vγ9Vδ2	Non-small cell lung cancer	I	Zoledronate and IL-2 *in vitro* expansion	(126)
Vγ9Vδ2	Non-small cell lung cancer	I	Zoledronate and IL-2 *in vitro* expansion	(127)
Vγ9Vδ2	Breast cancer	I	Zoledronate and IL-2 *in vivo*	(128)
Vγ9Vδ2	Various solid tumors	Unknown	zoledronic acid *in vitro*	(129)
Vγ9Vδ2	Breast cancer	Unknown	zoledronic acid *in vivo*	(130)
Vγ9Vδ3	Multiple myeloma	Pilot study	Zoledronate and IL-2 *in vitro* expansion	(131)
γδ T	Pancreatic cancer	I	Combination of gemcitabine (GEM) and autologous γδ T-cell therapy	(132)
γδ T	Locally advanced pancreatic cancer	II	Irreversible electroporation plus allogeneic γδ T cells	(133)
γδ T	Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC).	I/II	Locoregional therapy followed by adoptive transfer of allogeneic γδ T cells	(134)
γδ T	Non-muscle invasive bladder cancer	II	Rapamycin and BCG instillations	(135)

tumor immune responses of γδT cells (149). Immune checkpoint blockade using anti-PD-1 mAb promoted Vγ9Vδ2 T cell cytotoxicity against PC-2 tumors in immunodeficient NSG mice (150). Furthermore, combination of Tim-3 blocking antibody and bispecific antibody MT110 (anti-CD3 and anti-EpCAM) enhanced the anti-tumor efficacy of the adoptively transferred γδT cells (151). However, autologous γδT cells combined with gemcitabine therapy for patients with curatively resected pancreatic cancer revealed no significant difference compared with those receiving gemcitabine alone (132), suggesting better understanding of the mechanism of action during different treatment is required to achieved effective combination treatment outcome with γδT cells. Cytokine combinations promoting γδT cell function revealed in pre-clinical studies are yet to be evaluated in clinical trials.

CHALLENGES AND POTENTIAL STRATEGIES TARGETING CYTOKINE SIGNALS TO IMPROVE γδT CELL-BASED IMMUNOTHERAPY

γδT cell-based immunotherapy mainly faces three challenges in achieving improved outcomes for cancer patients. The first challenge is the *in vitro* generation/expansion of activated γδT cells with superior cytotoxicity. Although adoptive transfer or *in vivo* expanded human Vγ9Vδ2 T cells exhibited good safety profile, it did not achieve clinical benefit in some patients (123). To boost the cytotoxicity of expanded γδT cells and overcome the immune suppressive tumor microenvironment, cytokine stimulated allogeneic Vγ9Vδ2 cells or Vδ1 cells have been used for clinical trials. A recent study on 132 late-stage cancer patients confirmed the safety and efficacy of IL-15 and VC activated allogeneic Vγ9Vδ2 T cells (89). The addition of IL-15 resulted in the activation, proliferation and increased cytotoxic capacity of γδ T cells (152). To activate cytokine signals, expanded Vδ1 T cells were engineered with a GPC-3 CAR and secreted IL-15 (sIL-15) which significantly controlled tumor growth without inducing GVHD. Moreover, GPC-3-CAR/sIL-15 Vδ1 T cells displayed greater proliferation and stronger anti-tumor responses when compared with GPC-3-CAR Vδ1 T cells lacking sIL-15, suggesting IL-15 signal was critical for CAR Vδ1 T cell function (153). The adoptive transfer of IL-7-expanded human Vδ1 cells also displayed improved cytotoxicity and prolonged the survival of human colon carcinoma xenografted mice (73).

Secondly, rapid exhaustion is a big challenge for maintaining survival and durable anti-tumor functions of γδT cells. Persistent stimulation of human γδT cells with PAgs often induces γδT cell exhaustion (154). It was demonstrated that CD137 costimulation promoted the proliferation and prolonged the survival of Vγ9Vδ2 T cells *in vitro* and *in vivo* (145). Moreover, Endogenous IL-15 acted as a potential factor to support the survival of human Vγ9Vδ2 T cells *in vivo* in the absence of exogenous IL-2 (120). The dysfunction of T cells is also associated with the immunosuppressive tumor microenvironment which will be discussed in the following session.

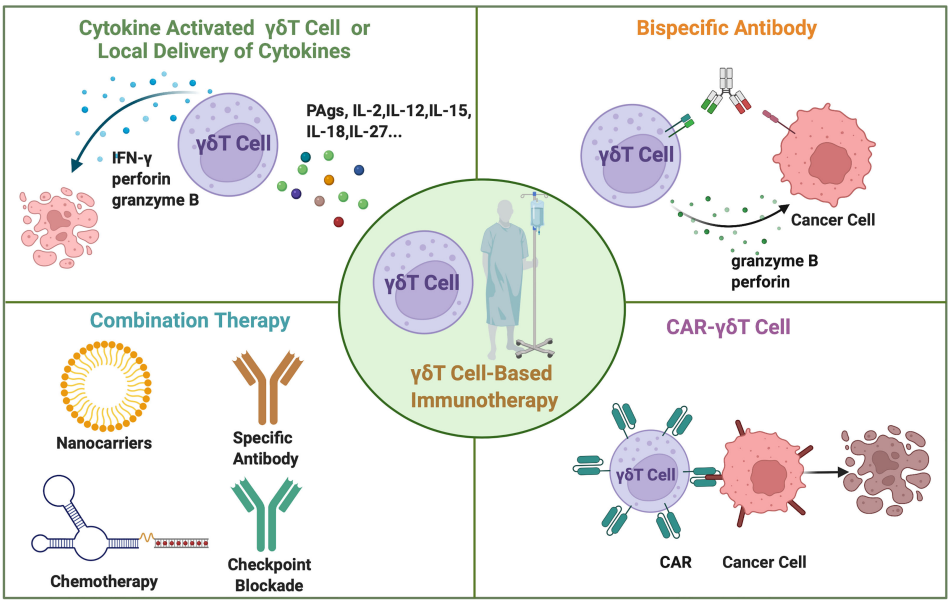


FIGURE 4 | The current approaches for γδT cell-based cancer immunotherapy. The adoptive transfer of cytokine-activated γδT cells *in vitro* or locally administration of cytokines *in vivo*. Combination therapy includes γδT cell transfer combined with specific antibody therapy, immune checkpoint blockade, chemotherapy, and nanoparticles. Bispecific antibodies simultaneously bind to γδT cells and cancer cells. Gene modified CAR-γδT cells directly recognize the cancer cells and mediate cancer cell lysis.

The third challenge is the immunosuppression mechanisms in cancer patients that can impair the anti-tumor functions of the infused/activated γδT cells. The lack of IL-2 and IL-21 in HCC patients was associated with the PD-1 expression and reduced cytotoxicity of human γδT cells (93). In a murine HCC model, IL-23 overexpression in the liver induced the polarization of γδT cells to IL-17-producing γδT cells (155). Then γδT17 cells promoted tumor growth *via* recruiting immunosuppressive myeloid-derived suppressor cells (MDSCs). TGF-β is a pivotal immunosuppressive cytokine that secreted by immunosuppressive cell subsets (such as MDSCs and Treg) and tumor cells (156). Mouse Foxp3$^+$ γδT cells can be induced by TGF-β and inhibit T cell activation (157). To avoid γδT cell exhaustion and circumvent tumour immunosuppressive microenvironment, it is a feasible approach to target cytokine signals *via* administering exogenous stimulating cytokines or blocking the immunosuppressive cytokines. As systemic administration of cytokines usually induces toxicity in patients (158, 159), local delivery of cytokine can limit the systemic toxicity and offer an approach to benefit from the therapeutic effects of the activating cytokines. The local delivery of mRNAs encoding interleukin-12 (IL-12) single chain, interferon-α, granulocyte-macrophage colony-stimulating factor, or IL-15 sushi led to robust anti-tumor immune responses and tumor regression in multiple murine models (160). These findings provided preclinical evidence for modifying the tumor microenvironment *via* local administration of cytokines. It is possible to induce highly cytotoxic γδT cells through modulations of tumor microenvironment through the induction or delivery of cytokines that can specially promote the anti-tumor functions of γδT cells (**Figure 4**: top left).

CONCLUSION AND FUTURE DIRECTIONS

Taken together, γδT cells are promising cellular products for adoptive cancer immunotherapy. γδT cells mediate anti-tumor effects by direct killing and indirect immune regulatory function to other immune cells. γδTCR can recognize specific molecules often in an antigen-independent manner. γδT cells can differentiate into various subsets producing signature cytokines, which can have anti-tumor or pro-tumor functions. In the meantime, this differentiation is greatly influenced by the cytokines present in the microenvironment. γδT cell-based cancer immunotherapy has a good safety profile in the clinical trials but its clinical efficacy needs further improvement. Combination therapies involving γδT cells have had some clinical successes, including chemotherapy, CAR therapy, and checkpoint blockade therapy. IL-2 and IL-15 have been explored for their functions to activate γδT cells in clinical trials. However, other cytokines and combinations that can activate γδT cells are yet to be evaluated in clinical trials. First, cytokines or cytokine combinations can be used to expand, activate, and polarize γδT cells *ex vivo* to generate potent cellular products for adoptive therapy. Cytokine signals can also be modulated to prolong the survival of the transferred γδT cells *in vivo*. Second, cytokine can be incorporated into CAR γδT cell therapy to facilitate CAR γδT cell function and prolong their survival *in vivo via* autocrine mechanism, which can avoid the toxicity induced by systemic cytokine treatment. Third, cytokine signal on γδT cells can be triggered *via* antibody binding in the form of bi-specific or tri-specific antibody targeting tumor antigens. The additional cytokine signal can facilitate γδT cell function and survival. Thus, detailed understanding of the effects of cytokines and cytokine combinations

on γδT cell anti-tumor function is critical for designing effective therapeutic strategies to incorporate cytokine signals into various γδT cell-based cancer immunotherapy to achieve superior clinical efficacy.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work has been supported by Singapore National Research Foundation grant NRF-CRP19-2017-04.

ACKNOWLEDGMENTS

Figures in this paper were drawn using BioRender (@biorender.com).

REFERENCES

1. Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3a1 Binds Phosphorylated Antigens and Stimulates Human Gammadelta T Cells. *Nat Immunol* (2013) 14(9):908–16. doi: 10.1038/ni.2665
2. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2a1 Is Essential for Phosphoantigen Reactivity by Gammadelta T Cells. *Science* (2020) 367(6478):1–10. doi: 10.1126/science.aay5516
3. Laplagne C, Ligat L, Foote J, Lopez F, Fournie JJ, Laurent C, et al. Self-Activation of Vgamma9vdelta2 T Cells by Exogenous Phosphoantigens Involves Tcr and Butyrophilins. *Cell Mol Immunol* (2021) 18(8):1861–70. doi: 10.1038/s41423-021-00720-w
4. Toutirais O, Cabillic F, Le Friec G, Salot S, Loyer P, Le Gallo M, et al. Dnax Accessory Molecule-1 (Cd226) Promotes Human Hepatocellular Carcinoma Cell Lysis by Vgamma9vdelta2 T Cells. *Eur J Immunol* (2009) 39(5):1361–8. doi: 10.1002/eji.200838409
5. Sandberg Y, Almeida J, Gonzalez M, Lima M, Barcena P, Szczepanski T, et al. Tcrgammadelta+ Large Granular Lymphocyte Leukemias Reflect the Spectrum of Normal Antigen-Selected Tcrgammadelta+ T-Cells. *Leukemia* (2006) 20 (3):505–13. doi: 10.1038/sj.leu.2404112
6. Davey MS, Willcox CR, Joyce SP, Ladell K, Kasatskaya SA, McLaren JE, et al. Clonal Selection in the Human Vdelta1 T Cell Repertoire Indicates Gammadelta Tcr-Dependent Adaptive Immune Surveillance. *Nat Commun* (2017) 8:14760. doi: 10.1038/ncomms14760
7. Kaminski H, Marseres G, Cosentino A, Guerville F, Pitard V, Fournie JJ, et al. Understanding Human Gammadelta T Cell Biology Toward a Better Management of Cytomegalovirus Infection. *Immunol Rev* (2020) 298(1):264–88. doi: 10.1111/imr.12922
8. Pizzolato G, Kaminski H, Tosolini M, Franchini DM, Pont F, Martins F, et al. Single-Cell Rna Sequencing Unveils the Shared and the Distinct Cytotoxic Hallmarks of Human Tcrvdelta1 and Tcrvdelta2 Gammadelta T Lymphocytes. *Proc Natl Acad Sci USA* (2019) 116(24):11906–15. doi: 10.1073/pnas.1818488116
9. Scheper W, van Dorp S, Kersting S, Pietersma F, Lindemans C, Hol S, et al. Gammadeltat Cells Elicited by Cmv Reactivation After Allo-Sct Cross-Recognize Cmv and Leukemia. *Leukemia* (2013) 27(6):1328–38. doi: 10.1038/leu.2012.374
10. Pitard V, Roumanes D, Lafarge X, Couzi L, Garrigue I, Lafon ME, et al. Long-Term Expansion of Effector/Memory Vdelta2-Gammadelta T Cells Is a Specific Blood Signature of Cmv Infection. *Blood* (2008) 112(4):1317–24. doi: 10.1182/blood-2008-01-136713
11. Reijneveld JF, Ocampo TA, Shahine A, Gully BS, Vantourout P, Hayday AC, et al. Human Gammadelta T Cells Recognize Cd1b by Two Distinct Mechanisms. *Proc Natl Acad Sci USA* (2020) 117(37):22944–52. doi: 10.1073/pnas.2010545117
12. Pellicci DG, Uldrich AP, Le Nours J, Ross F, Chabrol E, Eckle SB, et al. The Molecular Bases of Delta/Alphabeta T Cell-Mediated Antigen Recognition. *J Exp Med* (2014) 211(13):2599–615. doi: 10.1084/jem.20141764
13. Roy S, Ly D, Castro CD, Li NS, Hawk AJ, Altman JD, et al. Molecular Analysis of Lipid-Reactive Vdelta1 Gammadelta T Cells Identified by Cd1c Tetramers. *J Immunol* (2016) 196(4):1933–42. doi: 10.4049/jimmunol.1502202
14. Luoma AM, Castro CD, Mayassi T, Bembinster LA, Bai L, Picard D, et al. Crystal Structure of Vdelta1 T Cell Receptor in Complex With Cd1d-Sulfatide Shows Mhc-Like Recognition of a Self-Lipid by Human Gammadelta T Cells. *Immunity* (2013) 39(6):1032–42. doi: 10.1016/j.immuni.2013.11.001
15. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, McPherson KG, Lim RT, et al. Cd1d-Lipid Antigen Recognition by the Gammadelta Tcr. *Nat Immunol* (2013) 14(11):1137–45. doi: 10.1038/ni.2713
16. Di Marco Barros R, Roberts NA, Dart RJ, Vantourout P, Jandke A, Nussbaumer O, et al. Epithelia Use Butyrophilin-Like Molecules to Shape Organ-Specific Gammadelta T Cell Compartments. *Cell* (2016) 167(1):203–18.e17. doi: 10.1016/j.cell.2016.08.030
17. Marlin R, Pappalardo A, Kaminski H, Willcox CR, Pitard V, Netzer S, et al. Sensing of Cell Stress by Human Gammadelta Tcr-Dependent Recognition of Annexin A2. *Proc Natl Acad Sci USA* (2017) 114(12):3163–8. doi: 10.1073/pnas.1621052114
18. Harly C, Joyce SP, Domblides C, Bachelet T, Pitard V, Mannat C, et al. Human Gammadelta T Cell Sensing of Ampk-Dependent Metabolic Tumor Reprogramming Through Tcr Recognition of Epha2. *Sci Immunol* (2021) 6 (61):1–14. doi: 10.1126/sciimmunol.aba9010
19. Hudecek R, Kohlova B, Siskova I, Piskacek M, Knight A. Blocking of Epha2 on Endometrial Tumor Cells Reduces Susceptibility to Vdelta1 Gamma-Delta T-Cell-Mediated Killing. *Front Immunol* (2021) 12:752646. doi: 10.3389/fimmu.2021.752646
20. Le Nours J, Gherardin NA, Ramarathinam SH, Awad W, Wiede F, Gully BS, et al. A Class of Gammadelta T Cell Receptors Recognize the Underside of the Antigen-Presenting Molecule Mr1. *Science* (2019) 366(6472):1522–7. doi: 10.1126/science.aav3900
21. Groh V, Steinle A, Bauer S, Spies T. Recognition of Stress-Induced Mhc Molecules by Intestinal Epithelial Gammadelta T Cells. *Science* (1998) 279 (5357):1737–40. doi: 10.1126/science.279.5357.1737
22. Poggi A, Venturino C, Catellani S, Clavio M, Miglino M, Gobbi M, et al. Vdelta1 T Lymphocytes From B-Cll Patients Recognize Ulbp3 Expressed on Leukemic B Cells and Up-Regulated by Trans-Retinoic Acid. *Cancer Res* (2004) 64(24):9172–9. doi: 10.1158/0008-5472.CAN-04-2417
23. Wu J, Groh V, Spies T. T Cell Antigen Receptor Engagement and Specificity in the Recognition of Stress-Inducible Mhc Class I-Related Chains by Human Epithelial Gamma Delta T Cells. *J Immunol* (2002) 169(3):1236–40. doi: 10.4049/jimmunol.169.3.1236
24. Hunter S, Willcox CR, Davey MS, Kasatskaya SA, Jeffery HC, Chudakov DM, et al. Human Liver Infiltrating Gammadelta T Cells Are Composed of Clonally Expanded Circulating and Tissue-Resident Populations. *J Hepatol* (2018) 69(3):654–65. doi: 10.1016/j.jhep.2018.05.007
25. Petrasca A, Melo AM, Breen EP, Doherty DG. Human Vdelta3(+) Gammadelta T Cells Induce Maturation and Igm Secretion by B Cells. *Immunol Lett* (2018) 196:126–34. doi: 10.1016/j.imlet.2018.02.002
26. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, O'Shea D, et al. Cutting Edge: Cd1d Restriction and Th1/Th2/Th17 Cytokine Secretion by Human Vdelta3 T Cells. *J Immunol* (2013) 191(1):30–4. doi: 10.4049/jimmunol.1300121
27. Mohan Rao LV, Esmon CT, Pendurthi UR. Endothelial Cell Protein C Receptor: A Multiliganded and Multifunctional Receptor. *Blood* (2014) 124 (10):1553–62. doi: 10.1182/blood-2014-05-578328
28. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and Tumor Stress Surveillance by Binding of a Human Gammadelta T Cell Antigen Receptor to Endothelial Protein C Receptor. *Nat Immunol* (2012) 13(9):872–9. doi: 10.1038/ni.2394
29. Born WK, Yin Z, Hahn YS, Sun D, O'Brien RL. Analysis of Gamma Delta T Cell Functions in the Mouse. *J Immunol* (2010) 184(8):4055–61. doi: 10.4049/jimmunol.0903679
30. Castillo-Gonzalez R, Cibrian D, Sanchez-Madrid F. Dissecting the Complexity of Gammadelta T-Cell Subsets in Skin Homeostasis, Inflammation, and

Malignancy. *J Allergy Clin Immunol* (2021) 147(6):2030–42. doi: 10.1016/j.jaci.2020.11.023
31. Itohara S, Farr AG, Lafaille JJ, Bonneville M, Takagaki Y, Haas W, et al. Homing of a Gamma Delta Thymocyte Subset With Homogeneous T-Cell Receptors to Mucosal Epithelia. *Nature* (1990) 343(6260):754–7. doi: 10.1038/343754a0
32. Marchitto MC, Dillen CA, Liu H, Miller RJ, Archer NK, Ortines RV, et al. Clonal Vgamma6(+)Vdelta4(+) T Cells Promote Il-17-Mediated Immunity Against Staphylococcus Aureus Skin Infection. *Proc Natl Acad Sci USA* (2019) 116(22):10917–26. doi: 10.1073/pnas.1818256116
33. Melandri D, Zlatareva I, Chaleil RAG, Dart RJ, Chancellor A, Nussbaumer O, et al. The Gammadeltatcr Combines Innate Immunity With Adaptive Immunity by Utilizing Spatially Distinct Regions for Agonist Selection and Antigen Responsiveness. *Nat Immunol* (2018) 19(12):1352–65. doi: 10.1038/s41590-018-0253-5
34. Crowley MP, Reich Z, Mavaddat N, Altman JD, Chien Y. The Recognition of the Nonclassical Major Histocompatibility Complex (Mhc) Class I Molecule, T10, by the Gammadelta T Cell, G8. *J Exp Med* (1997) 185(7):1223–30. doi: 10.1084/jem.185.7.1223
35. Shin S, El-Diwany R, Schaffert S, Adams EJ, Garcia KC, Pereira P, et al. Antigen Recognition Determinants of Gammadelta T Cell Receptors. *Science* (2005) 308(5719):252–5. doi: 10.1126/science.1106480
36. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. Gammadelta T Cells Recognize a Microbial Encoded B Cell Antigen to Initiate a Rapid Antigen-Specific Interleukin-17 Response. *Immunity* (2012) 37(3):524–34. doi: 10.1016/j.immuni.2012.06.011
37. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, et al. Thymic Selection Determines Gammadelta T Cell Effector Fate: Antigen-Naive Cells Make Interleukin-17 and Antigen-Experienced Cells Make Interferon Gamma. *Immunity* (2008) 29(1):90–100. doi: 10.1016/j.immuni.2008.04.022
38. Jandke A, Melandri D, Monin L, Ushakov DS, Laing AG, Vantourout P, et al. Butyrophilin-Like Proteins Display Combinatorial Diversity in Selecting and Maintaining Signature Intraepithelial Gammadelta T Cell Compartments. *Nat Commun* (2020) 11(1):3769. doi: 10.1038/s41467-020-17557-y
39. Vantourout P, Hayday A. Six-Of-the-Best: Unique Contributions of Gammadelta T Cells to Immunology. *Nat Rev Immunol* (2013) 13(2):88–100. doi: 10.1038/nri3384
40. Silva-Santos B, Serre K, Norell H. Gammadelta T Cells in Cancer. *Nat Rev Immunol* (2015) 15(11):683–91. doi: 10.1038/nri3904
41. Cui K, Mei X, Cheng M. Increased Interleukin-17a-Producing Gammadeltat Cells Predict Favorable Survival in Elderly Patients With Luad and Lusc. *J Cancer Res Clin Oncol* (2021) 147(11):3289–98. doi: 10.1007/s00432-021-03742-z
42. Cheng M, Chen Y, Huang D, Chen W, Xu W, Chen Y, et al. Intrinsically Altered Lung-Resident Gammadeltat Cells Control Lung Melanoma by Producing Interleukin-17a in the Elderly. *Aging Cell* (2020) 19(2):e13099. doi: 10.1111/acel.13099
43. Pennington DJ, Vermijlen D, Wise EL, Clarke SL, Tigelaar RE, Hayday AC. The Integration of Conventional and Unconventional T Cells That Characterizes Cell-Mediated Responses. *Adv Immunol* (2005) 87:27–59. doi: 10.1016/S0065-2776(05)87002-6
44. Roessner K, Wolfe J, Shi C, Sigal LH, Huber S, Budd RC. High Expression of Fas Ligand by Synovial Fluid-Derived Gamma Delta T Cells in Lyme Arthritis. *J Immunol* (2003) 170(5):2702–10. doi: 10.4049/jimmunol.170.5.2702
45. Tawfik D, Groth C, Gundlach JP, Peipp M, Kabelitz D, Becker T, et al. Trail-Receptor 4 Modulates Gammadelta T Cell-Cytotoxicity Toward Cancer Cells. *Front Immunol* (2019) 10:2044. doi: 10.3389/fimmu.2019.02044
46. Braakman E, van de Winkel JG, van Krimpen BA, Jansze M, Bolhuis RL. Cd16 on Human Gamma Delta T Lymphocytes: Expression, Function, and Specificity for Mouse Igg Isotypes. *Cell Immunol* (1992) 143(1):97–107. doi: 10.1016/0008-8749(92)90008-d
47. Wang X, Xiang Z, Liu Y, Huang C, Pei Y, Wang X, et al. Exosomes Derived From Vdelta2-T Cells Control Epstein-Barr Virus-Associated Tumors and Induce T Cell Antitumor Immunity. *Sci Transl Med* (2020) 12(563):1–16. doi: 10.1126/scitranslmed.aaz3426
48. Wang X, Zhang Y, Mu X, CR Tu, Chung Y, Tsao SW, et al. Exosomes Derived From Gammadelta-T Cells Synergize With Radiotherapy and Preserve Antitumor Activities Against Nasopharyngeal Carcinoma in Immunosuppressive Microenvironment. *J Immunother Cancer* (2022) 10(2):e003832. doi: 10.1136/jitc-2021-003832
49. Brandes M, Willimann K, Moser B. Professional Antigen-Presentation Function by Human Gammadelta T Cells. *Science* (2005) 309(5732):264–8. doi: 10.1126/science.1110267
50. Meuter S, Eberl M, Moser B. Prolonged Antigen Survival and Cytosolic Export in Cross-Presenting Human Gammadelta T Cells. *Proc Natl Acad Sci USA* (2010) 107(19):8730–5. doi: 10.1073/pnas.1002769107
51. Brandes M, Willimann K, Bioley G, Levy N, Eberl M, Luo M, et al. Cross-Presenting Human Gammadelta T Cells Induce Robust Cd8+ Alphabeta T Cell Responses. *Proc Natl Acad Sci USA* (2009) 106(7):2307–12. doi: 10.1073/pnas.0810059106
52. Holmen Olofsson G, Idorn M, Carnaz Simoes AM, Aehnlich P, Skadborg SK, Noessner E, et al. Vgamma9vdelta2 T Cells Concurrently Kill Cancer Cells and Cross-Present Tumor Antigens. *Front Immunol* (2021) 12:645131. doi: 10.3389/fimmu.2021.645131
53. Maniar A, Zhang X, Lin W, Gastman BR, Pauza CD, Strome SE, et al. Human Gammadelta T Lymphocytes Induce Robust Nk Cell-Mediated Antitumor Cytotoxicity Through Cd137 Engagement. *Blood* (2010) 116(10):1726–33. doi: 10.1182/blood-2009-07-234211
54. Wang B, Tian Q, Guo D, Lin W, Xie X, Bi H. Activated Gammadelta T Cells Promote Dendritic Cell Maturation and Exacerbate the Development of Experimental Autoimmune Uveitis (Eau) in Mice. *Immunol Invest* (2021) 50(2-3):164–83. doi: 10.1080/08820139.2020.1716786
55. Petrasca A, Doherty DG. Human Vdelta2(+) Gammadelta T Cells Differentially Induce Maturation, Cytokine Production, and Alloreactive T Cell Stimulation by Dendritic Cells and B Cells. *Front Immunol* (2014) 5:650. doi: 10.3389/fimmu.2014.00650
56. Ismaili J, Olislagers V, Poupot R, Fournie JJ, Goldman M. Human Gamma Delta T Cells Induce Dendritic Cell Maturation. *Clin Immunol* (2002) 103(3 Pt 1):296–302. doi: 10.1006/clim.2002.5218
57. Ullrich L, Lueder Y, Juergens AL, Wilharm A, Barros-Martins J, Bubke A, et al. Il-4-Producing Vgamma1(+)/Vdelta6(+) Gammadelta T Cells Sustain Germinal Center Reactions in Peyer's Patches of Mice. *Front Immunol* (2021) 12:729607. doi: 10.3389/fimmu.2021.729607
58. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, et al. Contribution of Il-17-Producing Gamma Delta T Cells to the Efficacy of Anticancer Chemotherapy. *J Exp Med* (2011) 208(3):491–503. doi: 10.1084/jem.20100269
59. Daley D, Zambirinis CP, Seifert L, Akkad N, Mohan N, Werba G, et al. Gammadelta T Cells Support Pancreatic Oncogenesis by Restraining Alphabeta T Cell Activation. *Cell* (2016) 166(6):1485–99.e15. doi: 10.1016/j.cell.2016.07.046
60. Brauneck F, Weimer P, Schulze Zur Wiesch J, Weisel K, Leypoldt L, Vohwinkel G, et al. Bone Marrow-Resident Vdelta1 T Cells Co-Express Tigit With Pd-1, Tim-3 or Cd39 in Aml and Myeloma. *Front Med (Lausanne)* (2021) 8:763773. doi: 10.3389/fmed.2021.763773
61. Beatty GL, Gladney WL. Immune Escape Mechanisms as a Guide for Cancer Immunotherapy. *Clin Cancer Res* (2015) 21(4):687–92. doi: 10.1158/1078-0432.CCR-14-1860
62. Park JH, Kim HJ, Kim CW, Kim HC, Jung Y, HS L, et al. Tumor Hypoxia Represses Gammadelta T Cell-Mediated Antitumor Immunity Against Brain Tumors. *Nat Immunol* (2021) 22(3):336–46. doi: 10.1038/s41590-020-00860-7
63. Sureshbabu SK, Chaukar D, Chiplunkar SV. Hypoxia Regulates the Differentiation and Anti-Tumor Effector Functions of Gammadeltat Cells in Oral Cancer. *Clin Exp Immunol* (2020) 201(1):40–57. doi: 10.1111/cei.13436
64. Ma S, Cheng Q, Cai Y, Gong H, Wu Y, Yu X, et al. Il-17a Produced by Gammadelta T Cells Promotes Tumor Growth in Hepatocellular Carcinoma. *Cancer Res* (2014) 74(7):1969–82. doi: 10.1158/0008-5472.CAN-13-2534
65. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. Il-17-Producing Gammadelta T Cells and Neutrophils Conspire to Promote Breast Cancer Metastasis. *Nature* (2015) 522(7556):345–8. doi: 10.1038/nature14282
66. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. Gammadeltat17 Cells Promote the Accumulation and Expansion of Myeloid-Derived Suppressor Cells in Human Colorectal Cancer. *Immunity* (2014) 40(5):785–800. doi: 10.1016/j.immuni.2014.03.013

67. Rei M, Goncalves-Sousa N, Lanca T, Thompson RG, Mensurado S, Balkwill FR, et al. Murine Cd27(-) Vgamma6(+) Gammadelta T Cells Producing Il-17a Promote Ovarian Cancer Growth Via Mobilization of Protumor Small Peritoneal Macrophages. *Proc Natl Acad Sci USA* (2014) 111(34):E3562–70. doi: 10.1073/pnas.1403424111
68. Wakita D, Sumida K, Iwakura Y, Nishikawa H, Ohkuri T, Chamoto K, et al. Tumor-Infiltrating Il-17-Producing Gammadelta T Cells Support the Progression of Tumor by Promoting Angiogenesis. *Eur J Immunol* (2010) 40(7):1927–37. doi: 10.1002/eji.200940157
69. Kuhl AA, Pawlowski NN, Grollich K, Blessenohl M, Westermann J, Zeitz M, et al. Human Peripheral Gammadelta T Cells Possess Regulatory Potential. *Immunology* (2009) 128(4):580–8. doi: 10.1111/j.1365-2567.2009.03162.x
70. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-Infiltrating Gammadelta T Cells Suppress T and Dendritic Cell Function *Via* Mechanisms Controlled by a Unique Toll-Like Receptor Signaling Pathway. *Immunity* (2007) 27(2):334–48. doi: 10.1016/j.immuni.2007.05.020
71. Hu G, Wu P, Cheng P, Zhang Z, Wang Z, Yu X, et al. Tumor-Infiltrating Cd39 (+)Gammadeltatregs Are Novel Immunosuppressive T Cells in Human Colorectal Cancer. *Oncoimmunology* (2017) 6(2):e1277305. doi: 10.1080/2162402X.2016.1277305
72. Hu G, Cheng P, Pan J, Wang S, Ding Q, Jiang Z, et al. An Il6-Adenosine Positive Feedback Loop Between Cd73(+) Gammadeltatregs and Cafs Promotes Tumor Progression in Human Breast Cancer. *Cancer Immunol Res* (2020) 8(10):1273–86. doi: 10.1158/2326-6066.CIR-19-0923
73. Wu D, Wu P, Wu X, Ye J, Wang Z, Zhao S, et al. Ex Vivo Expanded Human Circulating Vdelta1 Gammadeltat Cells Exhibit Favorable Therapeutic Potential for Colon Cancer. *Oncoimmunology* (2015) 4(3):e992749. doi: 10.4161/2162402X.2014.992749
74. Siegers GM, Dhamko H, Wang XH, Mathieson AM, Kosaka Y, Felizardo TC, et al. Human Vdelta1 Gammadelta T Cells Expanded From Peripheral Blood Exhibit Specific Cytotoxicity Against B-Cell Chronic Lymphocytic Leukemia-Derived Cells. *Cytotherapy* (2011) 13(6):753–64. doi: 10.3109/14653249.2011.553595
75. Kakimi K, Matsushita H, Murakawa T, Nakajima J. Gammadelta T Cell Therapy for the Treatment of Non-Small Cell Lung Cancer. *Transl Lung Cancer Res* (2014) 3(1):23–33. doi: 10.3978/j.issn.2218-6751.2013.11.01
76. Bachmann MF, Oxenius A. Interleukin 2: From Immunostimulation to Immunoregulation and Back Again. *EMBO Rep* (2007) 8(12):1142–8. doi: 10.1038/sj.embor.7401099
77. Yui MA, Sharp LL, Havran WL, Rothenberg EV. Preferential Activation of an Il-2 Regulatory Sequence Transgene in Tcr Gamma Delta and Nkt Cells: Subset-Specific Differences in Il-2 Regulation. *J Immunol* (2004) 172(8):4691–9. doi: 10.4049/jimmunol.172.8.4691
78. Xiang Z, Liu Y, Zheng J, Liu M, Lv A, Gao Y, et al. Targeted Activation of Human Vgamma9vdelta2-T Cells Controls Epstein-Barr Virus-Induced B Cell Lymphoproliferative Disease. *Cancer Cell* (2014) 26(4):565–76. doi: 10.1016/j.ccr.2014.07.026
79. Zysk A, DeNichilo MO, Panagopoulos V, Zinonos I, Liapis V, Hay S, et al. Adoptive Transfer of Ex Vivo Expanded Vgamma9vdelta2 T Cells in Combination With Zoledronic Acid Inhibits Cancer Growth and Limits Osteolysis in a Murine Model of Osteolytic Breast Cancer. *Cancer Lett* (2017) 386:141–50. doi: 10.1016/j.canlet.2016.11.013
80. Wada I, Matsushita H, Noji S, Mori K, Yamashita H, Nomura S, et al. Intraperitoneal Injection of *in Vitro* Expanded Vgamma9vdelta2 T Cells Together With Zoledronate for the Treatment of Malignant Ascites Due to Gastric Cancer. *Cancer Med* (2014) 3(2):362–75. doi: 10.1002/cam4.196
81. Kondo M, Sakuta K, Noguchi A, Ariyoshi N, Sato K, Sato S, et al. Zoledronate Facilitates Large-Scale Ex Vivo Expansion of Functional Gammadelta T Cells From Cancer Patients for Use in Adoptive Immunotherapy. *Cytotherapy* (2008) 10(8):842–56. doi: 10.1080/14653240802419328
82. Pressey JG, Adams J, Harkins L, Kelly D, You Z, Lamb LSJr. *In Vivo* Expansion and Activation of Gammadelta T Cells as Immunotherapy for Refractory Neuroblastoma: A Phase 1 Study. *Med (Baltimore)* (2016) 95(39):e4909. doi: 10.1097/MD.0000000000004909
83. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting Human {Gamma}Delta} T Cells With Zoledronate and Interleukin-2 for Immunotherapy of Hormone-Refractory Prostate Cancer. *Cancer Res* (2007) 67(15):7450–7. doi: 10.1158/0008-5472.CAN-07-0199
84. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, et al. Gammadelta T Cells for Immune Therapy of Patients With Lymphoid Malignancies. *Blood* (2003) 102(1):200–6. doi: 10.1182/blood-2002-12-3665
85. Wang Y, Zhang Y, Yi P, Dong W, Nalin AP, Zhang J, et al. The Il-15-Akt-Xbp1s Signaling Pathway Contributes to Effector Functions and Survival in Human Nk Cells. *Nat Immunol* (2019) 20(1):10–7. doi: 10.1038/s41590-018-0265-1
86. Mikulak J, Oriolo F, Bruni E, Roberto A, FS C, Villa A, et al. Nkp46-Expressing Human Gut-Resident Intraepithelial Vdelta1 T Cell Subpopulation Exhibits High Antitumor Activity Against Colorectal Cancer. *JCI Insight* (2019) 4(24):1–19. doi: 10.1172/jci.insight.125884
87. Correia DV, Fogli M, Hudspeth K, da Silva MG, Mavilio D, Silva-Santos B. Differentiation of Human Peripheral Blood Vdelta1+ T Cells Expressing the Natural Cytotoxicity Receptor Nkp30 for Recognition of Lymphoid Leukemia Cells. *Blood* (2011) 118(4):992–1001. doi: 10.1182/blood-2011-02-339135
88. Kouakanou L, Xu Y, Peters C, He J, Wu Y, Yin Z, et al. Vitamin C Promotes the Proliferation and Effector Functions of Human Gammadelta T Cells. *Cell Mol Immunol* (2020) 17(5):462–73. doi: 10.1038/s41423-019-0247-8
89. Xu Y, Xiang Z, Alnaggar M, Kouakanou L, Li J, He J, et al. Allogeneic Vgamma9vdelta2 T-Cell Immunotherapy Exhibits Promising Clinical Safety and Prolongs the Survival of Patients With Late-Stage Lung or Liver Cancer. *Cell Mol Immunol* (2021) 18(2):427–39. doi: 10.1038/s41423-020-0515-7
90. Colpitts SL, Puddington L, Lefrancois L. Il-15 Receptor Alpha Signaling Constrains the Development of Il-17-Producing Gammadelta T Cells. *Proc Natl Acad Sci USA* (2015) 112(31):9692–7. doi: 10.1073/pnas.1420741112
91. Mao Y, Yin S, Zhang J, Hu Y, Huang B, Cui L, et al. A New Effect of Il-4 on Human Gammadelta T Cells: Promoting Regulatory Vdelta1 T Cells *Via* Il-10 Production and Inhibiting Function of Vdelta2 T Cells. *Cell Mol Immunol* (2016) 13(2):217–28. doi: 10.1038/cmi.2015.07
92. Michel ML, Pang DJ, Haque SF, Potocnik AJ, Pennington DJ, Hayday AC. Interleukin 7 (Il-7) Selectively Promotes Mouse and Human Il-17-Producing Gammadelta Cells. *Proc Natl Acad Sci USA* (2012) 109(43):17549–54. doi: 10.1073/pnas.1204327109
93. Jiang H, Yang Z, Song Z, Green M, Song H, Shao Q. Gammadelta T Cells in Hepatocellular Carcinoma Patients Present Cytotoxic Activity But Are Reduced in Potency Due to Il-2 and Il-21 Pathways. *Int Immunopharmacol* (2019) 70:167–73. doi: 10.1016/j.intimp.2019.02.019
94. Bansal RR, Mackay CR, Moser B, Eberl M. Il-21 Enhances the Potential of Human Gammadelta T Cells to Provide B-Cell Help. *Eur J Immunol* (2012) 42(1):110–9. doi: 10.1002/eji.201142017
95. Barjon C, Michaud HA, Fages A, Dejou C, Zampieri A, They L, et al. Il-21 Promotes the Development of a Cd73-Positive Vgamma9vdelta2 T Cell Regulatory Population. *Oncoimmunology* (2017) 7(1):e1379642. doi: 10.1080/2162402X.2017.1379642
96. Song Y, Hu B, Liu Y, Jin Z, Zhang Y, Lin D, et al. Il-12/Il-18-Preactivated Donor Nk Cells Enhance Gvl Effects and Mitigate Gvhd After Allogeneic Hematopoietic Stem Cell Transplantation. *Eur J Immunol* (2018) 48(4):670–82. doi: 10.1002/eji.201747177
97. Romee R, Rosario M, Berrien-Elliott MM, Wagner JA, Jewell BA, Schappe T, et al. Cytokine-Induced Memory-Like Natural Killer Cells Exhibit Enhanced Responses Against Myeloid Leukemia. *Sci Transl Med* (2016) 8(357):357ra123. doi: 10.1126/scitranslmed.aaf2341
98. Romee R, Schneider SE, Leong JW, Chase JM, Keppel CR, Sullivan RP, et al. Cytokine Activation Induces Human Memory-Like Nk Cells. *Blood* (2012) 120(24):4751–60. doi: 10.1182/blood-2012-04-419283
99. Yoshimoto T, Takeda K, Tanaka T, Ohkusu K, Kashiwamura S, Okamura H, et al. Il-12 Up-Regulates Il-18 Receptor Expression on T Cells, Th1 Cells, and B Cells: Synergism With Il-18 for Ifn-Gamma Production. *J Immunol* (1998) 161(7):3400–7.
100. Schilbach K, Welker C, Krickeberg N, Kaisser C, Schleicher S, Hashimoto H. In the Absence of a Tcr Signal Il-2/Il-12/18-Stimulated Gammadelta T Cells Demonstrate Potent Anti-Tumoral Function Through Direct Killing and Senescence Induction in Cancer Cells. *Cancers (Basel)* (2020) 12(1):130. doi: 10.3390/cancers12010130
101. Domae E, Hirai Y, Ikeo T, Goda S, Shimizu Y. Cytokine-Mediated Activation of Human Ex Vivo-Expanded Vgamma9vdelta2 T Cells. *Oncotarget* (2017) 8(28):45928–42. doi: 10.18632/oncotarget.17498
102. Schofield L, Ioannidis LJ, Karl S, Robinson LJ, Tan QY, Poole DP, et al. Synergistic Effect of Il-12 and Il-18 Induces Tim3 Regulation of Gammadelta

T Cell Function and Decreases the Risk of Clinical Malaria in Children Living in Papua New Guinea. *BMC Med* (2017) 15(1):114. doi: 10.1186/s12916-017-0883-8
103. Hibbert L, Pflanz S, De Waal Malefyt R, Kastelein RA. Il-27 and Ifn-Alpha Signal *Via* Stat1 and Stat3 and Induce T-Bet and Il-12rbeta2 in Naive T Cells. *J Interferon Cytokine Res* (2003) 23(9):513–22. doi: 10.1089/10799900360708632
104. Morandi F, Prigione I, Airoldi I. Human Tcrgammadelta+ T Cells Represent a Novel Target for Il-27 Activity. *Eur J Immunol* (2012) 42(6):1547–52. doi: 10.1002/eji.201142241
105. McGeachy MJ, Cua DJ. Th17 Cell Differentiation: The Long and Winding Road. *Immunity* (2008) 28(4):445–53. doi: 10.1016/j.immuni.2008.03.001
106. Revu S, Wu J, Henkel M, Rittenhouse N, Menk A, Delgoffe GM, et al. Il-23 and Il-1beta Drive Human Th17 Cell Differentiation and Metabolic Reprogramming in Absence of Cd28 Costimulation. *Cell Rep* (2018) 22(10):2642–53. doi: 10.1016/j.celrep.2018.02.044
107. Muschaweckh A, Petermann F, Korn T. Il-1beta and Il-23 Promote Extrathymic Commitment of Cd27(+)Cd122(-) Gammadelta T Cells to Gammadeltat17 Cells. *J Immunol* (2017) 199(8):2668–79. doi: 10.4049/jimmunol.1700287
108. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and Il-23 Induce Innate Il-17 Production From Gammadelta T Cells, Amplifying Th17 Responses and Autoimmunity. *Immunity* (2009) 31(2):331–41. doi: 10.1016/j.immuni.2009.08.001
109. Do JS, PJ F, Li L, Spolski R, Robinson J, WJ L, et al. Cutting Edge: Spontaneous Development of Il-17-Producing Gamma Delta T Cells in the Thymus Occurs *Via* a Tgf-Beta 1-Dependent Mechanism. *J Immunol* (2010) 184(4):1675–9. doi: 10.4049/jimmunol.0903539
110. Ness-Schwickerath KJ, Jin C, Morita CT. Cytokine Requirements for the Differentiation and Expansion of Il-17a- and Il-22-Producing Human Vgamma2vdelta2 T Cells. *J Immunol* (2010) 184(12):7268–80. doi: 10.4049/jimmunol.1000600
111. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, et al. Differentiation, Phenotype, and Function of Interleukin-17-Producing Human Vgamma9vdelta2 T Cells. *Blood* (2011) 118(1):129–38. doi: 10.1182/blood-2011-01-331298
112. Beatson RE, AC P-P, Halim L, Cozzetto D, Hull C, LM W, et al. Tgf-Beta1 Potentiates Vgamma9vdelta2 T Cell Adoptive Immunotherapy of Cancer. *Cell Rep Med* (2021) 2(12):100473. doi: 10.1016/j.xcrm.2021.100473
113. Peters C, Meyer A, Kouakanou L, Feder J, Schricker T, Lettau M, et al. Tgf-Beta Enhances the Cytotoxic Activity of Vdelta2 T Cells. *Oncoimmunology* (2019) 8(1):e1522471. doi: 10.1080/2162402X.2018.1522471
114. Zhou J, Kang N, Cui L, Ba D, He W. Anti-Gammadelta Tcr Antibody-Expanded Gammadelta T Cells: A Better Choice for the Adoptive Immunotherapy of Lymphoid Malignancies. *Cell Mol Immunol* (2012) 9(1):34–44. doi: 10.1038/cmi.2011.16
115. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K. Phase I/Ii Study of Adoptive Transfer of Gammadelta T Cells in Combination With Zoledronic Acid and Il-2 to Patients With Advanced Renal Cell Carcinoma. *Cancer Immunol Immunother* (2011) 60(8):1075–84. doi: 10.1007/s00262-011-1021-7
116. Bennouna J, Levy V, Sicard H, Senellart H, Audrain M, Hiret S, et al. Phase I Study of Bromohydrin Pyrophosphate (Brhpp, Iph 1101), a Vgamma9vdelta2 T Lymphocyte Agonist in Patients With Solid Tumors. *Cancer Immunol Immunother* (2010) 59(10):1521–30. doi: 10.1007/s00262-010-0879-0
117. Bennouna J, Bompas E, Neidhardt EM, Rolland F, Philip I, Galea C, et al. Phase-I Study of Innacell Gammadelta, an Autologous Cell-Therapy Product Highly Enriched in Gamma9delta2 T Lymphocytes, in Combination With Il-2, in Patients With Metastatic Renal Cell Carcinoma. *Cancer Immunol Immunother* (2008) 57(11):1599–609. doi: 10.1007/s00262-008-0491-8
118. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety Profile and Anti-Tumor Effects of Adoptive Immunotherapy Using Gamma-Delta T Cells Against Advanced Renal Cell Carcinoma: A Pilot Study. *Cancer Immunol Immunother* (2007) 56(4):469–76. doi: 10.1007/s00262-006-0199-6
119. Sugie T, Suzuki E, Yamauchi A, Yamagami K, Masuda N, Gondo N, et al. Combined Effects of Neoadjuvant Letrozole and Zoledronic Acid on Gammadeltat Cells in Postmenopausal Women With Early-Stage Breast Cancer. *Breast* (2018) 38:114–9. doi: 10.1016/j.breast.2017.12.017
120. Izumi T, Kondo M, Takahashi T, Fujieda N, Kondo A, Tamura N, et al. Ex Vivo Characterization of Gammadelta T-Cell Repertoire in Patients After Adoptive Transfer of Vgamma9vdelta2 T Cells Expressing the Interleukin-2 Receptor Beta-Chain and the Common Gamma-Chain. *Cytotherapy* (2013) 15(4):481–91. doi: 10.1016/j.jcyt.2012.12.004
121. Fazzi R, Petrini I, Giuliani N, Morganti R, Carulli G, Dalla Palma B, et al. Phase Ii Trial of Maintenance Treatment With Il2 and Zoledronate in Multiple Myeloma After Bone Marrow Transplantation: Biological and Clinical Results. *Front Immunol* (2020) 11:573156. doi: 10.3389/fimmu.2020.573156
122. Wilhelm M, Smetak M, Schaefer-Eckart K, Kimmel B, Birkmann J, Einsele H, et al. Successful Adoptive Transfer and *in Vivo* Expansion of Haploidentical Gammadelta T Cells. *J Transl Med* (2014) 12:45. doi: 10.1186/1479-5876-12-45
123. Kunzmann V, Smetak M, Kimmel B, Weigang-Koehler K, Goebeler M, Birkmann J, et al. Tumor-Promoting Versus Tumor-Antagonizing Roles of Gammadelta T Cells in Cancer Immunotherapy: Results From a Prospective Phase I/Ii Trial. *J Immunother* (2012) 35(2):205–13. doi: 10.1097/CJI.0b013e318245bb1e
124. Lang JM, Kaikobad MR, Wallace M, Staab MJ, Horvath DL, Wilding G, et al. Pilot Trial of Interleukin-2 and Zoledronic Acid to Augment Gammadelta T Cells as Treatment for Patients With Refractory Renal Cell Carcinoma. *Cancer Immunol Immunother* (2011) 60(10):1447–60. doi: 10.1007/s00262-011-1049-8
125. Sumi E, Sugie T, Yoshimura K, Tada H, Ikeda T, Suzuki E, et al. Effects of Zoledronic Acid and the Association Between Its Efficacy and Gammadeltat Cells in Postmenopausal Women With Breast Cancer Treated With Preoperative Hormonal Therapy: A Study Protocol. *J Transl Med* (2014) 12:310. doi: 10.1186/s12967-014-0310-2
126. Sakamoto M, Nakajima J, Murakawa T, Fukami T, Yoshida Y, Murayama T, et al. Adoptive Immunotherapy for Advanced Non-Small Cell Lung Cancer Using Zoledronate-Expanded Gammadeltatcells: A Phase I Clinical Study. *J Immunother* (2011) 34(2):202–11. doi: 10.1097/CJI.0b013e318207ecfb
127. Nakajima J, Murakawa T, Fukami T, Goto S, Kaneko T, Yoshida Y, et al. A Phase I Study of Adoptive Immunotherapy for Recurrent Non-Small-Cell Lung Cancer Patients With Autologous Gammadelta T Cells. *Eur J Cardiothorac Surg* (2010) 37(5):1191–7. doi: 10.1016/j.ejcts.2009.11.051
128. Meraviglia S, Eberl M, Vermijlen D, Todaro M, Buccheri S, Cicero G, et al. *In Vivo* Manipulation of Vgamma9vdelta2 T Cells With Zoledronate and Low-Dose Interleukin-2 for Immunotherapy of Advanced Breast Cancer Patients. *Clin Exp Immunol* (2010) 161(2):290–7. doi: 10.1111/j.1365-2249.2010.04167.x
129. Noguchi A, Kaneko T, Kamigaki T, Fujimoto K, Ozawa M, Saito M, et al. Zoledronate-Activated Vgamma9gammadelta T Cell-Based Immunotherapy Is Feasible and Restores the Impairment of Gammadelta T Cells in Patients With Solid Tumors. *Cytotherapy* (2011) 13(1):92–7. doi: 10.3109/14653249.2010.515581
130. Santini D, Martini F, Fratto ME, Galluzzo S, Vincenzi B, Agrati C, et al. *In Vivo* Effects of Zoledronic Acid on Peripheral Gammadelta T Lymphocytes in Early Breast Cancer Patients. *Cancer Immunol Immunother* (2009) 58(1):31–8. doi: 10.1007/s00262-008-0521-6
131. Abe Y, Muto M, Nieda M, Nakagawa Y, Nicol A, Kaneko T, et al. Clinical and Immunological Evaluation of Zoledronate-Activated Vgamma9gammadelta T-Cell-Based Immunotherapy for Patients With Multiple Myeloma. *Exp Hematol* (2009) 37(8):956–68. doi: 10.1016/j.exphem.2009.04.008
132. Aoki T, Matsushita H, Hoshikawa M, Hasegawa K, Kokudo N, Kakimi K. Adjuvant Combination Therapy With Gemcitabine and Autologous Gammadelta T-Cell Transfer in Patients With Curatively Resected Pancreatic Cancer. *Cytotherapy* (2017) 19(4):473–85. doi: 10.1016/j.jcyt.2017.01.002
133. Lin M, Zhang X, Liang S, Luo H, Alnaggar M, Liu A, et al. Irreversible Electroporation Plus Allogenic Vgamma9vdelta2 T Cells Enhances Antitumor Effect for Locally Advanced Pancreatic Cancer Patients. *Signal Transduct Target Ther* (2020) 5(1):215. doi: 10.1038/s41392-020-00260-1
134. Zhang T, Chen J, Niu L, Liu Y, Ye G, Jiang M, et al. Clinical Safety and Efficacy of Locoregional Therapy Combined With Adoptive Transfer of

Allogeneic Gammadelta T Cells for Advanced Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. *J Vasc Interv Radiol* (2022) 33(1):19–27.e3. doi: 10.1016/j.jvir.2021.09.012

135. Ji N, Mukherjee N, Reyes RM, Gelfond J, Javors M, Meeks JJ, et al. Rapamycin Enhances Bcg-Specific Gammadelta T Cells During Intravesical Bcg Therapy for Non-Muscle Invasive Bladder Cancer: A Random Double-Blind Study. *J Immunother Cancer* (2021) 9(3):e001941. doi: 10.1136/jitc-2020-001941
136. Nada MH, Wang H, Workalemahu G, Tanaka Y, Morita CT. Enhancing Adoptive Cancer Immunotherapy With Vgamma2vdelta2 T Cells Through Pulse Zoledronate Stimulation. *J Immunother Cancer* (2017) 5:9. doi: 10.1186/s40425-017-0209-6
137. Nishimoto KP, Barca T, Azameera A, Makkouk A, Romero JM, Bai L, et al. Allogeneic Cd20-Targeted Gammadelta T Cells Exhibit Innate and Adaptive Antitumor Activities in Preclinical B-Cell Lymphoma Models. *Clin Transl Immunol* (2022) 11(2):e1373. doi: 10.1002/cti2.1373
138. Zhai X, You F, Xiang S, Jiang L, Chen D, Li Y, et al. Muc1-Tn-Targeting Chimeric Antigen Receptor-Modified Vgamma9vdelta2 T Cells With Enhanced Antigen-Specific Anti-Tumor Activity. *Am J Cancer Res* (2021) 11(1):79–91.
139. Juraske C, Wipa P, Morath A, Hidalgo JV, Hartl FA, Raute K, et al. Anti-Cd3 Fab Fragments Enhance Tumor Killing by Human Gammadelta T Cells Independent of Nck Recruitment to the Gammadelta T Cell Antigen Receptor. *Front Immunol* (2018) 9:1579. doi: 10.3389/fimmu.2018.01579
140. De Gassart A, KS Le, Brune P, Agaugue S, Sims J, Goubard A, et al. Development of Ict01, a First-In-Class, Anti-Btn3a Antibody for Activating Vgamma9vdelta2 T Cell-Mediated Antitumor Immune Response. *Sci Transl Med* (2021) 13(616):eabj0835. doi: 10.1126/scitranslmed.abj0835
141. Ganesan R, Chennupati V, Ramachandran B, Hansen MR, Singh S, Grewal IS. Selective Recruitment of Gammadelta T Cells by a Bispecific Antibody for the Treatment of Acute Myeloid Leukemia. *Leukemia* (2021) 35(8):2274–84. doi: 10.1038/s41375-021-01122-7
142. Oberg HH, Kellner C, Gonnermann D, Sebens S, Bauerschlag D, Gramatzki M, et al. Tribody [(Her2)2xcd16] Is More Effective Than Trastuzumab in Enhancing Gammadelta T Cell and Natural Killer Cell Cytotoxicity Against Her2-Expressing Cancer Cells. *Front Immunol* (2018) 9:814. doi: 10.3389/fimmu.2018.00814
143. Oberg HH, Kellner C, Gonnermann D, Peipp M, Peters C, Sebens S, et al. Gammadelta T Cell Activation by Bispecific Antibodies. *Cell Immunol* (2015) 296(1):41–9. doi: 10.1016/j.cellimm.2015.04.009
144. Hoeres T, Pretscher D, Holzmann E, Smetak M, Birkmann J, Triebel J, et al. Improving Immunotherapy Against B-Cell Malignancies Using Gammadelta T-Cell-Specific Stimulation and Therapeutic Monoclonal Antibodies. *J Immunother* (2019) 42(9):331–44. doi: 10.1097/CJI.0000000000000289
145. Pei Y, Wen K, Xiang Z, Huang C, Wang X, Mu X, et al. Cd137 Costimulation Enhances the Antiviral Activity of Vgamma9vdelta2-T Cells Against Influenza Virus. *Signal Transduct Target Ther* (2020) 5(1):74. doi: 10.1038/s41392-020-0174-2
146. Pan Y, Chiu YH, Chiu SC, Cho DY, Lee LM, Wen YC, et al. Gamma/Delta T-Cells Enhance Carboplatin-Induced Cytotoxicity Towards Advanced Bladder Cancer Cells. *Anticancer Res* (2020) 40(9):5221–7. doi: 10.21873/anticanres.14525
147. Chitadze G, Lettau M, Luecke S, Wang T, Janssen O, Furst D, et al. Nkg2d- and T-Cell Receptor-Dependent Lysis of Malignant Glioma Cell Lines by Human Gammadelta T Cells: Modulation by Temozolomide and a Disintegrin and Metalloproteases 10 and 17 Inhibitors. *Oncoimmunology* (2016) 5(4):e1093276. doi: 10.1080/2162402X.2015.1093276
148. Hu Y, Liu T, Li J, Mai F, Li J, Chen Y, et al. Selenium Nanoparticles as New Strategy to Potentiate Gammadelta T Cell Anti-Tumor Cytotoxicity Through Upregulation of Tubulin-Alpha Acetylation. *Biomaterials* (2019) 222:119397. doi: 10.1016/j.biomaterials.2019.119397
149. Lin L, He J, Li J, Xu Y, Li J, Wu Y. Chitosan Nanoparticles Strengthen Vgamma9vdelta2 T-Cell Cytotoxicity Through Upregulation of Killing Molecules and Cytoskeleton Polarization. *Int J Nanomedicine* (2019) 14:9325–36. doi: 10.2147/IJN.S212898
150. Nada MH, Wang H, Hussein AJ, Tanaka Y, Morita CT. Pd-1 Checkpoint Blockade Enhances Adoptive Immunotherapy by Human Vgamma2vdelta2 T Cells Against Human Prostate Cancer. *Oncoimmunology* (2021) 10 (1):1989789. doi: 10.1080/2162402X.2021.1989789
151. Guo Q, Zhao P, Zhang Z, Zhang J, Zhang Z, Hua Y, et al. Tim-3 Blockade Combined With Bispecific Antibody Mt110 Enhances the Anti-Tumor Effect of Gammadelta T Cells. *Cancer Immunol Immunother* (2020) 69(12):2571–87. doi: 10.1007/s00262-020-02638-0
152. Van Acker HH, Anguille S, Willemen Y, Van den Bergh JM, Berneman ZN, Lion E, et al. Interleukin-15 Enhances the Proliferation, Stimulatory Phenotype, and Antitumor Effector Functions of Human Gamma Delta T Cells. *J Hematol Oncol* (2016) 9(1):101. doi: 10.1186/s13045-016-0329-3
153. Makkouk A, Yang XC, Barca T, Lucas A, Turkoz M, Wong JTS, et al. Off-The-Shelf Vdelta1 Gamma Delta T Cells Engineered With Glypican-3 (Gpc-3)-Specific Chimeric Antigen Receptor (Car) and Soluble Il-15 Display Robust Antitumor Efficacy Against Hepatocellular Carcinoma. *J Immunother Cancer* (2021) 9(12):e003411. doi: 10.1136/jitc-2021-003441
154. Oberg HH, Kellner C, Peipp M, Sebens S, Adam-Klages S, Gramatzki M, et al. Monitoring Circulating Gammadelta T Cells in Cancer Patients to Optimize Gammadelta T Cell-Based Immunotherapy. *Front Immunol* (2014) 5:643. doi: 10.3389/fimmu.2014.00643
155. Liu Y, Song Y, Lin D, Lei L, Mei Y, Jin Z, et al. Ncr(-) Group 3 Innate Lymphoid Cells Orchestrate Il-23/Il-17 Axis to Promote Hepatocellular Carcinoma Development. *EBioMedicine* (2019) 41:333–44. doi: 10.1016/j.ebiom.2019.02.050
156. Yang L, Pang Y, Moses HL. Tgf-Beta and Immune Cells: An Important Regulatory Axis in the Tumor Microenvironment and Progression. *Trends Immunol* (2010) 31(6):220–7. doi: 10.1016/j.it.2010.04.002
157. Kawamoto K, Pahuja A, Hering BJ, Bansal-Pakala P. Transforming Growth Factor Beta 1 (Tgf-Beta1) and Rapamycin Synergize to Effectively Suppress Human T Cell Responses *Via* Upregulation of Foxp3+ Tregs. *Transpl Immunol* (2010) 23(1-2):28–33. doi: 10.1016/j.trim.2010.03.004
158. Zaharoff DA, Hoffman BS, Hooper HB, Benjamin CJJr., Khurana KK, Hance KW, et al. Intravesical Immunotherapy of Superficial Bladder Cancer With Chitosan/Interleukin-12. *Cancer Res* (2009) 69(15):6192–9. doi: 10.1158/0008-5472.CAN-09-1114
159. Cohen J. Il-12 Deaths: Explanation and a Puzzle. *Science* (1995) 270 (5238):908. doi: 10.1126/science.270.5238.908a
160. Hotz C, Wagenaar TR, Gieseke F, Bangari DS, Callahan M, Cao H, et al. Local Delivery of Mrna-Encoded Cytokines Promotes Antitumor Immunity and Tumor Eradication Across Multiple Preclinical Tumor Models. *Sci Transl Med* (2021) 13(610):eabc7804. doi: 10.1126/scitranslmed.abc7804

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.

frontiers | Frontiers in Immunology

MINI REVIEW
published: 16 June 2022
doi: 10.3389/fimmu.2022.915837

Gamma Delta T-Cell Based Cancer Immunotherapy: Past-Present-Future

*José Saura-Esteller[1], Milon de Jong[1], Lisa A. King[1], Erik Ensing[2], Benjamin Winograd[3], Tanja D. de Gruijl[1], Paul W. H. I. Parren[2,4] and Hans J. van der Vliet[1,2]**

[1] Department of Medical Oncology, Cancer Center Amsterdam, Amsterdam Infection and Immunity Institute, Amsterdam University Medical Center (UMC), Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [2] LAVA Therapeutics, Utrecht, Netherlands, [3] LAVA Therapeutics, Philadelphia, PA, United States, [4] Department of Immunology, Leiden University Medical Center, Leiden, Netherlands

γδ T-cells directly recognize and kill transformed cells independently of HLA-antigen presentation, which makes them a highly promising effector cell compartment for cancer immunotherapy. Novel γδ T-cell-based immunotherapies, primarily focusing on the two major γδ T-cell subtypes that infiltrate tumors (*i.e.* Vδ1 and Vδ2), are being developed. The Vδ1 T-cell subset is enriched in tissues and contains both effector T-cells as well as regulatory T-cells with tumor-promoting potential. Vδ2 T-cells, in contrast, are enriched in circulation and consist of a large, relatively homogeneous, pro-inflammatory effector T-cell subset. Healthy individuals typically harbor in the order of 50-500 million Vγ9Vδ2 T-cells in the peripheral blood alone (1-10% of the total $CD3^+$ T-cell population), which can rapidly expand upon stimulation. The Vγ9Vδ2 T-cell receptor senses intracellular phosphorylated metabolites, which accumulate in cancer cells as a result of mevalonate pathway dysregulation or upon pharmaceutical intervention. Early clinical studies investigating the therapeutic potential of Vγ9Vδ2 T-cells were based on either *ex vivo* expansion and adoptive transfer or their systemic activation with aminobisphosphonates or synthetic phosphoantigens, either alone or combined with low dose IL-2. Immune-related adverse events (irAE) were generally \mild, but the clinical efficacy of these approaches provided overall limited benefit. In recent years, critical advances have renewed the excitement for the potential of Vγ9Vδ2 T-cells in cancer immunotherapy. Here, we review γδ T-cell-based therapeutic strategies and discuss the prospects of those currently evaluated in clinical studies in cancer patients as well as future therapies that might arise from current promising pre-clinical results.

Keywords: gamma delta T-cell, cancer, immunotherapy, phosphoantigens, aminobisphosphonates, adoptive cell transfer, bispecific t-cell engager, chimeric antigen receptor

OPEN ACCESS

Edited by:
Alice Cheung, Singapore General Hospital, Singapore

Reviewed by:
Alessandro Poggi, San Martino Hospital (IRCCS), Italy
Emmanuel Scotet, Université de Nantes, France

***Correspondence:**
Hans J. van der Vliet
h.vandervliet@lavatherapeutics.com;
jj.vandervliet@amsterdamumc.nl

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *08 April 2022*
Accepted: *05 May 2022*
Published: *16 June 2022*

Citation:
Saura-Esteller J, de Jong M, King LA, Ensing E, Winograd B, de Gruijl TD, Parren PWHI and van der Vliet HJ (2022) Gamma Delta T-Cell-Based Cancer Immunotherapy: Past-Present-Future. Front. Immunol. 13:915837. doi: 10.3389/fimmu.2022.915837

INTRODUCTION

In humans, γδ T-cells represent 1 to 10% of total $CD3^+$ T-cells (1, 2), and express a combination of either of 7 different Vγ TCR chains (Vγ2, 3, 4, 5, 8, 9, and 11), paired with either of 4 Vδ (Vδ1, 2, 3, and 5) chains (2–4). γδ T-cells are considered to bridge the innate and adaptive immune systems (3). Activated γδ T-cells display strong cytotoxic activity through the release of granzyme B and perforin, by membrane bound TRAIL and Fas (CD95) ligands or production of IFNγ or TNFα to

amplify the immune response (12), thereby counteracting tumor development. Using γδ T-cell-deficient mice in a cutaneous carcinogenesis model, γδ T-cells were first shown to prevent malignancy formation (5). High γδ T-cell frequency in tumor infiltrates from cancer patients correlates with better clinical outcome in different malignancies (6–10) and γδ T-cells were identified as the prognostically most favorable immune cell subset in tumor infiltrates from 18,000 tumors across 39 malignancies (11). A more recent study confirmed the relative abundance of Vγ9Vδ2 T-cells in TILs and their association with improved patient outcome (12). These results highlight the relevance of γδ T-cells in tumor control and their potential for cancer therapy. γδ T-cells express several receptors shared with natural killer (NK) cells that participate in enhanced tumor cell recognition of which FcγRIIIa (CD16a), DNAM-1, and NKG2D are a few examples (13) (**Figure 1A**).

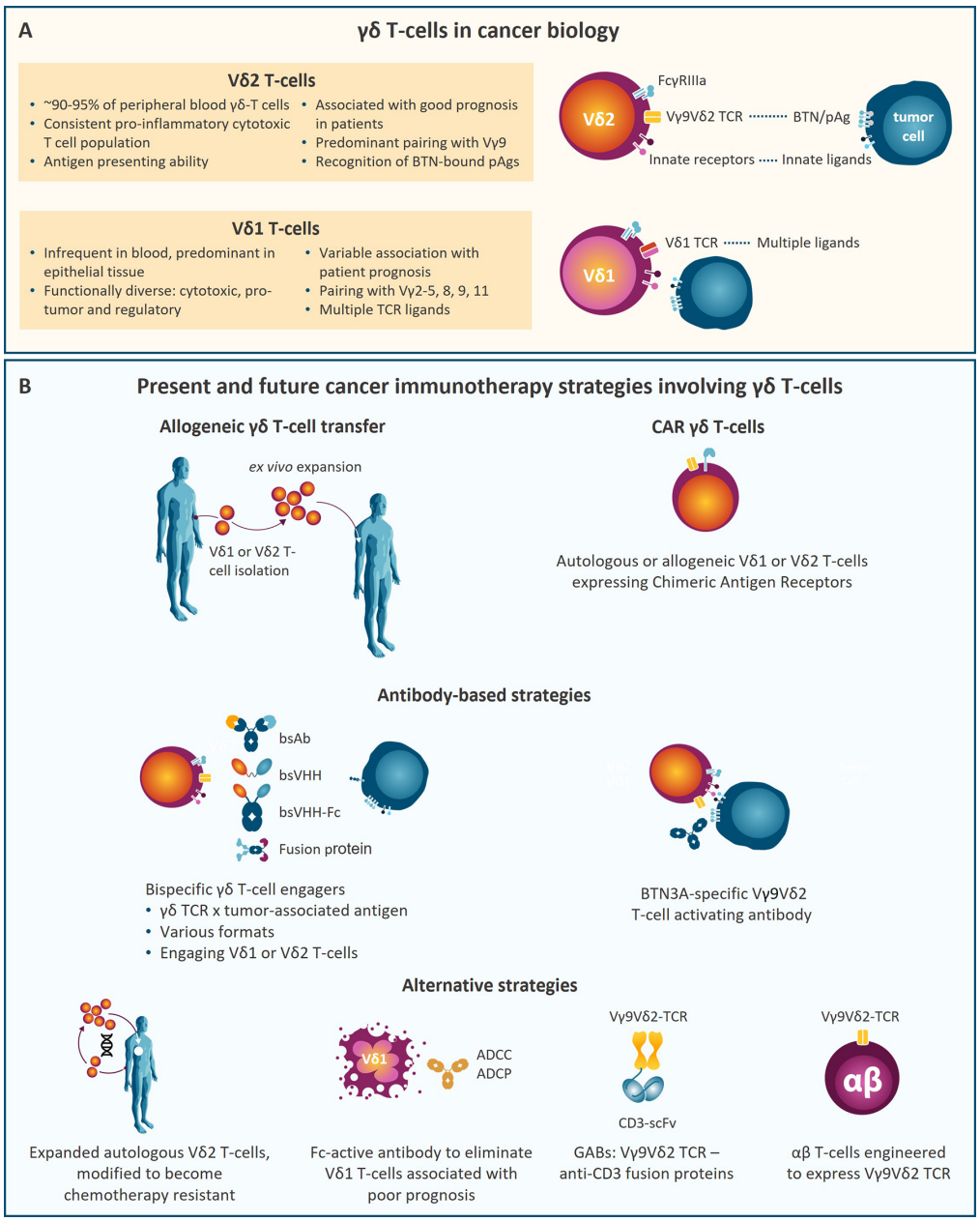


FIGURE 1 | **(A)** Key characteristics of the two main γδ T-cell subsets, Vδ2 and Vδ1 T-cells, in cancer biology. **(B)** Schematic representation of therapeutic strategies involving γδ T-cells that are currently being developed. ADCC, Antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; bsAb, bispecific antibody; bsVHH, bispecific variable domain of heavy-chain only antibody; BTN, Butyrophilin; CAR, chimeric antigen receptor; pAg, phosphoantigen; scFv, single-chain variable fragment.

The complete repertoire of antigens recognized by γδ-TCRs and the specificity of each γδ T subset is still not fully understood. Vγ9Vδ2 T-cells represent the predominant γδ T-cell subset (95%) in peripheral blood (14). Vγ9Vδ2 T-cells participate in the defense against malignant cells by sensing small phosphorylated metabolites (phosphoantigen (pAg) molecules) produced in cholesterol synthesis [isopentenyl pyrophosphate (IPP)] or by pathogens [*e.g.* (*E*)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMBPP)] (5, 15–19). Unlike conventional αβ T-cells, ligand recognition by Vγ9Vδ2 and most γδ T-cells does not involve antigen presentation by human leukocyte antigen (HLA) molecules (15, 20). Ligand recognition by Vγ9Vδ2 T-cells requires butyrophilin (BTN) 3A1 (21) and BTN2A1 (22–24). Intracellular pAg levels are increased under stress conditions like infection or malignant transformation or by aminobisphosphonates (ABP) (16, 17, 25–27). Vγ9Vδ2 T-cells sense increased intracellular pAg levels causing their activation and target cell killing. Recent studies show that pAg-bound BTN3A1 associates with BTN2A1 which directly interacts with non-variable regions of the Vγ9 chain on γδ T-cells. Besides Vγ9Vδ2 T-cell recognition of pAgs, some subsets of Vδ1 and Vδ3 T-cells detect pathogenic and self-lipids presented by CD1d through their TCR (28, 29). Vδ1 T-cells are less abundant in circulation than Vγ9Vδ2 T-cells, but they are enriched in epithelia (30) and among tumor infiltrating lymphocytes (TILs). While cultured Vδ1 T-cells may have higher cytotoxic capacity than Vγ9Vδ2 T-cells, Vδ1 T-cells can be pro-tumoral in certain malignancies (6, 31, 32) (**Figure 1A**).

In this review we discuss γδ T-cell-based therapeutic strategies with a focus on recent developments of bispecific γδ T-cell engagers (bsTCEs) and chimeric antigen receptor (CAR) γδ T-cells, and point towards approaches that may develop into therapies in the near future (**Figure 1B**).

PAST CLINICAL STUDIES WITH Vγ9Vδ2 T-CELLS

In the year 2000, ABP drugs, already approved to treat patients with excessive bone resorption, were shown to cause systemic Vγ9Vδ2 T-cell stimulation and to increase their antitumor activity in a preclinical study (26). Following this observation, studies explored ABP treatment as a systemic γδ T-cell stimulant or as an *ex vivo* tool to expand them for subsequent adoptive cell transfer (ACT) for cancer immunotherapy.

The ABPs pamidronate (PAM) and zoledronate (ZOL), and synthetic pAg analogues, mainly bromohydrin pyrophosphate (BrHPP) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), have been used alone or in combination with IL-2 to activate Vγ9Vδ2 T-cells (33, 34). ABP treatment has been evaluated in cancer patients (*e.g.* with multiple myeloma (MM), non-Hodgkin lymphoma (NHL), acute myeloid leukemia (AML), prostate cancer, renal cell carcinoma, colorectal cancer, breast cancer, melanoma or neuroblastoma) (33, 35–39). Additionally, *ex vivo* expansion of autologous γδ T-cells with ABPs or synthetic pAg followed by ACT has been tested in a wide range of malignancies (*e.g.* in MM, renal cell carcinoma, non-small cell lung cancer, gastric cancer, hepatocellular carcinoma, melanoma, ovarian cancer, colon cancer and pancreatic cancer) (40–51). While these approaches were well tolerated, clinical responses typically were found to be infrequent and not long-lasting, though sporadic meaningful responses were achieved (52–54). The overall moderate clinical antitumor effect of systemic γδ T-cell activation with ABP or synthetic pAg and of autologous γδ T transfer, negatively impacted further development of these Vγ9Vδ2 T-cell-directed cancer immunotherapeutic approaches.

PRESENT AND FUTURE STUDIES INVOLVING γδ T-CELLS

γδ T-Cell-Based Cellular Strategies

Allogeneic γδ T-Cell Transfer

As mentioned above, most γδ T-cells recognize target cells independently of HLA antigen presentation, suggesting that allogeneic donor derived γδ T-cells can be relatively safe for ACT due to low risk of graft-versus-host disease (GvHD). Taking advantage of this, current strategies exploring the use of *ex vivo* expanded γδ T-cell infusion have shifted towards allogeneic origin (**Table 1**). Increased frequency of γδ T-cells in leukemia patients that underwent αβ-depleted allogeneic stem cell transplantation from partially HLA-mismatched donors, was associated with a higher 5-year and overall survival (OS) (55, 56). A single infusion of allogeneic Vγ9Vδ2 T-cells, expanded *ex vivo* with ZOL plus IL-2, is being administered in a clinical trial (NCT03533816) to maximize antitumor response and reduce GvHD, after allogeneic hematopoietic cell transplant (alloHCT) and cyclophosphamide for hematologic malignancies. Moreover, allogeneic Vγ9Vδ2 T-cell infusion after lymphodepletion is being tested independently of alloHCT for hematologic malignancies and solid tumors. Some of these studies have already been completed with no major adverse effects reported, highlighting the safety of Vγ9Vδ2 T-cell transfer (57, 58). Importantly, patients receiving Vγ9Vδ2 T-cell infusion had increased OS compared to control patients and repeated Vγ9Vδ2 T-cell infusions resulted in higher OS when compared to single infusion. Future approaches are based on allogeneic γδ T-cells derived from healthy donors, either unmodified or CAR-transfected (see below) (**Table 2**).

Application of non-Vγ9Vδ2 T-cell subsets, like Vδ1 T-cells, is of interest but lagged behind because of lack of proper expansion protocols. In 2016, Almeida *et al.* described a 3 week culture protocol based on stimulation of γδ T-cells from healthy donors or CLL patients with a combination of cytokines and anti-CD3 monoclonal antibody (mAb) clone OKT-3, resulting in 2000-fold expansion and 60-80% enrichment of Vδ1 T-cells (59). Expanded cells expressed the NK receptors NKp30 and NKp40, displayed cytotoxic activity, produced IFNγ, TNFα and no IL-17. Application of this protocol led to the development of different "delta one T" (DOT) cell products. Gamma Delta Therapeutics initiated a first-in-human phase I clinical trial in AML patients after lymphodepletion with fludarabine and cyclophosphamide (NCT05001451) (**Table 1**). This study will analyse safety and

TABLE 1 | Ongoing clinical trials based on γδ T-cells.

Title	Intervention	Malignancy	Organization	Phase	Initial Date	Status	Study Identifier
Allogeneic γδ T-cell transfer							
TCRαβ-depleted Progenitor Cell Graft With Additional Memory T-cell DLI, Plus Selected Use of Blinatumomab, in Naive T-cell Depleted Haploidentical Donor Hematopoietic Cell Transplantation for Hematologic Malignancies	HPC-A Infusion (TCRα/β$^+$ and CD19+ depleted)	ALL, AML, MDS, NK-CL, HL, NHL, JMML,CML	St. Jude Children's Research Hospital	II	January 31, 2019	Recruiting	NCT03849651
Ex-vivo Expanded γδ T Lymphocytes in Patients With Refractory/Relapsed Acute Myeloid Leukaemia	Ex-vivo expanded allogeneic γδ T-cells from blood of related donors	AML	Wuhan Union Hospital and Jinan University, China	I	September 1, 2019	Recruiting	NCT04008381
Expanded/Activated Gamma Delta T-cell Infusion Following Hematopoietic Stem Cell Transplantation and Post-transplant Cyclophosphamide	EAGD T-cell infusion	AML,CML, ALL, MDS	University of Kansas Medical Center and In8bio Inc.	I	January 31, 2020	Recruiting	NCT03533816
Allogeneic "Gammadelta T Cells (γδ T Cells)" Cell Immunotherapy in Phase 1 Hepatocellular Carcinoma Clinical Trial	Ex-vivo expanded allogeneic γδ-T cells from related donors	HCC	Beijing 302 Hospital	I	August 15, 2020	Recruiting	NCT04518774
Gamma Delta T-cell Infusion for AML at High Risk of Relapse After Allo HCT	AlloHCT + AAPC-expanded donor T-cells	AML	H. Lee Moffitt Cancer Center and Research Institute	I/Ib	August 13, 2021	Recruiting	NCT05015426
Study of GDX012 in Patients With MRD Positive AML	GDX012. Allogeneic cell therapy enriched for Vδ1+	AML	GammaDelta Therapeutics Limited	I	August 13, 2021	Recruiting	NCT05001451
Allogeneic γδ T Cells Immunotherapy in r/r Non-Hodgkin's Lymphoma (NHL) or Peripheral T Cell Lymphomas (PTCL) Patients	Ex-vivo expanded allogeneic γδ T-cells from related donors	NHL, PTCL	Institute of Hematology & Blood Diseases Hospital	I	January 6, 2021	Recruiting	NCT04696705
Safety and Efficiency of γδ T Cell Against Hematological Malignancies After Allo-HSCT	Ex-vivo expanded γδ T-cell infusion	AML, ALL, MDS	Chinese PLA General Hospital	I/II	September 2021	Recruiting	NCT04764513
γδ CAR-T-cells							
Immunotherapy With CD19 CAR γδT-cells for B-Cell Lymphoma, ALL and CLL	Allogeneic γδ CAR-T-cells (anti-CD19)	RR ALL, CLL, B-NHL	Beijing Doing Biomedical Co., Ltd.	I	October 2017	Active, not recruiting	NCT02656147
Haplo/Allogeneic NKG2DL-targeting Chimeric Antigen Receptor-grafted γδ T Cells for Relapsed or Refractory Solid Tumour	Haploidentical or allogeneic Vδ2 CAR-T-cells (anti-NKG2DL) (CTM-N2D)	RR solid tumors of different types	CytoMed Therapeutics Pte Ltd.	I	December 1, 2019	Active, not recruiting	NCT04107142
A Study of ADI-001 in B Cell Malignancies (GLEAN-1)	Lymphodepletion + ADI-001 (Anti-CD20 γδ CAR-T-cells) in monotherapy and combined with IL-2	B-NHL	Adicet Bio, Inc	I	March 4, 2021	Recruiting	NCT04735471
Antibody-based strategies							
First-in-Human Study of ICT01 in Patients With Advanced Cancer (EVICTION)	ICT01. monoclonal antibody targeting BTN3A	Solid Tumor, Adult Hematopoietic/ Lymphoid Cancer	ImCheck Therapeutics	I/II	February 10, 2020	Recruiting	NCT04243499
Trial With LAVA-051 in Patients With Relapsed/ Refractory CD1d (Cluster of Differentiation (CD)1d)-Positive CLL, MM, AML	LAVA-051. Bispecific γδ T-cell engager	CLL, AML, MM	Lava Therapeutics	I/II	July 12, 2021	Recruiting	NCT04887259
Trial of LAVA-1207 in Patients With Therapy Refractory Metastatic Castration Resistant Prostate Cancer	LAVA-1207. Bispecific γδ T-cell engager	Prostate Cancer	Lava Therapeutics	I/IIa	January 31, 2022	Recruiting	NCT05369000

(Continued)

TABLE 1 | Continued

Title	Intervention	Malignancy	Organization	Phase	Initial Date	Status	Study Identifier
Alternative γδ T-cell-related strategies							
Safety of TEG001 in patients with r/r AML, high-risk MDS or MM	TEG001	RR AML, high-risk MDS, MM	Gadeta B.V.	I	June 01, 2017	Recruiting	NTR6541
Novel Gamma-Delta (γδ)T Cell Therapy for Treatment of Patients With Newly Diagnosed Glioblastoma	DRI γδ T-cells modified to be resistant to TMZ + TMZ	Glioblastoma multiforme	University of Alabama at Birmingham and IN8Bio Inc.	I	February 11, 2020	Recruiting	NCT04165941
A Study to Investigate the Safety and Efficacy of TEG002 in Relapsed/Refractory Multiple Myeloma Patients	TEG002	RR MM	Gadeta B.V.	I	May 13, 2021	Recruiting	NCT04688853

AAPC, Artificial antigen presenting cell; ALL, acute lymphocytic leukemia; AlloHCT, Allogeneic hematopoietic cell transplantation; AML, Acute myeloid leukemia; B-NHL, B cell Non-Hodgkin lymphoma; CAR, Chimeric antigen receptor; CLL, Chronic lymphocytic leukemia; CML, Chronic myeloid leukaemia; DLI, Donor lymphocyte infusion; DRI, Drug resistant immunotherapy; EAGDT, Expanded/Activated γδ T-cell; HCC, Hepatocellular carcinoma; HL, Hodgkin lymphoma; HPC-A, Hematopoietic progenitor cells apheresis; HSCT, haematopoietic stem cell transplantation; JMML, Juvenile myelomonocytic leukemia; MM, Multiple myeloma; MDS, Myelodysplastic syndrome; NHL, Non-Hodgkin lymphoma; NKCL, Natural killer cell leukemia; PBMC, peripheral blood mononuclear cell; PTCL, peripheral T cell lymphoma. RR, Relapsed/Refractory; TMZ, temozolomide. Initial date, Date of first patient enrolment.

maximum tolerated dose of GDX012 and its effect on minimal residual disease, progression free survival (PFS) and OS.

Chimeric Antigen Receptor γδ T-Cells

Another therapeutic approach to harness the potent anti-tumor effects of γδ T-cells consists of adoptive transfer of γδ CAR-T-cells (60). CARs are chimeric antigen-recognition receptors, consisting of an ectodomain, which binds a tumor specific cell surface receptor, and endodomains, consisting of CD3ζ as the signaling domain with co-stimulatory domains to provide robust activation (*e.g.* CD28, 4-1BB, or ICOS) (61). In recent years, CAR-T-cell therapy has been extensively investigated in preclinical and clinical studies, primarily focused on conventional αβ T-cells (62–64). These autologous CAR-T-cells have triggered encouraging remission rates in patients refractory to standard treatments against, in particular, B-lymphoid malignancies. This resulted in FDA approvals of CAR-T-cell therapies for the treatment of B-cell NHL, ALL,

TABLE 2 | Companies developing γδ T-cell-based or γδ T-cell-engaging therapies.

Organization	γδ T-cell subtype	Approach
γδ T-cell-based therapy		
Acepodia	information not available	Allogeneic mAb-conjugated γδ-cells
Adicet Bio	Vδ1	Allogeneic γδ CAR-T-cells
Expression Therapeutics	Vδ2	Allogeneic γδ CAR-T-cells
GammaDelta Therapeutics (acquired by Takeda)	Vδ1	Allogeneic unmodified or engineered Vδ1$^+$ T-cells
Immatics	information not available	Allogeneic γδ CAR-T-cells
IN8bio (previously Incysus Therapeutics)	Vδ2	Expanded γδ T-cells engineered to achieve drug resistant immunotherapy (DRI)
Kiromic BioPharma	information not available	Allogeneic γδ CAR-T-cells genetically engineered using ABBIE non-viral gene editing technology
PersonGen BioTherapeutics	information not available	Allogeneic universal CAR (UCAR) based γδ-cells
TC BioPharm	Vδ1/Vδ2	Allogeneic unmodified γδ–cells or engineered γδ CAR-T-cells
One Chain Immunotherapeutics	Vδ1	Expanded allogeneic Vδ1$^+$ T-cells for ACT
Beroni group	information not available	Allogeneic γδ ACT
γδ T-cell-based antibody therapy		
Organization	**γδ T-cell subtype**	**Approach**
Adaptate Biotherapeutics (acquired by Takeda)	Vδ1	Vδ1 bispecific T-cell engagers
ImCheck Therapeutics	Vδ2	mAbs targeting BTN isoforms to modulate γδ T-cell activation
LAVA Therapeutics	Vδ2	Vδ2 bispecific T-cell engagers
PureTech Health	Vδ1	mAb against Vδ1 to induce pro-tumoral Vδ1 T-cell killing
Shattuck Labs	Vδ2	Recombinant proteins containing heterodimeric BTN extracellular domains and a tumor targeting scFv
Other γδ T-cell-based therapies		
Organization	**γδ-T-cell subtype**	**Approach**
American Gene Technologies	Vδ2	Lentivirus to increase pAg levels in tumor cells

ACT, Adoptive cell transfer; bsTCE, bispecific T cell engager; bsVHH, bispecific Variable Heavy chain-only antibody; BTN, Butyrophilin; CAR, Chimeric antigen receptor; mAb, monoclonal antibody; pAg, phosphoantigen; scFv, Single chain variable fragment.

and MM (65–69). The remarkable success of CAR-T-cell therapy revolutionized the field of adoptive cell therapy for treating hematologic malignancies and resulted in numerous ongoing clinical trials. However, CAR-T-cell therapy can be complicated by severe, potentially life-threatening, toxicities such as cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS) and other 'on-target off-tumor' toxicities (70). Moreover, in contrast to the results seen in hematologic malignancies, only limited antitumor effects have been obtained in patients with solid tumors.

It was hypothesized that the efficacy of CAR-T-cells could be improved and its side effects mitigated by harnessing the innate properties of γδ T-cells as a backbone for CAR. CAR-modified γδ T-cells were first described by Rischer *et al.* (71), demonstrating specific *in vitro* tumor cell lysis using ZOL-expanded Vγ9Vδ2 T-cells with CD19- or GD2-directed CARs, followed by other studies confirming these findings using γδ T-cells containing CARs against a variety of targets (72–77). Interestingly, CAR-modified Vγ9Vδ2 T-cells maintained their ability to cross-present tumor antigens to αβ T-cells *in vitro*, which may prolong the anti-tumor efficacy (76). Furthermore, γδ T-cells bearing a CD19-CAR, unlike standard CD19-αβ CAR-T-cells, had reactivity against CD19-positive and negative tumor cells *in vitro* and *in vivo*, an effect that was enhanced by ZOL (78), suggesting that CD19-directed γδ CAR-T-cells may target leukemic cells also after antigen loss and retain pAg specificity *via* their TCR. More recently, Wallet *et al.* described the generation of induced pluripotent stem cell-derived γδ CAR-T-cells (γδ CAR-iT) (79). They demonstrated sustained *in vitro* tumor cell killing by γδ CAR-iT-cells in the presence of IL-15, with markedly less IFN-γ and other inflammatory cytokines being produced compared to conventional αβ CAR-T-cells, potentially resulting in lower risk of CRS. Moreover, a single dose of γδ CAR-iT-cells resulted in potent tumor growth inhibition in a xenograft mouse model (79). **Table 2** summarizes the companies currently developing γδ CAR-T-cells.

Pre-clinical research on γδ CAR-T-cell based therapy initially focused on Vγ9Vδ2 T-cells, due to their dominant frequency in blood and their unique pAg response that allowed the specific expansion of this subset (80). Makkouk *et al.* recently showed the first example of genetically modified Vδ1 T-cells. They expanded PBMC-derived Vδ1 T-cells using an agonistic anti-Vδ1 antibody and genetically modified them to express a GPC-3 targeted CAR and to secrete IL-15 (81). In a HepG2 mouse model, these allogeneic Vδ1 CAR-T-cells primarily accumulated in the tumor and a single dose efficiently controlled tumor growth without evidence of xenogeneic GvHD. ADI-001 consists of CD20-targeting Vδ1 CAR-T-cells generated by a similar procedure by Adicet Bio (82) and is currently being used in a phase I clinical trial (NCT04735471). Recently reported interim data from this dose-escalation study showed complete responses in two and a partial response in one out of four evaluable patients already with low doses ($30x10^6$ cells) of ADI-001, indicating that relatively low amounts of γδ T-cells may suffice for activity (press release). To date, no dose-limiting toxicities, GvHD, or grade 3 or higher CRS has been reported. These encouraging first results underscore the potential of Vδ1 CAR-T-cell therapy in the clinic. A complete overview of the ongoing clinical trials evaluating CAR-modified γδ T-cells is listed in **Table 1**.

Antibody-Based Strategies

Imcheck develops ICT01, a Vγ9Vδ2 T-cell activating humanized IgG1 with a silent Fc that binds to all three BTN3A isoforms to trigger Vγ9Vδ2 T-cell activation and increased cytotoxicity against $BTN3A^+$ tumor cell lines from diverse origin (21). However, this approach is not tumor specific as BTN3A is broadly expressed and could also be hampered by soluble BTN3A molecules potentially acting as decoy receptors (83). In immunodeficient NSG mice, treatment with ICT01 resulted in *in vivo* activation of adoptively transferred human Vγ9Vδ2 T-cells and delayed outgrowth of the AML cell line MOLM14 (84). The EVICTION trial is a Phase I/IIa clinical trial currently testing the effect of ICT01 in relapsed/refractory advanced-stage hematologic malignancies as a monotherapy and in a broad range of solid tumors as monotherapy or in combination with pembrolizumab (NCT04243499). Preliminary results show a good safety profile with activation of Vγ9Vδ2 T-cells and increased tumor infiltration in one melanoma patient. Stable disease has been achieved in 31% of patients treated with ICT01 as a monotherapy and in 62% in combination with pembrolizumab (84).

BsTCEs have emerged as a promising therapeutic approach for immune-oncology (85) and consist of a tumor antigen binding antibody linked to a T-cell engaging antibody fragment aiming to crosslink tumor cells and T-cells to elicit T-cell-mediated anti-tumor cytotoxicity (86, 87). Most efforts to generate bsTCEs have made use of CD3 as a T-cell engaging domain due to its role in T-cell activation. For CD3-based TCEs, proteins that are uniquely expressed or specifically overexpressed by tumor cells are the most attractive candidates for targeting, as this reduces on-target off-tumor toxicity. After approval of the CD19-CD3 bsTCE blinatumomab (88), multiple CD3-directed TCEs have been developed (89), but in many cases development has been complicated by the occurrence of adverse events such as on-target off-tumor toxicity, CRS or ICANS, highlighting the need for more tumor-selective targeting (90–92). Considering the clinical safety observed following systemic γδ T-cell activation and γδ T ACT, specific engagement of γδ T-cells using γδ bsTCEs might have an improved safety profile due to their tumor selectivity compared to CD3-bsTCEs. By avoiding detrimental co-activation of regulatory $CD3^+$ T-cells observed with CD3 pan T-cell engagers (93) and their ability to bridge and engage components of both the innate and adaptive immune system, γδ bsTCEs could potentially result in increased antitumor activity.

Several γδ T-cell engaging formats are being developed and evaluated preclinically. Vγ9-TCR specific engagers directed against Her2 (94–96) and CD123 (97) were shown to cause killing of Her2 expressing cell lines and AML cell lines, respectively. The GADLEN platform (Shattuck Labs) consists of fusion proteins containing BTN heterodimers, to engage and activate Vγ9Vδ2 T-cells, bound to a tumor targeting scFv

domain through an Fc linker (98). Vδ1 bsTCEs are also being developed by Adaptate Biotherapeutics. Heavy chain only antibodies occur naturally in camelids (99). Their antigen-binding fragments or variable heavy chain-only antibodies (VHH), are small, stable and with low inherent immunogenicity (100, 101). Lava Therapeutics` Gammabody™ platform combines Vδ2-specific and tumor-targeting VHHs as modules to generate bsTCE (102–105). In pre-clinical studies, Gammabody™ molecules targeting CD40, CD1d and EGFR efficiently engage Vγ9Vδ2 T-cells to kill tumor cells expressing these antigens (102–105). Two Gammabody™ molecules, are currently evaluated in clinical trials. LAVA-051, a Gammabody™ targeting CD1d is tested in a Phase I/IIa clinical trial (NCT04887259) in patients with therapy-refractory CLL, AML or MM. Preliminary data of the first 3 cohorts from this study showed a thus far good safety profile with no dose-limiting toxicities or CRS. In addition, LAVA-1207, a Gammabody™ targeting PSMA is tested in a phase I/IIa clinical trial (NCT05369000) in patients suffering from therapy-refractory metastatic castration-resistant prostate cancer. **Table 2** summarizes companies developing antibody-based γδ T-cell therapies, and **Table 1** contains clinical trials involving antibody-based γδ T-cell approaches.

Alternative γδ T-Cell-Related Strategies

A new γδ T-cell based approach being tested in clinical trials is DeltEx drug-resistant immunotherapy (DRI). IN8Bio`s first DeltEx DRI product, INB-200, consists of expanded autologous Vγ9Vδ2 T-cells genetically modified to express a methylguanine DNA methyltransferase (MGMT). MGMT confers them resistance to temozolomide (TMZ) allowing for simultaneous treatment with TMZ and immunotherapy (106). TMZ, which is the current standard of care for glioblastoma multiforme (GBM) together with radiotherapy after resection, might sensitize tumor cells to γδ T-cell recognition through upregulation of NKG2D ligands but it also causes lymphocytopenia that is avoided by MGMT expression (107). An ongoing clinical trial (NCT04165941) is testing intracranial administration of INB-200 to the tumor site after surgical resection, followed by TMZ treatment (**Table 1**). All 4 GBM patients enrolled in this study have been reported to exceed the expected PFS for TMZ alone treatment. This technology is based on expansion and modification of autologous γδ T-cells, however, other DeltEx DRI based on allogeneic γδ T-cells (INB-400) and γδ CAR-T-cells (INB-300) are being developed.

Interestingly, although Vδ1$^+$ T-cells have cytotoxic capacity, Vδ1$^+$ TIL associate with poor prognosis in certain malignancies, possibly through production of IL-17 (6, 32). LYT-210 is a mAb directed towards the Vδ1$^+$ TCR with the aim of eliminating these pathogenic cells (**Table 2**). Gamma-delta TCR bispecific molecules (GABs) combine the extracellular domain of the Vγ9Vδ2 TCR fused with a CD3 binding domain, allowing conventional T-cells to recognize the presence of pAg on tumor cells (108). In the presence of GABs, αβ T-cells recognized and killed the squamous cell carcinoma cell line SCC9 in a pAg dependent manner and produced increased amounts of IFNγ when exposed to patient-derived AML blasts but not with healthy hematopoietic cells indicating preferential recognition of tumor cells.

Two phase I dose-escalation clinical trials (NCT04688853; NTR6541) initiated by Gadeta are assessing the safety and tolerability of αβ T-cells engineered to express a defined Vγ9Vδ2 TCR (TEGs) in relapsed/refractory AML, MM, and high-risk myelodysplastic syndrome patients. These T-cells combine the tumor specificity of γδ T-cells with the tumor cell killing potential of αβ T-cells and show promising antitumor reactivity both *in vitro* and *in vivo*. Furthermore, chimeric PD-1 receptor (chPD1) γδ T-cells, turn PD-1 immune suppression into T-cell activation (109). The chPD1 γδ T-cells selectively killed PD-L1$^+$ tumor cells in a xenograft murine model, without lysis of normal PD-L1$^+$ cells or significant elevation of CRS-related cytokines. The authors reported that chPD1 γδ T-cell therapy will be assessed in a phase I/II clinical trial.

CONCLUSION

Past clinical trials have demonstrated that systemic activation of Vγ9Vδ2 T-cells or adoptive transfer of autologous Vγ9Vδ2 T-cells were well tolerated and could trigger antitumor immunity. These studies have been followed by a number of trials based on Vγ9Vδ2 and the first study with Vδ1 allogeneic T-cell transfer, which would allow for donor-derived therapies. Up to this date, these trials have not resulted in major adverse effects. Most strategies that are currently under evaluation profit from the safety of γδ T-cell activation and incorporate tumor-targeting mechanisms, *e.g.* CARs or bsTCEs, which might be key to obtain more robust and consistent clinical responses. Initial results from these targeted approaches, both cell and antibody-based, show great promise and confirm the safety of Vγ9Vδ2 and Vδ1 T-cell-based strategies. However, cell-based products present challenges that are not shared by antibody-based therapies, such as high cost, difficulty of production or need of specialized facilities, and preparatory lymphodepleting chemotherapy regimens. In the near future, the results obtained by the trials described in this review will determine whether the potential of γδ T-cells can be translated into clinical benefit.

AUTHOR CONTRIBUTIONS

JS-E and MJ wrote the manuscript. HV co-wrote and reviewed the manuscript. LK, PP, EE, BW and TG reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The authors declare that this study received funding from LAVA therapeutics. The funder had the following involvement with the study: providing research funding to Amsterdam UMC and in designing, writing and revising the text of the mini-review.

REFERENCES

1. Kozbor D, Trinchieri G, Monos DS, Isobe M, Russo G, Haney JA, et al. Human TCR-Gamma+/Delta+, CD8+ T Lymphocytes Recognize Tetanus Toxoid in an MHC-Restricted Fashion. *J Exp Med* (1989) 169(5):1847–51. doi: 10.1084/jem.169.5.1847
2. Pistoia V, Tumino N, Vacca P, Veneziani I, Moretta A, Locatelli F, et al. Human γδ T-Cells: From Surface Receptors to the Therapy of High-Risk Leukemias. *Front Immunol* (2018) 9:984. doi: 10.3389/fimmu.2018.00984
3. Adams EJ, Gu S, Luoma AM. Human Gamma Delta T Cells: Evolution and Ligand Recognition. *Cell Immunol* (2015) 296(1):31–40. doi: 10.1016/j.cellimm.2015.04.008
4. Zhao Y, Niu C, Cui J. Gamma-Delta (Gammadelta) T Cells: Friend or Foe in Cancer Development? *J Transl Med* (2018) 16(1):3. doi: 10.1186/s12967-017-1378-2
5. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. Regulation of Cutaneous Malignancy by Gammadelta T Cells. *Science* (2001) 294(5542):605–9. doi: 10.1126/science.1063916
6. Meraviglia S, Lo Presti E, Tosolini M, La Mendola C, Orlando V, Todaro M, et al. Distinctive Features of Tumor-Infiltrating γδ T Lymphocytes in Human Colorectal Cancer. *OncoImmunology* (2017) 6(10):e1347742. doi: 10.1080/2162402X.2017.1347742
7. Wang J, Lin C, Li H, Li R, Wu Y, Liu H, et al. Tumor-Infiltrating γδt Cells Predict Prognosis and Adjuvant Chemotherapeutic Benefit in Patients With Gastric Cancer. *OncoImmunology* (2017) 6(11):e1353858. doi: 10.1080/2162402X.2017.1353858
8. Donia M, Ellebaek E, Andersen MH, Straten PT, Svane IM. Analysis of Vδ1 T Cells in Clinical Grade Melanoma-Infiltrating Lymphocytes. *OncoImmunology* (2012) 1(8):1297–304. doi: 10.4161/onci.21659
9. Lu H, Dai W, Guo J, Wang D, Wen S, Yang L, et al. High Abundance of Intratumoral γδ T Cells Favors a Better Prognosis in Head and Neck Squamous Cell Carcinoma: A Bioinformatic Analysis. *Front Immunol* (2020) 11. doi: 10.3389/fimmu.2020.573920
10. Zhao N, Dang H, Ma L, Martin SP, Forgues M, Ylaya K, et al. Intratumoral γδ T-Cell Infiltrates, Chemokine (C-C Motif) Ligand 4/Chemokine (C-C Motif) Ligand 5 Protein Expression and Survival in Patients With Hepatocellular Carcinoma. *Hepatology* (2020) 73(3):2021. doi: 10.1002/hep.31412
11. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. *Nat Med* (2015) 21(8):938–45. doi: 10.1038/nm.3909
12. Tosolini M, Pont F, Poupot M, Vergez F, Nicolau-Travers ML, Vermijlen D, et al. Assessment of Tumor-Infiltrating Tcrvγ9vδ2 γδ Lymphocyte Abundance by Deconvolution of Human Cancers Microarrays. *OncoImmunology* (2017) 6(3):e1284723. doi: 10.1080/2162402X.2017.1284723
13. Silva-Santos B, Serre K, Norell H. γδt Cells in Cancer. *Nat Rev Immunol* (2015) 15(11):683–91. doi: 10.1038/nri3904
14. Hinz T, Wesch D, Halary F, Marx S, Choudhary A, Arden B, et al. Identification of the Complete Expressed Human TCR V γ Repertoire by Flow Cytometry. *Int Immunol* (1997) 9(8):1065–72. doi: 10.1093/intimm/9.8.1065
15. Janis EM, Kaufmann SH, Schwartz RH, Pardoll DM. Activation of Gamma Delta T Cells in the Primary Immune Response to Mycobacterium Tuberculosis. *Science* (1989) 244(4905):713–6. doi: 10.1126/science.2524098
16. Gruenbacher G, Nussbaumer O, Gander H, Steiner B, Leonhartsberger N, Thurnher M. Stress-Related and Homeostatic Cytokines Regulate Vγ9vδ2 T-Cell Surveillance of Mevalonate Metabolism. *OncoImmunology* (2014) 3(8):1–12. doi: 10.4161/21624011.2014.953410
17. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and Synthetic non-Peptide Antigens Recognized by Human Gamma Delta T Cells. *Nature* (1995) 375(6527):155–8. doi: 10.1038/375155a0
18. Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, Jomaa H. Microbial Isoprenoid Biosynthesis and Human γδ T Cell Activation. *FEBS Lett* (2003) 544(1-3):4–10. doi: 10.1016/S0014-5793(03)00483-6
19. Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind RM, Kollas A-K, et al. Identi¢cation of (E)-4-Hydroxy-3-Methyl-But-2-Enyl Pyrophosphate as a Major Activator for Human QN T Cells in Escherichia Coli. *FEBS Lett* (2001) 509(2):317–22. doi: 10.1016/S0014-5793(01)03191-X
20. La Gruta NL, Gras S, Daley SR, Thomas PG, Rossjohn J. Understanding the Drivers of MHC Restriction of T Cell Receptors. *Nat Rev Immunol* (2018) 18(7):467–78. doi: 10.1038/s41577-018-0007-5
21. Harly C, Guillaume Y, Nedellec S, Peigné CM, Mönkkönen H, Mönkkönen J, et al. Key Implication of CD277/butyrophilin-3 (BTN3A) in Cellular Stress Sensing by a Major Human γδ T-Cell Subset. *Blood* (2012) 120(11):2269–79. doi: 10.1182/blood-2012-05-430470
22. Cano CE, Pasero C, De Gassart A, Kerneur C, Gabriac M, Fullana M, et al. BTN2A1, an Immune Checkpoint Targeting Vγ9vδ2 T Cell Cytotoxicity Against Malignant Cells. *Cell Rep* (2021) 36(2):109359. doi: 10.1016/j.celrep.2021.109359
23. Karunakaran MM, Willcox CR, Salim M, Paletta D, Fichtner AS, Noll A, et al. Butyrophilin-2a1 Directly Binds Germline-Encoded Regions of the Vγ9vδ2 TCR and Is Essential for Phosphoantigen Sensing. *Immunity* (2020) 52(3):487–98.e6. doi: 10.1016/j.immuni.2020.02.014
24. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 Is Essential for Phosphoantigen Reactivity by Gd T Cells. *Science* (2020) 367(6478):eaay5516. doi: 10.1126/science.aay5516
25. Gober HJ, Kistowska M, Angman L, Jenö P, Mori L, De Libero G. Human T Cell Receptor γδ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells. *J Exp Med* (2003) 197(2):163–8. doi: 10.1084/jem.20021500
26. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of Gammadelta T Cells by Aminobisphosphonates and Induction of Antiplasma Cell Activity in Multiple Myeloma. *Blood* (2000) 96(2):384–92. doi: 10.1182/blood.V96.2.384
27. Dhar MK, Koul A, Kaul S. Farnesyl Pyrophosphate Synthase: A Key Enzyme in Isoprenoid Biosynthetic Pathway and Potential Molecular Target for Drug Development. *N Biotechnol* (2013) 30(2):114–23. doi: 10.1016/j.nbt.2012.07.001
28. Luoma AM, Castro CD, Mayassi T, Bembinster LA, Bai L, Picard D, et al. Crystal Structure of Vδ1t Cell Receptor in Complex With CD1d-Sulfatide Shows MHC-Like Recognition of a Self-Lipid by Human γδ T Cells. *Immunity* (2013) 39(6):1032–42. doi: 10.1016/j.immuni.2013.11.001
29. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, O'Shea D, et al. Cutting Edge: CD1d Restriction and Th1/Th2/Th17 Cytokine Secretion by Human Vδ3 T Cells. *J Immunol* (2013) 191(1):30–4. doi: 10.4049/jimmunol.1300121
30. Deusch K, Pfeffer K, Reich K, Gstettenbauer M, Daum S, Luling F, et al. Phenotypic and Functional Characterization of Human TCR Gamma Delta+ Intestinal Intraepithelial Lymphocytes. *Curr Top Microbiol Immunol* (1991) 173:279–83.
31. Wu D, Wu P, Wu X, Ye J, Wang Z, Zhao S, et al. Ex Vivo Expanded Human Circulating Vdelta1 gammadeltaT Cells Exhibit Favorable Therapeutic Potential for Colon Cancer. *Oncoimmunology* (2015) 4(3):e992749. doi: 10.4161/2162402X.2014.992749
32. Li Y, Li G, Zhang J, Wu X, Chen X. The Dual Roles of Human γδ T Cells: Anti-Tumor or Tumor-Promoting. *Front Immunol* (2021) 11:619954. doi: 10.3389/fimmu.2020.619954
33. Bennouna J, Levy V, Sicard H, Senellart H, Audrain M, Hiret S, et al. Phase I Study of Bromohydrin Pyrophosphate (BrHPP, IPH 1101), a Vγ9vδ2 T Lymphocyte Agonist in Patients With Solid Tumors. *Cancer Immunol Immunother* (2010) 59(10):1521–30. doi: 10.1007/s00262-010-0879-0
34. Tanaka Y, Kobayashi H, Terasaki T, Toma H, Aruga A, Uchiyama T, et al. Synthesis of Pyrophosphate-Containing Compounds That Stimulate Vgamma2Vdelta2 T Cells: Application to Cancer Immunotherapy. *Med Chem* (2007) 3(1):85–99. doi: 10.2174/157340607779317544
35. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, et al. T Cells for Immune Therapy of Patients With Lymphoid Malignancies. *Blood* (2003) 102:200–6. doi: 10.1182/blood-2002-12-3665
36. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting Human γδ T Cells With Zoledronate and Interleukin-2 for Immunotherapy of Hormone-Refractory Prostate Cancer. *Cancer Res* (2007) 67(15):7450–7. doi: 10.1158/0008-5472.CAN-07-0199
37. Lang JM, Kaikobad MR, Wallace M, Staab MJ, Horvath DL, Wilding G, et al. Pilot Trial of Interleukin-2 and Zoledronic Acid to Augment γδ T Cells as Treatment for Patients With Refractory Renal Cell Carcinoma. *Cancer Immunol Immunother* (2011) 60(10):1447–60. doi: 10.1007/s00262-011-1049-8

38. Kunzmann V, Smetak M, Kimmel B, Weigang-Koehler K, Goebeler M, Birkmann J, et al. Tumor-Promoting Versus Tumor-Antagonizing Roles of Gd T Cells in Cancer Immunotherapy: Results From a Prospective Phase I/II Trial. *J Immunother* (2012) 35(2):205–13. doi: 10.1097/CJI.0b013e318245bb1e
39. Pressey JG, Adams J, Harkins L, Kelly D, You Z, Lamb LS. *In Vivo* Expansion and Activation of Gd T Cells as Immunotherapy for Refractory Neuroblastoma A Phase 1 Study. *Med (United States)* (2016) 95(39):e4909. doi: 10.1097/MD.0000000000004909
40. Abe Y, Muto M, Nieda M, Nakagawa Y, Nicol A, Kaneko T, et al. Clinical and Immunological Evaluation of Zoledronate-Activated Vγ9γδ T-Cell-Based Immunotherapy for Patients With Multiple Myeloma. *Exp Hematol* (2009) 37(8):956–68. doi: 10.1016/j.exphem.2009.04.008
41. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety Profile and Anti-Tumor Effects of Adoptive Immunotherapy Using Gamma-Delta T Cells Against Advanced Renal Cell Carcinoma: A Pilot Study. *Cancer Immunol Immunother* (2007) 56(4):469–76. doi: 10.1007/s00262-006-0199-6
42. Bennouna J, Bompas E, Neidhardt EM, Rolland F, Philip I, Galéa C, et al. Phase-I Study of Innacell γδ™, an Autologous Cell-Therapy Product Highly Enriched in γ9δ2 T Lymphocytes, in Combination With IL-2, in Patients With Metastatic Renal Cell Carcinoma. *Cancer Immunol Immunother* (2008) 57(11):1599–609. doi: 10.1007/s00262-008-0491-8
43. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K. Phase I/II Study of Adoptive Transfer of γδ T Cells in Combination With Zoledronic Acid and IL-2 to Patients With Advanced Renal Cell Carcinoma. *Cancer Immunol Immunother* (2011) 60(8):1075–84. doi: 10.1007/s00262-011-1021-7
44. Nakajima J, Murakawa T, Fukami T, Goto S, Kaneko T, Yoshida Y, et al. A Phase I Study of Adoptive Immunotherapy for Recurrent non-Small-Cell Lung Cancer Patients With Autologous γδ T Cells. *Eur J Cardio-thoracic Surgery* (2010) 37(5):1191–7. doi: 10.1016/j.ejcts.2009.11.051
45. Izumi T, Kondo M, Takahashi T, Fujieda N, Kondo A, Tamura N, et al. Ex Vivo Characterization of γδ T-Cell Repertoire in Patients After Adoptive Transfer of Vγ9vδ2 T Cells Expressing the Interleukin-2 Receptor β-Chain and the Common γ-Chain. *Cytotherapy* (2013) 15(4):481–91. doi: 10.1016/j.jcyt.2012.12.004
46. Kakimi K, Matsushita H, Masuzawa K, Karasaki T, Kobayashi Y, Nagaoka K, et al. Adoptive Transfer of Zoledronate-Expanded Autologous Vgamma9Vdelta2 T-Cells in Patients With Treatment-Refractory Non-Small-Cell Lung Cancer: A Multicenter, Open-Label, Single-Arm, Phase 2 Study. *J Immunother Cancer* (2s020) 8(2):e001185. doi: 10.1136/jitc-2020-001185
47. Sakamoto M, Nakajima J, Murakawa T, Fukami T, Yoshida Y, Murayama T, et al. Adoptive Immunotherapy for Advanced Non-Small Cell Lung Cancer Using Zoledronate-Expanded γδ T Cells: A Phase I Clinical Study. *J Immunother* (2011) 34(2):202–11. doi: 10.1097/CJI.0b013e318207ecfb
48. Wada I, Matsushita H, Noji S, Mori K, Yamashita H, Nomura S, et al. Intraperitoneal Injection of *In Vitro* Expanded Vγ9vδ2 T Cells Together With Zoledronate for the Treatment of Malignant Ascites Due to Gastric Cancer. *Cancer Med* (2014) 3(2):362–75. doi: 10.1002/cam4.196
49. Cui J, Wang N, Zhao H, Jin H, Wang G, Niu C, et al. Combination of Radiofrequency Ablation and Sequential Cellular Immunotherapy Improves Progression-Free Survival for Patients With Hepatocellular Carcinoma. *Int J Cancer* (2014) 134(2):342–51. doi: 10.1002/ijc.28372
50. Nicol AJ, Tokuyama H, Mattarollo SR, Hagi T, Suzuki K, Yokokawa K, et al. Clinical Evaluation of Autologous Gamma Delta T Cell-Based Immunotherapy for Metastatic Solid Tumours. *Br J Cancer* (2011) 105(6):778–86. doi: 10.1038/bjc.2011.293
51. Aoki T, Matsushita H, Hoshikawa M, Hasegawa K, Kokudo N, Kakimi K. Adjuvant Combination Therapy With Gemcitabine and Autologous γδ T-Cell Transfer in Patients With Curatively Resected Pancreatic Cancer. *Cytotherapy* (2017) 19(4):473–85. doi: 10.1016/j.jcyt.2017.01.002
52. Sebestyen Z, Prinz I, Déchanet-Merville J, Silva-Santos B, Kuball J. Translating Gammadelta (γδ) T Cells and Their Receptors Into Cancer Cell Therapies. *Nat Rev Drug Discovery* (2020) 19(3):169–84. doi: 10.1038/s41573-019-0038-z
53. Hoeres T, Smetak M, Pretscher D, Wilhelm M. Improving the Efficiency of Vγ9vδ2 T-Cell Immunotherapy in Cancer. *Front Immunol* (2018) 9:800. doi: 10.3389/fimmu.2018.00800
54. Wilhelm M, Smetak M, Schaefer-Eckart K, Kimmel B, Birkmann J, Einsele H, et al. Successful Adoptive Transfer and *In Vivo* Expansion of Haploidentical Gammadelta T Cells. *J Transl Med* (2014) 12:45. doi: 10.1186/1479-5876-12-45
55. Lamb LSJr., Henslee-Downey PJ, Parrish RS, Godder K, Thompson J, Lee C, et al. Increased Frequency of TCR Gamma Delta + T Cells in Disease-Free Survivors Following T Cell-Depleted, Partially Mismatched, Related Donor Bone Marrow Transplantation for Leukemia. *J Hematother* (1996) 5(5):503–9. doi: 10.1089/scd.1.1996.5.503
56. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, et al. Long Term Disease-Free Survival in Acute Leukemia Patients Recovering With Increased γδ T Cells After Partially Mismatched Related Donor Bone Marrow Transplantation. *Bone Marrow Transplantation* (2007) 39(12):751–7. doi: 10.1038/sj.bmt.1705650
57. Lin M, Zhang X, Liang S, Luo H, Alnaggar M, Liu A, et al. Irreversible Electroporation Plus Allogenic Vγ9vδ2 T Cells Enhances Antitumor Effect for Locally Advanced Pancreatic Cancer Patients. *Signal Transduction Targeted Ther* (2020) 5(1):215. doi: 10.1038/s41392-020-00260-1
58. Xu Y, Xiang Z, Alnaggar M, Kouakanou L, Li J, He J, et al. Allogeneic Vγ9vδ2 T-Cell Immunotherapy Exhibits Promising Clinical Safety and Prolongs the Survival of Patients With Late-Stage Lung or Liver Cancer. *Cell Mol Immunol* (2021) 18(2):427–39. doi: 10.1038/s41423-020-0515-7
59. Almeida AR, Correia DV, Fernandes-Platzgummer A, da Silva CL, da Silva MG, Anjos DR, et al. Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept. *Clin Cancer Res* (2016) 22(23):5795–804. doi: 10.1158/1078-0432.CCR-16-0597
60. Mirzaei HR, Mirzaei H, Lee SY, Hadjati J, Till BG. Prospects for Chimeric Antigen Receptor (CAR) γδ T Cells: A Potential Game Changer for Adoptive T Cell Cancer Immunotherapy. *Cancer Letters* (2016) 380(2):413–23. doi: 10.1016/j.canlet.2016.07.001
61. Sadelain M, Riviere I, Riddell S. Therapeutic T Cell Engineering. *Nature* (2017) 545(7655):423–31. doi: 10.1038/nature22395
62. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T Cells Expressing CD19 Chimeric Antigen Receptors for Acute Lymphoblastic Leukaemia in Children and Young Adults: A Phase 1 Dose-escalation Trial. *Lancet* (2015) 385(9967):517–28. doi: 10.1016/S0140-6736(14)61403-3
63. Park JH, Riviere I, Gonen M, Wang X, Senechal B, Curran KJ, et al. Long-Term Follow-Up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N Engl J Med* (2018) 378(5):449–59. doi: 10.1056/NEJMoa1709919
64. Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, et al. Chemotherapy-Refractory Diffuse Large B-Cell Lymphoma and Indolent B-Cell Malignancies can be Effectively Treated With Autologous T Cells Expressing an Anti-CD19 Chimeric Antigen Receptor. *J Clin Oncol* (2015) 33(6):540–9. doi: 10.1200/JCO.2014.56.2025
65. Wang M, Munoz J, Goy A, Locke FL, Jacobson CA, Hill BT, et al. KTE-X19 CAR T-Cell Therapy in Relapsed or Refractory Mantle-Cell Lymphoma. *N Engl J Med* (2020) 382(14):1331–42. doi: 10.1056/NEJMoa1914347
66. Munshi NC, Anderson LDJr., Shah N, Madduri D, Berdeja J, Lonial S, et al. Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. *N Engl J Med* (2021) 384(8):705–16. doi: 10.1056/NEJMoa2024850
67. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med* (2017) 377(26):2531–44. doi: 10.1056/NEJMoa1707447
68. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma. *N Engl J Med* (2019) 380(1):45–56. doi: 10.1056/NEJMoa1804980
69. Abramson JS, Palomba ML, Gordon LI, Lunning MA, Wang M, Arnason J, et al. Lisocabtagene Maraleucel for Patients With Relapsed or Refractory Large B-Cell Lymphomas (TRANSCEND NHL 001): A Multicentre Seamless Design Study. *Lancet* (2020) 396(10254):839–52. doi: 10.1016/S0140-6736(20)31366-0
70. Sterner RC, Sterner RM. CAR-T-Cell Therapy: Current Limitations and Potential Strategies. *Blood Cancer J* (2021) 11(4):69. doi: 10.1038/s41408-021-00459-7

71. Rischer M, Pscherer S, Duwe S, Vormoor J, Jurgens H, Rossig C. Human Gammadelta T Cells as Mediators of Chimaeric-Receptor Redirected Anti-Tumour Immunity. *Br J Haematol* (2004) 126(4):583–92. doi: 10.1111/j.1365-2141.2004.05077.x
72. Harrer DC, Simon B, Fujii S-I, Shimizu K, Uslu U, Schuler G, et al. RNA-Transfection of γ/δ T Cells With a Chimeric Antigen Receptor or an α/β T-Cell Receptor: A Safer Alternative to Genetically Engineered α/β T Cells for the Immunotherapy of Melanoma. *BMC Cancer* (2017) 17(1):551. doi: 10.1186/s12885-017-3539-3
73. Ang WX, Ng YY, Xiao L, Chen C, Li Z, Chi Z, et al. Electroporation of NKG2D RNA CAR Improves Vgamma9Vdelta2 T Cell Responses Against Human Solid Tumor Xenografts. *Mol Ther Oncolytics* (2020) 17:421–30. doi: 10.1016/j.omto.2020.04.013
74. Xiao L, Chen C, Li Z, Zhu S, Tay JC, Zhang X, et al. Large-Scale Expansion of Vγ9vδ2 T Cells With Engineered K562 Feeder Cells in G-Rex Vessels and Their Use as Chimeric Antigen Receptor–Modified Effector Cells. *Cytotherapy* (2018) 20(3):420–35. doi: 10.1016/j.jcyt.2017.12.014
75. Deniger DC, Switzer K, Mi T, Maiti S, Hurton L, Singh H, et al. Bispecific T-Cells Expressing Polyclonal Repertoire of Endogenous Gammadelta T-Cell Receptors and Introduced CD19-Specific Chimeric Antigen Receptor. *Mol Ther* (2013) 21(3):638–47. doi: 10.1038/mt.2012.267
76. Capsomidis A, Benthall G, Van Acker HH, Fisher J, Kramer AM, Abeln Z, et al. Chimeric Antigen Receptor-Engineered Human Gamma Delta T Cells: Enhanced Cytotoxicity With Retention of Cross Presentation. *Mol Ther* (2018) 26(2):354–65. doi: 10.1016/j.ymthe.2017.12.001
77. Fleischer LC, Becker SA, Ryan RE, Fedanov A, Doering CB, Spencer HT. Non-Signaling Chimeric Antigen Receptors Enhance Antigen-Directed Killing by Gammadelta T Cells in Contrast to Alphabeta T Cells. *Mol Ther Oncolytics* (2020) 18:149–60. doi: 10.1016/j.omto.2020.06.003
78. Rozenbaum M, Meir A, Aharony Y, Itzhaki O, Schachter J, Bank I, et al. Gamma-Delta CAR-T-Cells Show CAR-Directed and Independent Activity Against Leukemia. *Front Immunol* (2020) 11:1347. doi: 10.3389/fimmu.2020.01347
79. Wallet M, Nishimura T, Del Casale C, Lebid A, Salantes B, Santostefano K, et al. Induced Pluripotent Stem Cell-Derived Gamma Delta CAR-T-Cells for Cancer Immunotherapy. *Blood* (2021) 138Supplement 1):2771. doi: 10.1182/blood-2021-149095
80. Siegers GM, Lamb LSJr. Cytotoxic and Regulatory Properties of Circulating Vdelta1+ Gammadelta T Cells: A New Player on the Cell Therapy Field? *Mol Ther* (2014) 22(8):1416–22. doi: 10.1038/mt.2014.104
81. Makkouk A, Yang XC, Barca T, Lucas A, Turkoz M, Wong JTS, et al. Off-The-Shelf Vdelta1 Gamma Delta T Cells Engineered With Glypican-3 (GPC-3)-Specific Chimeric Antigen Receptor (CAR) and Soluble IL-15 Display Robust Antitumor Efficacy Against Hepatocellular Carcinoma. *J Immunother Cancer* (2021) 9(12):e003441. doi: 10.1136/jitc-2021-003441
82. Nishimoto KP, Barca T, Azameera A, Makkouk A, Romero JM, Bai L, et al. Allogeneic CD20-Targeted Gammadelta T Cells Exhibit Innate and Adaptive Antitumor Activities in Preclinical B-Cell Lymphoma Models. *Clin Transl Immunol* (2022) 11(2):e1373. doi: 10.1002/cti2.1373
83. Benyamine A, Loncle C, Foucher E, Blazquez JL, Castanier C, Chretien AS, et al. BTN3A Is a Prognosis Marker and a Promising Target for Vgamma9Vdelta2 T Cells Based-Immunotherapy in Pancreatic Ductal Adenocarcinoma (PDAC). *Oncoimmunology* (2017) 7(1):e1372080. doi: 10.1080/2162402X.2017.1372080
84. De Gassart A, Le KS, Brune P, Agaugue S, Sims J, Goubard A, et al. Development of ICT01, a First-in-Class, Anti-BTN3A Antibody for Activating Vgamma9Vdelta2 T Cell-Mediated Antitumor Immune Response. *Sci Transl Med* (2021) 13(616):eabj0835. doi: 10.1126/scitranslmed.abj0835
85. Wang S, Chen K, Lei Q, Ma P, Yuan AQ, Zhao Y, et al. The State of the Art of Bispecific Antibodies for Treating Human Malignancies. *EMBO Mol Med* (2021) 13(9):e14291. doi: 10.15252/emmm.202114291
86. Singh A, Dees S, Grewal IS. Overcoming the Challenges Associated With CD3+ T-Cell Redirection in Cancer. *Br J Cancer* (2021) 124(6):1037–48. doi: 10.1038/s41416-020-01225-5
87. Labrijn AF, Janmaat ML, Reichert JM, Parren P. Bispecific Antibodies: A Mechanistic Review of the Pipeline. *Nat Rev Drug Discovery* (2019) 18(8):585–608. doi: 10.1038/s41573-019-0028-1
88. Topp MS, Gokbuget N, Zugmaier G, Degenhard E, Goebeler ME, Klinger M, et al. Long-Term Follow-Up of Hematologic Relapse-Free Survival in a Phase 2 Study of Blinatumomab in Patients With MRD in B-Lineage ALL. *Blood* (2012) 120(26):5185–7. doi: 10.1182/blood-2012-07-441030
89. Einsele H, Borghaei H, Orlowski RZ, Subklewe M, Roboz GJ, Zugmaier G, et al. The BiTE (Bispecific T-Cell Engager) Platform: Development and Future Potential of a Targeted Immuno-Oncology Therapy Across Tumor Types. *Cancer* (2020) 126(14):3192–201. doi: 10.1002/cncr.32909
90. Jain T, Litzow MR. Management of Toxicities Associated With Novel Immunotherapy Agents in Acute Lymphoblastic Leukemia. *Ther Adv Hematol* (2020) 11:2040620719899897. doi: 10.1177/2040620719899897
91. Strohl WR, Naso M. Bispecific T-Cell Redirection Versus Chimeric Antigen Receptor (CAR)-T Cells as Approaches to Kill Cancer Cells. *Antibodies (Basel)* (2019) 8(3):41. doi: 10.3390/antib8030041
92. Teachey DT, Rheingold SR, Maude SL, Zugmaier G, Barrett DM, Seif AE, et al. Cytokine Release Syndrome After Blinatumomab Treatment Related to Abnormal Macrophage Activation and Ameliorated With Cytokine-Directed Therapy. *Blood* (2013) 121(26):5154–7. doi: 10.1182/blood-2013-02-485623
93. Koristka S, Cartellieri M, Arndt C, Feldmann A, Seliger B, Ehninger G, et al. Tregs Activated by Bispecific Antibodies: Killers or Suppressors? *OncoImmunology* (2015) 4(3):1–3. doi: 10.4161/2162402X.2014.994441
94. Oberg HH, Janitschke L, Sulaj V, Weimer J, Gonnermann D, Hedemann N, et al. Bispecific Antibodies Enhance Tumor-Infiltrating T Cell Cytotoxicity Against Autologous HER-2-Expressing High-Grade Ovarian Tumors. *J Leukoc Biol* (2020) 107(6):1081–95. doi: 10.1002/JLB.5MA1119-265R
95. Oberg HH, Kellner C, Gonnermann D, Peipp M, Peters C, Sebens S, et al. Gammadelta T Cell Activation by Bispecific Antibodies. *Cell Immunol* (2015) 296(1):41–9. doi: 10.1016/j.cellimm.2015.04.009
96. Oberg HH, Peipp M, Kellner C, Sebens S, Krause S, Petrick D, et al. Novel Bispecific Antibodies Increase Gammadelta T-Cell Cytotoxicity Against Pancreatic Cancer Cells. *Cancer Res* (2014) 74(5):1349–60. doi: 10.1158/0008-5472.CAN-13-0675
97. Ganesan R, Chennupati V, Ramachandran B, Hansen MR, Singh S, Grewal IS. Selective Recruitment of Gammadelta T Cells by a Bispecific Antibody for the Treatment of Acute Myeloid Leukemia. *Leukemia* (2021) 35(8):2274–84. doi: 10.1038/s41375-021-01122-7
98. de Silva S, Lai A, Patel A, Evans KJ, Yoo K, Jin K, et al. Antigen-Specific Targeting of Tissue-Resident Gamma Delta T Cells With Recombinant Butyrophilin Heterodimeric Fusion Proteins. *Am Assoc Cancer Res* (2021) 81(13_Supplement):1736. doi: 10.1158/1538-7445.AM2021-1736
99. Arbabi-Ghahroudi M. Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook. *Front Immunol* (2017) 8(NOV). doi: 10.3389/fimmu.2017.01589
100. Muyldermans S. Nanobodies: Natural Single-Domain Antibodies. *Annu Rev Biochem* (2013) 82:775–97. doi: 10.1146/annurev-biochem-063011-092449
101. Kunz P, Zinner K, Mucke N, Bartoschik T, Muyldermans S, Hoheisel JD. The Structural Basis of Nanobody Unfolding Reversibility and Thermoresistance. *Sci Rep* (2018) 8(1):e1375641. doi: 10.1038/s41598-018-26338-z
102. de Bruin RCG, Veluchamy JP, Lougheed SM, Schneiders FL, Lopez-Lastra S, Lameris R, et al. A Bispecific Nanobody Approach to Leverage the Potent and Widely Applicable Tumor Cytolytic Capacity of Vγ9vδ2-T Cells. *OncoImmunology* (2018) 7(1). doi: 10.1080/2162402X.2017.1375641
103. de Weerdt I, Lameris R, Scheffer GL, Vree J, de Boer R, Stam AG, et al. A Bispecific Antibody Antagonizes Prosurvival CD40 Signaling and Promotes Vγ9vδ2 T Cell-Mediated Antitumor Responses in Human B-Cell Malignancies. *Cancer Immunol Res* (2021) 9(1):50–61. doi: 10.1158/2326-6066.CIR-20-0138
104. de Weerdt I, Lameris R, Ruben JM, de Boer R, Kloosterman J, King LA, et al. A Bispecific Single-Domain Antibody Boosts Autologous Vgamma9Vdelta2-T Cell Responses Toward CD1d in Chronic Lymphocytic Leukemia. *Clin Cancer Res* (2021) 27(6):1744–55. doi: 10.1158/1078-0432.CCR-20-4576
105. Lameris R, de Bruin RC, van Bergen En Henegouwen PM, Verheul HM, Zweegman S, de Gruijl TD, et al. Generation and Characterization of CD1d-Specific Single-Domain Antibodies With Distinct Functional Features. *Immunology* (2016) 149(1):111–21. doi: 10.1111/imm.12635
106. Lamb LS, Pereboeva L, Youngblood S, Gillespie GY, Nabors LB, Markert JM, et al. A Combined Treatment Regimen of MGMT-Modified γδ T Cells

and Temozolomide Chemotherapy Is Effective Against Primary High Grade Gliomas. *Sci Rep* (2021) 11(1):21133. doi: 10.1038/s41598-021-00536-8

107. Lamb LS, Bowersock J, Dasgupta A, Gillespie GY, Su Y, Johnson A, et al. Engineered Drug Resistant γδ T Cells Kill Glioblastoma Cell Lines During a Chemotherapy Challenge: A Strategy for Combining Chemo- and Immunotherapy. *PloS One* (2013) 8(1):e51805. doi: 10.1371/journal.pone.0051805
108. van Diest E, Hernandez Lopez P, Meringa AD, Vyborova A, Karaiskaki F, Heijhuurs S, et al. Gamma Delta TCR Anti-CD3 Bispecific Molecules (GABs) as Novel Immunotherapeutic Compounds. *J Immunother Cancer* (2021) 9(11):e003850. doi: 10.1136/jitc-2021-003850
109. Barber A, Wang X, Gopisetty A, Mirandola L, Chiriva-Internati M. Abstract LB148: Gamma Delta T Cells Engineered With a Chimeric PD-1 Receptor Effectively Control PD-L1 Positive Tumors In Vitro and *In Vivo* With Minimal Toxicities. *Cancer Res* (2021) 81Supplement):LB148–LB. doi: 10.1158/1538-7445.AM2021-LB148

Conflict of Interest: JS-E, MJ and LK are funded by Lava therapeutics. HV, PP, EE, BW are employed by and hold stock of LAVA Therapeutics. TG holds stock of LAVA Therapeutics.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 17 June 2022
doi: 10.3389/fimmu.2022.872122

CD137 Costimulation Enhances the Antitumor Activity of Vγ9Vδ2-T Cells in IL-10-Mediated Immunosuppressive Tumor Microenvironment

*Yujun Pei[1,2], Zheng Xiang[1], Kun Wen[1], Chloe Ran Tu[3], Xiwei Wang[1], Yanmei Zhang[1], Xiaofeng Mu[1], Yinping Liu[1] and Wenwei Tu[1]**

[1] Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong SAR, China, [2] Bioland Laboratory (Guangzhou Regenerative Medicine and Health Guangdong Laboratory), Guangzhou, China, [3] Computational and Systems Biology Interdepartmental Program, University of California, Los Angeles, Los Angeles, CA, United States

OPEN ACCESS

Edited by:
Andy Hee-Meng Tan, Bioprocessing Technology Institute (A*STAR), Singapore

Reviewed by:
Graham Robert Leggatt, The University of Queensland, Australia
Alice Cheung, Singapore General Hospital, Singapore

***Correspondence:**
Wenwei Tu
wwtu@hku.hk

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 09 February 2022
Accepted: 17 May 2022
Published: 17 June 2022

Citation:
Pei Y, Xiang Z, Wen K, Tu CR, Wang X, Zhang Y, Mu X, Liu Y and Tu W (2022) CD137 Costimulation Enhances the Antitumor Activity of Vγ9Vδ2-T Cells in IL-10-Mediated Immunosuppressive Tumor Microenvironment. Front. Immunol. 13:872122. doi: 10.3389/fimmu.2022.872122

Although γδ-T cell-based tumor immunotherapy using phosphoantigens to boost γδ-T cell immunity has shown success in some cancer patients, the clinical application is limited due to the rapid exhaustion of Vγ9Vδ2-T cells caused by repetitive stimulation from phosphoantigens and the profoundly immunosuppressive tumor microenvironment (TME). In this study, using a cell culture medium containing human and viral interleukin-10 (hIL-10 and vIL-10) secreted from EBV-transformed lymphoblastoid B cell lines (EBV-LCL) to mimic the immunosuppressive TEM, we found that the antitumor activity of Vγ9Vδ2-T cells was highly suppressed by endogenous hIL-10 and vIL-10 within the TME. CD137 costimulation could provide an anti-exhaustion signal to mitigate the suppressive effects of IL-10 in TME by suppressing IL-10R1 expression on Vγ9Vδ2-T cells. CD137 costimulation also improved the compromised antitumor activity of Vγ9Vδ2-T cells in TME with high levels of IL-10 in Rag2$^{-/-}$ γc$^{-/-}$ mice. In humanized mice, CD137 costimulation boosted the therapeutic effects of aminobisphosphonate pamidronate against EBV-induced lymphoma. Our study offers a novel approach to overcoming the obstacle of the hIL-10 and vIL-10-mediated immunosuppressive microenvironment by costimulating CD137 and enhancing the efficacy of γδ-T cell-based tumor therapy.

Keywords: CD137, γδ-T cells, antitumor acitivity, IL-10, immunotherapy

INTRODUCTION

Epstein-Barr virus (EBV) is a predominant type of human herpesviruses. It infects over 95% of the population by adulthood (1, 2). EBV infection is highly correlated with several human malignancies (1–3). As the first known human tumor virus, the carcinogenesis of EBV has been identified in various hematopoietic and epithelial cell cancers, including EBV-associated tumors and lymphoproliferative disorder (1, 2, 4). Current therapeutic approaches for EBV-associated tumors are restricted by undesirable side effects and ineffectiveness for refractory or relapsed

diseases (5, 6). It was reported that EBV-specific CTL-based therapy is effective in the control of EBV-associated malignancy (5, 6). However, its clinical application is hampered due to insufficient quantity of EBV-specific CTL generated *ex vivo* (7).

As a major subset of human γδ-T cells, Vγ9Vδ2-T cells have been extensively demonstrated to have promising anti-tumor effects (8–13). Vγ9Vδ2-T cells can be activated specifically by phosphoantigens from isoprenoid biosynthesis in an MHC-unrestricted manner. Aminobisphosphonates pamidronate (PAM) and zoledronate (ZOL) are commonly used pharmacological phosphoantigens for osteoporosis and Paget's disease treatment (11, 14). Previously, we demonstrated that direct administration of PAM could expand Vγ9Vδ2-T cells *in vivo* and thus control EBV-induced lymphoma in humanized mice, suggesting that Vγ9Vδ2-T cell-based immunotherapy is promising for treating EBV-associated tumors (15). A recent meta-analysis of about 18,000 human cancers revealed that tumor-infiltrating γδ T cells are the most favorable cancer-wide prognostic marker (16). However, the clinical application was limited by the rapid exhaustion of Vγ9Vδ2-T cells caused by the repetitive stimulation from phosphoantigens *in vivo* (17) and the profoundly immunosuppressive tumor microenvironment (TME) (18–20).

Interleukin (IL)-10, as a major immunosuppressive cytokine in TME secreted by tumor cells, can help tumor cells escape immunological recognition and destruction (21–24). Current evidence indicates that EBV codes a homologue of human IL-10 (vIL-10) with immunosuppressive properties to evade immunity and establish persistent/latent infections (25–28). EBV-LCL also express and release various amounts of human IL-10 (hIL-10) (29, 30). hIL-10 and vIL-10 are crucial for B cell transformation of B cell (31, 32) and oncogenesis of EBV-associated tumors (33). However, whether the antitumor activity of Vγ9Vδ2-T cells was suppressed by IL-10 in TME remained largely unknown.

CD137 (4-1BB), a membrane-bound receptor, is a costimulatory molecule expressed in many lymphocytes (34–36). Recently, we demonstrated that CD137 costimulation enhanced the activation and cytolytic activity of Vγ9Vδ2-T cells against virus-infected cells (37). Importantly, boosting cancer immunotherapy with agonistic CD137 antibodies has been demonstrated to be a promising therapeutic strategy for different tumors (38, 39). However, the roles of CD137 signaling for human Vγ9Vδ2-T cells in the immunosuppressive TME remained to be determined.

In this study, we aim to clarify whether IL-10 in the TME is responsible for the exhaustion of Vγ9Vδ2-T cells and determine whether targeting CD137 can enhance the antitumor activity of Vγ9Vδ2-T cells compromised by the immunosuppressive TME.

MATERIALS AND METHODS

Vγ9Vδ2-T Cell Cultures

hPBMC were isolated from buffy coats by Ficoll-Hypaque gradient centrifugation of EBV^+ healthy donors after informed consents were obtained. PAM-expanded Vγ9Vδ2-T cells were prepared according to the protocol we established before (40). Briefly, hPBMC were cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) in the presence of PAM from day 0 to day 3 at a concentration of 9μg/ml. Recombinant human IL-2 was added to medium from day 3 to day 14 at a concentration of 500 IU/ml. After 2 weeks, the γδ-T cells were purified by positive selection with α-TCRγ/δ MicroBead (Miltenyi Biotec).

Cytotoxic Assay

Purified Vγ9Vδ2-T cells were cultured with $IL\text{-}10^{low}$ or $IL\text{-}10^{high}$ conditioned medium for 24h, RPMI 1640 with 10% FBS medium (plain medium, PM) as a control. The pretreated Vγ9Vδ2-T cells (effector cells, E) were cocultured with autologous EBV-LCL (target cells, T) at an E: T ratio of 10:1 for 4 to 6 h in the $IL\text{-}10^{low/high}$ CM or PM, and then the death of target cells was analyzed with flow cytometry. Cells were stained with anti-CD3 to identify Vγ9Vδ2-T cells and propidium iodide (PI) was used to identify dead cells. The death of EBV-LCL was shown as the percentage of PI^+ cells in the $CD3^-$ population (40). In some experiments, neutralizing antibody against IL-10 (abcam) was added to block IL-10 mediated pathways. To confirm the suppressive role of IL-10 in the CM, recombinant hIL-10 (Peprotech) or recombinant vIL-10 (R&D systems) was added to culture medium at the indicated concentration.

Establishment of EBV-LCL

EBV-secreting cell lines B95-8 and B95.8EBfaV-GFP were cultured and EBV-containing supernatants were collected for the following infection. hPBMC were incubated with EBV-containing supernatants, and then cultured in the RPMI 1640 medium containing 15% FBS with the addition of cyclosporine-A (1μg/ml) as we describe before (15).

Collection of EBV-LCL Conditioned Medium

EBV-LCL were cultured in RPMI1640 medium for 24 h. The conditioned medium (CM) was collected, centrifuged at 5000 rpm at 4°C for 10 min to remove cell debris and then frozen at −80°C in aliquots. Stored CM was passed through a 0.22-μm syringe filter (Millipore) before use. Plain medium (PM) collected from complete medium without cell incubation under the same experimental conditions served as the control for CM.

Determination of hIL-10 and vIL-10 Levels

For hIL-10, the concentrations in conditioned medium were measured by ELISA. The procedures for human IL-10 ELISA kits (Biolegend, San Diego, CA, USA) were performed based on the manufacturer's instructions. For vIL-10, the concentrations in conditioned medium were measured according to the method described before (41). The conditioned medium was concentrated by Amicon-Ultra centrifugation filters (Millipore) following the manufacturer's instructions. Then, the concentrated conditioned medium was used for performing Western blot assay. Mouse monoclonal antibody against vIL-10 (R&D) was used as primary antibody for incubating transferred membranes at 4°C overnight. Horseradish peroxide

conjugated goat anti-mouse secondary antibody (R&D) was used as secondary antibody for detecting vIL-10 levels. The bands of Western blot were quantified by "Gels" analysis tool of ImageJ. Recombinant vIL-10 was used as a standard to quantify the vIL-10 level in conditioned medium.

Establishment and Treatment of EBV-Associated Lymphoma in Mice

All animal studies were approved and performed in compliance with the guidelines for the use of experimental animals by the Committee on the Use of Live Animals in the Teaching and Research, the University of Hong Kong. $Rag2^{-/-}\gamma c^{-/-}$ mice were bred in Centre for Comparative Medicine Research of the University of Hong Kong. Humanized mice were generated according to the protocol we established before (14, 15). $Rag2^{-/-}\gamma c^{-/-}$ or humanized mice were inoculated with EBV-LCL expressing high or low level of IL-10 (0.1×10^6/mouse) by subcutaneous injection to establish the EBV-associated lymphoma model. For $Rag2^{-/-}\gamma c^{-/-}$ mice, PAM-expanded Vγ9Vδ2-T cells (5×10^6/mouse) with or without the addition of SA-hCD137L (5μg/mouse) were adoptively transferred intravenously into EBV-associated lymphoma murine model at indicated time. For humanized mice, PAM (5mg/kg body weight) and SA-hCD137L (15μg/mouse) were injected intraperitoneally at the indicated time. The mice treated with an equivalent volume of PBS or SA were used as controls. The tumor volume and mice survival were monitored every day and calculated at the indicated time. Mice were counted as dying when their subcutaneous tumor diameter was larger than 17 mm and thus sacrificed according to the regulation of Centre for Comparative Medicine Research of the University of Hong Kong. Otherwise, mice were monitored for 100 days before being sacrificed. The tumor tissues were reserved for immunohistochemical evaluation.

Preparation of the Recombinant SA-hCD137L Protein

Recombinant SA-hCD137L proteins were generated as described before (37). Briefly, the DNA sequences were synthesized encoding the extracellular domain of human CD137L (a.a. 58-254) and the core streptavidin (SA; a.a. 16-133) with an N-terminal 6×His tag. The recombinant SA-hCD137L protein was expressed in *E. coli* by inserting the SA-hCD137L DNA fragments into the pETH expression vector and transforming into competent cells. After purifying with Ni-nitrilotriacetic acid affinity chromatography (QIAGEN, Germany), the recombinant SA-hCD137L protein was filtered a and quantitated by BCA Protein Assay Kit (Pierce, USA).

Flow Cytometric Analysis

Cells were stained for surface molecules with the following antibodies: αIL10R (Miltenyi Biotec, clone REA239), αCD3 (Biolegend, clone HIT3a), αTCRγ9 (Biolegend, clone B3), αTCRVδ2 (Biolegend, clone B6), and αCD137 (Biolegend, clone 4B4-1). All samples were performed with a FACS LSR II (BD). The results were analyzed with FlowJo software.

Histological Staining and Immunohistochemical Assays

The tumor tissues were fixed with 10% formalin for 24 h and maintained in 70% ethanol. Fixed tumor tissues were embedded in paraffin and sectioned. The tumor sections were performed immunohistochemistry staining with αIL-10 antibody (abcam) (42).

Statistics

Data are shown in the form of mean ± standard error of the mean (SEM). All data were tested by Shapiro-Wilk test to verify the normality. For data that did not meet normal distribution, Mann-Whitney U test was used for analysis. For data that met normal distribution, one-way analysis of variance (ANOVA) with Bonferroni correction was used for analysis. For multiple variables, two-way ANOVA was used. Kaplan-Meier log-rank test was used for comparing survival among different groups. Two-tailed test was used for all analyses. $P < 0.05$ was regarded as significant.

RESULTS

Antitumor Activity of Vγ9Vδ2-T Cells Was Inhibited by IL-10 Secreted From EBV-LCL *In Vitro*

To investigate the effects of IL-10 in TME on the antitumor activity of Vγ9Vδ2-T cells, conditioned medium (CM) was obtained by collecting the supernatant of EBV-LCL culture for modeling TME *in vitro*. As shown in **Figure 1A**, CM from EBV-LCL culture established from different donors contained distinct levels of IL-10, and vIL-10 accounted for about 9.56 ± 5.74% of total IL-10. CM collected from EBV-LCL1 and EBV-LCL6, which contained the lowest and highest concentrations of IL-10, was used as $IL\text{-}10^{low}$ CM and $IL\text{-}10^{high}$ CM, respectively, in the following experiments. Importantly, the cytotoxic activity of $IL\text{-}10^{high}$ CM-treated Vγ9Vδ2-T cells against EBV-LCL was significantly lower than $IL\text{-}10^{low}$ CM- or PM-treated Vγ9Vδ2-T cells (**Figure 1B**). To verify the immunosuppressive role of IL-10 in the CM, an IL-10 neutralizing mAb was applied to block IL-10 signaling during Vγ9Vδ2-T cells exposed to $IL\text{-}10^{high/low}$ CM. The reduced cytotoxicity of Vγ9Vδ2-T cells against EBV-LCL was significantly abrogated when blocked with the IL-10 neutralizing mAb (**Figure 1C**). Furthermore, both hIL-10 and vIL-10 recombinant proteins showed dose-dependent inhibitions in the cytotoxicity of Vγ9Vδ2-T cells against EBV-LCL in the PM (**Figures 1D, E**). Taken together, our data indicate that the antitumor activity of Vγ9Vδ2-T cells against EBV-LCL was suppressed by both the hIL-10 and vIL-10 in the CM from EBV-LCL *in vitro*.

Antitumor Activity of Vγ9Vδ2-T Cells Against EBV-Induced Lymphoma Was Decreased Under $IL\text{-}10^{high}$ TME *In Vivo*

To determine whether the therapeutic effects of Vγ9Vδ2-T cells on EBV-induced B cell lymphoma were inhibited by IL-10 within the TME, EBV-LCL1 expressing low levels of IL-10 ($IL\text{-}10^{low}$ LCL) and EBV-LCL6 expressing high levels of IL-10 ($IL\text{-}10^{high}$ LCL) were inoculated into $Rag2^{-/-}$ $\gamma c^{-/-}$ mice, respectively

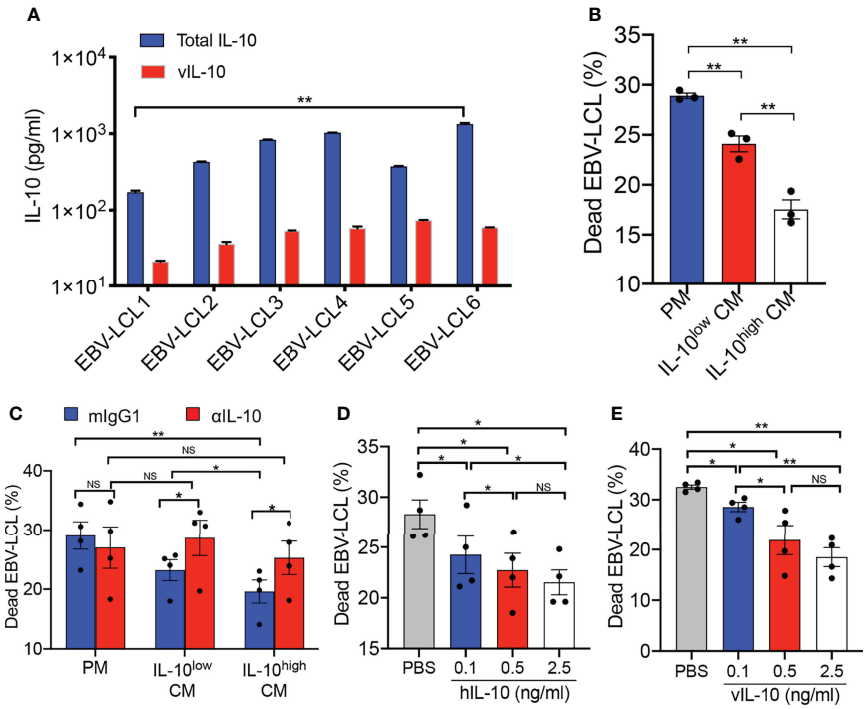


FIGURE 1 | Antitumor activity of Vγ9Vδ2-T cells was inhibited by IL-10 secreted from EBV-LCL *in vitro*. **(A)** The concentration of vIL-10 and total IL-10 in conditioned medium (CM) collected from EBV-LCL established from different donors were detected. **(B)** Purified Vγ9Vδ2-T cells were pretreated in the IL-10^{low} CM and IL-10^{high} CM separately for 24 h, RPMI 1640 with 10% FBS medium (plain medium, PM) as a control. Pretreated Vγ9Vδ2-T cells then cocultured with autologous EBV-LCL at an effector: target (E:T) ratio of 10:1 for 4–6 h in the IL-10$^{low/high}$ CM and PM, respectively. Cytotoxicity was calculated as the proportion of dead EBV-LCL (CD3$^-$PI$^+$). **(C)** Purified Vγ9Vδ2-T cells were pretreated with IL-10^{low} CM, IL-10^{high} CM or PM in the presence of a neutralizing anti-IL-10 mAb (αIL-10, 5μg/ml) or isotype control (mIgG1, 5μg/ml), then cocultured with autologous EBV-LCL at an E:T ratio of 10:1 for 4–6 h in the IL-10$^{low/high}$ CM and PM respectively. Cytotoxicity was calculated as the proportion of dead EBV-LCL (CD3$^-$ PI$^+$). **(D, E)** Purified Vγ9Vδ2-T cells were pretreated with recombinant hIL-10 **(D)** or vIL-10 **(E)** at different concentration, then cocultured with autologous EBV-LCL at an E:T ratio of 10:1 for 4–6h. The proportion of dead EBV-LCL (CD3$^-$ PI$^+$) were detected by flow cytometry. All data are shown as mean ± SEM and representative of three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

(**Figure 2A**). After 21 days, large subcutaneous tumors developed in all the mice as detected by *in vivo* imaging (**Figures 2B, C**). The expressions of IL-10 in the tumor tissues generated from IL-10^{low} LCL and IL-10^{high} LCL were detected by immunohistochemistry (**Figure 2D**). Consistent with our previous results (15), Vγ9Vδ2-T cell treatment constrained tumor growth and prolonged the survival of tumor-bearing mice in contrast with the mice treated with PBS as the control (**Figures 2E, F**). Importantly, Vγ9Vδ2-T cells showed less efficacy in controlling EBV-induced lymphoma developed from IL-10^{high} LCL compared with that developed from IL-10^{low} LCL, along with larger tumor volume and lower survival rates (**Figures 2E, F**). These results suggest that the decreased antitumor activity of Vγ9Vδ2-T cells against EBV-induced lymphoma may be associated with IL-10^{high} TME *in vivo*.

CD137 Costimulation Suppressed IL-10R1 Expression and Restored the Antitumor Activity of Vγ9Vδ2-T Cells

IL-10 mediates its biological effects mainly through a heterodimeric membrane receptor composed of IL-10R1 and IL-10R2 (43). Since IL-10R2 is shared by more than five IL-10 family cytokines (44), we investigated the expression of IL-10R1 on Vγ9Vδ2-T cells exposed to the IL-10^{high} CM upon γδ-TCR activation *in vitro*. Importantly, we found that following activation, IL-10R1$^+$ Vγ9Vδ2-T cell subset expressed high levels of CD137 compared with IL-10R1$^{-/lo}$ Vγ9Vδ2-T cell subset in the IL-10^{high} CM, indicating that CD137 could be an effective costimulatory signaling to restore the antitumor activity of Vγ9Vδ2-T cells compromised by the IL-10 in TME (**Figures 3A, B**).

To determine whether CD137 costimulation could provide an anti-exhaustion signal to mitigate the inhibiting effects mediated by IL-10 in TME, a recombinant SA-hCD137L protein containing a core streptavidin (SA) molecule with the extracellular domains of human CD137L (hCD137L) was generated as we reported previously (37). We found that the addition of the recombinant SA-hCD137L protein significantly inhibited the surface expression of IL-10R1 in total and CD137$^+$ Vγ9Vδ2-T cells in IL-10^{high} CM in terms of both percentage and expression level (MFI) changes (**Figures 3C–E**). These results indicate that CD137 costimulation suppressed IL-10R1

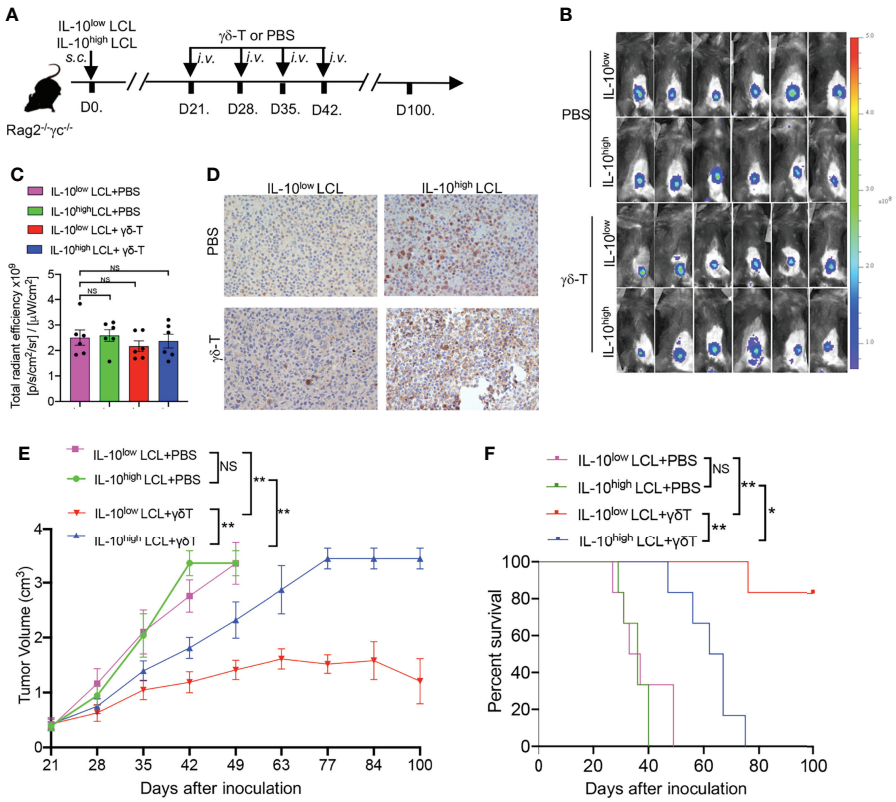


FIGURE 2 | Antitumor activity of Vγ9Vδ2-T cells against EBV-induced lymphoma was decreased under IL-10^{high} TME *in vivo*. **(A)** IL-10^{low} LCL and IL-10^{high} LCL were injected *s.c.* in Rag2$^{-/-}$γc$^{-/-}$ mice separately. After 21 days, mice that had developed subcutaneous tumor were randomly divided into two groups respectively followed by the treatment with allogeneic Vγ9Vδ2-T cells or PBS at indicated time (six mice per group). **(B, C)** Whole-body fluorescence images **(B)** and total radiant efficiency **(C)** of mice before treatment with Vγ9Vδ2-T cells or PBS. **(D)** Representative histology of IL-10 in tumor sections that developed from IL-10^{low} LCL and IL-10^{high} LCL. **(E, F)** The tumor volume **(E)** and mouse survival **(F)** were determined at the indicated time. The tumor volume was compared using two-way ANOVA analysis, and mice survival was compared using Kaplan-Meier log-rank test. Data are representative for three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

expression in CD137^{+} Vγ9Vδ2-T cells, and thereby was able to reduce their sensitivity to endogenous IL-10 in the immunosuppressive TME.

To determine whether CD137 costimulation could rescue the impaired antitumor efficacy of Vγ9Vδ2-T cells in suppressive TME, the recombinant SA-hCD137L protein was added to the coculture of Vγ9Vδ2-T cells with EBV-LCL in IL-10^{high} CM for mimicking the tumor milieu. As shown in **Figure 3F**, the SA-hCD137L protein significantly increased the cytotoxicity of Vγ9Vδ2-T cells against EBV-LCL under both the immunosuppressive and normal microenvironments mimicked by the IL-10^{high} CM and the PM. Importantly, CD137 costimulation not only completely restored the reduced cytotoxicity of Vγ9Vδ2-T cells in the IL-10^{high} CM to normal levels, but also had a better effect to enhance the cytotoxic activity of Vγ9Vδ2-T cells in IL-10^{high} CM than that in PM (**Figure 3F**). These data demonstrate that CD137 engagement enables Vγ9Vδ2-T cells to withstand the hostile environment mediated by endogenous IL-10, resulting in the increase of the antitumor activity of Vγ9Vδ2-T cells *in vitro*.

CD137 Costimulation Enhanced the Compromised Antitumor Activity of Vγ9Vδ2-T Cells With IL-10^{high} TME in Rag2$^{-/-}$ γc$^{-/-}$ Mice

Previously we have demonstrated that Vγ9Vδ2-T cells could control EBV-inducing lymphoma (15), and their antitumor activity in controlling EBV-induced lymphoma developed from IL-10^{high} LCL was lower than that developed from IL-10^{low} LCL (**Figures 2E, F**). To further elucidate the roles of CD137 costimulation in the compromised antitumor activity of Vγ9Vδ2-T cells in IL-10^{high} TME *in vivo*, EGFP-expressing IL-10^{high} LCL was inoculated in Rag2$^{-/-}$ γc$^{-/-}$ mice, *s.c.* (**Figure 4A**). Twenty-one days later, mice bearing subcutaneous tumors were randomly divided into three groups as detected by *in vivo* imaging (**Figure 4B**). No significant differences were found in fluorescent density from tumor cells among the three groups after 21 days of tumor cell inoculation (**Figure 4C**). PAM-expanded Vγ9Vδ2-T cells were adoptively transferred to one group of the tumor-bearing mice with the recombinant SA-hCD137L protein weekly from day 21 to day 42. The other two groups of mice were adoptively

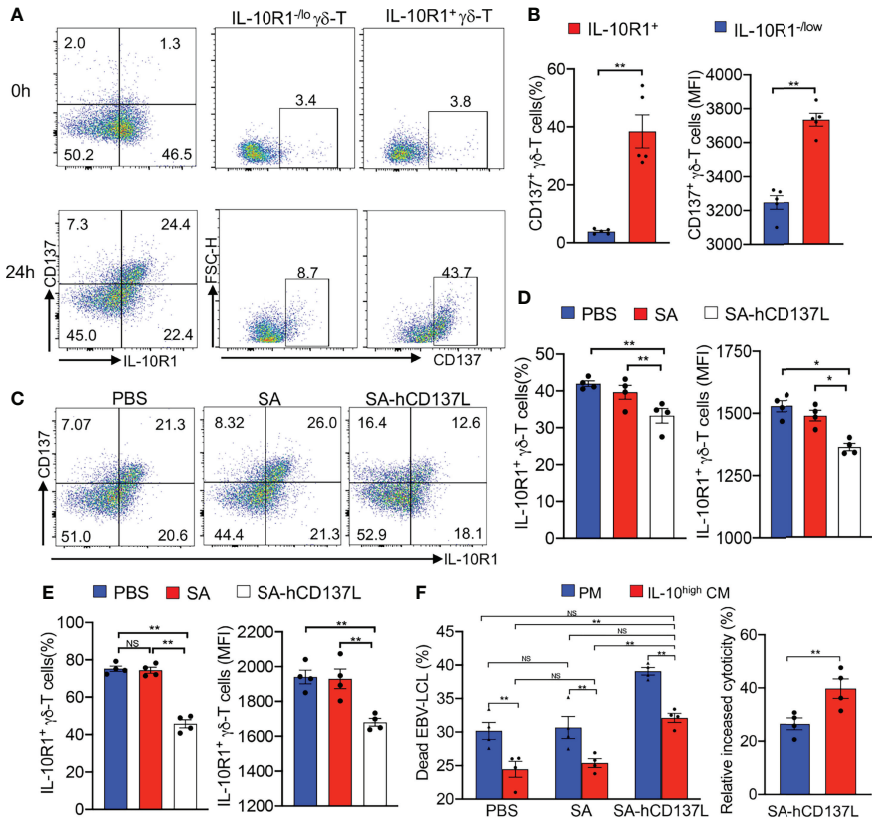


FIGURE 3 | CD137 costimulation suppressed IL-10R1 expression and restored the antitumor activity of Vγ9Vδ2-T cells. **(A)** The expression of CD137 on IL-10R1$^{-/lo}$ and IL-10R1^{+} Vγ9Vδ2-T cells before (0h) and after stimulation with anti-γδ-TCR mAb for 24h (24h) in IL-10^{high} CM. **(B)** The percentages and expression levels (mean fluorescence intensities, MFI) of CD137 on IL-10R1$^{-/lo}$ and IL-10R1^{+} Vγ9Vδ2-T cells upon stimulation for 24h in IL-10^{high} CM. **(C, E)** The FACS patterns of CD137 and IL-10R1 expressions **(C)**, surface expression of IL-10R1 in total Vγ9Vδ2-T cells **(D)** and in CD137^{+} Vγ9Vδ2-T cells **(E)** upon stimulation by anti-γδ-TCR mAb supplemented with SA-CD137L (500ng/ml), PBS and SA in IL-10^{high} CM for 24h were detected by flow cytometry after surface staining of IL-10R1. **(F)** Purified Vγ9Vδ2-T cells were pretreated with SA-hCD137L (500ng/ml), PBS or SA for 24 h in the PM and IL-10^{high} CM, and then cocultured with autologous EBV-LCL at an E: T ratio of 10:1 for 4-6h. The proportions of dead EBV-LCL (CD3^{-} PI^{+}, left), and the relative increase of cytotoxicity after being treated with SA-hCD137L (right) are shown. All data are shown as mean ± SEM and representative of three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

transferred with Vγ9Vδ2-T cells in the presence of PBS or SA as the controls. Importantly, Vγ9Vδ2-T cells in combination with SA-hCD137L treatment significantly limited tumor growth (**Figure 4D**) and improved mouse survival (**Figure 4E**) compared to treatments of Vγ9Vδ2-T cells with PBS or SA. These data indicate that the costimulation of CD137 efficiently enhanced the antitumor activity of Vγ9Vδ2-T cells in the highly immunosuppressive microenvironment mediated by IL-10 *in vivo*.

CD137 Costimulation Improved the Therapeutic Effect of PAM in Controlling EBV-Induced Lymphoma With IL-10^{high} TME in Humanized Mice

Previously we had demonstrated that PAM could expand Vγ9Vδ2-T cells *in vivo* to control EBV-induced lymphoma in humanized mice with functional hPBMC (15). We then investigated the role of CD137 costimulation on the therapeutic effect of PAM in controlling EBV-induced lymphoma with IL-10^{high} TME in humanized mice. EBV-induced lymphoma with IL-10^{high} TME model was generated by inoculation *s.c.* of IL-10^{high} EBV-LCL in humanized mice (**Figure 5A**) (15). All humanized mice developed subcutaneous tumors after IL-10^{high} EBV-LCL inoculation for 28 days with similar fluorescent density from tumor cells as detected by *in vivo* imaging (**Figures 5B, C**). PAM, SA-hCD137L, or the combination of these two agents were injected intraperitoneally (*i.p.*) at days 28, 35, 42, and 49 after IL-10^{high} EBV-LCL inoculation (**Figure 5A**). PBS- and SA-treated mice were controls. As a result, PAM administration alone decreased the tumor volume significantly and extended the survival of the tumor-bearing humanized mice compared with the treatment with PBS, SA, or SA-hCD137L protein alone, respectively (**Figures 5D, E**). Importantly, the combination treatment of PAM with SA-hCD137L was more potent than PAM alone to control the development of EBV-induced lymphoma with IL-10^{high} TME in humanized mice, in terms of tumor growth and survival (**Figures 5D, E**).

Humanized mice reconstituted with Vγ9Vδ2-T-cell-depleted hPBMC were also used to confirm whether the effect of SA-hCD137L costimulation on the control of EBV-induced

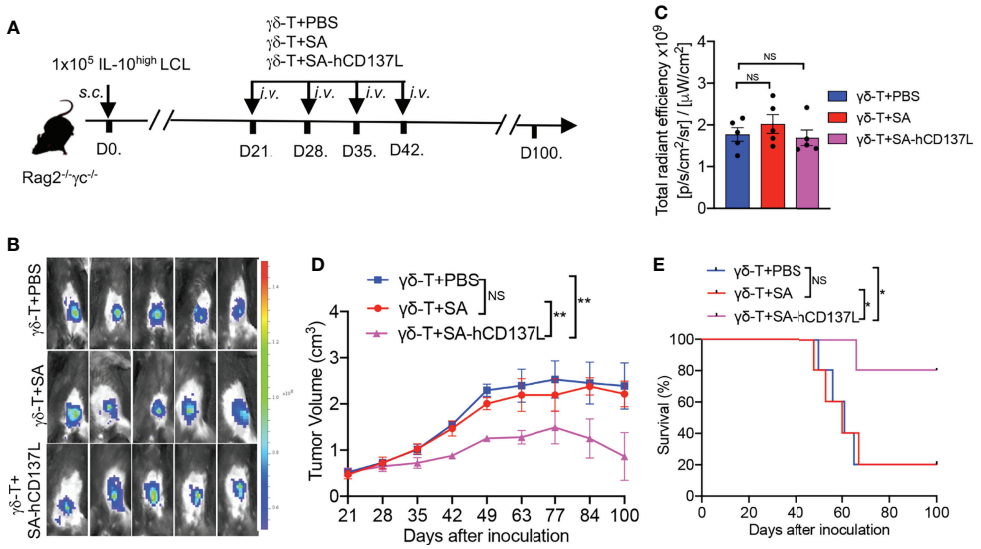


FIGURE 4 | CD137 costimulation enhanced the compromised antitumor activity of Vγ9Vδ2-T cells with IL-10^{high} TME in Rag2$^{-/-}$ γc$^{-/-}$ mice. **(A)** Protocol for evaluation of the synergistic therapeutic effect of Vγ9Vδ2-T cells and SA-hCD137L on EBV-induced lymphoma in Rag2$^{-/-}$ γc$^{-/-}$ mice (five mice per group). **(B, C)** Whole-body fluorescence images **(B)** and total radiant efficiency **(C)** of mice before treatment with PAM, SA-hCD137, SA, and PBS. **(D, E)** The tumor volume **(D)** and mouse survival **(E)** were determined at the indicated time. The tumor volume was analyzed by two-way ANOVA test, and mice survival was analyzed by Kaplan-Meier log-rank test. Data are representative for three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

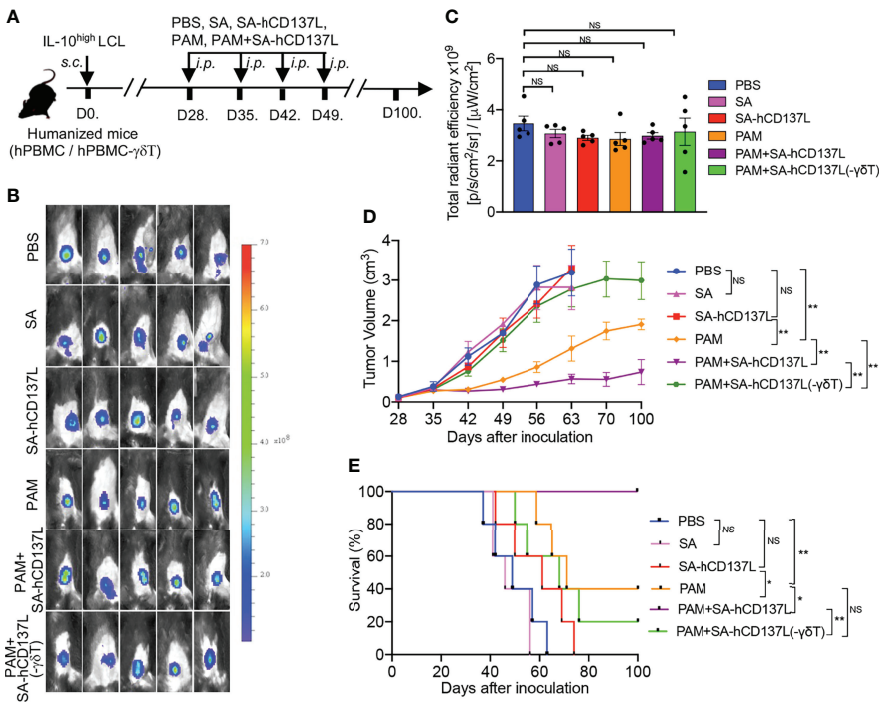


FIGURE 5 | CD137 costimulation improved the therapeutic effect of PAM in controlling EBV-induced lymphoma with IL-10^{high} TME in humanized mice. **(A)** The evaluation protocol of the synergistic therapeutic effect of PAM and SA-hCD137L on EBV-induced lymphoma in humanized mice (five mice per group). **(B, C)** Whole-body fluorescence images **(B)** and total radiant efficiency **(C)** of mice before treatment with PAM, SA-hCD137, SA and PBS. **(D, E)** The tumor volume **(D)** and mouse survival **(E)** were determined at the indicated time. The tumor volume was analyzed by two-way ANOVA test, and mice survival was analyzed by Kaplan-Meier log-rank test. Data are representative for three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

lymphoma with IL-10^{high} TME was mediated by Vγ9Vδ2-T cells (**Figure 5A**). As shown in **Figures 5D, E**, there were no therapeutic effects by the combination treatment of PAM with SA-hCD137L in humanized mice reconstituted with Vγ9Vδ2-T-cell-depleted hPBMC. These data demonstrate that the recombinant SA-hCD137L protein had a synergistic effect with PAM to overcome the barriers of IL-10^{high} TME *in vivo* and this synergistic effect was mainly mediated by Vγ9Vδ2-T cells.

DISCUSSION

In this study, we demonstrated that the antitumor activities of Vγ9Vδ2-T cells against EBV-LCL were inhibited by both hIL-10 and vIL-10 *in vitro* and *in vivo*. Importantly, we found that IL-10R1 was highly expressed on CD137^{+} Vγ9Vδ2-T cells compared to CD137$^{-/lo}$ Vγ9Vδ2-T cells following activation. CD137 engagement significantly suppressed IL-10R1 expression in Vγ9Vδ2-T cells, therefore reducing the Vγ9Vδ2-T cells' sensitivity to IL-10 in the TME. We further demonstrated that SA-hCD137L in a tetrameric form of human CD137L protein obviously enhanced the therapeutic effects of adoptive transfer of *ex vivo* expanded Vγ9Vδ2-T cell in Rag2$^{-/-}$γc$^{-/-}$ mice and direct administration of PAM in humanized mice for the treatment of EBV-induced lymphoma with IL-10^{high} TME.

IL-10 is important as an immunoregulatory cytokine to suppress inflammatory responses. However, its effects on tumorigenesis and development are controversial (45). IL-10 can inhibit the process of antigen presentation by downregulating the expression of MHC-II in APCs (46) and MHC-I in tumor cells (47). Thus, IL-10 can facilitate tumor escape by contributing to an immunosuppressive environment. A meta-analysis of 1788 cancer patients also revealed that the elevated serum IL-10 can predict poor prognosis (48). Paradoxically, it was reported that IL-10 can also induce immune-dependent antitumor effects (15, 49–51). Therefore, the roles of IL-10 on tumor development are dependent on the local environment and physiopathological states. The inhibitory role of IL-10 on APCs, CD8^{+} T cells, and CD4^{+} T cells has been clearly defined, but its impact on Vγ9Vδ2-T cells remains unclear. In this study, our data supported that hIL-10 and vIL-10 derived from tumor cells and EBV significantly inhibited the cytotoxicity of Vγ9Vδ2-T cells, which substantially limits the antitumor efficacy of Vγ9Vδ2-T cells.

EBV has evolved to express vIL-10, thereby providing a suitable microenvironment for itself to evade immunity and cause tumorigenesis (52, 53). The structurally homologous viral and human IL-10 perform similarly in several biological properties, including inhibition of IFN-γ production, suppression of T cell proliferation in response to antigens and mitogens, and stimulation of B cell growth (54). This similarity has raised the possibility that EBV might have captured the IL-10 gene during evolution. Furthermore, IL-10 has been shown to be involved in the pathogenesis of lymphoid disorders (55, 56). Elevated IL-10 levels are correlated with shorter survival and adverse disease features in patients with EBV-associated tumors (33, 57). Thus, we reasoned that hIL-10 and vIL-10 may be associated with the suppression of Vγ9Vδ2-T cells' antitumor activity. Such an interaction would provide a suitable microenvironment for viruses to evade immunity and cause tumorigenesis. Here, our *in vitro* data revealed that vIL-10 derived from EBV and hIL-10 derived from EBV-LCL were the dominant factors for inhibiting the antitumor activity of Vγ9Vδ2-T cells in TME. Our *in vivo* data also suggested that the reduced antitumor activity of Vγ9Vδ2-T cells against EBV-induced lymphoma may be associated with IL-10^{high} TME. Further study using IL-10 neutralizing mAb or IL-10 knockout mice is required to determine the exact role of IL-10 in antitumor activity of Vγ9Vδ2-T cells *in vivo*. Of note, additional factors in the CM might also contribute to suppressing Vγ9Vδ2-T cells activity because a smaller extent of cytotoxicity reduction after treatment with recombinant hIL-10 and vIL-10 proteins was observed when compared with IL-10^{high} CM (**Figure 1**).

Recently, clinical trials utilizing bisphosphonates, such as PAM and ZOL, to expand γδ-T cell *in vivo* in combination with IL-2 therapy or adoptive transfer of *ex vivo* cultured γδ-T cells were performed in patients with tumors and virus infections (11, 15, 58, 59). Administration of bisphosphonates with IL-2 and the transfer of expanded autologous Vγ9Vδ2 T-cells have been demonstrated to be safe with limited adverse events (60). However, there is only a modest efficacy in the treatment of some tumors (61, 62). One drawback of γδ-T cell-based immunotherapy is the rapid exhaustion of proliferation and effector responses due to repeated phosphoantigen treatments (17). Another drawback of this therapy is the impaired antitumor activity of γδ-T cells caused by the tumor immunosuppressive microenvironment (18, 63).

CD137 is a promising costimulatory immunologic target for enhancing antitumor immune responses (39). CD137 costimulation, known as "stepping on the accelerator," is believed to be a compelling complement for "removing the brakes" *via* blocking inhibitory signaling. Importantly, it is now appreciated that CD137 signaling not only works as an "accelerator" to provide costimulation, but also breaks and reverses the established anergy in cytotoxic T lymphocytes (CTLs) (64, 65). However, the role of CD137 signaling in Vγ9Vδ2-T cells within the context of IL-10-mediated TME is not clear. Here, we revealed that IL-10R1^{+} Vγ9Vδ2 T-cell subset expressed high levels of CD137. Moreover, CD137 costimulation suppressed IL-10R1 in Vγ9Vδ2-T cells, suggesting that CD137 engagement possessed the potential to ameliorate the exhaustion and dysfunction of Vγ9Vδ2-T cells.

Ligation of CD137 is correlated with effective antitumor responses; however, the application of anti-CD137 agonistic antibodies in patients is limited by a variety of side effects (66). The natural CD137 ligand is an alternative to the CD137-specific antibodies to stimulate antitumor T cell responses. Shirwan lab reported that a streptavidin-conjugated murine CD137L (SA-mCD137L) complex could induce effective antitumor immune responses (67, 68). SA-mCD137L induces less pathological side effects than anti-CD137 agonistic antibody therapy, suggesting a

higher therapeutic index of SA-mCD137L. Previously, we demonstrated that recombinant SA-hCD137L enhanced the cytotoxic effect of Vγ9Vδ2- T cells against influenza virus infection (37). Here, we further found that SA-hCD137L restored the antitumor activity of Vγ9Vδ2-T cells compromised by the IL-10-mediated TME. These data indicate that SA-hCD137L can provide an alternative to anti-CD137 agonistic for anti-tumor therapy.

There are no Vγ9Vδ2-T cells in mice, thus it is impossible to study these cells in mouse models (69). Previously, we successfully established humanized mice with a similar proportion of Vγ9Vδ2-T cells in murine peripheral blood to that in humans (12, 14, 70, 71). Importantly, here the synergistic effect of PAM and recombinant SA-hCD137L Vγ9Vδ2-T cells was verified in humanized mice.

In conclusion, our study further elucidates the role of CD137 in the antitumor activity of human Vγ9Vδ2-T cells in the IL-10-mediated immunosuppressive TME. The combination of a phosphoantigen and CD137 agonist also provides a novel strategy for treating EBV-induced tumors by avoiding Vγ9Vδ2-T cell exhaustion and enhancing the efficacy of Vγ9Vδ2-T cell-based therapy.

AUTHOR CONTRIBUTORS

YP, WT, and YL conceived and designed the study, interpreted the results, wrote and edited the manuscript; YP, KW, ZX, CT, XW, YZ, and XM designed and performed the experiments, analyzed the results. WT supervised this study. All authors contributed to the article and approved the submitted version.

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

The studies involving human participants were reviewed and approved by the institutional review board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

FUNDING

This study was supported partially by GRF, RGC (17122519, 17126317); Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (18192021); Seed Funding for Strategic Interdisciplinary Research Scheme, the University of Hong Kong; Hong Kong SAR, China.

REFERENCES

1. Cohen JI, Fauci AS, Varmus H, Nabel GJ. Epstein-Barr Virus: An Important Vaccine Target for Cancer Prevention. *Sci Transl Med* (2011) 3(107):107fs7. doi: 10.1126/scitranslmed.3002878
2. Young LS, Yap LF, Murray PG. Epstein-Barr Virus: More Than 50 Years Old and Still Providing Surprises. *Nat Rev Cancer* (2016) 16(12):789–802. doi: 10.1038/nrc.2016.92
3. Zhu QY, Zhao GX, Li Y, Talakatta G, Mai HQ, Le QT, et al. Advances in Pathogenesis and Precision Medicine for Nasopharyngeal Carcinoma. *MedComm (2020)* (2021) 2(2):175–206. doi: 10.1002/mco2.32
4. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD. The Immunology of Epstein-Barr Virus-Induced Disease. *Annu Rev Immunol* (2015) 33:787–821. doi: 10.1146/annurev-immunol-032414-112326
5. Israel BF, Kenney SC. Virally Targeted Therapies for EBV-Associated Malignancies. *Oncogene* (2003) 22(33):5122–30. doi: 10.1038/sj.onc.1206548
6. Dharnidharka VR, Mohanakumar T. New Approaches to Treating B-Cell Cancers Induced by Epstein-Barr Virus. *N Engl J Med* (2015) 372(6):569–71. doi: 10.1056/NEJMcibr1415117
7. Bollard CM, Rooney CM, Heslop HE. T-Cell Therapy in the Treatment of Post-Transplant Lymphoproliferative Disease. *Nat Rev Clin Oncol* (2012) 9 (9):510–9. doi: 10.1038/nrclinonc.2012.111
8. Hayday AC. Gammadelta T Cell Update: Adaptate Orchestrators of Immune Surveillance. *J Immunol* (2019) 203(2):311–20. doi: 10.4049/jimmunol.1800934
9. Silva-Santos B, Mensurado S, Coffelt SB. Gammadelta T Cells: Pleiotropic Immune Effectors With Therapeutic Potential in Cancer. *Nat Rev Cancer* (2019) 19(7):392–404. doi: 10.1038/s41568-019-0153-5
10. Xiang Z, Tu W. Dual Face of Vgamma9Vdelta2-T Cells in Tumor Immunology: Anti- Versus Pro-Tumoral Activities. *Front Immunol* (2017) 8:1041. doi: 10.3389/fimmu.2017.01041
11. Tanaka Y, Murata-Hirai K, Iwasaki M, Matsumoto K, Hayashi K, Kumagai A, et al. Expansion of Human Gammadelta T Cells for Adoptive Immunotherapy Using a Bisphosphonate Prodrug. *Cancer Sci* (2018) 109(3):587–99. doi: 10.1111/cas.13491
12. Wang X, Xiang Z, Liu Y, Huang C, Pei Y, Wang X, et al. Exosomes Derived From Vδ2-T Cells Control Epstein-Barr Virus-Associated Tumors and Induce T Cell Antitumor Immunity. *Sci Transl Med* (2020) 12(563):eaaz3426. doi: 10.1126/scitranslmed.aaz3426
13. Wang X, Zhang Y, Mu X, Tu CR, Chung Y, Tsao SW, et al. Exosomes Derived From Gammadelta-T Cells Synergize With Radiotherapy and Preserve Antitumor Activities Against Nasopharyngeal Carcinoma in Immunosuppressive Microenvironment. *J Immunother Cancer* (2022) 10(2): e003832. doi: 10.1136/jitc-2021-003832
14. Tu W, Zheng J, Liu Y, Sia SF, Liu M, Qin G, et al. The Aminobisphosphonate Pamidronate Controls Influenza Pathogenesis by Expanding a Gammadelta T Cell Population in Humanized Mice. *J Exp Med* (2011) 208(7):1511–22. doi: 10.1084/jem.20110226
15. Xiang Z, Liu Y, Zheng J, Liu M, Lv A, Gao Y, et al. Targeted Activation of Human Vgamma9Vdelta2-T Cells Controls Epstein-Barr Virus-Induced B Cell Lymphoproliferative Disease. *Cancer Cell* (2014) 26(4):565–76. doi: 10.1016/j.ccr.2014.07.026
16. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. *Nat Med* (2015) 21(8):938–45. doi: 10.1038/nm.3909
17. Sicard H, Ingoure S, Luciani B, Serraz C, Fournié JJ, Bonneville M, et al. *In Vivo* Immunomanipulation of V Gamma 9V Delta 2 T Cells With a Synthetic

Phosphoantigen in a Preclinical Nonhuman Primate Model. *J Immunol* (2005) 175(8):5471–80. doi: 10.4049/jimmunol.175.8.5471
18. Yi Y, He HW, Wang JX, Cai XY, Li YW, Zhou J, et al. The Functional Impairment of HCC-Infiltrating γδ T Cells, Partially Mediated by Regulatory T Cells in a Tgfβ- and IL-10-Dependent Manner. *J Hepatol* (2013) 58(5):977–83. doi: 10.1016/j.jhep.2012.12.015
19. Park JH, Lee HK. Function of γδ T Cells in Tumor Immunology and Their Application to Cancer Therapy. *Exp Mol Med* (2021) 53(3):318–27. doi: 10.1038/s12276-021-00576-0
20. Pitt JM, Marabelle A, Eggermont A, Soria JC, Kroemer G, Zitvogel L. Targeting the Tumor Microenvironment: Removing Obstruction to Anticancer Immune Responses and Immunotherapy. *Ann Oncol* (2016) 27 (8):1482–92. doi: 10.1093/annonc/mdw168
21. Dennis KL, Blatner NR, Gounari F, Khazaie K. Current Status of Interleukin-10 and Regulatory T-Cells in Cancer. *Curr Opin Oncol* (2013) 25(6):637–45. doi: 10.1097/CCO.0000000000000006
22. Kajino K, Nakamura I, Bamba H, Sawai T, Ogasawara K. Involvement of IL-10 in Exhaustion of Myeloid Dendritic Cells and Rescue by CD40 Stimulation. *Immunology* (2007) 120(1):28–37. doi: 10.1111/j.1365-2567.2006.02474.x
23. Gassa A, Jian F, Kalkavan H, Duhan V, Honke N, Shaabani N, et al. IL-10 Induces T Cell Exhaustion During Transplantation of Virus Infected Hearts. *Cell Physiol Biochem* (2016) 38(3):1171–81. doi: 10.1159/000443067
24. Shi R, Tang YQ, Miao H. Metabolism in Tumor Microenvironment: Implications for Cancer Immunotherapy. *MedComm* (2020) 1(1):47–68. doi: 10.1002/mco2.6
25. Stuart AD, Stewart JP, Arrand JR, Mackett M. The Epstein-Barr Virus Encoded Cytokine Viral Interleukin-10 Enhances Transformation of Human B Lymphocytes. *Oncogene* (1995) 11(9):1711–9.
26. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB. Interleukin-10 Determines Viral Clearance or Persistence *In Vivo*. *Nat Med* (2006) 12(11):1301–9. doi: 10.1038/nm1492
27. Ejrnaes M, Filippi CM, Martinic MM, Ling EM, Togher LM, Crotty S, et al. Resolution of a Chronic Viral Infection After Interleukin-10 Receptor Blockade. *J Exp Med* (2006) 203(11):2461–72. doi: 10.1084/jem.20061462
28. Jochum S, Moosmann A, Lang S, Hammerschmidt W, Zeidler R. The EBV Immunoevasins vIL-10 and BNLF2a Protect Newly Infected B Cells From Immune Recognition and Elimination. *PLos Pathog* (2012) 8(5):e1002704. doi: 10.1371/journal.ppat.1002704
29. Wilson AD, Hopkins JC, Morgan AJ. *In Vitro* Cytokine Production and Growth Inhibition of Lymphoblastoid Cell Lines by CD4+ T Cells From Epstein-Barr Virus (EBV) Seropositive Donors. *Clin Exp Immunol* (2001) 126 (1):101–10. doi: 10.1046/j.1365-2249.2001.01641.x
30. Miyauchi K, Urano E, Yoshiyama H, Komano J. Cytokine Signatures of Transformed B Cells With Distinct Epstein-Barr Virus Latencies as a Potential Diagnostic Tool for B Cell Lymphoma. *Cancer Sci* (2011) 102(6):1236–41. doi: 10.1111/j.1349-7006.2011.01924.x
31. Lambert SL, Martinez OM. Latent Membrane Protein 1 of EBV Activates Phosphatidylinositol 3-Kinase to Induce Production of IL-10. *J Immunol* (2007) 179(12):8225–34. doi: 10.4049/jimmunol.179.12.8225
32. Miyazaki I, Cheung RK, Dosch HM. Viral Interleukin 10 is Critical for the Induction of B Cell Growth Transformation by Epstein-Barr Virus. *J Exp Med* (1993) 178(2):439–47. doi: 10.1084/jem.178.2.439
33. Lech-Maranda E, Bienvenu J, Michallet AS, Houot R, Robak T, Coiffier B, et al. Elevated IL-10 Plasma Levels Correlate With Poor Prognosis in Diffuse Large B-Cell Lymphoma. *Eur Cytokine Netw* (2006) 17(1):60–6.
34. Wang C, Lin GH, McPherson AJ, Watts TH. Immune Regulation by 4-1BB and 4-1BBL: Complexities and Challenges. *Immunol Rev* (2009) 229(1):192–215. doi: 10.1111/j.1600-065X.2009.00765.x
35. Stoll A, Bruns H, Fuchs M, Völkl S, Nimmerjahn F, Kunz M, et al. CD137 (4-1BB) Stimulation Leads to Metabolic and Functional Reprogramming of Human Monocytes/Macrophages Enhancing Their Tumoricidal Activity. *Leukemia* (2021) 35(12):3482–96. doi: 10.1038/s41375-021-01287-1
36. Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, et al. 4-1BB Costimulatory Signals Preferentially Induce CD8+ T Cell Proliferation and Lead to the Amplification *In Vivo* of Cytotoxic T Cell Responses. *J Exp Med* (1997) 186(1):47–55. doi: 10.1084/jem.186.1.47
37. Pei Y, Wen K, Xiang Z, Huang C, Wang X, Mu X, et al. CD137 Costimulation Enhances the Antiviral Activity of Vγ9vδ2-T Cells Against Influenza Virus. *Signal Transduct Target Ther* (2020) 5(1):74. doi: 10.1038/s41392-020-0174-2
38. Chester C, Sanmamed MF, Wang J, Melero I. Immunotherapy Targeting 4-1BB: Mechanistic Rationale, Clinical Results, and Future Strategies. *Blood* (2018) 131(1):49–57. doi: 10.1182/blood-2017-06-741041
39. Hashimoto K. CD137 as an Attractive T Cell Co-Stimulatory Target in the TNFRSF for Immuno-Oncology Drug Development. *Cancers (Basel)* (2021) 13(10):2288–303. doi: 10.3390/cancers13102288
40. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan PL, et al. Phosphoantigen-Expanded Human Gammadelta T Cells Display Potent Cytotoxicity Against Monocyte-Derived Macrophages Infected With Human and Avian Influenza Viruses. *J Infect Dis* (2009) 200(6):858–65. doi: 10.1086/605413
41. Jog NR, Chakravarty EF, Guthridge JM, James JA. Epstein Barr Virus Interleukin 10 Suppresses Anti-Inflammatory Phenotype in Human Monocytes. *Front Immunol* (2018) 9:2198. doi: 10.3389/fimmu.2018.02198
42. Vahl JM, Friedrich J, Mittler S, Trump S, Heim L, Kachler K, et al. Interleukin-10-Regulated Tumour Tolerance in Non-Small Cell Lung Cancer. *Br J Cancer* (2017) 117(11):1644–55. doi: 10.1038/bjc.2017.336
43. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the Interleukin-10 Receptor. *Annu Rev Immunol* (2001) 19:683–765. doi: 10.1146/annurev.immunol.19.1.683
44. Donnelly RP, Sheikh F, Kotenko SV, Dickensheets H. The Expanded Family of Class II Cytokines That Share the IL-10 Receptor-2 (IL-10R2) Chain. *J Leukoc Biol* (2004) 76(2):314–21. doi: 10.1189/jlb.0204117
45. Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The Paradoxical Role of IL-10 in Immunity and Cancer. *Cancer Lett* (2015) 367(2):103–7. doi: 10.1016/j.canlet.2015.07.009
46. Steinbrink K, Jonuleit H, Müller G, Schuler G, Knop J, Enk AH. Interleukin-10-Treated Human Dendritic Cells Induce a Melanoma-Antigen-Specific Anergy in CD8(+) T Cells Resulting in a Failure to Lyse Tumor Cells. *Blood* (1999) 93(5):1634–42. doi: 10.1182/blood.V93.5.1634
47. Adris S, Klein S, Jasnis M, Chuluyan E, Ledda M, Bravo A, et al. IL-10 Expression by CT26 Colon Carcinoma Cells Inhibits Their Malignant Phenotype and Induces a T Cell-Mediated Tumor Rejection in the Context of a Systemic Th2 Response. *Gene Ther* (1999) 6(10):1705–12. doi: 10.1038/sj.gt.3301012
48. Zhao S, Wu D, Wu P, Wang Z, Huang J. Serum IL-10 Predicts Worse Outcome in Cancer Patients: A Meta-Analysis. *PLos One* (2015) 10(10): e0139598. doi: 10.1371/journal.pone.0139598
49. Wang Y, Sun SN, Liu Q, Yu YY, Guo J, Wang K, et al. Autocrine Complement Inhibits IL10-Dependent T-Cell-Mediated Antitumor Immunity to Promote Tumor Progression. *Cancer Discov* (2016) 6(9):1022–35. doi: 10.1158/2159-8290.CD-15-1412
50. Fujii S, Shimizu K, Shimizu T, Lotze MT. Interleukin-10 Promotes the Maintenance of Antitumor CD8(+) T-Cell Effector Function in Situ. *Blood* (2001) 98(7):2143–51. doi: 10.1182/blood.V98.7.2143
51. Emmerich J, Mumm JB, Chan IH, LaFace D, Truong H, McClanahan T, et al. IL-10 Directly Activates and Expands Tumor-Resident CD8(+) T Cells Without *De Novo* Infiltration From Secondary Lymphoid Organs. *Cancer Res* (2012) 72(14):3570–81. doi: 10.1158/0008-5472.CAN-12-0721
52. Salek-Ardakani S, Arrand JR, Mackett M. Epstein-Barr Virus Encoded Interleukin-10 Inhibits HLA-Class I, ICAM-1, and B7 Expression on Human Monocytes: Implications for Immune Evasion by EBV. *Virology* (2002) 304(2):342–51. doi: 10.1006/viro.2002.1716
53. Yoon SI, Jones BC, Logsdon NJ, Harris BD, Kuruganti S, Walter MR. Epstein-Barr Virus IL-10 Engages IL-10R1 by a Two-Step Mechanism Leading to Altered Signaling Properties. *J Biol Chem* (2012) 287(32):26586–95. doi: 10.1074/jbc.M112.376707
54. Slobedman B, Barry PA, Spencer JV, Avdic S, Abendroth A. Virus-Encoded Homologs of Cellular Interleukin-10 and Their Control of Host Immune Function. *J Virol* (2009) 83(19):9618–29. doi: 10.1128/JVI.01098-09
55. Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M. The Multifaceted Relationship Between IL-10 and Adaptive Immunity: Putting Together the Pieces of a Puzzle. *Cytokine Growth Factor Rev* (2004) 15(1):61–76. doi: 10.1016/j.cytogfr.2003.11.001

56. Benjamin D, Knobloch TJ, Dayton MA. Human B-Cell Interleukin-10: B-Cell Lines Derived From Patients With Acquired Immunodeficiency Syndrome and Burkitt's Lymphoma Constitutively Secrete Large Quantities of Interleukin-10. *Blood* (1992) 80(5):1289–98. doi: 10.1182/blood.V80.5.1289.1289
57. Nacinović-Duletić A, Stifter S, Dvornik S, Skunca Z, Jonjić N. Correlation of Serum IL-6, IL-8 and IL-10 Levels With Clinicopathological Features and Prognosis in Patients With Diffuse Large B-Cell Lymphoma. *Int J Lab Hematol* (2008) 30(3):230–9. doi: 10.1111/j.1751-553X.2007.00951.x
58. Kabelitz D, Serrano R, Kouakanou L, Peters C, Kalyan S. Cancer Immunotherapy With Gammadelta T Cells: Many Paths Ahead of Us. *Cell Mol Immunol* (2020) 17(9):925–39. doi: 10.1038/s41423-020-0504-x
59. Zheng J, Liu Y, Lau YL, Tu W. Gammadelta-T Cells: An Unpolished Sword in Human Anti-Infection Immunity. *Cell Mol Immunol* (2013) 10(1):50–7. doi: 10.1038/cmi.2012.43
60. Xu Y, Xiang Z, Alnaggar M, Kouakanou L, Li J, He J, et al. Allogeneic Vgamma9Vdelta2 T-Cell Immunotherapy Exhibits Promising Clinical Safety and Prolongs the Survival of Patients With Late-Stage Lung or Liver Cancer. *Cell Mol Immunol* (2021) 18(2):427–39. doi: 10.1038/s41423-020-0515-7
61. Kakimi K, Matsushita H, Masuzawa K, Karasaki T, Kobayashi Y, Nagaoka K, et al. Adoptive Transfer of Zoledronate-Expanded Autologous Vγ9vδ2 T-Cells in Patients With Treatment-Refractory Non-Small-Cell Lung Cancer: A Multicenter, Open-Label, Single-Arm, Phase 2 Study. *J Immunother Cancer* (2020) 8(2):e001185. doi: 10.1136/jitc-2020-001185
62. Sakamoto M, Nakajima J, Murakawa T, Fukami T, Yoshida Y, Murayama T, et al. Adoptive Immunotherapy for Advanced Non-Small Cell Lung Cancer Using Zoledronate-Expanded γδtcells: A Phase I Clinical Study. *J Immunother* (2011) 34(2):202–11. doi: 10.1097/CJI.0b013e318207ecfb
63. Lafont V, Sanchez F, Laprevotte E, Michaud HA, Gros L, Eliaou JF, et al. Plasticity of γδ T Cells: Impact on the Anti-Tumor Response. *Front Immunol* (2014) 5:622. doi: 10.3389/fimmu.2014.00622
64. Wilcox RA, Tamada K, Flies DB, Zhu G, Chapoval AI, Blazar BR, et al. Ligation of CD137 Receptor Prevents and Reverses Established Anergy of CD8+ Cytolytic T Lymphocytes *In Vivo*. *Blood* (2004) 103(1):177–84. doi: 10.1182/blood-2003-06-2184
65. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB Costimulation Ameliorates T Cell Exhaustion Induced by Tonic Signaling of Chimeric Antigen Receptors. *Nat Med* (2015) 21(6):581–90. doi: 10.1038/nm.3838
66. Ascierto PA, Simeone E, Sznol M, Fu YX, Melero I. Clinical Experiences With Anti-CD137 and Anti-PD1 Therapeutic Antibodies. *Semin Oncol* (2010) 37(5):508–16. doi: 10.1053/j.seminoncol.2010.09.008
67. Barsoumian HB, Batra L, Shrestha P, Bowen WS, Zhao H, Egilmez NK, et al. A Novel Form of 4-1BBL Prevents Cancer Development *via* Nonspecific Activation of CD4(+) T and Natural Killer Cells. *Cancer Res* (2019) 79(4):783–94. doi: 10.1158/0008-5472.CAN-18-2401
68. Srivastava AK, Dinc G, Sharma RK, Yolcu ES, Zhao H, Shirwan H. SA-4-1BBL and Monophosphoryl Lipid A Constitute an Efficacious Combination Adjuvant for Cancer Vaccines. *Cancer Res* (2014) 74(22):6441–51. doi: 10.1158/0008-5472.CAN-14-1768-A
69. Born WK, Reardon CL, O'Brien RL. The Function of Gammadelta T Cells in Innate Immunity. *Curr Opin Immunol* (2006) 18(1):31–8. doi: 10.1016/j.coi.2005.11.007
70. Chen Q, Wen K, Lv A, Liu M, Ni K, Xiang Z, et al. Human Vgamma9Vdelta2-T Cells Synergize CD4(+) T Follicular Helper Cells to Produce Influenza Virus-Specific Antibody. *Front Immunol* (2018) 9:599. doi: 10.3389/fimmu.2018.00599
71. Ni K, Liu M, Zheng J, Wen L, Chen Q, Xiang Z, et al. PD-1/PD-L1 Pathway Mediates the Alleviation of Pulmonary Fibrosis by Human Mesenchymal Stem Cells in Humanized Mice. *Am J Respir Cell Mol Biol* (2018) 58(6):684–95. doi: 10.1165/rcmb.2017-0326OC

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.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 07 July 2022
doi: 10.3389/fimmu.2022.915366

γ9δ2 T-Cell Expansion and Phenotypic Profile Are Reflected in the CDR3δ Repertoire of Healthy Adults

*Anna Vyborova[1], Anke Janssen[1], Lucrezia Gatti[1], Froso Karaiskaki[1], Austin Yonika[1], Sanne van Dooremalen[1], Jasper Sanders[1], Dennis X. Beringer[1], Trudy Straetemans[1,2], Zsolt Sebestyen[1] and Jürgen Kuball[1,2]**

[1] Center for Translational Immunology, University Medical Center (UMC) Utrecht, Utrecht University, Utrecht, Netherlands, [2] Department of Hematology, University Medical Center (UMC) Utrecht, Utrecht University, Utrecht, Netherlands

OPEN ACCESS

Edited by:
Andy Hee-Meng Tan, Bioprocessing Technology Institute (A*STAR), Singapore

Reviewed by:
David Vermijlen, Université libre de Bruxelles, Belgium
Haiyan Liu, National University of Singapore, Singapore

***Correspondence:**
Jürgen Kuball
j.h.e.kuball@umcutrecht.nl

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 07 April 2022
Accepted: 06 June 2022
Published: 07 July 2022

Citation:
Vyborova A, Janssen A, Gatti L, Karaiskaki F, Yonika A, van Dooremalen S, Sanders J, Beringer DX, Straetemans T, Sebestyen Z and Kuball J (2022) γ9δ2 T-Cell Expansion and Phenotypic Profile Are Reflected in the CDR3δ Repertoire of Healthy Adults. Front. Immunol. 13:915366. doi: 10.3389/fimmu.2022.915366

γ9δ2T cells fill a distinct niche in human immunity due to the unique physiology of the phosphoantigen-reactive γ9δ2TCR. Here, we highlight reproducible TCRδ complementarity-determining region 3 (CDR3δ) repertoire patterns associated with γ9δ2T cell proliferation and phenotype, thus providing evidence for the role of the CDR3δ in modulating *in vivo* T-cell responses. Features that determine γ9δ2TCR binding affinity and reactivity to the phosphoantigen-induced ligand *in vitro* appear to similarly underpin *in vivo* clonotypic expansion and differentiation. Likewise, we identify a CDR3δ bias in the γ9δ2T cell natural killer receptor (NKR) landscape. While expression of the inhibitory receptor CD94/NKG2A is skewed toward cells bearing putative high-affinity TCRs, the activating receptor NKG2D is expressed independently of the phosphoantigen-sensing determinants, suggesting a higher net NKR activating signal in T cells with TCRs of low affinity. This study establishes consistent repertoire–phenotype associations and justifies stratification for the T-cell phenotype in future research on γ9δ2TCR repertoire dynamics.

Keywords: human γ9δ2 T cells, CDR3δ, adult Vδ2 repertoire, differentiation profile, public clonotypes, NKG2D, CD94/NKG2A(B)

INTRODUCTION

From generation onward, the T-cell receptor repertoire accumulates footprints of selection and clonotypic expansion that reduce its diversity and lead to skewing. As a result, the repertoire of antigen-experienced T cells represents only a modest fraction of the repertoire of their newly generated naïve precursors, limited to the antigenic specificities relevant to the individual's immunological history [reviewed in (1)]. A γ9δ2TCR is notably different from an αβTCR, as it senses the accumulation of intracellular phosphorylated non-peptide antigens (phosphoantigens or pAgs) through modification of surface butyrophilin family members BTN3A1 and BTN2A1, orchestrated by interaction with the small GTPase RhoB, rather than sensing peptide–MHC complexes (2–6). Nevertheless, despite the unique antigen specificity and recognition mode, the γ9δ2TCR repertoire equally acquires imprints of selection and expansion throughout the human lifetime (7, 8), starting with a wave of polyclonal expansion immediately after birth (9, 10) and

ultimately leading to a characteristic "selected," highly private pAg-reactive γ9δ2TCR repertoire in adults [reviewed in (11)]. While the TCRγ9 chain harbors motifs that are critical for binding the non-polymorphic BTN ligand, the TCRδ2 chain contains the yet unresolved clue to pAg reactivity (4, 12), possibly through the influence of the CDR3δ on the γ9δ2TCR binding affinity in this multiple-ligand system (13, 14). Therefore, the most dramatic repertoire changes are seen in the CDR3δ repertoire from T-cell generation to maturity.

Phenotypic maturation in T cells parallels repertoire maturation and eventually reaches terminal differentiation defined by the dominance of cytotoxic responses over the proliferative, acquisition of natural cytotoxicity markers and reduced TCR signaling (15, 16). γ9δ2T cells mature across a similar continuum of transcriptional, phenotypic, and functional changes as αβT cells do (17, 18). The CD27+CD28+ T cells ("early-stage differentiation profile," proliferative, characteristic of T cells isolated from cord blood or thymus) or CD27-CD28-CD16+ cells ("late-stage differentiation profile," readily cytotoxic, seen in selected healthy adults) dominate the two distinct poles of the continuum. Unlike αβT cells, however, γδT cells are characterized already at the fetal stage of T-cell development by a prominent natural killer receptor (NKR) signature, present independently of other differentiation markers (19), and continuing to distinguish γδT cells from their αβ counterparts during adulthood (20, 21).

In early infancy, massive γ9δ2T cell expansion and phenotypic maturation are evidently reflected by enrichment of the γ9δ2TCR repertoire in features that underpin recognition of the pAg-driven changes in BTN2 and BTN3, such as TRGJP rearrangements in the γ chain and a hydrophobic amino acid at position 5 of the CDR3 of the δ chain, suggestive of the key role of these γ9δ2TCR traits in the effector responses of the γ9δ2T cells at that developmental stage (10). As to already highly selected adult γ9δ2TCR repertoires, a clonotypic bias in association with T-cell expansion and differentiation profile has been reported (7); however, it remains unclear whether any distinct TCR features explain this bias. In addition, the expression of certain NKRs has been associated with a CDR3δ bias (22). Considering the growing interest in γδT cells and their TCRs as therapeutics (23), and in reliance on a model in which the γ9δ2TCR diversity is assumed to underpin a range of ligand-binding affinities (13, 14), the question of whether the repertoire changes are related to the phenotypic changes is particularly relevant, since it clears the way for isolating the TCRs with the highest affinity to the antigen and the T-cell populations most potent for therapeutic use (14, 24). In this study, we deconstruct the adult CDR3δ repertoire in relation to the phenotype of the γ9δ2T cells in an attempt to understand the functional role of the γ9δ2TCR diversity in adults.

MATERIALS AND METHODS

Isolation of Peripheral Blood Mononuclear Cells and Cell Phenotyping

Buffy coats were obtained from Sanquin blood bank (Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque PLUS gradient centrifugation (GE Healthcare, Chicago, IL, USA). The cells at the interface were harvested, washed 4× with phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA), and further stained with the following monoclonal antibodies (mAbs): CD3 (CD3-PB Clone UCHT1, BD Biosciences, San Jose, CA, USA; cat. no. 558117), TCR panγδ (TCR panγδ-PE-Cy7 Clone IMMU510, Beckman Coulter, Brea, CA, USA; cat. no. B10247), Vδ2 (TCR Vδ2-FITC Clone B6, BioLegend, San Diego, CA, USA; cat. no. 331406), CD8a (CD8a-PerCP-Cy5.5 Clone RPA-T8, BioLegend; cat. no. 301031), CD4 (CD4-APC Clone RPA-T4, BioLegend; cat. no. 300514), CD27 (CD27-APC-eF780 clone O323, eBioscience, San Diego, CA, USA; cat. no. 47-0279-42) and isotype control (IgG1-APC-eF780 Clone P3.6.2.8.1, eBioscience; cat. no. 47-4714-80), CD45RA (CD45RA-BV650 Clone HI100, BD Horizon, Franklin Lakes, NJ, USA; cat. no. 563963), CD28 (CD28-BV605 Clone CD28.2, BD Horizon; cat. no. 562976), CD16 (CD16-PE Clone eBioCB16, eBioscience; cat. no. 12-0168-41), CD16-BV785 Clone 3G8, BioLegend; cat. no. 302046), CCR7 (CCR7-APC Clone G043H7 Sony Biotechnology, San Jose, CA, USA; cat. no. 2366070), NKG2D (NKG2D-PE clone 1D11, eBioscience; cat. no. 12-5878-42), CD94 (CD94-PE clone HP-3B1, Beckman Coulter; cat. no. IM2276), NKG2A (human CD159a (NKG2A)-APC/Fire™ 750 clone S19004C, BioLegend; cat. no. 375115), and NKG2C (CD159c(NKG2C)-AF700clone 134591, R&D Systems, Minneapolis, MN, USA; cat. no. FAB138N-025).

Live/dead aqua stain (LIVE/DEAD stain kit Aqua fluorescent, Life Technologies, Carlsbad, CA, USA; cat. no. L34957) was used to exclude dead cells.

Cell phenotyping was performed on BD Fortessa and BD Canto, and all fluorescence-activated cell sorting (FACS) processes were performed on BD ARIAII. Sorted T-cell subpopulations were collected in equal numbers in at least duplicates, which were further processed separately as independent biological replicates. We sorted 50,000 cells per population for the late-stage profile donors; for the early-stage donors, there were 12,000–30,000 cells. Cells were collected in ice-cold Roswell Park Memorial Institute (RPMI) medium (Gibco, Grand Island, NY, USA) supplemented with 20% fetal calf serum (FCS) and spun down; cell pellets were resuspended in RLTplus buffer (Qiagen, Valencia, CA, USA; art 1053393) supplemented with fresh β-mercaptoethanol (Life Technologies; art 11528926) (10 μl of βME per 1 ml of RLTplus) for 2 min for lysis at RT and stored at −80°C until RNA isolation.

TCRδ Chain Sequencing and Data Analysis

RNA isolation, cDNA synthesis, PCR of the TCRδ chain, library preparation, high-throughput sequencing, and analysis of the raw sequencing files were performed at the University Medical Center Utrecht as described earlier (13). In-house R scripts were used for repertoire analysis; data were filtered to include clonotypes with a frequency higher than one read/clonotype. The tcR package (25) was used as the framework of the repertoire analysis pipeline.

All head-to-head comparisons of quantitative repertoire features, such as diversity, were made on samples with equal numbers of sorted cells.

Sequences were annotated as public if they were shared among at least 2 donors in the current study. In order to reduce the uncertainty intrinsic to the detection of low-frequency sequences, which might impair the robust detection of rare public clonotypes, we analyzed only the nucleotypes that had been detected at least in two samples from the same donor. When analyzing public AA clonotypes, multiple (both private and public) nucleotypes encoding for the same AA sequence were collapsed to a single entry with a read count equaling the total of the read counts of all nucleotypes. Several previously published datasets containing high-throughput TCRδ chain sequencing data were used to identify highly common public Vδ2 CDR3 sequences in healthy individuals (9, 10, 26–28).

Analysis of the number of N insertions was performed using the IMGT Junction Analysis tool (29).

Cells and Cell Lines

Daudi and Phoenix-Ampho cells were obtained from ATCC (Manassas, VA, USA). The TCRβ−/− Jurkat76 cell line was a kind gift from Miriam Heemskerk (LUMC, Leiden, the Netherlands).

Retroviral Vector Generation

The γ chain of the TCR clone 5 cloned into a pBullet-IRES-neomycin vector *via* 5′ *Nco*I and 3′ *Bam*HI restriction sites was previously available (30). Public and private CDR3δ chain retroviral plasmid vectors were generated based on the previously available retroviral vector encoding the δ chain of the TCR clone 5 (30) by introducing the new CDR3δ sequence using overlap extension PCR as described earlier (13). For the overhang primer pairs encoding the target CDR3δ NT sequences, see **Supplementary Table 3**.

Retroviral Transduction of T Cells

All public and private TCR δ2 chains were paired with clone 5 TCRγ chain and were transduced into TCR-deficient T-cell line Jurkat76.

For the generation of transduced Jurkat76 cells, Phoenix Ampho cells were transfected with the pBullet retroviral constructs containing TCRγ and TCRδ using FuGENE HD (Promega, Madison, WI, USA). Next, Jurkat76 cells were transduced with the viral supernatants in the presence of polybrene (4 μg/ml; Sigma-Aldrich). The transduced Jurkat76 cells were magnetic-activated cell sorting (MACS)-selected using anti-CD3 microbeads (Miltenyi, Bergisch Gladbach, Germany) and cultured for at least 5 days before functional assay.

CD69 Upregulation Assay

10^5 transduced Jurkat76 cells were cocultured overnight in a 1:1 ratio with Daudi cells as a target, with or without 100 μM of pamidronate treatment. The cells were then stained with the following antibody mix for 30 min on ice: Vδ2-FITC clone B6 (BioLegend), CD69-APC clone FN50 (Sony Biotechnology; cat. no. 2154550), and CD20-eFluor450 clone 2H7 (eBioscience; cat. no. 48-0209-42). After being washed, the cells were fixed using 1% paraformaldehyde and analyzed with BD FACS Canto II (BD Biosciences). Readouts were analyzed using FlowJo software.

Statistical Analysis

Statistical analysis was performed using R Studio. The two-sided Wilcoxon rank-sum test was used to calculate the median difference between two independent groups (31) and its significance.

Code Availability

Details of the code can be requested from the corresponding author.

RESULTS

Clonotypic Expansion Involves Concentration of the CDR3δ Phosphoantigen-Sensing Determinants

As a framework for the study of the repertoire fingerprints of the T-cell expansion, we used the publicly available repertoire data of healthy preterm infants and young children in whom γ9δ2T cells are actively proliferating (10), focusing in this study on the TCRδ chain. Intending to define the CDR3δ features associated with *in vivo* expansion, we classified clonotypes according to their abundance, or clonotype frequency f into non-expanded ($f < 0.1\%$), expanded ($0.1\% \leq f < 1\%$), and hyperexpanded ($f \geq 1\%$) and further analyzed their qualitative traits. Clonotypic expansion associated unambiguously with J1 region usage, and the presence of the "invariant T" nucleotide (invT) (32), or, more generally, a hydrophobic amino acid at position 5 (hAA5) of the CDR3δ (position 109 according to IMGT) (12), features that define the transition from poorly reactive neonatal (8, 19) to adult-type highly pAg-reactive repertoires (7, 9–11, 32) (**Supplementary Figure 1A**). Next to these well-defined pAg-sensing determinants, we noted a marked enrichment in shorter CDR3δ sequences in expanding clones, seen most clearly in the J1-rearranged subrepertoires (**Supplementary Figures 1B, C**). As clonotype frequency might be determined not solely by the expansion of a T-cell clone upon antigen encounter but by generation biases such as short-homology-repeat recombination (33–36) or convergent recombination [reviewed in (11)], we performed a separate analysis of the infant repertoires at the moment of postnatal "γ9δ2T cell burst" (3–5 weeks of age) (10) while splitting the data according to the possible origin of bias. We analyzed independently the germline-encoded sequences (no N insertions) and the sequences with N insertions, thus segregating the effect of the short-homology-repeat recombination. A separate analysis of the sequences shared between the subjects (public sequences) and private sequences allowed to account for the effect of convergent recombination since the generation of most if not all public clonotypes relies on this mechanism [(11); see **Supplementary Table 1**]. Although the highly prevalent "hyperexpanded" clonotypes at this developmental stage are almost exclusively germline-encoded and public, emphasizing the recombination effect on clonotype frequency, enrichment in sequences featuring

hAA5 and in shorter sequences with increased clonotype frequency remained equally evident in all independent analyses (**Supplementary Figures 1D, E**), suggesting that both features have a functional implication in T-cell proliferation and do not solely reflect a production bias.

Similar to the proliferative burst seen in early childhood, a marked γ9δ2T cell expansion associated with loss of CD27 expression has been occasionally noted in adults, resulting in γ9δ2TCR repertoires focused on a few (hyper)expanded clones (7). We wondered whether CDR3δ features that are associated with expansion in infant repertoires similarly underpin clonotypic expansion and T-cell maturation in adults. Therefore, we sorted $V\delta2^{pos}CD27^{pos}$ and $V\delta2^{pos}CD27^{neg}$ populations in a cohort of 9 adult donors (**Supplementary Figure 2A**) and performed high-throughput sequencing of the TCRδ chain of the sorted subsets, first focusing the analysis on the phenotypically younger $CD27^{pos}$ cells. The gating strategy at sorting did not include an antibody directed against the Vγ9 chain (**Supplementary Figure 2A**); however, we confirmed that $V\gamma9^{neg}V\delta2^{pos}$ cells represented less than 3.5% (median 1.75%) of the total $V\delta2^{pos}$ sorted cells and less than 10% (median 6.8%) of the $V\delta2^{pos}CD27^{pos}$ subset. $V\gamma9^{neg}V\delta2^{pos}$ cells were mainly $V\delta2^{dim}$ cells, a population gated out during sorting (**Supplementary Figure 2B**). As seen with the infant and the child repertoires, in the $CD27^{pos}$ cells of the healthy adults, clonotype frequency was positively associated with rearrangement to the J1 region, the invT nucleotide, and the hAA5 (**Figures 1A–C**); however, the effect sizes observed were smaller in comparison to changes seen in early ontogeny. Within the dominant subset of clonotypes rearranged to the shorter J1 region, a far less notable but still discernible enrichment in shorter sequences was seen with increased clonotype frequency, largely due to the fact that none of the long (>20 AA) sequences reached *f* of 1% (**Figures 1D, E**). Not surprisingly, as parts of the variable stretch between the conserved motifs of the CDR3δ are encoded by N nucleotides, we saw a similar decrease in the average number of the N insertions (**Figure 1F**). Germline-encoded sequences and public sequences are fewer in number and occupied space in adult repertoires compared to the infant ones (**Supplementary Figures 2C, D**); nevertheless, we repeated the analysis of private subrepertoires and subrepertoires generated with N insertions to account for the possible generation bias in these phenotypically "young" cells. We obtained overall similar trends for enrichment in J1 rearrangements, invT, and hAA5 (**Supplementary Figure 2E**) and equally in the shorter sequences, despite an overall shift toward slightly greater mean CDR3 lengths after sorting out the public sequences (**Supplementary Figures 2F, G**; see **Figure 3** and the corresponding text for the characteristics of the public sequences).

Thus, CDR3δ features postulated as determinants of sensing pAg-driven changes in BTN2 and BTN3 likely underpin the *in vivo* clonotypic expansions seen in infancy and adulthood, although the enrichment patterns are less pronounced in adult repertoires that are nearly saturated with pAg-reactive clones. Clonotypic expansion is further associated with a narrowing range of CDR3δ lengths. Enrichment in shorter sequences in expanded clones, translating, in part, from fewer N insertions, is readily apparent at the time of active T-cell expansion in infants and, as a reminiscence of the patterns seen in infancy, is still discernible in the repertoires of $CD27^{pos}$ cells in adults.

Phenotypic Maturation Reflects Repertoire Focusing, Which Is Independent of the CDR3δ Traits

We next focused on the repertoire traits associated with phenotypic maturation. Assuming that the phenotype shifts

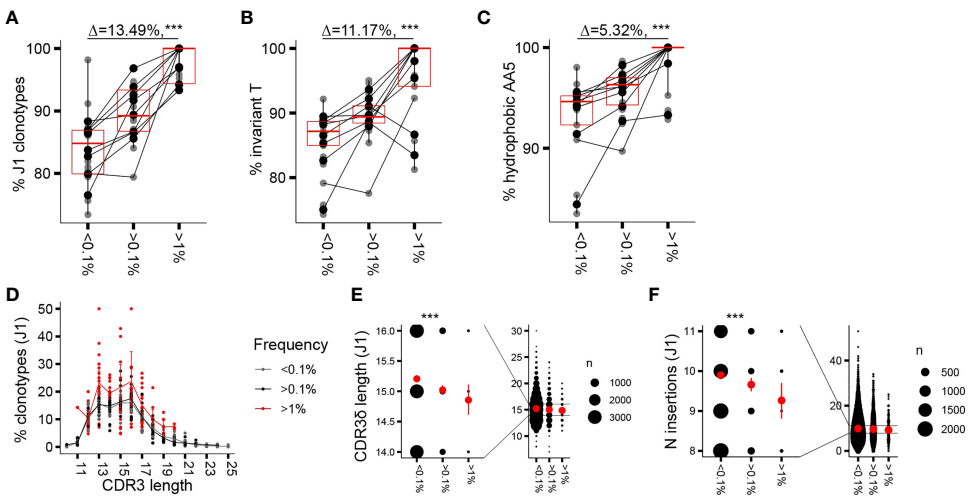


FIGURE 1 | Clonotypic expansion involves concentration of the CDR3δ pAg-sensing determinants. **(A–C)** Proportion of sequences featuring the determinants of pAg reactivity: rearrangement to J1 region **(A)**, the invT **(B)**, and a hAA5 (A/V/L/I/P/W/F/M) **(C)** among clonotypes with increasing *f* in the $CD27^{pos}$ cells. Lines connect the average of the replicates in each donor. Bars represent median values and interquartile range (IQR). The median difference and the p-values were obtained using Wilcoxon rank-sum test. **(D)** CDR3δ length distribution among J1-rearranged clonotypes in the $CD27^{pos}$ cells. Sequences were classified by *f*. Lines connect the mean abundances of clonotypes of each respective length within the frequency category. **(E, F)** CDR3δ length **(E)** and number of N insertions **(F)** in clonotypes rearranged to J1 region. Mean values and error bars representing 95% CI for the mean are shown in red ***P ≤ 0.001 (Wilcoxon rank-sum test).

result from an antigenic trigger in the periphery (37), we thus aimed to identify CDR3δ traits underlying peripheral repertoire selection. As adult donors are extremely heterogeneous with respect to the proportions of cells at distinct stages of differentiation (17), we concentrated on the part of the cohort in which a sizable fraction of the Vδ2+ compartment has completed differentiation to CD27−CD28−CD45RA++CD16+ phenotype, designated here as late-stage differentiation profile donors (n = 6 of the 20 donors phenotyped, cells from 4 out of 6 donors sorted, and repertoires sequenced; see **Supplementary Figure 2A**, an example of a healthy donor (HD) 94, and **Supplementary Figure 3A**). The rest of the cohort (n = 14, cells from 5 donors sorted and repertoires sequenced) was collectively characterized by the dominance of $CD27^{pos}$ cells (**Supplementary Figure 2A**, example of HD101), as well as by a less-differentiated state of the sorted $CD27^{neg}$ population, and was categorized here as belonging to the early-stage differentiation profile (**Supplementary Figure 3A**). Late-stage profile donors had a higher proportion of Vδ2TCR+ T cells among CD3+ cells (**Figure 2A**), and the expression of each differentiation marker in the cohort correlated positively (CD16) or negatively (CD27, CD28) with the percentage of Vδ2TCR+ T cells among total T cells (**Figure 2B**). The $CD27^{neg}$ population showed a markedly lower CDR3δ repertoire diversity (estimated here using Shannon entropy) compared to the $CD27^{pos}$ cells (**Figure 2C**). This resulted from a lower number of clonotypes in the $CD27^{neg}$ subset, suggesting a selection process as cells mature from "young" $CD27^{pos}$ to terminally differentiated $CD27^{neg}$ phenotype (**Figure 2D**), as well as from lower evenness, indicating a greater degree of clonotypic expansion in the terminally differentiated cells (evenness was estimated using D75, the percentage of unique clonotypes that occupy 75% of the sample library, **Figure 2E**). The same metrics in early-stage profile individuals showed less profound and less consistent demarcation between the populations sorted according to CD27 expression (**Supplementary Figures 3B–D**). In general, cells with larger distances in phenotype showed more CDR3δ repertoire dissimilarity. Thus, the TCRδ repertoire in phenotypically differentiated cells is shaped by both selection of clonotypes and clonotypic expansion, the latter arguably effectuating the observed inflation of the Vδ2+ compartment as a whole in the late-stage profile donors.

We then analyzed the qualitative repertoire traits that could denote repertoire selection, performing first a head-to-head comparison of the entire repertoires of the sorted subsets. Only a marginal enrichment in the pAg reactivity determinants (**Supplementary Figures 4A–C**) a slight shift towards longer CDR3 length (**Supplementary Figure 4D**) and no change in the number of N insertions (**Supplementary Figure 4E**) characterized the transition from $CD27^{pos}$ to $CD27^{neg}$ phenotype at the level of the complete repertoires. Considering the extreme focusing in the $CD27^{neg}$ compartment (median D75 of 9.43%), we were particularly interested in the characteristics of the high-frequency clonotypes. The "hyperexpanded" repertoires of the $CD27^{neg}$ cells appeared to be permissive to occasional J3 rearrangements, and sequences featuring "non-invT encoded" amino acids, mostly alanine and glycine (**Figures 2F–H**). Further, the CDR3δ length distribution that was centered around the 13 and 16 AA in the $CD27^{pos}$ subset here peaked at 14 and 17 AA (**Figure 2I**). Thus, although terminally differentiated cells harbor a TCR repertoire slightly more "saturated" in sequences sensing the pAg-driven ligand than cells of $CD27^{pos}$ phenotype, the extreme clonotypic expansions that skew the CDR3δ repertoires in the late-stage donors to near oligoclonality do not strictly rely on the pAg-sensing determinants, and therefore non- or poorly pAg-reactive T-cell clones may take prominent positions in the repertoire.

Public Delta Clonotypes Expand *In Vivo*, But Their Number Diminishes Upon Maturation

CDR3δ clonotypes shared between unrelated subjects (public δ clonotypes) have been shown to play a prominent role in the first wave of postnatal expansion of the γ9δ2T cells in newborns (9, 10). However, their number decreases with advancing age in children (10) and decreases even further in adults, possibly as a result of peripheral repertoire selection (7, 27), changes in thymic output after birth (36), or both. Here we aimed to explore the expansion potential and the evidence for peripheral selection of the public sequences in adults, by tracing the sequences shared between at least two individual donors in the current study. The proportion of public clonotypes increased with increasing *f* in the $CD27^{pos}$ population in the majority of the donors (**Figure 3A**). More widely shared clonotypes were encoded by larger numbers of nucleotypes, emphasizing that convergent recombination is the foundation of publicity (**Figure 3B**); however, we detected no tendency toward more common clonotypes having higher frequencies (**Figure 3C**), unlike the observations made for the public sequences of fetal origin in infants (10). We then narrowed the analysis down to public sequences with a high degree of sharing, i.e., sequences detected in >50% of the donors included in this study and present in multiple published studies (**Table 1**; see **Supplementary Table 1** for the encoding nucleotypes). We looked at the impact of peripheral repertoire selection on the maintenance of the public sequences, again using the model of transition from the $CD27^{pos}$ phenotype to terminal differentiation in the late-stage profile donors. We observed a loss of the highly common public sequences as the cells evolve from $CD27^{pos}$ to the $CD27^{neg}$ phenotype (**Figures 3D, E**), suggesting their deletion in the periphery as cells mature. Thus, public TCRδ clonotypes persist in the repertoires of healthy adults and expand *in vivo* but are only partly maintained when cells undergo terminal differentiation.

Public Delta Clonotype Function: The Beauty of Brevity

The published evidence for the expansion of public Vδ2 clonotypes in infants (10), our current data on their persistence into adulthood, the high degree of sharing and *in vivo* proliferation in adults made us wonder whether these sequences may share properties that would confer high TCR affinity to the pAg-driven ligand. We first characterized the

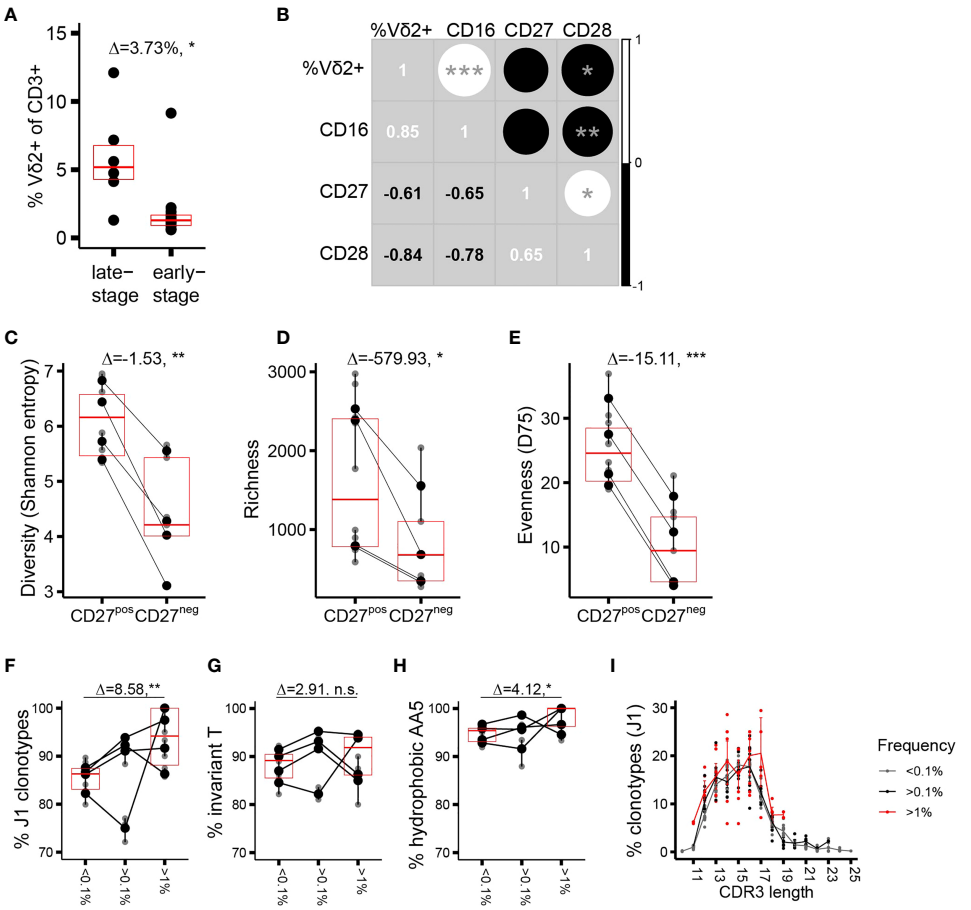


FIGURE 2 | Phenotypic maturation reflects repertoire focusing which is independent of the CDR3δ traits. **(A)** Proportion of Vδ2TCR+ T cells among CD3+ cells in the donors of different phenotypic profiles. **(B)** Correlation matrix showing Spearman's correlation coefficients for the proportion of Vδ2+ T cells among CD3+ T cells, and the proportion of CD27+, CD28+, or CD16+ T cells among Vδ2+ T cells, measured in an independent set of 10 healthy donors. **(C–E)** Measures of diversity: Shannon entropy **(C)**, repertoire richness (number of individual clonotypes, **(D)**, and repertoire evenness, defined as D75 **(E)** in the late-stage profile donors (n = 4). **(F–H)** Proportion of sequences featuring the determinants of pAg reactivity: rearrangement to J1 region **(F)**, the invT **(G)**, and a hydrophobic AA5 **(H)** among the clonotypes with increasing frequency *f* in the $CD27^{neg}$ cell population of the late-stage donors. Bars represent median values and IQRs. The median difference and the p-values were determined using Wilcoxon rank-sum test. *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. **(I)** CDR3δ length distribution among J1-rearranged clonotypes in the $CD27^{neg}$ cells. Sequences were classified by *f*. Lines connect the mean abundances of clonotypes of each respective length within the frequency category.

qualitative features of adult public CDR3δ sequences, looking for distinct traits that could potentially underpin their functionality. Public sequences were most notable for their short average length, which peaked at 12AA for the most common public clonotypes versus 16AA for the private sequences (**Figure 3F**), and the underlying low number of N insertions (**Figure 3G**). Public and private sequences did not differ in J region usage (**Figure 3H**), while the CDR3δ AA5 was exclusively hydrophobic in common public sequences, with relative enrichment in non-invT-encoded amino acids A and W (**Figures 3I, J**).

In order to assess the impact of the public and private TCRδ chain properties on TCR functionality, we selected four common public CDR3δ sequences present in this study, as well as in previously published repertoire studies (9, 10, 26–28) (**Table 1**), which all have 12AA length, and expressed them alongside a fixed γ chain of the TCR clone 5 (30). A set of private sequences of various lengths (median length 16AA), all rearranged to the J1 region and all harboring a hAA5, was taken along as a control (**Supplementary Table 2**). All constructs were transduced into TCR-deficient Jurkat76 (J76) cells, yielding similar γδTCR surface expression levels (**Supplementary Figure 5A**), and CD69 upregulation by J76 cells upon co-culture with the tumor cell line Daudi, was used as a marker for TCR-mediated T-cell activation. We saw higher T-cell activation with the short public δ sequences in the condition without N-amino bisphosphonate (NBP), while NBP treatment eliminated the difference (**Figure 3K**). There was no confounding effect of the TCR surface expression level on the observed differences (**Supplementary Figure 5B**). In summary, a shorter length of public CDR3δ sequences, in addition to the consistent positivity for hAA5, is associated with a higher functional avidity, as demonstrated in the TCR gene transfer experiment.

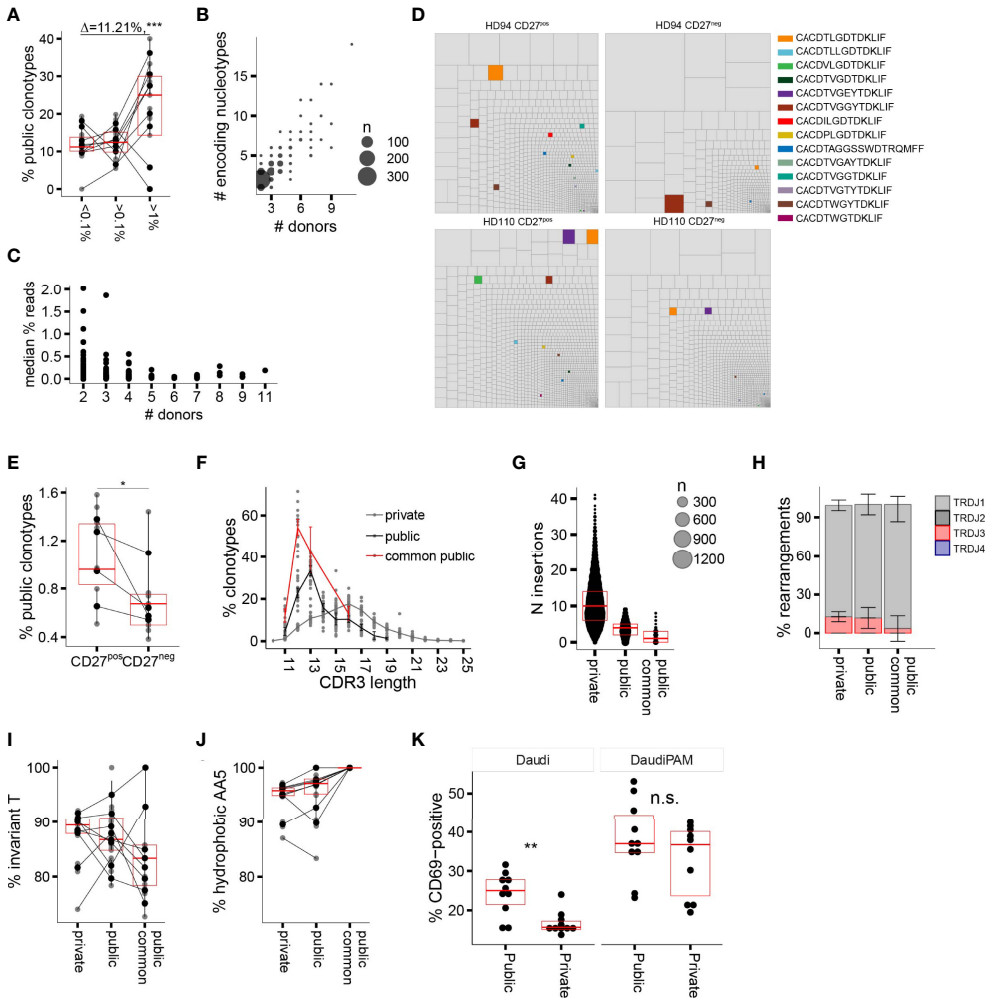


FIGURE 3 | Public delta sequences: persistence in the adult repertoire, qualitative traits, and functionality. **(A)** Percentage of public AA CDR3δ sequences among clonotypes with increasing *f*. **(B)** Number of individual nucleotypes encoding for individual public AA sequence in relation to the sequence "publicity" (number of individual donors sharing a given sequence). **(C)** The relationship between the number of donors sharing a public sequence and the median *f* (all donors pooled together; each dot represents an individual sequence). **(D)** Representative treemaps of the repertoires of $CD27^{pos}$ and $CD27^{neg}$ populations of two late-stage profile donors. Public AA clonotypes are highlighted in color; private sequences are shown in gray. **(E)** Percentage of highly common public AA CDR3δ sequences in the repertoires of $CD27^{pos}$ and $CD27^{neg}$ populations in the late-stage profile donors (n = 4). **(F)** CDR3δ length distribution of private vs. public AA sequences; the highly common public clonotypes are shown separately in red. **(G)** Number of total N insertions in private vs. public AA sequences. **(H–J)** J region usage **(H)**, invT **(I)**, and hAA5 **(J)** in private versus public AA sequences. **(K)** Percentage $CD69^{pos}$ TCR-transduced Jurkat76 cells upon overnight incubation with target cell line Daudi without or in the presence of 100 μM of PAM. In all panels horizontal bars represent median values and IQRs. The median differences and the p-values were determined using Wilcoxon rank-sum test. *p < 0.05; **p ≤ 0.01; ***p ≤ 0.001. Panels **(A, C, F–J)** refer to the $CD27^{pos}$ cell populations.

Surface NKG2D Expression Declines With T-Cell Maturation and Is Independent of TCR Clonality

Despite the general trend for preferential in vivo expansion of putative high-affinity CDR3δ clones containing the traits associated with sensing pAg-driven changes in BTN2 and BTN3, we occasionally witnessed (hyper)expanded clonotypes harboring polar, or even charged amino acids at position 5 of the CDR3δ (**Figure 4A**), which according to the existing experimental evidence are non- or poorly pAg-reactive (13, 14). We hypothesized that pAg-unrelated stimuli could trigger *in vivo* proliferation of such T-cell clones and that poorly pAg-reactive TCRs could possibly benefit from the expression of non-clonally restricted activating receptors. In exploring this hypothesis, we focused on NKG2D, the best-studied activating NKR on γδT cells, previously reported to play both an autonomous stimulatory role (38–40) and a co-stimulatory role next to a γδTCR (41, 42).

We found the absolute majority of the Vδ2+ T cells in adult blood to express NKG2D (**Supplementary Figure 6A**). NKG2D expression correlated with T-cell differentiation phenotype within a donor, as, in general, more differentiated $CD27^{neg}CD28^{neg}$ T cells

TABLE 1 | Public clonotypes shared among >50% of donors in this study and their occurrence in other published repertoire studies.

Public CDR3δ clonotypes	CDR3δ length	Nucleotypes		# donors in this study	Adult repertoires			Infant and child repertoires	
		Public	Private		(27)	(26)	(28)	(10)	(9)
CACDTLGDTDKLIF*	12	6	13	11	+	+	+	+	+
CACDTLLGDTDKLIF	13	5	9	9	+	+	–	+	+
CACDVLGDTDKLIF*	12	2	4	9	+	+	+	+	+
CACDTLGVYTDKLIF	13	1	8	9	–	–	+	+	+
CACDTVGDTDKLIF*	12	2	8	8	+	+	+	+	+
CACDTVGEYTDKLIF	13	2	5	8	+	+	+	+	+
CACDTVGGYTDKLIF	13	3	11	8	+	+	–	+	+
CACDILGDTDKLIF	12	2	3	7	+	+	+	+	+
CACDPLGDTDKLIF	12	2	10	7	–	+	+	+	+
CACDTAGGSSWDTRQMFF	16	2	4	7	–	+	+	+	+
CACDTVGGTDKLIF	12	3	6	7	–	+	+	+	–
CACDTVGTYTDKLIF	13	0	8	7	+	+	+	+	–
CACDTWGTDKLIF	11	1	6	7	+	+	+	+	+
CACDTWGYTDKLIF*	12	2	3	7	+	+	+	+	+

Sequences used for the TCR transfer experiment (see below) are marked with an asterisk.

expressed less surface NKG2D (**Supplementary Figure 6B**). The trend for declining surface expression with T-cell maturation was in line with lower NKG2D mRNA expression levels in more differentiated populations, detected in an independent dataset that we extracted from the study by Ryan et al. (17) (**Supplementary Figure 6C**). Conversely, gating on NKG2D^{bright} vs. NKG2D$^{dim/neg}$ cells (**Supplementary Figure 6D**) revealed an overall "younger" phenotype of the NKG2D^{bright} cells in the majority of donors, with notably higher expression of CCR7, CD27, and TCRγδ (**Supplementary Figures 6E, F**), with the NKG2D^{intermediate} population lying between two extremes (**Supplementary Figures 6G, H**).

To examine the repertoire characteristics of Vδ2+ T cells in the context of NKG2D expression, we sorted NKG2D$^{dim/neg}$ and NKG2D^{bright} Vδ2+ T cells as shown in **Supplementary Figure 6D** and again performed sequencing of the TRDV2 repertoires as described above. Unlike the T-cell differentiation profile, the NKG2D expression level did not denote any change in the TCR diversity metrics (**Figures 4B–D**). When we looked into the set of qualitative CDR3δ features that could define the unique clonotypes, we detected no difference between the NKG2D$^{neg/dim}$ and NKG2D^{bright} cells, except for a bias in J1 region usage in the low-frequency clones (**Figure 4E**), which, however, did not translate into a shorter average CDR3δ length (**Supplementary Figure 6I**).

Thus, although the expression level of the activating receptor NKG2D in adult γ9δ2T cells shows a discernible relation to the T-cell differentiation status, it is not associated with a bias toward putative low-affinity or pAg-unreactive TCRs.

Expression of Inhibitory NKR CD94/NKG2A(B) Associates With a Bias Toward Putative High-Affinity γ9δ2TCRs

The net total of the inhibitory and activating signals from the environment will direct the γ9δ2T cell effector functions (43). Although we did not find compelling evidence for skewing in NKG2D expression toward putative pAg-unreactive clones, there could be still an effect of the signal strength through the γδTCR, generally considered "signal one" (44), on the landscape of the remaining NKRs and thus on the net NKR signal. We therefore aimed to investigate the TCR repertoire features in the context of surface expression of CD94/NKG2A(B), an inhibitory C-type lectin receptor broadly expressed on the γ9δ2T cells and recently reported to identify a highly pAg- and tumor-reactive γ9δ2T cell subset (45).

We sorted Vδ2+ T cells from peripheral blood based on CD94 expression into a CD94^{bright} subpopulation, in which CD94 is known to heterodimerize with NKG2A(B) (22, 46), and a CD94^{neg} subpopulation (**Supplementary Figures 6J, K**), and performed a similar comparative CDR3δ repertoire analysis. CD94^{bright} cells were collectively characterized by a higher percentage of sequences featuring an invT-encoded or generally hydrophobic AA5 in the repertoire (**Figure 4F**), in line with an earlier low-throughput sequencing study (22). CDR3δ bias according to the invT/hAA5 as one of the strong predictors of the TCR reactivity is further exemplified here by the differential clonotypic expansion of a single pAg-unreactive clonotype (**Figure 4G**). Moreover, we observed a trend toward enrichment in shorter CDR3δ sequences in CD94^{bright} cells (**Supplementary Figure 6L**).

In summary, the CDR3δ features known to underpin pAg-sensing and γ9δ2TCR signal strength appear to impact the surface expression of an inhibitory NKR CD94/NKG2A(B), with the putative high-affinity CDR3δ sequences being accompanied by high CD94/NKG2A(B) surface expression, while low-affinity TCR-bearing clones remained predominantly CD94-negative.

DISCUSSION

The discovery of the germline region-mediated interactions at the basis of the mechanism of antigen recognition by a γ9δ2TCR (2, 4) provides a rationale for the observed *in vitro* (47) and *in vivo* (9, 10) polyclonal γ9δ2T cell responses to antigenic stimuli, fostering the paradigm of the γ9δ2TCR as a pattern recognition

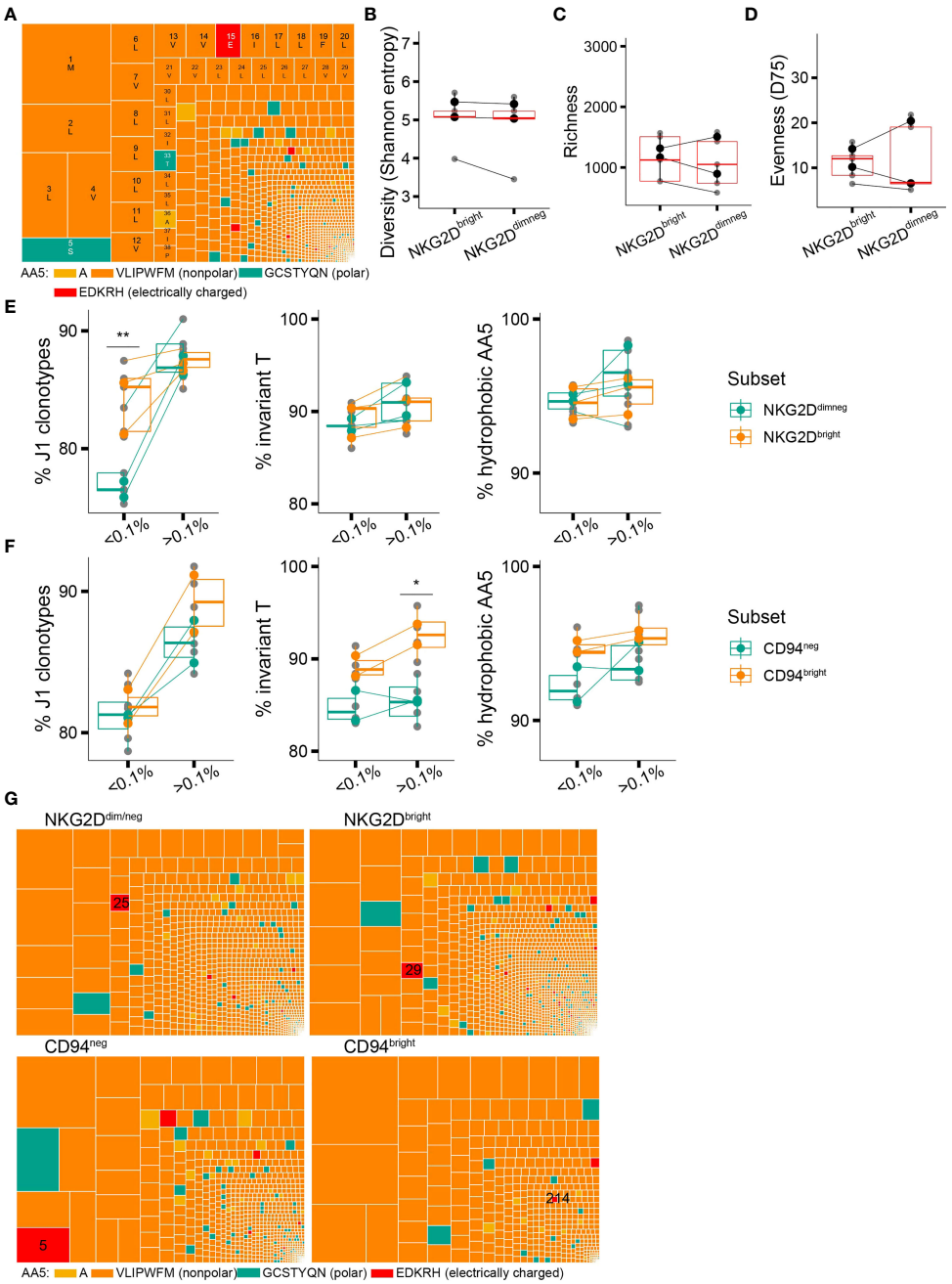


FIGURE 4 | Non-clonally restricted NKG2D expression versus a CDR3δ bias in surface expression of CD94/NKG2A(B). **(A)** An example of a repertoire of a healthy donor where a putative non-pAg-reactive clonotype occupies a prominent position (position 16, CACDT**E**GTPTLLIF). **(B–D)** TCR repertoire diversity of the NKG2D$^{dim/neg}$ and NKG2D^{bright} cell populations estimated with Shannon entropy **(B)**, species richness **(C)**, and evenness(D75) **(D)** (n = 3 donors). y-Axis limits are set equal to those in **Figures 2C–E**. **(E)** Qualitative features of the repertories of the NKG2D$^{dim/neg}$ and NKG2D^{bright} cells in relation to clonotype frequency: proportion of sequences rearranged to J1 region and proportion of sequences featuring the invT or a hAA5. **(F)** Qualitative features of the repertories of the CD94^{neg} and CD94^{bright} cells in relation to clonotype frequency: proportion of sequences rearranged to J1 region and proportion of sequences featuring the invT or a hAA5 (n = 2 donors). **(G)** Treemaps showing the CDR3δ repertoires of the NKG2D$^{dim/neg}$, NKG2D^{bright}, CD94^{bright}, and CD94^{neg}-sorted populations in the same healthy donor as in **(A)** Position of the putative non-pAg reactive clonotype CACDT**E**GTPTLLIF is indicated. **(B–F)** Bars represent median values and interquartile range (IQR) (Wilcoxon rank-sum test, *p < 0.05; **p ≤ 0.01; ***p ≤ 0.001).

receptor. The absence of a known polymorphic ligand leaves the role of the highly diverse CDR3 regions currently undefined. Nevertheless, particularly in the case of the CDR3δ repertoire, a distinct evolution pattern seen during ontogeny (11) hints at the still significant role of the CDR3δ in antigen recognition. Here we expand the evidence on the role of CDR3δ in γ9δ2TCR-mediated responses. We show that *in vivo* γ9δ2T cell expansion and phenotypic maturation are associated with repertoire

enrichment in the CDR3δ determinants of sensing pAg-induced changes (12, 48, 49), presumably equating to the concentration of the γ9δ2TCRs with higher binding affinity and signal strength (13, 14). We find that the number of the highly common public TCRδ sequences that combine all known pAg reactivity features, known to diminish with the transition from infant to adult repertoires, diminishes even further within an individual upon T-cell maturation, despite optimal signaling. Lastly, we detect skewing in the inhibitory NKR CD94/NKG2A(B) surface expression toward cells expressing putative high-affinity TCRs. In contrast, expression of the activating NKR NKG2D emerges as independent of CDR3δ traits, suggesting possibly higher net NKR activating signal in T cells with low γ9δ2TCR affinity.

Prevalence of a clonotype in the repertoire, especially in the repertoires of infants and of the "younger" $CD27^{pos}$ cells in adults, had the most apparent association with the features of the Vδ2 chain known as "sensors" of the pAg-induced changes in the target. Such features include rearrangement to the J1 region, a hydrophobic AA5, and, notably, an optimal (shorter) CDR3δ length. Although generation biases such as short-homology-repeat recombination (33–36) and convergent recombination (11) play a role in shaping the repertoire, especially that of the immature cells, the above-described enrichment patterns remained evident in the analysis of subrepertoires generated by either mechanism, suggesting the true implication of the pAg-sensing features in T-cell proliferation, and not solely a reflection of a generation bias. The enrichment was less pronounced in adult repertoires, compared to the repertoires of infants, presumably as a result of the switch from the fetal-type to adult-type thymic output (36), which is nearly saturated with pAg-reactive clones.

Analysis of the $CD27^{pos}$ and $CD27^{neg}$ populations in the late-stage profile donors allowed us to correlate distances in phenotype to CDR3δ repertoire dissimilarity. A change in phenotype from young $CD27^{pos}CD28^{pos}$ cells to a mature $CD27^{neg}CD28^{neg}CD16^{pos}$ profile was associated with extreme repertoire focusing, suggesting a major proliferation event preceding maturation, or, alternatively, expansion of the already mature cells (20). Such inflation remained visible at the level of the entire Vδ2 compartment in these donors, and both the visible inflation and mature phenotypes of these cells suggested preceding antigenic triggers rather than possible generation bias. Although the enrichment in putative high-affinity clonotypes was equally evident with increasing clonotype frequency in the mature $CD27^{neg}CD28^{neg}CD16^{pos}$ cells, the hyperexpanded clonotypes in the terminally differentiated subpopulation occasionally encompassed sequences that in theory mediate submaximal pAg reactivity.

Earlier studies of the αβT cells highlighted the mechanistic differences between homeostatic proliferation with the maintenance of the T-cell memory phenotype that depends on the strength of tonic TCR signaling, in comparison to massive expansion with differentiation to the effector phenotype as a result of antigenic stimulation [reviewed in (37)]. We suggest the possibility of a similar disparity here: while expansion in the "young" (naïve and central memory) cells, defined here as $CD27^{pos}$, clearly showcases the enrichment in the CDR3δ traits associated with TCR binding affinity, the repertoire of the terminally differentiated $CD27^{neg}$ population might reflect additional imprints of the stimuli other than BTNs/pAgs, which bypass signaling through the TCR. Another argument supporting the hypothesis of critical (co-)stimulants involved in massive oligoclonal proliferation seen in late-stage profile donors is the maintenance of the once-established profile over long time periods, even under repetitive NBP stimulation (17). Likewise, *in vitro* stimulation with pAg or NBPs could not induce such massive oligoclonal outgrowth from PBMCs (47); however, the latter study did not specify the phenotype of the cells used for stimulation experiments.

Sequences that universally possess the key pAg reactivity features are the (near-)germline public TCRδ sequences of high generation probability (50) shared between the repertoires of multiple unrelated individuals. The repertoire studies published to date (7–10, 27, 28, 36, 47) define public sequences at the AA rather than at the NT level, to highlight the functionality of a sequence rather than its generation probability, and to account for convergent recombination (11). Thus, public Vδ2 clonotypes have recently been shown to expand *in vivo* shortly after birth (9, 10) and *in vitro* after stimulation with NBP of cord blood-derived γ9δ2 T cells (47). The more common sequences appear to take more prominent positions in the infant repertoires, pointing to the functional relevance of the public sequences at this stage of development (10). Here we found highly common sequences seen in children across continents, back in the adult repertoires, whether due to persistence from early childhood or *de novo* thymic synthesis at a later stage. The more common sequences had a higher number of encoding individual nucleotypes, emphasizing the role of convergent recombination in sequence sharing. However, we did not find the most common public sequences to occupy more repertoire space in adults, suggesting that recombination bias, while explaining occurrence in multiple unrelated donors, does not necessarily govern repertoire focusing in an individual donor. Being uniformly positive for a hAA5, and except for an occasional J3-rearranged sequence distinguished by a short length of 11-13 AA, the highly common public δ sequences appeared to confer higher functional avidity when expressed alongside a fixed γ chain, as demonstrated by the TCR transfer experiment, suggesting their functional advantage. However, high-dose pamidronate treatment minimized the difference between the shorter public δ chains and the longer private ones, supposedly through conjugation enhancement and stabilization of the immunological synapse in case of putative low-affinity TCRs (13). Remarkably, as the cells differentiate to effector phenotype in the periphery, public sequences are partly disappearing. Thus, a decline in the number of public clonotypes set by the switch in thymic production from the neonatal to adult-type, more private output (36), is extended further, due to negative selection in the periphery.

Collectively, our results support the role of the specific features of the CDR3δ as a γ9δ2TCR signaling modulator by demonstrating their steady enrichment associated with

clonotypic expansion, and to a lesser extent with the peripheral repertoire selection *in vivo*. In addition, our results corroborate the paradigm of the orderly evolution of the CDR3δ repertoire, rather than its use for random barcoding. *In vivo* T-cell homeostatic proliferation is related to J region usage, hAA5, and, no less critically, CDR3δ length. At the same time, antigen-driven outgrowth might result in the endorsement of putative low-affinity sequences at the prominent positions in the repertoire and the loss of the high-affinity public sequences.

The expression of NK receptors on terminally differentiated CD8+ αβT cells has been associated with defective TCR signaling and a shift from TCR-mediated to NKR-mediated effector functions, including NKG2D-mediated responses (15, 16, 51). Surprisingly, in the majority of the donors, we found NKG2D expression to decline as γ9δ2T cells mature. NKG2D surface expression could not point to clonotypes with supposed weak γ9δ2TCR signaling, substantiating the notion of its constitutive expression that is uncoupled from the γ9δ2TCR signal strength. In contrast, we observed a bias toward high-affinity CDR3δ features in the surface expression of CD94/NKG2A(B), corroborating the results of the earlier low-throughput study (22). The skewed expression of CD94/NKG2A(B) found here is in line with the activation-inducible expression of this inhibitory NKR in CD8+ αβT cells (52) and its low expression on γ9δ2T cells at the early stages of development, compared to relatively high NKG2D expression already *in utero* (19). We consider the observed CDR3δ bias a likely explanation for the recently reported high antitumor responses of the NKG2A^{pos} γ9δ2T cells (45).

From the translational standpoint, with the advancing field of γδT cell- and γδTCR-based therapies in mind, the repertoire–phenotype associations explored in this study are crucial. Researchers exploring these areas should consider the fact that donors with the highest numbers of γ9δ2T cells in the periphery, which are likely to be selected for *in vitro* experiments, as it is easy to get sufficient cell numbers, will frequently harbor cells whose phenotype is largely skewed toward terminal differentiation and whose repertoire, in turn, will be skewed to near-oligoclonality, where potentially poorly pAg-reactive clonotypes may take prominent positions. Concerning the design of the high-affinity γ9δ2TCR-based therapies (14, 24), CDR3δ features such as the AA5 and CDR3 length are essential, while J1 region usage might reflect an advantage of the shorter length. Lastly, as a high-affinity γ9δ2TCR will influence the net NKR signaling through upregulation of inhibitory receptors, it is worth modifying this second signal in favor of the activating arm.

DATA AVAILABILITY STATEMENT

FASTQ files of the TRD sequences are deposited and available under the Sequence Read Archive (SRA) accession code PRJNA851385 (http://www.ncbi.nlm.nih.gov/bioproject/851385).

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AV, AJ, DB, and JK designed the experiments. AV, AJ, LG, AY, SD, and FK performed the experiments and analyzed the data. AV, AJ, and JS generated and analyzed the high-throughput sequencing data. AV and JK wrote the manuscript. DB, TS, ZS, and JK supervised the work. All authors provided critical reviews.

FUNDING

Funding for this study was provided by ZonMW 43400003 and VIDI-ZonMW 917.11.337, KWF UU 2010-4669, UU 2013-6426, UU 2014-6790, and UU 2015-7601, UU 2018-11393, UU 2018-11979, UU 2020-12586, and UU 2021-13043 to JK.

ACKNOWLEDGMENTS

We thank the staff of the Flow Core Facility at the UMC Utrecht for their kind assistance with cell sorting.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.915366/full#supplementary-material

REFERENCES

1. Attaf M, Huseby E, Sewell AK. Alphabeta T Cell Receptors as Predictors of Health and Disease. *Cell Mol Immunol* (2015) 12:391–9. doi: 10.1038/cmi.2014.134
2. Karunakaran M. M., Willcox C. R., Salim M., Paletta D., Fichtner A. S., Noll A., et al. Butyrophilin-2a1 Directly Binds Germline-Encoded Regions of the Vgamma9Vdelta2 TCR and Is Essential for Phosphoantigen Sensing. *Immunity* (2020) 52:487–498.e486. doi: 10.1016/j.immuni.2020.02.014
3. Gu S., Sachleben J. R., Boughter C. T., Nawrocka W. I., Borowska M. T., Tarrasch J. T., et al. Phosphoantigen-Induced Conformational Change of Butyrophilin 3A1 (BTN3A1) and its Implication on Vgamma9Vdelta2 T Cell Activation. *Proc Natl Acad Sci U.S.A.* (2017) 114:E7311–20. doi: 10.1073/pnas.1707547114
4. Rigau M., Ostrouska S., Fulford T. S., Johnson D. N., Woods K., Ruan Z., et al. Butyrophilin 2A1 is Essential for Phosphoantigen Reactivity by Gamma Delta T Cells. *Science* (2020) 367:642. doi: 10.1126/science.aay5516
5. Yang Y. Y., Li L. P., Yuan L. J., Zhou X. Y., Duan J. X., Xiao H. Y., et al . A Structural Change in Butyrophilin Upon Phosphoantigen Binding Underlies Phosphoantigen-Mediated V Gamma 9V Delta 2 T Cell Activation. *Immunity* (2019) 50:1043. doi: 10.1016/j.immuni.2019.02.016
6. Sebestyen Z., Scheper W., Vyborova A., Gu S., Rychnavska Z., Schiffler M., et al. RhoB Mediates Phosphoantigen Recognition by Vgamma9Vdelta2 T Cell Receptor. *Cell Rep* (2016) 15:1973–85. doi: 10.1016/j.celrep.2016.04.081

7. Davey M. S., Willcox C. R., Hunter S., Kasatskaya S. A., Remmerswaal E. B.M., Salim M., et al. The Human Vdelta2(+) T-Cell Compartment Comprises Distinct Innate-Like Vgamma9(+) and Adaptive Vgamma9(-) Subsets. *Nat Commun* (2018) 9:1760. doi: 10.1038/s41467-018-04076-0
8. Kallemeijn M. J., Kavelaars F. G., van derKlift M. Y., Wolvers-Tettero I. L.M., Valk P. J.M., van Dongen J. J.M., et al. Next-Generation Sequencing Analysis of the Human TCRgammadelta+ T-Cell Repertoire Reveals Shifts in Vgamma- and Vdelta-Usage in Memory Populations Upon Aging. *Front Immunol* (2018) 9:448. doi: 10.3389/fimmu.2018.00448
9. Papadopoulou M., Dimova T., Shey M., Briel L., Veldtsman H., Khomba N., et al. Fetal Public V Gamma 9V Delta 2 T Cells Expand and Gain Potent Cytotoxic Functions Early After Birth. *P Natl Acad Sci USA* (2020) 117:18638–48. doi: 10.1073/pnas.1922595117
10. Ravens S., Fichtner A. S., Willers M., Torkornoo D., Pirr S., Schoning J., et al. Microbial Exposure Drives Polyclonal Expansion of Innate Gammadelta T Cells Immediately After Birth. *Proc Natl Acad Sci U.S.A.* (2020) 117:18649–60. doi: 10.1073/pnas.1922588117
11. Willcox CR, Davey MS, Willcox BE. Development and Selection of the Human Vgamma9Vdelta2(+) T-Cell Repertoire. *Front Immunol* (2018) 9:1501. doi: 10.3389/fimmu.2018.01501
12. Wang H, Fang ZM, Morita CT. V Gamma 2V Delta 2 T Cell Receptor Recognition of Prenyl Pyrophosphates Is Dependent on All CDRs. *J Immunol* (2010) 184:6209–22. doi: 10.4049/jimmunol.1000231
13. Vyborova A., Beringer D. X., Fasci D., Karaiskaki F., van Diest E., Kramer L., et al. Gamma9delta2t Cell Diversity and the Receptor Interface With Tumor Cells. *J Clin Invest* (2020) 130:4637–51 . doi: 10.1172/JCI132489
14. van Diest E., Lopez P. H., Meringa A. D., Vyborova A., Karaiskaki F., Heijhuurs S., et al. Gamma Delta TCR Anti-CD3 Bispecific Molecules (GABs) as Novel Immunotherapeutic Compounds. *J Immunother Cancer* (2021) 9:e003850. doi: 10.1136/jitc-2021-003850
15. Pereira B. I., De Maeyer R. P.H., Covre L. P., Nehar-Belaid D., Lanna A., Ward S., et al. Sestrins Induce Natural Killer Function in Senescent-Like CD8(+) T Cells. *Nat Immunol* (2020) 21:684–94. doi: 10.1038/s41590-020-0643-3
16. Fann M., Chiu W. K., Wood W. H., 3rd Levine B. L., Becker K. G., Weng N. P., et al. Gene Expression Characteristics of CD28null Memory Phenotype CD8+ T Cells and its Implication in T-Cell Aging. *Immunol Rev* (2005) 205:190–206. doi: 10.1111/j.0105-2896.2005.00262.x
17. Ryan P. L., Sumaria N., Holland C. J., Bradford Izotova C. M.N., Grandjean C. L. Heterogeneous Yet Stable Vdelta2(+) T-Cell Profiles Define Distinct Cytotoxic Effector Potentials in Healthy Human Individuals. *Proc Natl Acad Sci U.S.A.* (2016) 113:14378–83. doi: 10.1073/pnas.1611098113
18. Angelini D. F., Borsellino G., Poupot M., Diamantini A., Poupot R., Bernardi G., et al. Fc Gamma RIII Discriminates Between 2 Subsets of V Gamma 9V Delta 2 Effector Cells With Different Responses and Activation Pathways. *Blood* (2004) 104:1801–7. doi: 10.1182/blood-2004-01-0331
19. Dimova T., Brouwer M., Gosselin F., Tassignon J., Leo O., Donner C., et al. Effector Vgamma9Vdelta2 T Cells Dominate the Human Fetal Gammadelta T-Cell Repertoire. *Proc Natl Acad Sci U.S.A.* (2015) 112:E556–565. doi: 10.1073/pnas.1412058112
20. Pizzolato G., Kaminski H., Tosolini M., Franchini D. M., Pont F., Martins F., et al. Single-Cell RNA Sequencing Unveils the Shared and the Distinct Cytotoxic Hallmarks of Human TCRV Delta 1 and TCRV Delta 2 Gamma Delta T Lymphocytes. *P Natl Acad Sci USA* (2019) 116:11906–15. doi: 10.1073/pnas.1818488116
21. Pont F., Familiades J., Dejean S., Fruchon S., Cendron D., Poupot M., et al. The Gene Expression Profile of Phosphoantigen-Specific Human Gammadelta T Lymphocytes is a Blend of Alphabeta T-Cell and NK-Cell Signatures. *Eur J Immunol* (2012) 42:228–40. doi: 10.1002/eji.201141870
22. Halary F., Peyrat M. A., Champagne E., Lopez-Botet M., Moretta A., Moretta L., et al. Control of Self-Reactive Cytotoxic T Lymphocytes Expressing Gamma Delta T Cell Receptors by Natural Killer Inhibitory Receptors. *Eur J Immunol* (1997) 27:2812–21. doi: 10.1002/eji.1830271111
23. Sebestyen Z, Prinz I, Dechanet-Merville J, Silva-Santos B, Kuball J. Translating Gammadelta (Gammadelta) T Cells and Their Receptors Into Cancer Cell Therapies. *Nat Rev Drug Discovery* (2020) 19:169–84. doi: 10.1038/s41573-019-0038-z
24. Marcu-Malina V., Heijhuurs S., van Buuren M., Hartkamp L., Strand S., Sebestyen Z., et al. Redirecting Alphabeta T Cells Against Cancer Cells by Transfer of a Broadly Tumor-Reactive gammadeltaT-Cell Receptor. *Blood* (2011) 118:50–9. doi: 10.1182/blood-2010-12-325993
25. Nazarov V. I., Pogorelyy M. V., Komech E. A., Zvyagin I. V., Bolotin D. A., Shugay M., et al. Tcr: An R Package for T Cell Receptor Repertoire Advanced Data Analysis. *BMC Bioinf* (2015) 16:175. doi: 10.1186/s12859-015-0613-1
26. Davey M. S., Willcox C. R., Joyce S. P., Ladell K., Kasatskaya S. A., McLaren J. E., et al. Clonal Selection in the Human Vdelta1 T Cell Repertoire Indicates Gammadelta TCR-Dependent Adaptive Immune Surveillance. *Nat Commun* (2017) 8:14760. doi: 10.1038/ncomms14760
27. Ravens S., Schultze-Florey C., Raha S., Sandrock I., Drenker M., Oberdorfer L., et al. Human Gammadelta T Cells are Quickly Reconstituted After Stem-Cell Transplantation and Show Adaptive Clonal Expansion in Response to Viral Infection. *Nat Immunol* (2017) 18:393–401. doi: 10.1038/ni.3686
28. Janssen A., Villacorta Hidalgo J., Beringer D. X., van Dooremalen S., Fernando F., van Diest E., et al. Gammadelta T-Cell Receptors Derived From Breast Cancer-Infiltrating T Lymphocytes Mediate Antitumor Reactivity. *Cancer Immunol Res* (2020) 8:530–43. doi: 10.1158/2326-6066.CIR-19-0513
29. Yousfi Monod M, Giudicelli V, Chaume D, Lefranc MP. IMGT/JunctionAnalysis: The First Tool for the Analysis of the Immunoglobulin and T Cell Receptor Complex V-J and V-D-J JUNCTIONs. *Bioinformatics* (2004) 20(Suppl 1):i379–385. doi: 10.1093/bioinformatics/bth945
30. Grunder C., van Dorp S., Hol S., Drent E., Scholten K., Heijhuurs S., et al. Gamma 9-and Delta 2-CDR3 Domains Regulate Functional Avidity of T Cells Harboring C9d2 T Cell Receptors. *J Immunother* (2012) 35:723–3. doi: 10.1182/blood-2012-05-432427
31. Hodges JL, Lehmann EL. Estimates of Location Based on Rank-Tests. *Ann Math Stat* (1963) 34:598. doi: 10.1214/aoms/1177704172
32. Breit TM, Wolvers-Tettero IL, van Dongen JJ. Unique Selection Determinant in Polyclonal V Delta 2-J Delta 1 Junctional Regions of Human Peripheral Gamma Delta T Lymphocytes. *J Immunol* (1994) 152:2860–4.
33. Ma L., Papadopoulou M., Taton M., Genco F., Marchant A., Meroni V., et al. Effector Vgamma9Vdelta2 T Cell Response to Congenital Toxoplasma Gondii Infection. *JCI Insight* (2021) 6. doi: 10.1172/jci.insight.138066
34. Papadopoulou M, Sanchez Sanchez G, Vermijlen D. Innate and Adaptive Gammadelta T Cells: How, When, and Why. *Immunol Rev* (2020) 298:99–116. doi: 10.1111/imr.12926
35. Zhang Y., Cado D., Asarnow D. M., Komori T., Alt F. W., Raulet D. H., et al. The Role of Short Homology Repeats and TdT in Generation of the Invariant Gamma Delta Antigen Receptor Repertoire in the Fetal Thymus. *Immunity* (1995) 3:439–47. doi: 10.1016/1074-7613(95)90173-6
36. Papadopoulou M., Tieppo P., McGovern N., Gosselin F., Chan J. K.Y., Goetgeluk G., et al. TCR Sequencing Reveals the Distinct Development of Fetal and Adult Human Vgamma9Vdelta2 T Cells. *J Immunol* (2019) 203:1468–79. doi: 10.4049/jimmunol.1900592
37. Surh CD, Sprent J. Homeostasis of Naive and Memory T Cells. *Immunity* (2008) 29:848–62. doi: 10.1016/j.immuni.2008.11.002
38. Rincon-Orozco B., Kunzmann V., Wrobel P., Kabelitz D., Steinle A., Herrmann T., et al. Activation of V Gamma 9V Delta 2 T Cells by NKG2D. *J Immunol* (2005) 175:2144–51. doi: 10.4049/jimmunol.175.4.2144
39. Wrobel P., Shojaei H., Schittek B., Gieseler F., Wollenberg B., Kalthoff H., et al. Lysis of a Broad Range of Epithelial Tumour Cells by Human Gamma Delta T Cells: Involvement of NKG2D Ligands and T-Cell Receptor- Versus NKG2D-Dependent Recognition. *Scand J Immunol* (2007) 66:320–8. doi: 10.1111/j.1365-3083.2007.01963.x
40. Wu Y., Kyle-Cezar F., Woolf R. T., Naceur-Lombardelli C., Owen J., Biswas D., et al. An Innate-Like V Delta 1(+) Gamma Delta T Cell Compartment in the Human Breast is Associated With Remission in Triple-Negative Breast Cancer. *Sci Trans Med* (2019) 11. doi: 10.1126/scitranslmed.aax9364
41. Das H., Groh V., Kuijl C., Sugita M., Morita C. T., Spies T., et al. MICA Engagement by Human V Gamma 2V Delta 2 T Cells Enhances Their Antigen-Dependent Effector Function. *Immunity* (2001) 15:83–93. doi: 10.1016/S1074-7613(01)00168-6
42. Nedellec S, Sabourin C, Bonneville M, Scotet E. NKG2D Costimulates Human V Gamma 9V Delta 2 T Cell Antitumor Cytotoxicity Through Protein Kinase C Theta-Dependent Modulation of Early TCR-Induced Calcium and Transduction Signals. *J Immunol* (2010) 185:55–63. doi: 10.4049/jimmunol.1000373
43. Hayday AC. Gammadelta T Cells and the Lymphoid Stress-Surveillance Response. *Immunity* (2009) 31:184–96. doi: 10.1016/j.immuni.2009.08.006

44. Ribeiro S, Ribot JC, Silva-Santos B. Five Layers of Receptor Signaling in Gamma Delta T-Cell Differentiation and Activation. *Front Immunol* (2015) 6. doi: 10.3389/fimmu.2015.00015
45. Cazzetta V., Bruni E., Terzoli S., Carenza C., Franzese S., Piazza R., et al. NKG2A Expression Identifies a Subset of Human Vdelta2 T Cells Exerting the Highest Antitumor Effector Functions. *Cell Rep* (2021) 37:109871. doi: 10.1016/j.celrep.2021.109871
46. Arlettaz L., Villard J., de Rham C., Degermann S., Chapuis B., Huard B., et al. Activating CD94:NKG2C and Inhibitory CD94:NKG2A Receptors are Expressed by Distinct Subsets of Committed CD8+ TCR Alphabeta Lymphocytes. *Eur J Immunol* (2004) 34:3456–64. doi: 10.1002/eji.200425210
47. Fichtner A. S., Bubke A., Rampoldi F., Wilharm A., Tan L., Steinbruck L., et al. TCR Repertoire Analysis Reveals Phosphoantigen-Induced Polyclonal Proliferation of Vgamma9Vdelta2 T Cells in Neonates and Adults. *J Leukoc Biol* (2020) 107:1023–32. doi: 10.1002/JLB.1MA0120-427RR
48. Davodeau F., Peyrat M. A., Hallet M. M., Houde I., Vie H., Bonneville M., et al. Peripheral Selection of Antigen Receptor Junctional Features in a Major Human Gamma Delta Subset. *Eur J Immunol* (1993) 23:804–8. doi: 10.1002/eji.1830230405
49. Yamashita S, Tanaka Y, Harazaki M, Mikami B, Minato N. Recognition Mechanism of non-Peptide Antigens by Human Gammadelta T Cells. *Int Immunol* (2003) 15:1301–7. doi: 10.1093/intimm/dxg129
50. Murugan A, Mora T, Walczak AM, Callan CGJr. Statistical Inference of the Generation Probability of T-Cell Receptors From Sequence Repertoires. *Proc Natl Acad Sci U.S.A.* (2012) 109:16161–6. doi: 10.1073/pnas.1212755109
51. Pereira BI, Akbar AN. Convergence of Innate and Adaptive Immunity During Human Aging. *Front Immunol* (2016) 7:445. doi: 10.3389/fimmu.2016.00445
52. Mingari M. C., Ponte M., Bertone S., Schiavetti F., Vitale C., Bellomo R., et al. HLA Class I-Specific Inhibitory Receptors in Human T Lymphocytes: Interleukin 15-Induced Expression of CD94/NKG2A in Superantigen- or Alloantigen-Activated CD8+ T Cells. *Proc Natl Acad Sci U.S.A.* (1998) 95:1172–7. doi: 10.1073/pnas.95.3.1172

Conflict of Interest: JK reports grants from Gadeta, Novartis, and Miltenyi Biotech and is the inventor of patents dealing with γδT cell-related aspects, as well as the co-founder and shareholder of Gadeta. AV, AJ, DB, and ZS are inventors of patents dealing with γδT cell-related aspects.

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

REVIEW
published: 11 July 2022
doi: 10.3389/fimmu.2022.894315

γδ T Cells in the Tumor Microenvironment—Interactions With Other Immune Cells

Kok Fei Chan[1], *Jessica Da Gama Duarte*[1], *Simone Ostrouska*[1] *and Andreas Behren*[1,2]*

[1] Olivia Newton-John Cancer Research Institute, and School of Cancer Medicine, La Trobe University, Heidelberg, VIC, Australia, [2] Department of Medicine, University of Melbourne, Parkville, VIC, Australia

A growing number of studies have shown that γδ T cells play a pivotal role in mediating the clearance of tumors and pathogen-infected cells with their potent cytotoxic, cytolytic, and unique immune-modulating functions. Unlike the more abundant αβ T cells, γδ T cells can recognize a broad range of tumors and infected cells without the requirement of antigen presentation *via* major histocompatibility complex (MHC) molecules. Our group has recently demonstrated parts of the mechanisms of T-cell receptor (TCR)-dependent activation of Vγ9Vδ2$^+$ T cells by tumors following the presentation of phosphoantigens, intermediates of the mevalonate pathway. This process is mediated through the B7 immunoglobulin family-like butyrophilin 2A1 (BTN2A1) and BTN3A1 complexes. Such recognition results in activation, a robust immunosurveillance process, and elicits rapid γδ T-cell immune responses. These include targeted cell killing, and the ability to produce copious quantities of cytokines and chemokines to exert immune-modulating properties and to interact with other immune cells. This immune cell network includes αβ T cells, B cells, dendritic cells, macrophages, monocytes, natural killer cells, and neutrophils, hence heavily influencing the outcome of immune responses. This key role in orchestrating immune cells and their natural tropism for tumor microenvironment makes γδ T cells an attractive target for cancer immunotherapy. Here, we review the current understanding of these important interactions and highlight the implications of the crosstalk between γδ T cells and other immune cells in the context of anti-tumor immunity.

Keywords: γδ T cells, αβ T cells, B cells, dendritic cells, macrophages, monocytes, natural killer cells, neutrophils

OPEN ACCESS

Edited by:
Dieter Kabelitz,
University of Kiel, Germany

Reviewed by:
Kenth Gustafsson,
Great Ormond Street Institute of Child Health, University College London, United Kingdom
Massimo Massaia,
University of Turin, Italy

***Correspondence:**
Andreas Behren
andreas.behren@onjcri.org.au

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *11 March 2022*
Accepted: *15 June 2022*
Published: *11 July 2022*

Citation:
Chan KF, Da Gama Duarte J, Ostrouska S and Behren A (2022) γδ T Cells in the Tumor Microenvironment—Interactions With Other Immune Cells. Front. Immunol. 13:894315. doi: 10.3389/fimmu.2022.894315

INTRODUCTION

For the past 37 years, since the first isolation of the TCR γ gene segment (1, 2), the knowledge accumulated about the γδ T-cell lineage has grown exponentially and received strong clinical interest, especially for cancer immunotherapy development (3–15). Similar to the other two lineages of lymphocytes in the jawed vertebrates that utilize somatically recombined receptors for immunosurveillance (B cells and αβ T cells) (16), TCR heterodimers of γδ T cells are generated through somatic rearrangements of genes encoding for TCR δ chain variable (V), diversity (D), joining (J), and constant (C) gene segments, and TCR γ chain V, J, and C gene segments at the thymus (17, 18). Hypothetically, such diverse gene rearrangements can result in a total of 10^{17}

possible distinct γδ TCRs (19). Despite the diverse theoretical γδ TCR repertoire, human γδ T cells can be classified into two major subsets according to their TCR Vδ chain usage: $Vδ2^+$ populations that are usually paired with Vγ9 chain, and $Vδ2^-$ populations with diversified Vγ chain usage (6, 20). Among all 8 TCR Vδ gene segments, Vδ1, Vδ2, and Vδ3 are three commonly used segments for δ chain rearrangement (21, 22).

$Vγ9Vδ2^+$ T cells are the most abundant Vδ cell population found in peripheral blood and are activated by phosphorylated non-protein metabolites called phosphoantigens *via* the BTN2A1/BTN3A1 complexes in a TCR-dependent manner (3, 11, 23, 24). Phosphoantigens are derived from the mevalonate pathway as an intermediate metabolite known as isopentenyl pyrophosphate (IPP) (25), or are generated in the microbial non-mevalonate isoprenoid synthesis pathway as (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMBPP) (26). Following phosphoantigen binding to the intracellular B30.2 domains of BTN3A1 in tumor or pathogen-infected cells (27), BTN3A1 undergoes a conformational change (28–30) and promotes the interaction between BTN2A1 and BTN3A1 intracellular domains (31). Subsequently, the germline-encoded regions of the TCR Vγ9 chain directly bind to BTN2A1 on tumor cells (3, 32, 33), as described by us and confirmed later by others (34–36). An additional but yet to be identified ligand is likely to bind to a separate region within the complementarity-determining region 2δ (CDR2δ) and CDR3γ of the $Vγ9Vδ2$ TCR for phosphoantigen-mediated $Vγ9Vδ2^+$ T-cell activation (3, 33). In concert with BTN2A1, the phosphoantigen-induced conformational change of BTN3A1 then leads to $Vγ9Vδ2^+$ T-cell activation (31, 33–36) (**Figure 1**). Accordingly, dysregulation of the mevalonate pathway in tumors was shown to cause activation of $Vγ9Vδ2^+$ T cells *via* IPP accumulation (37) and induced γδ T-cell chemotaxis toward tumor cells (38, 39). Activated $Vγ9Vδ2^+$ T cells are capable of inducing cytotoxicity *via* secretion of Th1 cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), pro-apoptotic protease granzyme B, and cytolytic granules containing pore-forming perforin molecules (40–44). Therefore, many clinical studies used aminobisphosphonates (e.g., zoledronate and pamidronate) to inhibit farnesyl pyrophosphate synthase in the

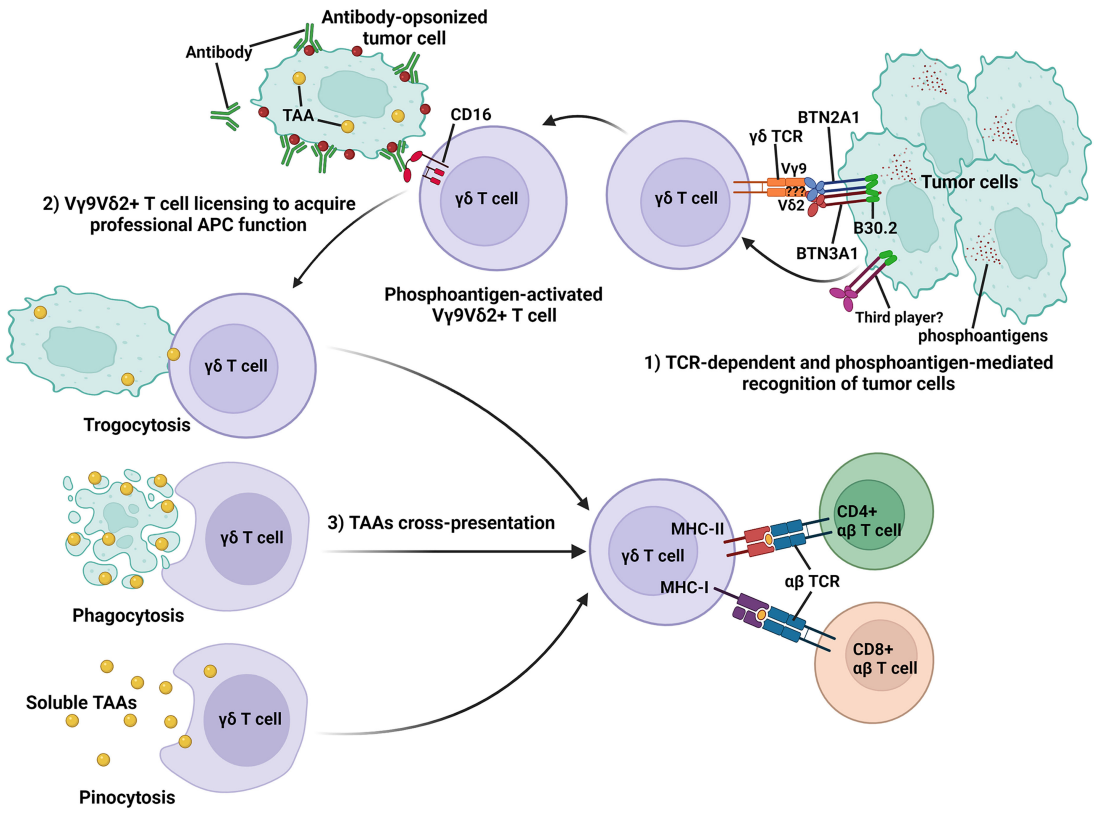


FIGURE 1 | Schematic representation of TCR-dependent and phosphoantigen-mediated recognition of tumor cells by $Vγ9Vδ2^+$ T cells and the acquisition of professional APC function by activated $Vγ9Vδ2^+$ T cells to cross-present TAAs to antigen-specific $CD4^+$ and $CD8^+$ αβ T cells. During the $Vγ9Vδ2^+$ T-cell activation process, accumulated phosphoantigens in tumor cells bind to the intracellular B30.2 domain of BTN3A1. Following phosphoantigen binding, BTN3A1 undergoes conformational changes and induces the interaction between the intracellular domains of BTN2A1 and BTN3A1. BTN2A1 directly binds the TCR Vγ9 chain and leads to T-cell activation in concert with at least one additional ligand. Activated $Vγ9Vδ2^+$ T cells can recognize antibody-opsonized tumor cell *via* CD16 (FcγRIII) and are licensed to acquire professional APC function *via* trogocytosis, phagocytosis, and pinocytosis and cross-present antigens from tumor cells to antigen-specific $CD4^+$ and $CD8^+$ αβ T cells.

mevalonate pathway to promote accumulation of IPP in cells, or synthetic phosphoantigen analogues such as bromohydrin pyrophosphate (BrHPP) and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), to activate Vγ9Vδ2$^+$ T cells in cancer patients (19, 45–47). In recent years, however, agonist antibodies against BTN3A such as clone 20.1 (48–51), CTX-2026 (52), and ICT-01 (53) have been explored as a phosphoantigen-independent approach to activate Vγ9Vδ2$^+$ T cells for targeted cell killing. Moreover, Vγ9Vδ2$^+$ T cells can be activated by other ligands including human MutS homolog 2, stress-induced MHC class I chain-related antigens A and B (MICA/MICB), UL16-binding proteins (ULBPs), nectin-like-5, staphylococcal enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1), and F1-ATPase-apolipoprotein-AI through surface receptors, natural killer group 2D (NKG2D), and DNAX accessory molecule-1 (DNAM-1) (12, 13, 17, 19, 54, 55). Other than direct targeted cell killing, activated Vγ9Vδ2$^+$ T cells have been implicated to directly or indirectly interact with a range of immune cells: αβ T cells (56–63), B cells (64–72), natural killer (NK) cells (73–75), monocytes (76–78), macrophages (79–82), neutrophils (78, 83–86), monocyte-derived dendritic cells (moDCs) (87–93), and DCs (72, 76, 94–96), and influence the outcome of the immune responses. The underlying mechanisms of such γδ T-cell crosstalk with other immune cells are summarized in **Table 1** and will be thoroughly discussed in the following sections.

The non-Vδ2 γδ T cells are mostly identified with Vδ1$^+$ or Vδ3$^+$ TCR chain usage and are localized in the skin, large intestine, spleen, and liver (6, 12, 54). Several studies have shown that Vδ1$^+$ γδ T cells recognize CD1c-phosphomycoketide (110), CD1d-α-GalCer (111), CD1d-sulfatide (112, 113), R-phycoerythrin (PE) (114), ephrin receptor A2 (EphA2) (115), and MHC-related

TABLE 1 | Summary of distinct γδ T-cell subset interactions with other immune cells.

γδ T-cell subset	Crosstalk target	Comments	References
Pan-γδ	CD4$^+$ and CD8$^+$ αβ T cells	Activated γδ T cells were capable of professional phagocytosis to mediate presentation of antigens to CD4$^+$ and CD8$^+$ αβ T cells	(62, 97, 98)
	CD4$^+$ and CD8$^+$ αβ T cells; CD4$^+$ CD25$^+$ Treg cells	Tumor-activated γδ T cells induced proliferation and differentiation of CD4$^+$ and CD8$^+$ αβ T cells, mediated cytotoxic function of CD8$^+$ αβ T cells and inhibited immunosuppression effect by CD4$^+$ CD25$^+$ Treg cells on CD4$^+$ CD25$^-$ αβ T cells	(99)
	B cells	Phosphoantigen-activated γδ T cells provided B-cell help for the downstream production of IgA, IgG, and IgM antibodies	(68)
	NK cells	IPP-activated γδ T cells upregulated CD137L expression and co-stimulated CD25^{hi}, CD54^{hi}, CD69^{hi}, CD137^{hi} NK cells *via* CD137/CD137L (4-1BB/4-1BBL) interactions to promote NK cell-mediated cytotoxicity against tumors	(73, 75)
	NK cells	IPP-activated γδ T cells expressed ICOS and co-stimulated NK cell activation through ICOS/ICOS-L interactions, leading to increased CD137/CD137L signaling and acquisition of NK cell-mediated DC editing function	(100, 101)
Vδ1$^+$	CD4$^+$ and CD8$^+$ αβ T cells; DCs	Activated Vδ1$^+$ γδ T cells suppressed proliferation and IL-2 production by both CD4$^+$ and CD8$^+$ αβ T cells and impaired the maturation and function of DCs. The suppressive activity of activated Vδ1$^+$ γδ T cells was mediated by TLR8 signaling pathway	(102)
	DCs	Tumor-derived CXCL10 increased the expansion of Vδ1$^+$ γδ Treg cells that infiltrated solid tumors and either induced immune-senescence in DCs or killed DCs	(102–107)
Vδ2$^+$	CD4$^+$ αβ T cells	IPP-activated Vγ9Vδ2$^+$ T cells acquired professional APC functions by upregulating expression of co-stimulatory (CD40, CD80, and CD86), MHC class II and lymph node-homing CCR7 receptors, presented exogenous antigen and induced naïve autologous CD4$^+$ αβ T cells to proliferate and differentiate into T helper, Th1 subset	(56)
	CD8$^+$ αβ T cells	IPP-activated HLA-A2$^+$ Vγ9Vδ2$^+$ T cells could uptake soluble antigens, processed and cross-presented immunodominant or subdominant HLA-A2-restricted peptides and primed naïve CD8$^+$ αβ T cells for proliferation and effector cell function	(57–61)
	CD8$^+$ αβ T cells	IPP-activated Vγ9Vδ2$^+$ T cells upregulated CD36 expression to mediate apoptotic and live tumor cells uptake, cross-presentation, and induction of TAA-specific CD8$^+$ αβ T-cell response	(108)
	B cells	Vγ9Vδ2$^+$ T cells promoted the development of antibody-producing B cells *via* immunoglobulin class switching	(65–67, 69)
	B cells	Activated Vγ9Vδ2$^+$ T cells with functional CCR7 expression induced transient lymph node-homing and clustering within B-cell zones of germinal centers in lymphoid tissues	(64, 68)
	NK cells	IPP-activated Vδ2$^+$ γδ T cells induced cytotoxicity against CD56$^+$ DC-like cells and prematurely terminated NK cell response	(74)
	Monocytes	IPP- or HMBPP-activated Vδ2$^+$ γδ T cells induced downregulation of CD14, and upregulation of CD40, CD86, and HLA-DR on monocytes	(76, 77)
	Macrophages	Macrophages recruited Vδ2$^+$ γδ T cells to the site of infection *via* IP-10 and CXCR3; once there they were able to drive the local cytotoxic response *via* granzyme and perforin release or Fas ligand binding	(79–82)
	Neutrophils	IPP- or HMBPP-activated Vγ9Vδ2$^+$ T cells can induce neutrophil recruitment, migration, adhesion, activation, phagocytosis, and degranulation	(78, 83, 86)
	Neutrophils	TNF-α secretion by γδ T cells induces reactive oxygen species, arginase-1, and serine protease production from neutrophils, which subsequently inhibits CD25 and CD69 expression, IFN-γ production, and cell proliferation of Vδ2$^+$ γδ T cells	(84–86)
	DCs	Activated Vγ9Vδ2$^+$ T cells secreted IFN-γ and TNF-α and promoted maturation of antigen-expressing immature moDCs in circulation	(87–91, 93)
Vδ3$^+$	DCs	Activated Vδ3$^+$ γδ T cells induced immature moDCs to upregulate APC markers CD40, CD83, CD86, and HLA-DR and secreted IL-10 and IL-12. Vδ3$^+$ γδ T cell-mediated moDC maturation involved CD1d recognition but not CD40/CD40L interaction. Vδ3$^+$ γδ T cell-matured moDCs induced activation of naïve allogeneic T cells.	(109)

protein 1 (MR1) (116) ligands, and play a crucial role for anti-tumor responses (117–124). Similar to Vδ2$^+$ γδ T cells, the NKG2D-expressing Vδ1$^+$ γδ T cells can be activated by stress-inducible MICA/MICB and ULBP1–6 family proteins, which are frequently upregulated in tumor cells (8, 11). Ligand-bound NKG2D induces cytolytic functions of γδ T cells *via* granzyme B and perforin secretion to mediate tumor cell killing (125). Several studies have utilized Vδ1$^+$ γδ T-cell populations for adoptive cancer immunotherapy (8, 10, 126), but the clinical outcome so far was limited. The less frequent Vδ3$^+$ γδ T cells were shown to recognize and kill CD1d$^+$ target cells (109) and are activated by annexin A2 ligands on tumor cells that are upregulated under oxidative stress conditions (127). Interestingly, the binding affinity of the Vδ1$^+$ and Vδ3$^+$ γδ TCR ligands identified thus far falls within the range of 3 to 150 μM (55, 128), comparable to the well-studied αβ TCR binding affinities for the peptide–MHC complex (129, 130), suggesting a possible shared TCR docking footprint on the bound ligand (131). With the increasing numbers of non-Vδ2 γδ T-cell ligands uncovered so far (8, 55, 116, 132), different strategies have been developed to utilize activated non-Vδ2 γδ T cells for cancer immunotherapy (10, 19, 128). Of note, activated non-Vδ2 γδ T cells have also been implicated to modulate other immune cells (**Table 1**) including αβ T cells (102), B cells (133–135), DCs (89, 102–107, 109, 136, 137), macrophages (70, 138), and neutrophils (139).

Human Vδ2$^+$ γδ T cells represent ~0.5% to 10% of all circulating T lymphocytes in healthy adults and can undergo rapid expansion of up to 60% in the periphery during infections, and form between 20% to 30% of total infiltrating CD3$^+$ T cells in the early stage of disease onset (11, 17). Activated Vδ1$^+$ and Vδ2$^+$ γδ T cells upregulate various C-C chemokine receptor (CCR) such as CCR1 and CCR8 (140), CCR2 (141), CCR5 (142), and C-X-C chemokine receptor 3 (CXCR3) (107) to mediate infiltration into the tumor microenvironment (TME). Additionally, tumor cells and tumor-derived fibroblasts express chemokine ligand 2 (CCL2) (141), IFN-γ-inducible protein 10 (IP-10) (107), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and regulated on activation, normal T cell expressed and secreted (RANTES) to promote recruitment of activated Vδ1$^+$ and Vδ2$^+$ γδ T cells to the TME (140). Once recruited into the TME, tumor-infiltrating Vδ1$^+$ and Vδ2$^+$ γδ T cells can eliminate tumor cells *via* TNF-related apoptosis-inducing ligand (TRAIL) (143), Fas/Fas ligand pathway (144), induction of antibody-dependent cellular cytotoxicity (ADCC) on antibody-opsonized tumor cells through CD16 (FcγRIII) (60, 145, 146), perforin/granzymes, IFN-γ/TNF-α secretion, and NKG2D-mediated cytotoxicity (13, 147). As a result of the complex interplay between TME and tumor-infiltrating γδ T cells, activated γδ T cells can be functionally polarized to become the anti-tumor Th1 and follicular Th (Tfh) cells or the pro-tumor Th17 and T regulatory (Treg) cells (12, 132). For example, IPP-activated Vγ9Vδ2$^+$ T cells can be polarized into three distinct subsets based on the presence of different cytokines in the microenvironment: Th1 [interleukin-12 (IL-12) and anti-IL-4 antibody] (148), Th2 (IL-4 and anti-IL-12 antibody) (148), and Th17 [IL-1β, transforming growth factor β (TGF-β), IL-6 and IL-23] (149). Recent reviews on the topic of γδ T-cell polarization has provided comprehensive insight into the different role of γδ Th1, Th2, Th17, Tfh, and Treg cells, and we refer readers to these excellent publications (7, 8, 11, 54, 150–153).

Importantly, the presence of tumor-infiltrating γδ T cells was shown to be the most favorable prognostic marker for overall cancer patients survival in 25 different cancer types and solid tumors (non-brain tumor) (4). Their role in cancer immunosurveillance was clearly evidenced and validated in many tumor models and clinical studies including cutaneous carcinoma (154), melanoma (119, 155, 156), lymphoma (157–159), leukemia (44, 117, 160, 161), gastric (162), colorectal (43, 163, 164), kidney (41), prostate (165, 166), and pancreatic (143) cancers. The ability of γδ T cells to produce large quantities of cytokines and chemokines rapidly and their tendency to reside in blood circulation or in non-lymphoid tissues (e.g., skin, intestines, and lungs) (8, 16, 17), helps to provide the first line of immunosurveillance against aberrant cell growth and infectious diseases, and bridges the innate and adaptive immune responses. Thus, it is important to understand the crosstalk between γδ T cells and other immune cells in the TME and to harness this knowledge for effective cancer immunotherapy development.

CROSSTALK BETWEEN γδ T CELLS AND αβ T CELLS

The role of antigen processing and presentation to αβ T cells is mostly associated with the classical professional antigen-presenting cells (APCs) like DCs, macrophages, and B cells (167, 168). However, with the unexpected discovery by Brandes et al., it was shown that activated but not resting human Vγ9Vδ2$^+$ T cells were also capable of acquiring professional APC functions (56). Indeed, activated Vγ9Vδ2$^+$ T cells isolated from both healthy individuals and cancer patients' peripheral blood mononuclear cell (PBMC) exhibited potent APC functions to stimulate robust antigen-specific αβ T-cell responses (169).

During the activation process, human Vγ9Vδ2$^+$ T cells can rapidly gain APC functions by upregulating co-stimulatory (CD40, CD80, and CD86), MHC class I and II molecules (56, 57, 61, 62, 97, 108, 169), and transiently expressed lymph node-homing markers, chemokine receptor CCR4 and CCR7 (62, 68, 97). This allows recruitment of activated γδ T cells from the peripheral sites to secondary lymphoid tissues for antigen presentation and bridges the early phase of rapid innate-like γδ T-cell response to microbial or tumor antigens with the later phase of adaptive immune response involving the antigen-specific CD4$^+$ and CD8$^+$ αβ T cells (14, 15, 17, 168, 170). In a study by Himoudi et al., it was shown that activated human Vγ9Vδ2$^+$ T cells were "licensed" to acquire their APC functions through recognition of antibody-opsonized tumor cells, mediated targeted cell killing by their innate cytotoxicity, and subsequently helped to release tumor-

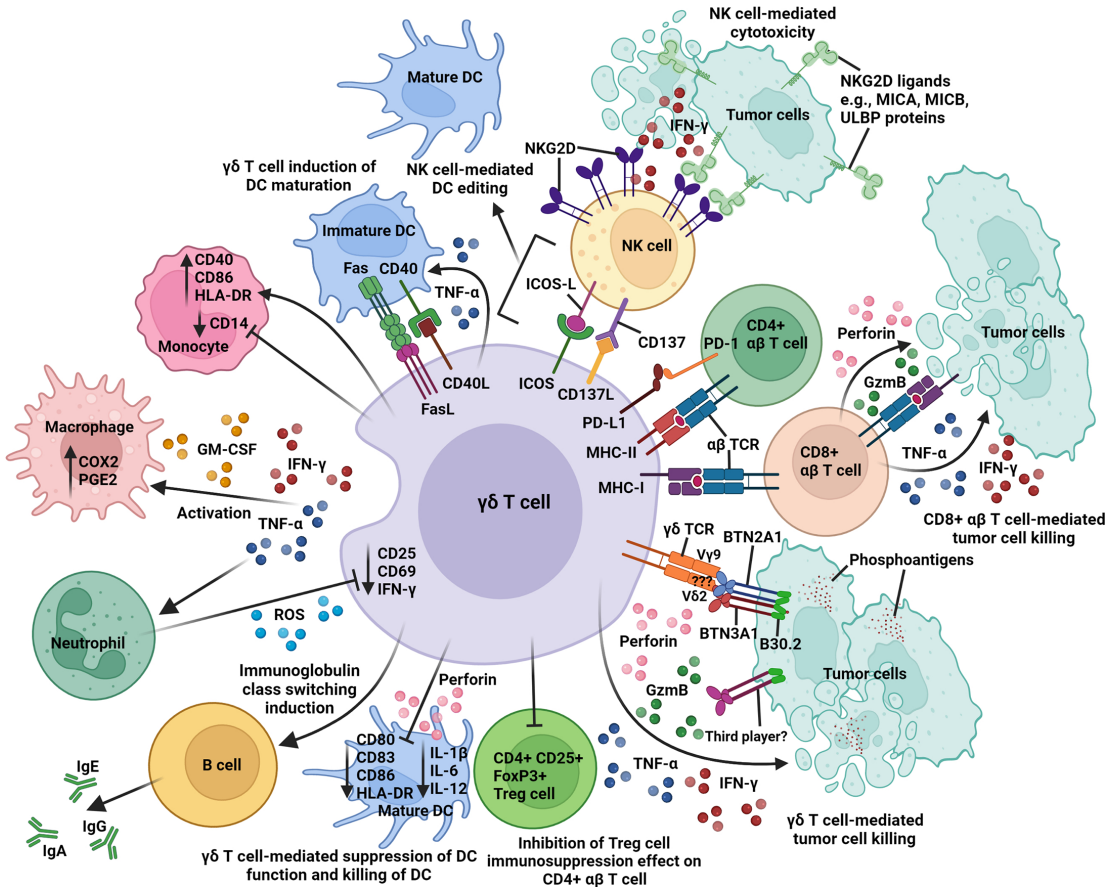


FIGURE 2 | An overview of the intricate network of immune interactions between γδ T cell and other immune cells in the tumor microenvironment. Activated γδ T cells express different surface receptors and molecules (γδ TCR, ICOS, MHC class I and II), ligands (CD40L, CD137L, FasL, and PD-L1), cytokines (IFN-γ and TNF-α), and GM-CSF for contact-dependent and independent crosstalk with tumor cells, $CD4^+$ and $CD8^+$ αβ T cells, NK cells, DCs, macrophages, and neutrophils. Activated γδ T cells cross-present antigens to $CD4^+$ and $CD8^+$ αβ T cells; induce B-cell immunoglobulin class switching; co-stimulate NK cells *via* CD137/CD137L and ICOS/ICOS-L interactions; induce upregulation of CD40, CD86, and HLA-DR expression on monocyte; promote DC maturation *via* CD40/CD40L and Fas/FasL interactions; and inhibit the immunosuppression function of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells on $CD4^+$ αβ T-cell activity. In contrast, activated γδ T cells can also suppress DC function (downregulation of CD80, CD83, CD86, HLA-DR, IL-1β, IL-6, and IL-12) and mediate DC killing *via* perforin release. Butyrophilin 2A1 and 3A1 (BTN2A1 and BTN3A1); cyclooxygenase-2 (COX2); granulocyte-macrophage colony stimulating factor (GM-CSF); granzyme B (GzmB); human leukocyte antigen-DR (HLA-DR); immunoglobulin A, E, or G (IgA, IgE, or IgG); inducible T-cell co-stimulator (ICOS); ICOS ligand (ICOS-L); interferon-γ (IFN-γ); major histocompatibility complex class I and II (MHC-I and -II); MHC class I chain-related antigens A and B (MICA and MICB); natural killer group 2D (NKG2D); programmed cell death 1 (PD-1); PD-1 ligand 1 (PD-L1); prostaglandin E2 (PGE2); reactive oxygen species (ROS); T-cell receptor (TCR); tumor necrosis factor-α (TNF-α); UL16-binding protein (ULBP).

associated antigens (TAAs) into the surrounding microenvironment (60). These TAAs can be taken up by activated γδ T cells *via* phagocytosis (62, 97, 98, 108), trogocytosis (171), or pinocytosis (57, 58), processed and presented on the cell surface for priming and induction of naïve αβ T cells (59, 60) (**Figure 1**). Furthermore, it was shown that $V\gamma9V\delta2^+$ γδ T cells can uptake microbes and soluble antigens *via* CD16-mediated phagocytosis, a process that can lead to functional antigen processing and presentation on MHC class II (98), and cross-presentation of immunodominant MHC class I peptides to antigen-specific $CD8^+$ αβ T cells (58, 60, 61). This notion was further supported by the identification of $V\gamma9V\delta2^+$ T cells in malaria patients that readily acquired APC functions upon infection and induced $CD4^+$ and $CD8^+$ αβ T-cell activation (61). Interestingly, it was also demonstrated that activated $V\gamma9V\delta2^+$ T cells can uptake CD1d-containing membrane fragments from phosphoantigen expressing $Cd1d^+$ target cells *via* trogocytosis, leading to the presentation of CD1d-restricted antigen and the activation of $V\alpha24V\beta11^+$ invariant natural killer T cells (iNKT) (172).

When compared to activated αβ T cells and monocytes, activated $V\gamma9V\delta2^+$ T cells were shown to be more efficient in presenting antigens and induced 100-fold higher proliferative

responses in naïve $CD4^+$ αβ T cells (56). Activated $V\gamma9V\delta2^+$ T cells were also able to cross-present antigens to $CD8^+$ αβ T cells with a higher efficiency and reproducibility (57), and induced less $CD4^+$ $CD25^{hi}$ $FoxP3^+$ Treg cell expansion than moDCs (59). Similar results were seen under pathological condition, when it was shown that γδ T cells isolated from gastric cancer patients can acquire APC functions upon activation with cells derived from autologous tumor tissues (99). These clinically relevant tumor-activated γδ T cells induced strong antigen-specific $CD4^+$ and $CD8^+$ αβ T-cell responses and prevented immunosuppression mediated by $CD4^+$ $CD25^+$ Treg cells (99) (**Figure 2**). Of note, Muto et al. showed that resting $V\gamma9V\delta2^+$ T cells can significantly upregulate the expression of scavenger receptor CD36 during activation and that this was mediated by a key transcription factor, CCAAT/enhancer-binding protein α (C/EBPα), that supports acquisition of APC functions in activated $V\gamma9V\delta2^+$ T cells (108). In contrast, resting αβ T cells expressed a low level of CD36 and did not upregulate it upon activation (108). In DCs and macrophages, the CD36 receptor was shown to facilitate the uptake of apoptotic cells and cross-presentation (173, 174), potentially explaining the induction of a stronger antigen-specific αβ T-cell response by activated $V\gamma9V\delta2^+$ T-cell APC.

The ability to migrate to the tumor site and cross-present TAAs to αβ T cells was also retained when $V\delta1^+$ and $V\delta2^+$ γδ T cells were engineered to express tumor-specific chimeric antigen receptors (CARs) and resulted in an increased cytotoxic level against tumor cells (175). Hence, activated $V\gamma9V\delta2^+$ T cells can process and present antigens and provide critical co-stimulatory signals to prime and induce naïve $CD4^+$ (56) and $CD8^+$ (57) αβ T cells for proliferation, differentiation, and cytokine production and to mediate cytotoxic responses against tumors and pathogen-infected cells (176–179). This remarkable ability of γδ T cells to uptake and present antigens and prime αβ T cells has been highlighted by Vantourout et al. (168), and the accumulated data so far have illustrated the potential of harnessing the APC functions of γδ T cells to crosstalk with αβ T cells for immunotherapy development.

Given their natural tropism for TME (14, 119, 175, 180–182), activated γδ T cells could hence be utilized to prolong the intratumoral immune response by cross-presenting TAAs to other tumor-infiltrating lymphocytes and provide an early source of IFN-γ to expand and increase immunogenicity of TAA-specific αβ T cells within the TME (155, 183, 184), and to upregulate expression of MHC class I and II on tumor cells (185, 186) for αβ T cell-mediated killing (**Figure 2**). The presence of tumor-infiltrating γδ T cells within the TME as revealed by genomic data analysis in over 18,000 human tumors has uncovered a strong correlation to good prognosis (4). In the context of cancer immunotherapy, the capability of activated γδ T cells to cross-present TAAs to αβ T cells could be further boosted through the "licensing" pathway (60, 187) by using therapeutic monoclonal antibodies against tumor cells, e.g., rituximab (anti-CD20) and trastuzumab (anti-HER2/neu) (145, 188, 189). Such combination treatment could greatly improve the outcome of γδ T-cell cancer immunotherapy.

Activated $V\gamma9V\delta2^+$ T cells can also modulate αβ T-cell activity indirectly by co-stimulating NK cells *via* inducible T-cell co-stimulator (ICOS)/ICOS-L and CD137/CD137L engagements to enhance IFN-γ and TNF-α production (100, 101), which, in turn, helps to support αβ T-cell activation (190). Another study has shown that activated $V\gamma9V\delta2^+$ T cells can induce B-cell and DC maturation and subsequently leads to alloreactive stimulation of αβ T-cell proliferation and IFN-γ production by mature B cells and DCs (72). The interactions between γδ T cells and other immune cells (B cells, DCs, and NK cells) will be discussed later in this review.

Despite their ability to exert positive immune modulation functions on αβ T cells, activated γδ T cells can also negatively regulate αβ T-cell response by upregulating an immune checkpoint inhibitory ligand, programmed cell death 1 ligand 1 (PD-L1) (11, 151, 191). The suppressive phenotype of activated $V\delta2^+$ γδ T cells on autologous αβ T cells was shown to be mediated by the PD-1/PD-L1 interactions and correlated well with the strength of $V\delta2^+$ γδ TCR signaling during the activation process but was independent of TGF-β and FoxP3 expression (192) (**Figure 2**). Daley et al. showed that tumor-infiltrating γδ T cells with high expression levels of checkpoint inhibitory ligands PD-L1 and Galectin-9 could inhibit αβ T-cell activation through checkpoint receptor ligation (193). The immunosuppressive effect can also be mediated by the interaction between CD86 on activated $V\delta2^+$ γδ T cell and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) on activated αβ T cells (191). Such γδ T cell-mediated immunosuppression of αβ T cells, however, can be significantly reduced by disrupting PD-1/PD-L1 and CTLA-4/CD86 interactions with blocking antibodies (191, 192). Furthermore, Peng et al. identified tumor-infiltrating $V\delta1^+$ γδ T cells that could suppress naïve/effector αβ T-cell proliferation and IL-2 production through the Toll-like receptor (TLR) 8 signaling pathway and may lead to tumor immune escape (102). The immunosuppressive activity of $V\delta1^+$ γδ T cells can be reversed using TLR8 ligands, and this signaling involved the myeloid differentiation primary response 88 (MyD88), TNFR-associated factor 6 (TRAF6), IKB kinase α (IKKα), IKKβ, and mitogen-activated protein kinase 14 (MAPK14), but not transforming growth factor-β-activated kinase 1 (TAK1), Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) molecules in $V\delta1^+$ γδ T cells (102). It was also reported that $\gamma\delta^+$ $NKG2A^+$ intraepithelial lymphocytes (IELs) can mediate suppression of $CD8^+$ $\alpha\beta^+$ IEL cytotoxic responses (IFN-γ and granzyme B) in patients with celiac disease through TGF-β secretion (194). The immunosuppressive effect on $CD8^+$ $\alpha\beta^+$ IELs can be further enhanced upon $\gamma\delta^+$ IELs NKG2A receptor ligation with the cognate ligand, human leukocyte antigen-E (HLA-E) (194). This immunosuppressive effect can be reduced by blocking NKG2A/HLA-E interaction and TGF-β with blocking antibodies (194). Therefore, it is important to consider these negative immunomodulatory roles of γδ T cells when designing novel immunotherapeutics.

Apart from the PD-1/PD-L1 and CTLA-4/CD86 immune checkpoint axes, other non-conventional checkpoint

receptors [killer Ig-like inhibitory receptors (KIRs), Ig-like transcript 2 (ILT-2), and NKG2A] can be expressed on Vγ9Vδ2$^+$ T cells, inhibit their cytotoxic function, and prevent tumor cell lysis upon recognition of specific HLA class I ligands on tumor cells (195–203). In this context, the presentation of HLA class I molecules on tumor cells can be a double-edged sword. On one hand, it facilitates the presentation of antigenic peptides to activate CD8$^+$ αβ T cells, but at the same time, it can also inhibit the activation of Vγ9Vδ2$^+$ T cells. Such inhibitory signals on immune cells mediated by KIRs, ILT-2, or NKG2A can be blocked using monoclonal antibodies targeting KIRs (lirilumab and IPH4102), ILT-2 (anti-ILT-2, anti-HLA-G1, anti-FasL), or NKG2A (monalizumab) (204, 205). In a study by André et al., treatment with monalizumab indeed led to enhanced anti-tumor immune responses elicited by T and NK cells (206). As a type 2 inhibitory membrane receptor, NKG2A carries cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and forms heterodimers with CD94 to recognize non-classical HLA-E molecule (207). Many human tumors have been shown to express HLA-E including in the colon, cervical, endometrial, head and neck, liver, lung, pancreas, ovarian, and stomach (206). Moreover, a majority of Vγ9Vδ2$^+$ T cells in healthy individuals express NKG2A/CD94 (197, 198, 200, 208), and the expression levels can be induced by IL-15 and TGF-β (209, 210). Therefore, treatments targeting these non-conventional checkpoint receptors on Vγ9Vδ2$^+$ T cells (KIRs, ILT-2, and NKG2A) to disrupt the interactions with their respective HLA class I ligands on tumor cells (HLA-C, HLA-G, and HLA-E) may help to enhance the effectiveness of Vγ9Vδ2$^+$ T cell-based tumor immunotherapy.

Recent work by Payne et al. suggests that BTN3A, itself part of the molecular complex required for phosphoantigen-mediated activation of Vγ9Vδ2$^+$ T cells, can also inhibit tumor-reactive CD8$^+$ αβ T cells when bound to N-mannosylated residues of CD45 by preventing its segregation from the immunological synapse (52). In this study, the suppression of αβ T-cell activation was shown to involve BTN3A1 but not BTN2A1, and the immunosuppressive effect could be blocked by BTN3A1-specific monoclonal antibodies such as clone 20.1, 103.2, and CTX-2026 (52). Targeting BTN3A1 with the agonistic antibody CTX-2026 induced BTN3A1 switching from immunosuppressive to immunostimulatory conformations and promoted coordinated Vγ9Vδ2$^+$ and CD8$^+$ αβ T-cell anti-tumor responses against BTN3A1$^+$ tumors (52). Hence, BTN3A1 may be an attractive immune target for intervention to orchestrate effective and coordinated γδ and αβ T-cell anti-tumor responses.

CROSSTALK BETWEEN γδ T CELLS AND B CELLS

γδ T cells have been previously reported to interact with B cells and modulate their immune functions (5, 8, 168, 211, 212). Vγ9Vδ2$^+$ T cells can adopt a role similar to T follicular helper (Tfh) cells and provide B-cell help, thereby regulating B-cell maturation. Specifically, a subset of CXCR5$^+$ Vγ9Vδ2$^+$ T cells present in circulation and in tonsil tissue expresses co-stimulatory molecules (ICOS and CD40L) upon antigen stimulation and secrete cytokines (IL-2, IL-4, and IL-10), which can promote the development of antibody-producing B cells *via* immunoglobulin class switching [including immunoglobulin A (IgA), IgE, IgG1, IgG2, IgG3, and IgG4] (8, 213, 214) in the extra-follicular or within germinal centers (65–67, 69) (**Figure 2**). Furthermore, upon stimulation with IL-21 and HMBPP, activated tonsillar Vγ9Vδ2$^+$ T cells can express CXCL13 receptor, CXCR5, induce lymphoid-homing phenotype and clustering in germinal centers, and sustain the production of germinal centers (70, 71). Similarly, IPP-stimulated Vδ2$^+$ γδ T cells with functional CCR7 expression can also induce transient lymph node-homing, migration, and clustering of Vδ2$^+$ γδ T cells within B-cell zones of germinal centers in lymphoid tissues (64, 68).

Phosphoantigen-activated Vδ2$^+$ γδ T cells can additionally induce the expression of B-cell co-stimulatory molecules (CD40L, OX40, CD70, and ICOS) and affect the downstream production of circulating IgA, IgG, and IgM antibodies by B cells (68). In patients with specific mutations (*RAG1* and *CD3D*) that impair αβ T-cell function, γδ T cells are responsible for hyper-IgE syndromes or the elevated production of circulating IgA, IgG, and IgM (215, 216). γδ T cells can also suppress antibody responses *via* the induction of CD4$^+$ Foxp3$^+$ Treg cells (217). Conversely, some B cells can express BTN2A1 and BTN3A1, required for Vγ9Vδ2$^+$ T-cell activation (33–35), thereby directly influencing Vδ2$^+$ γδ T-cell activation (218, 219) as shown by early studies using Daudi cells, a B-cell malignancy cell line (Burkitt's lymphoma) (220–226). Vγ9Vδ2$^+$ T cells can directly engage BTN2A1 expressed on B cells *via* the TCR Vγ9 chain (3, 32–36, 227), and in concert with BTN3A1, this results in Vγ9Vδ2$^+$ T-cell activation and expansion (101, 212). Hebbeler et al. showed that the Vγ9Vδ2$^+$ T cells activated and expanded by phosphoantigen or Daudi B lymphoma cells use public TCR Vγ9 clonotypes, and elicit comparable cytotoxic responses against tumor cells (228). Further investigations revealed that the germline-encoded region between TCR Vγ9 CDR2 and CDR3 is responsible for contacting BTN2A1 on target cells (33, 34). Such findings indicate the inherent property of TCR Vγ9 to recognize diverse range of cell types that express BTN2A1 including B cells (212, 227–229). In addition to BTN2A1 and BTN3A1, B cells also express other closely related BTN molecules such as BTN3A2 (in naïve or germinal center B cells), BTN3A3 (in memory B cells), BTN1A1, and BTN2A2 (3, 50). The contribution of these other BTN molecules in B cells for γδ T-cell activation remains elusive. Similarly, circulating activated B7$^+$ CD39$^+$ B cells can stimulate Vδ1$^+$ γδ T-cell proliferation (133, 134). The Vδ1$^+$ γδ T-cell stimulatory ligand is upregulated in B cells upon activation and can induce polyclonal Vδ1$^+$ γδ T-cell responses (133). This B cell-mediated immunostimulatory effect on Vδ1$^+$ γδ T cells can be blocked with antibodies against B7 and CD39 (133, 212).

In summary, Vγ9Vδ2$^+$ T cells can regulate B-cell maturation during development or initiation of an immune response, sustain the production of germinal centers within secondary and possibly tertiary lymphoid structures, and affect the production of circulating (auto)antibodies for humoral immunity (168, 211, 212), while B cells can activate Vδ1$^+$ and Vγ9Vδ2$^+$ T cells (230).

CROSSTALK BETWEEN γδ T CELLS AND NK CELLS

Human NK cells are important innate immune subset for controlling early tumor growth and metastasis through cell-mediated cytotoxicity and show broad reactivity to tumors that escaped immunosurveillance by loss or aberrant MHC class I expression (14, 231, 232). Being a specialized group of innate lymphoid cells (ILCs), NK cell functions are closely regulated by a range of cytokines such as IFN-γ, TNF-α, IL-2, IL-12, IL-15, IL-18, and IL-21 (233, 234). These effector molecules are important for the initiation of anti-viral and anti-tumor immune responses (235–238). However, more established tumors can evade NK cell surveillance by developing resistance to NK cell-mediated cytotoxicity, leading to tumor immune escape (239).

In order to overcome NK-resistant tumors, Maniar et al. showed that activated human NK cells (CD25^{hi}, CD54^{hi}, CD69^{hi}, and CD137^{hi}) increased surface expression of natural NKG2D receptors to promote tumor cytolysis and death (73). NKG2D is a lectin-like type 2 transmembrane receptor mostly expressed by human NK cells and binds to MHC-related ligands such as ULBPs, MICA, and MICB, which are highly expressed in tumor cells but rarely in healthy cells (231, 240). IPP-activated Vδ2$^+$ γδ T cells upregulate CD137L (4-1BBL), engage with CD137$^+$ NK cells, and can in turn lead to enhanced NKG2D expression and NK cell-mediated cytotoxicity against tumors (73) (**Figure 2**), highlighting a potential key role for γδ T cells in this process. CD137 or 4-1BB is a member of the tumor necrosis factor receptor superfamily (TNFRSF) and is expressed by a range of immune cells (190). Expression of CD137 on NK cells is induced by IL-2 and IL-15, and following CD137 signaling, it promotes NK cell proliferation and production of IFN-γ, which, in turn, can support NK tumor effector functions (101, 190). This finding was further corroborated by Liu et al., and they demonstrated that in the context of liver fibrosis, γδ T cells engaged with conventional and liver-resident NK cells through CD137/CD137L interactions to promote NK cell-mediated cytotoxicity against activated hepatic stellate cells and conferred immune protection (75).

Similar to NK and CD8$^+$ αβ T cells, human γδ T cells also express NKG2D to detect stress-inducible ligands on tumors and pathogen-infected cells (125, 241–245). Several studies have shown that NKG2D ligation to its cognate ligand can co-stimulate Vγ9Vδ2$^+$ T-cell activation (CD25 and CD69 upregulation) and promotes the release of IFN-γ, TNF-α, and cytolytic granules to mediate killing of NKG2D ligand-expressing tumors (163, 246–251). In the context of leukemia and lymphoma cell recognition by Vγ9Vδ2$^+$ T cells, it was reported that tumor-expressed ULBP1 was a strong marker for tumors susceptible to Vγ9Vδ2$^+$ T cell-mediated cytotoxicity (252). Similarly, it was shown that ULBP1 overexpression in tumor cells can lead to enhanced killing by Vγ9Vδ2$^+$ T cells (253). Hence, blocking NKG2D-mediated Vγ9Vδ2$^+$ T-cell recognition of tumor cells with anti-NKG2D and anti-MICA/B monoclonal antibodies inhibits tumor cell killing to varying degrees (247, 249, 253). Vδ1$^+$ γδ T cells can also recognize and kill NKG2D ligand-expressing tumors *via* NKG2D receptor (8, 11, 245, 254). The number of Vδ1$^+$ γδ T cells and ULBP3 expression level are negatively correlated with disease progression in chronic lymphocytic leukemia patients (254). A study reported by Kamei et al. demonstrated a longer overall survival in gastric cancer patients with high expression levels of NKG2D and ULBP1 (255). Hence, upregulation of stress-inducible NKG2D ligand in tumor cells and NKG2D receptor in tumor-infiltrating immune cells can help to orchestrate concerted NKG2D-mediated NK, CD8$^+$ αβ, and γδ T-cell anti-tumor responses within the TME. Of note, several anti-cancer drugs have been found to induce expression of NKG2D ligand in tumor cells, including the proteasome inhibitor bortezomib and the alkylating agent temozolomide, and these can help to promote tumor cell lysis by NK and γδ T cells (256, 257). Therefore, it is feasible to target NKG2D and its ligands for γδ T cell-based immunotherapy development.

It was later shown that IPP-activated Vγ9Vδ2$^+$ T cells can upregulate ICOS and signal NK cells *via* ICOS/ICOS-L engagement to promote CD69 and CD137 expression, which then leads to enhanced production of IFN-γ, TNF-α, MIP-1β, I-309, RANTES, and soluble Fas ligand by activated NK cells (100). Such ICOS/ICOS-L-mediated crosstalk enables NK cells to acquire the "license" to kill mature DCs that may play a role in inflammation and tumor growth (100). These studies have uncovered the immunomodulatory role of IPP-activated Vγ9Vδ2$^+$ T cells to circumvent NK-resistant tumors and to promote NK-mediated DC editing function by modulating NK cell cytotoxicity through CD137/CD137L and ICOS/ICOS-L engagements (73, 101) (**Figure 2**). Such findings will provide an alternative strategy for γδ T cell-based immunotherapy development against difficult-to-treat solid tumors or to prevent metastasis (239, 258, 259).

However, NK cell activity can also be negatively regulated by γδ T cells. Zoledronate-activated Vδ2$^+$ γδ T cells not only can co-stimulate early NK cell activation for IFN-γ production but also lead to premature ending of the response by inducing cytotoxicity against CD56$^+$ DC-like cells (74). In the absence of activated Vδ2$^+$ γδ T cells, CD56$^+$ DC-like cells survived (74) and maintained NK cell activity through secretion of NK cell-activating cytokines such as IL-1β and IL-18 (260, 261). Therefore, further studies will help to provide a better understanding of the immunosuppressive role of Vδ2$^+$ γδ T cells on NK cells.

CROSSTALK BETWEEN γδ T CELLS AND MONOCYTES/MACROPHAGES

γδ T cells share many of their innate functions with other immune cell subsets, including NK cells, monocytes, and macrophages (56, 98, 262, 263). These are integral to the innate inflammatory response against infectious pathogens and tumors, which, in turn, activates a strong and targeted adaptive immune response (170). While the hallmark of Vγ9Vδ2$^+$ T cell is recognition of phosphoantigens produced by bacteria-infected or tumor cells (25, 264), monocytes are adept at potentiating this process by taking up and accumulating phosphoantigen for subsequent presentation to γδ T cells (262, 263). Conversely, the prototypical roles of myeloid cells, such as phagocytosis and MHC class II presentation, are also shared by Vγ9Vδ2$^+$ T cells, which can act as professional APCs (56, 98). The close interconnection between these cell types and partial redundancy in functional properties denotes multiple implications for tumor immunity.

Vγ9Vδ2$^+$ T cells have been shown to activate monocytes, induce adhesion and aggregation, and increase their survival (76, 265). This occurs *via* production of inflammatory molecules including IFN-γ, TNF-α, granulocyte-macrophage colony stimulating factor (GM-CSF), lymphocyte function-associated antigen 1 (LFA-1), and CCL2 (76, 78). In turn, this leads to changes in monocyte markers such as downregulation of CD14, and upregulation of CD40, CD86, and HLA-DR (76, 77) (**Figure 2**). Bidirectionally, zoledronate- or HMBPP-primed monocytes can activate Vγ9Vδ2$^+$ T cells through phosphoantigen accumulation and presentation, leading to γδ T-cell proliferation and bacterial pathogen killing (76, 263). However, *in vitro*, it has also been reported that in the presence of zoledronate, monocytes and Vδ2$^+$ γδ T cells can negatively regulate each other by inducing apoptosis (266, 267). It is interesting to note that the contact-dependent stimulation of Vγ9Vδ2$^+$ T cells by monocytes *via* the intercellular adhesion molecule 1 (ICAM-1)/LFA-1 engagement can be disrupted by blocking CD11a with monoclonal antibody (78). In contrast to these *in vitro* results, *in vivo* treatment with zoledronate or other aminobisphosphonates has shown varying effects, with some studies reporting an increase in circulating monocyte numbers, while others found no difference (77, 268). This suggests that the relationship between these cells may be more nuanced and context-dependent than first thought and will require further investigation.

The crosstalk between γδ T cells and macrophages has not yet been thoroughly elucidated; however, the effects are again cell subtype- and context-dependent. Macrophages have been demonstrated to recruit Vγ9Vδ2$^+$ T cells to the site of infection *via* IP-10 and CXCR3 receptor–ligand interactions (80). Once this occurs, Vδ2$^+$ γδ T cells can drive the local cytotoxic response *via* granzyme and perforin release or Fas ligand binding (79, 81, 82). Both Vδ1$^+$ cells and Vδ2$^+$ cells have been shown to produce CCL3, CCL4 (MIP-1α and MIP-1β), and CXCL10, which find their respective cognate receptors expressed by macrophages (70, 138). *In vitro*, the supernatant of cultured γδ T cells has been shown to induce macrophage activation *via* IFN-γ, TNF-α, and GM-CSF production, arguing for a tightly regulated and balanced interplay between these immune cell populations (265). This was further demonstrated by studies showing that IFN-γ and TNF-α released by activated Vγ9Vδ2$^+$ T cells can induce cyclooxygenase-2 (COX2) expression and prostaglandin E2 (PGE2) release by both macrophages (**Figure 2**) and tumor cells, and this downregulates the cytotoxic response of γδ T cells (269, 270) and plays a major role in tumor immune escape (271, 272). Furthermore, galectin-9 on both γδ T cells and pancreatic tumor cells has been shown to bind dectin-1 on tumor-infiltrating macrophages, leading to M2 macrophage polarization and subsequent downregulation of IFN-γ and TNF-α production by γδ T cells (273, 274).

CROSSTALK BETWEEN γδ T CELLS AND NEUTROPHILS

Neutrophils are another immune cell population with complex interactions with γδ T cells at peripheral sites of inflammation and in the TME. Zoledronate-activated Vγ9Vδ2$^+$ T cells release cytokines and chemokines such as IFN-γ, TNF-α, IL-6, and MCP-2, and these have been demonstrated *in vitro* to induce neutrophil migration, activation, phagocytosis, degranulation, and release of α-defensins (83). In a differing context using a bacterial phosphoantigen, HMBPP-activated Vγ9Vδ2$^+$ T cells produce CXCL8 and TNF-α, which together mediate neutrophil recruitment, induce CD11b upregulation and prevent apoptosis, and downregulate CD62L, allowing neutrophil adhesion (78). This finding was further corroborated by Sabbione et al., showing that HMBPP-activated Vδ2$^+$ γδ T cells can stimulate CD11b expression and myeloperoxidase production by neutrophils (86), all of which imply a stimulatory role of γδ T cells towards these granulocytes. In another study, tissue-resident Vδ1$^+$ γδ T cells were shown to regulate the recruitment of neutrophils to the site of bacterial infection *via* IL-17 secretion (275). In the absence of Vδ1$^+$ γδ T cells, the production of IL-17 is reduced and leads to lower numbers of neutrophil recruitment to the site of infection (275).

Interestingly, activated neutrophils can inhibit CD25 and CD69 expression, IFN-γ production, and cell proliferation of Vδ2$^+$ γδ T cells either spontaneously or in response to HMBPP (86). This is dependent on initial TNF-α production by γδ T cells, which then induces reactive oxygen species (ROS) secretion from neutrophils (86) (**Figure 2**). These processes can be independent of cell–cell contact; however, the inhibition is more potent if cells are allowed to interact and form conjugates (86). Neutrophils can take up zoledronate, and despite also expressing BTN2A1 and BTN3A1, they do not have the capability of activating Vγ9Vδ2$^+$ T cells, which may be due to their extremely limited production and accumulation of IPP (276–278). Rather, these zoledronate-activated neutrophils inhibit TNF-α and IFN-γ production and proliferation of Vγ9Vδ2$^+$ T cells *via* ROS, arginase-1, and serine protease production. Some serine proteases are also able to downregulate BTN3A1 expression on PBMCs, which has

downstream consequences for BTN-mediated activation of Vδ2$^+$ γδ T cells (84, 85). Furthermore, Vδ1$^+$ γδ T cells have been shown to exhibit reduced proliferation in the presence of hydrogen peroxide as well as decreased glutathione production, which may be indicative of ROS-dependent neutrophil inhibition (139). In some instances, however, neutrophils that have phagocytosed HMBPP-producing bacteria subsequently release HMBPP, which is then able to activate Vγ9Vδ2$^+$ T cells. This results in CD25, CD69, LFA-1, IFN-γ, and TNF-α production and is crucial for initiating an immediate anti-inflammatory response (78).

Functionally, pancreatic tumor cell killing by γδ T cells within a PBMC context is decreased in the presence of neutrophils, in both unstimulated and zoledronate-activated conditions (279). However, when pancreatic tumor cells are co-cultured with purified, expanded γδ T cells and neutrophils, tumor cell lysis is increased compared to co-culture with γδ T cells alone, which can be attributed to elevated granzyme B and IFN-γ production. These conflicting observations may be explained by differences in immune cell subpopulation crosstalk within PBMCs, or by differing polarization of neutrophils: N1 neutrophils are tumor suppressive while N2 neutrophils have a pro-tumoral phenotype (280). It is worth noting that a higher neutrophil-to-lymphocyte ratio in a cohort study of 1,714 cancer patients treated with immune checkpoint inhibitors was recently reported to significantly correlate with low progression-free survival, poor response rates, and low clinical benefit (281). Considering the immunosuppressive functions of activated neutrophils on γδ T-cell activation as discussed above, this may partly contribute to the poor outcomes in cancer patients with higher neutrophil-to-lymphocyte ratios.

CROSSTALK BETWEEN γδ T CELLS AND DENDRITIC CELLS

DCs are professional APCs, and consist of classical or conventional DCs (cDCs), including cDC1 (CD11c$^+$ and CD141$^+$) and cDC2 (CD11c$^+$ and CD1c$^+$), and plasmacytoid DCs (pDCs, CD11c$^-$, CD123$^+$, and CD303$^+$) (282, 283). Their key role in anti-tumor immunity is well described, but the interactions between DCs and γδ T cells is lacking behind. It has been shown that upon recognition of bacteria-infected or tumor cells, activated Vγ9Vδ2$^+$ T cells can aid DC maturation through cytokine secretion (IFN-γ and TNF-α) (87, 88), and promote maturation of antigen-expressing immature DCs (monocyte-derived) in circulation *via* contact-dependent mechanisms (Fas/FasL, CD40/CD40L, and TCR/CD1) independent from TLR signaling (89–91, 93) (**Figure 2**). These Vγ9Vδ2$^+$ T cell-matured DCs upregulate HLA-DR, CD25, CD40, CD80, CD83, and CD86, and are capable of cytokine production (TNF-α, IL-12, and IL-15, but not IL-10), antigen presentation, and stimulation of naïve CD4$^+$ αβ T cells (76, 87, 89, 92, 284–288). In addition, Vγ9Vδ2$^+$ T cell-derived cytokines (IFN-γ and TNF-α) can also enhance TLR-dependent DC maturation, upregulate CCR7 (lymph node-homing receptor), and facilitate their migration to lymphoid tissues for CD4$^+$ αβ T-cell priming (289, 290).

In contrast, the tumor-derived chemokine ligand CXCL10 can promote the expansion of Vδ1$^+$ γδ Treg cells that infiltrate solid tumors and induce immune senescence in DCs, and prevent DC maturation (by inhibiting CD80, CD83, CD86, and HLA-DR expression), DC function (decreased IL-6 and IL-12 production), and DC phenotype (inability to stimulate naïve T-cell proliferation) *via* the TLR8 signaling pathway or by killing of DCs through a perforin-mediated pathway (102–107) (**Figure 2**).

In turn, DCs can mediate Vγ9Vδ2$^+$ T-cell activation by sensing/presenting HMBPP and induce γδ T-cell proliferation in the presence of IL-2, IL-15, and IL-21 (76, 94–96). Immature DCs can enhance the ability of Vγ9Vδ2$^+$ T cells to secrete inflammatory cytokines necessary for γδ T-cell maturation (TNF-α) in part due to the ability of DCs to upregulate and/or sense phosphoantigens (88). Mature cDCs and pDCs (monocyte-derived) can secrete cytokines (IL-1β, IL-12, IL-18, IFN-γ, and TNF-α) that activate Vγ9Vδ2$^+$ T cells, enhancing their proliferation and cytotoxic function (IL-18-mediated cytotoxicity against tumor cells) (287, 291–296). In the presence of phosphoantigen, IL-15-producing DCs (monocyte-derived) can also activate γδ T cells in a contact-dependent manner (CD86) and induce secretion of IFN-γ (284, 297, 298). Zoledronate-treated immature and mature DCs (monocyte-derived) can induce phosphoantigen-mediated activation and expansion of effector Vγ9Vδ2$^+$ T cells capable of co-stimulatory and cytotoxic functions *via* the expression of CD40L (299–303).

In summary, different γδ T-cell subsets can either aid and promote or inhibit DC maturation and function (7, 13, 304, 305), while DCs can activate and expand Vγ9Vδ2$^+$ T cells (7, 13, 304–307). The crosstalk between γδ T cells and DCs can thus have downstream anti- or pro-tumoral effects with therapeutic potential, albeit warranting further investigation using DCs that are not monocyte-derived (8, 150, 308).

OUTLOOK AND FUTURE PERSPECTIVE

Our understanding on γδ T cells continues to expand and their contributions in bridging the innate and adaptive anti-tumor immune responses are becoming more evident. Multiple studies are now highlighting their role in interacting with and orchestrating a variety of other immune cell subsets as reviewed here. Traditionally, γδ T cell-based cancer immunotherapies have been focused on assessing the efficacy of activated γδ T cells alone in mediating tumor clearance (41–46, 145, 157, 163, 165, 309). Although these past clinical trials have shown that γδ T cell-based immunotherapies were safe and well tolerated in patients, given the limited success to date (8, 10, 19, 101, 310–312), more innovative strategies aiming to overcome the challenges and immunosuppression within the TME should be thoroughly explored. Notably, with the ever-increasing numbers of studies demonstrating the intricate network of immune interactions within the TME, it is high time to deeply explore some of these interactions and to gain valuable insights into the unique immunomodulatory functions of γδ T cells in the context of cancer immunotherapy. Such acquired knowledge can be fully

harnessed to develop a multipronged γδ T cell-based immunotherapy focusing on γδ T cells' capability to influence the activities of other tumor-infiltrating immune cells *via* rapid cytokine and chemokine secretion, expression of various co-stimulatory molecules, and the professional APC functions in cross-priming and presenting antigens to αβ T cells.

For example, we are now armed with several potent therapeutic agents including the agonist antibodies against BTN3A1 (clone 20.1, CTX-2026, and ICT-01) and BTN2A1 (ICT-0302) that are capable of activating and enhancing the immunomodulatory functions of $V\gamma9V\delta2^+$ T cells (48–53, 227, 313, 314). Treatment targeting BTN3A1 (CTX-2026) can induce coordinated $V\gamma9V\delta2^+$ and αβ T-cell responses for tumor cell killing and represents a promising therapeutic approach that could be combined with other immune checkpoint inhibitors targeting PD-1/PD-L1 (nivolumab and pembrolizumab), CTLA-4/CD86 (ipilimumab and tremelimumab), KIRs (lirilumab and IPH4102), ILT-2 (anti-ILT-2, anti-HLA-G1, anti-FasL), and NKG2A (monalizumab) to circumvent potential immunosuppression in TME (11, 204, 205). These anti-tumor responses could potentially be further enhanced by inducing the expression of NKG2D ligands in tumor cells using a proteasome inhibitor (bortezomib) and an alkylating agent (temozolomide) to promote orchestrated NKG2D-mediated tumor cell lysis by tumor-infiltrating NK, $CD8^+$ αβ, and γδ T cells (240, 256, 257). Moreover, CD137 (4-1BB) co-stimulation with recombinant human CD137L can boost the therapeutic effect of $V\gamma9V\delta2^+$ T cell-based immunotherapy and lead to heightened NK cell-mediated cytotoxicity (73, 75, 101, 315). Taken together, such combined therapeutic treatment will be a powerful approach to elicit concerted anti-tumor responses in different tumor-infiltrating immune cells and help to maximize the efficacy of future γδ T cell-based immunotherapy treatments in cancer patients.

AUTHOR CONTRIBUTIONS

KFC, JDGD, SO, and AB wrote and prepared the manuscript draft. KFC prepared the figures. All authors contributed to the article and approved the submitted version.

FUNDING

AB is supported by a fellowship from the DHHS acting through the Victorian Cancer Agency. KFC is awarded the ECRs and MCRs Awards (ABC scheme 2022-2023) funding from La Trobe University (WBS: 3.2515.01.20). JDGD was supported by Cure Cancer Australia through the Cancer Australia Priority—Driven Cancer Research Scheme (#1187815). The contents of the published material are solely the responsibility of La Trobe University and do not reflect the views of Cancer Australia.

ACKNOWLEDGMENTS

In loving memory of the departed ones, author KFC would like to dedicate this article to commemorate his beloved uncle, Liew Yau Kong and uncle's son, Liew Sam Weng who passed away from COVID-19 in July 2021. **Figures 1**, **2** are created with BioRender.com.

REFERENCES

1. Chien Y-H, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM. A Third Type of Murine T-Cell Receptor Gene. *Nature* (1984) 312(5989):31–5. doi: 10.1038/312031a0
2. Hayday AC, Saito H, Gillies SD, Kranz DM, Tanigawa G, Eisen HN, et al. Structure, Organization, and Somatic Rearrangement of T Cell Gamma Genes. *Cell* (1985) 40(2):259–69. doi: 10.1016/0092-8674(85)90140-0
3. Rigau M, Uldrich AP, Behren A. Targeting Butyrophilins for Cancer Immunotherapy. *Trends Immunol* (2021) 42(8):670–80. doi: 10.1016/j.it.2021.06.002
4. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. *Nat Med* (2015) 21(8):938–45. doi: 10.1038/nm.3909
5. Zou C, Zhao P, Xiao Z, Han X, Fu F, Fu L. γδ T Cells in Cancer Immunotherapy. *Oncotarget* (2017) 8(5):8900. doi: 10.18632/oncotarget.13051
6. Godfrey DI, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T Cell Targets for Cancer Immunotherapy. *Immunity* (2018) 48(3):453–73. doi: 10.1016/j.immuni.2018.03.009
7. Zhao Y, Niu C, Cui J. Gamma-Delta (γδ) T Cells: Friend or Foe in Cancer Development? *J Trans Med* (2018) 16(1):1–13. doi: 10.1186/s12967-017-1378-2
8. Silva-Santos B, Mensurado S, Coffelt SB. γδ T Cells: Pleiotropic Immune Effectors With Therapeutic Potential in Cancer. *Nat Rev Cancer* (2019) 19(7):392–404. doi: 10.1038/s41568-019-0153-5
9. Hayday AC. γδ T Cell Update: Adaptate Orchestrators of Immune Surveillance. *J Immunol* (2019) 203(2):311–20. doi: 10.4049/jimmunol.1800934
10. Garber K. γδ T Cells Bring Unconventional Cancer-Targeting to the Clinic - Again. *Nat Biotechnol* (2020) 38(4):389–92. doi: 10.1038/s41587-020-0487-2
11. Kabelitz D, Serrano R, Kouakanou L, Peters C, Kalyan S. Cancer Immunotherapy With γδ T Cells: Many Paths Ahead of Us. *Cell Mol Immunol* (2020) 17(9):925–39. doi: 10.1038/s41423-020-0504-x
12. Lee HW, Chung YS, Kim TJ. Heterogeneity of Human γδ T Cells and Their Role in Cancer Immunity. *Immune Network* (2020) 20(1):e5. doi: 10.4110/in.2020.20.e5
13. Liu Y, Zhang C. The Role of Human γδ T Cells in Anti-Tumor Immunity and Their Potential for Cancer Immunotherapy. *Cells* (2020) 9(5):1206. doi: 10.3390/cells9051206
14. Morandi F, Yazdanifar M, Cocco C, Bertaina A, Airoldi I. Engineering the Bridge Between Innate and Adaptive Immunity for Cancer Immunotherapy: Focus on γδ T and NK Cells. *Cells* (2020) 9(8):1757. doi: 10.3390/cells9081757
15. Park JH, Lee HK. Function of γδ T Cells in Tumor Immunology and Their Application to Cancer Therapy. *Exp Mol Med* (2021) 53(3):318–27. doi: 10.1038/s12276-021-00576-0
16. Ribot JC, Lopes N, Silva-Santos B. γδ T Cells in Tissue Physiology and Surveillance. *Nat Rev Immunol* (2021) 21(4):221–32. doi: 10.1038/s41577-020-00452-4
17. Chien Y-H, Meyer C, Bonneville M. γδ T Cells: First Line of Defense and Beyond. *Annu Rev Immunol* (2014) 32:121–55. doi: 10.1146/annurev-immunol-032713-120216
18. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The Burgeoning Family of Unconventional T Cells. *Nat Immunol* (2015) 16(11):1114–23. doi: 10.1038/ni.3298
19. Sebestyen Z, Prinz I, Déchanet-Merville J, Silva-Santos B, Kuball J. Translating Gammadelta (γδ) T Cells and Their Receptors Into Cancer Cell Therapies. *Nat Rev Drug Discov* (2020) 19(3):169–84. doi: 10.1038/s41573-019-0038-z

20. Kabelitz D, Kalyan S, Oberg H-H, Wesch D. Human Vδ2 Versus non-Vδ2 γδ T Cells in Antitumor Immunity. *Oncoimmunology* (2013) 2(3):e23304. doi: 10.4161/onci.23304
21. Adams EJ, Gu S, Luoma AM. Human Gamma Delta T Cells: Evolution and Ligand Recognition. *Cell Immunol* (2015) 296(1):31–40. doi: 10.1016/j.cellimm.2015.04.008
22. Qi C, Wang Y, Li P, Zhao J. Gamma Delta T Cells and Their Pathogenic Role in Psoriasis. *Front Immunol* (2021) 12. doi: 10.3389/fimmu.2021.627139
23. Peigné C-M, Léger A, Gesnel M-C, Konczak F, Olive D, Bonneville M, et al. The Juxtamembrane Domain of Butyrophilin BTN3A1 Controls Phosphoantigen-Mediated Activation of Human Vγ9vδ2 T Cells. *J Immunol* (2017) 198(11):4228–34. doi: 10.4049/jimmunol.1601910
24. Wang H, Nada MH, Tanaka Y, Sakuraba S, Morita CT. Critical Roles for Coiled-Coil Dimers of Butyrophilin 3A1 in the Sensing of Prenyl Pyrophosphates by Human Vγ2vδ2 T Cells. *J Immunol* (2019) 203 (3):607–26. doi: 10.4049/jimmunol.1801252
25. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and Synthetic non-Peptide Antigens Recognized by Human γδ T Cells. *Nature* (1995) 375(6527):155–8. doi: 10.1038/375155a0
26. Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind RM, Kollas A-K, et al. Identification of (E)-4-Hydroxy-3-Methyl-But-2-Enyl Pyrophosphate as a Major Activator for Human γδ T Cells in Escherichia Coli. *FEBS Lett* (2001) 509(2):317–22. doi: 10.1016/S0014-5793(01)03191-X
27. Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 Binds Phosphorylated Antigens and Stimulates Human γδ T Cells. *Nat Immunol* (2013) 14(9):908–16. doi: 10.1038/ni.2665
28. Sandstrom A, Peigné C-M, Léger A, Crooks JE, Konczak F, Gesnel M-C, et al. The Intracellular B30. 2 Domain of Butyrophilin 3A1 Binds Phosphoantigens to Mediate Activation of Human Vγ9vδ2 T Cells. *Immunity* (2014) 40(4):490–500. doi: 10.1016/j.immuni.2014.03.003
29. Gu S, Sachleben JR, Boughter CT, Nawrocka WI, Borowska MT, Tarrasch JT, et al. Phosphoantigen-Induced Conformational Change of Butyrophilin 3A1 (BTN3A1) and its Implication on Vγ9vδ2 T Cell Activation. *Proc Natl Acad Sci* (2017) 114(35):E7311–E20. doi: 10.1073/pnas.1707547114
30. Yang Y, Li L, Yuan L, Zhou X, Duan J, Xiao H, et al. A Structural Change in Butyrophilin Upon Phosphoantigen Binding Underlies Phosphoantigen-Mediated Vγ9vδ2 T Cell Activation. *Immunity* (2019) 50(4):1043–53.e5. doi: 10.1016/j.immuni.2019.02.016
31. Hsiao C-HC, Nguyen K, Jin Y, Vinogradova O, Wiemer AJ. Ligand-Induced Interactions Between Butyrophilin 2A1 and 3A1 Internal Domains in the HMBPP Receptor Complex. *Cell Chem Biol* (2022) 29(6):985–95.e5. doi: 10.1016/j.chembiol.2022.01.004
32. Behren A, Anaka M, Lo PH, Vella LJ, Davis ID, Catimel J, et al. The Ludwig Institute for Cancer Research Melbourne Melanoma Cell Line Panel. *Pigment Cell Melanoma Res* (2013) 26(4):597–600. doi: 10.1111/pcmr.12097
33. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 is Essential for Phosphoantigen Reactivity by γδ T Cells. *Science* (2020) 367(6478):eaay5516. doi: 10.1126/science.aay5516
34. Karunakaran MM, Willcox CR, Salim M, Paletta D, Fichtner AS, Noll A, et al. Butyrophilin-2A1 Directly Binds Germline-Encoded Regions of the Vγ9vδ2 TCR and is Essential for Phosphoantigen Sensing. *Immunity* (2020) 52(3):487–98.e6. doi: 10.1016/j.immuni.2020.02.014
35. Vyborova A, Beringer DX, Fasci D, Karaiskaki F, van Diest E, Kramer L, et al. γ9δ2t Cell Diversity and the Receptor Interface With Tumor Cells. *J Clin Invest* (2020) 130(9):4637–51. doi: 10.1172/JCI132489
36. Cano CE, Pasero C, De Gassart A, Kerneur C, Gabriac M, Fullana M, et al. BTN2A1, an Immune Checkpoint Targeting Vγ9vδ2 T Cell Cytotoxicity Against Malignant Cells. *Cell Rep* (2021) 36(2):109359. doi: 10.1016/j.celrep.2021.109359
37. Gober H-J, Kistowska M, Angman L, Jenoü P, Mori L, De Libero G. Human T Cell Receptor γδ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells. *J Exp Med* (2003) 197(2):163–8. doi: 10.1084/jem.20021500
38. Benzaïd I, Mönkkönen H, Stresing V, Bonnelye E, Green J, Mönkkönen J, et al. High Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Promote Vγ9vδ2 T-Cell Chemotaxis and Cytotoxicity In Vivo. *Cancer Res* (2011) 71(13):4562–72. doi: 10.1158/0008-5472.CAN-10-3862
39. Ashihara E, Munaka T, Kimura S, Nakagawa S, Nakagawa Y, Kanai M, et al. Isopentenyl Pyrophosphate Secreted From Zoledronate-Stimulated Myeloma Cells, Activates the Chemotaxis of γδt Cells. *Biochem Biophys Res Commun* (2015) 463(4):650–5. doi: 10.1016/j.bbrc.2015.05.118
40. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of γδ T Cells by Aminobisphosphonates and Induction of Antiplasma Cell Activity in Multiple Myeloma. *Blood J Am Soc Hematol* (2000) 96(2):384–92. doi: 10.1182/blood.V96.2.384.013k07_384_392
41. Viey E, Fromont G, Escudier B, Morel Y, Da Rocha S, Chouaib S, et al. Phosphostim-Activated γδ T Cells Kill Autologous Metastatic Renal Cell Carcinoma. *J Immunol* (2005) 174(3):1338–47. doi: 10.4049/jimmunol.174.3.1338
42. Mattarollo SR, Kenna T, Nieda M, Nicol AJ. Chemotherapy and Zoledronate Sensitize Solid Tumour Cells to Vγ9vδ2 T Cell Cytotoxicity. *Cancer Immunol Immunother* (2007) 56(8):1285–97. doi: 10.1007/s00262-007-0279-2
43. Bouet-Toussaint F, Cabillic F, Toutirais O, Le Gallo M, de la Pintière CT, Daniel P, et al. Vγ9vδ2 T Cell-Mediated Recognition of Human Solid Tumors. Potential for Immunotherapy of Hepatocellular and Colorectal Carcinomas. *Cancer Immunol Immunother* (2008) 57(4):531–9. doi: 10.1007/s00262-007-0391-3
44. Gertner-Dardenne J, Castellano R, Mamessier E, Garbit S, Kochbati E, Etienne A, et al. Human Vγ9vδ2 T Cells Specifically Recognize and Kill Acute Myeloid Leukemic Blasts. *J Immunol* (2012) 188(9):4701–8. doi: 10.4049/jimmunol.1103710
45. Abe Y, Muto M, Nieda M, Nakagawa Y, Nicol A, Kaneko T, et al. Clinical and Immunological Evaluation of Zoledronate-Activated Vγ9γδ T-Cell-Based Immunotherapy for Patients With Multiple Myeloma. *Exp Hematol* (2009) 37(8):956–68. doi: 10.1016/j.exphem.2009.04.008
46. Meraviglia S, Eberl M, Vermijlen D, Todaro M, Buccheri S, Cicero G, et al. *In Vivo* Manipulation of Vγ9vδ2 T Cells With Zoledronate and Low-Dose Interleukin-2 for Immunotherapy of Advanced Breast Cancer Patients. *Clin Exp Immunol* (2010) 161(2):290–7. doi: 10.1111/j.1365-2249.2010.04167.x
47. Yazdanifar M, Barbarito G, Bertaina A, Airoldi I. γδ T Cells: The Ideal Tool for Cancer Immunotherapy. *Cells* (2020) 9(5):1305. doi: 10.3390/cells9051305
48. Harly C, Guillaume Y, Nedellec S, Peigné C-M, Mönkkönen H, Mönkkönen J, et al. Key Implication of CD277/butyrophilin-3 (BTN3A) in Cellular Stress Sensing by a Major Human γδ T-Cell Subset. *Blood J Am Soc Hematol* (2012) 120(11):2269–79. doi: 10.1182/blood-2012-05-430470
49. Palakodeti A, Sandstrom A, Sundaresan L, Harly C, Nedellec S, Olive D, et al. The Molecular Basis for Modulation of Human Vγ9vδ2 T Cell Responses by CD277/butyrophilin-3 (BTN3A)-Specific Antibodies. *J Biol Chem* (2012) 287(39):32780–90. doi: 10.1074/jbc.M112.384354
50. Arnett HA, Viney JL. Immune Modulation by Butyrophilins. *Nat Rev Immunol* (2014) 14(8):559–69. doi: 10.1038/nri3715
51. Starick L, Riano F, Karunakaran MM, Kunzmann V, Li J, Kreiss M, et al. Butyrophilin 3a (BTN3A, CD277)-Specific Antibody 20.1 Differentially Activates Vγ9vδ2 TCR Clonotypes and Interferes With Phosphoantigen Activation. *Eur J Immunol* (2017) 47(6):982–92. doi: 10.1002/eji.201646818
52. Payne KK, Mine JA, Biswas S, Chaurio RA, Perales-Puchalt A, Anadon CM, et al. BTN3A1 Governs Antitumor Responses by Coordinating αβ and γδ T Cells. *Science* (2020) 369(6506):942–9. doi: 10.1126/science.aay2767
53. De Gassart A, Le K-S, Brune P, Agaugué S, Sims J, Goubard A, et al. Development of ICT01, a First-in-Class, Anti-BTN3A Antibody for Activating Vγ9vδ2 T Cell–Mediated Antitumor Immune Response. *Sci Trans Med* (2021) 13(616):eabj0835. doi: 10.1126/scitranslmed.abj0835
54. Wu D, Wu P, Qiu F, Wei Q, Huang J. Human γδt-Cell Subsets and Their Involvement in Tumor Immunity. *Cell Mol Immunol* (2017) 14(3):245–53. doi: 10.1038/cmi.2016.55
55. Willcox BE, Willcox CR. γδ TCR Ligands: The Quest to Solve a 500-Million-Year-Old Mystery. *Nat Immunol* (2019) 20(2):121–8. doi: 10.1038/s41590-018-0304-y
56. Brandes M, Willimann K, Moser B. Professional Antigen-Presentation Function by Human γδ T Cells. *Science* (2005) 309(5732):264–8. doi: 10.1126/science.1110267

57. Brandes M, Willimann K, Bioley G, Lévy N, Eberl M, Luo M, et al. Cross-Presenting Human γδ T Cells Induce Robust CD8+ αβ T Cell Responses. *Proc Natl Acad Sci* (2009) 106(7):2307–12. doi: 10.1073/pnas.0810059106
58. Meuter S, Eberl M, Moser B. Prolonged Antigen Survival and Cytosolic Export in Cross-Presenting Human γδ T Cells. *Proc Natl Acad Sci* (2010) 107 (19):8730–5. doi: 10.1073/pnas.1002769107
59. Altvater B, Pscherer S, Landmeier S, Kailayangiri S, Savoldo B, Juergens H, et al. Activated Human γδ T Cells Induce Peptide-Specific CD8+ T-Cell Responses to Tumor-Associated Self-Antigens. *Cancer Immunol Immunother* (2012) 61(3):385–96. doi: 10.1007/s00262-011-1111-6
60. Himoudi N, Morgenstern DA, Yan M, Vernay B, Saraiva L, Wu Y, et al. Human γδ T Lymphocytes are Licensed for Professional Antigen Presentation by Interaction With Opsonized Target Cells. *J Immunol* (2012) 188(4):1708–16. doi: 10.4049/jimmunol.1102654
61. Howard J, Loizon S, Tyler CJ, Duluc D, Moser B, Mechain M, et al. The Antigen-Presenting Potential of Vγ9vδ2 T Cells During Plasmodium Falciparum Blood-Stage Infection. *J Infect Dis* (2017) 215(10):1569–79. doi: 10.1093/infdis/jix149
62. Barisa M, Kramer A, Majani Y, Moulding D, Saraiva L, Bajaj-Elliott M, et al. E. Coli Promotes Human Vγ9vδ2 T Cell Transition From Cytokine-Producing Bactericidal Effectors to Professional Phagocytic Killers in a TCR-Dependent Manner. *Sci Rep* (2017) 7(1):1–12. doi: 10.1038/s41598-017-02886-8
63. Tyler CJ, McCarthy NE, Lindsay JO, Stagg AJ, Moser B, Eberl M. Antigen-Presenting Human γδ T Cells Promote Intestinal CD4+ T Cell Expression of IL-22 and Mucosal Release of Calprotectin. *J Immunol* (2017) 198(9):3417–25. doi: 10.4049/jimmunol.1700003
64. Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, et al. Human Lymphocytes Bearing T Cell Receptor Gamma/Delta are Phenotypically Diverse and Evenly Distributed Throughout the Lymphoid System. *J Exp Med* (1989) 169(4):1277–94. doi: 10.1084/jem.169.4.1277
65. Gascan H, Gauchat J-F, Roncarolo M-G, Yssel H, Spits H, De Vries JE. Human B Cell Clones can be Induced to Proliferate and to Switch to IgE and IgG4 Synthesis by Interleukin 4 and a Signal Provided by Activated CD4+ T Cell Clones. *J Exp Med* (1991) 173(3):747–50. doi: 10.1084/jem.173.3.747
66. Horner AA, Jabara H, Ramesh N, Geha RS. γ/δ T Lymphocytes Express CD40 Ligand and Induce Isotype Switching in B Lymphocytes. *J Exp Med* (1995) 181(3):1239–44. doi: 10.1084/jem.181.3.1239
67. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 Receptor: Signaling Mechanisms and Biologic Functions. *Annu Rev Immunol* (1999) 17(1):701–38. doi: 10.1146/annurev.immunol.17.1.701
68. Brandes M, Willimann K, Lang AB, Nam K-H, Jin C, Brenner MB, et al. Flexible Migration Program Regulates γδ T-Cell Involvement in Humoral Immunity. *Blood* (2003) 102(10):3693–701. doi: 10.1182/blood-2003-04-1016
69. Caccamo N, Battistini L, Bonneville M, Poccia F, Fournié JJ, Meraviglia S, et al. CXCR5 Identifies a Subset of Vγ9vδ2 T Cells Which Secrete IL-4 and IL-10 and Help B Cells for Antibody Production. *J Immunol* (2006) 177 (8):5290–5. doi: 10.4049/jimmunol.177.8.5290
70. Vermijlen D, Ellis P, Langford C, Klein A, Engel R, Willimann K, et al. Distinct Cytokine-Driven Responses of Activated Blood γδ T Cells: Insights Into Unconventional T Cell Pleiotropy. *J Immunol* (2007) 178(7):4304–14. doi: 10.4049/jimmunol.178.7.4304
71. Bansal RR, Mackay CR, Moser B, Eberl M. IL-21 Enhances the Potential of Human γδ T Cells to Provide B-Cell Help. *Eur J Immunol* (2012) 42(1):110–9. doi: 10.1002/eji.201142017
72. Petrasca A, Doherty DG. Human Vδ2+ γδ T Cells Differentially Induce Maturation, Cytokine Production and Alloreactive T Cell Stimulation by Dendritic Cells and B Cells. *Front Immunol* (2014) 5:650. doi: 10.3389/fimmu.2014.00650
73. Maniar A, Zhang X, Lin W, Gastman BR, Pauza CD, Strome SE, et al. Human γδ T Lymphocytes Induce Robust NK Cell–Mediated Antitumor Cytotoxicity Through CD137 Engagement. *Blood J Am Soc Hematol* (2010) 116(10):1726–33. doi: 10.1182/blood-2009-07-234211
74. Nussbaumer O, Gruenbacher G, Gander H, Thurnher M. DC-Like Cell-Dependent Activation of Human Natural Killer Cells by the Bisphosphonate Zoledronic Acid is Regulated by γδ T Lymphocytes. *Blood J Am Soc Hematol* (2011) 118(10):2743–51. doi: 10.1182/blood-2011-01-328526
75. Liu M, Hu Y, Yuan Y, Tian Z, Zhang C. γδt Cells Suppress Liver Fibrosis *via* Strong Cytolysis and Enhanced NK Cell-Mediated Cytotoxicity Against Hepatic Stellate Cells. *Front Immunol* (2019) 10:477. doi: 10.3389/fimmu.2019.00477
76. Eberl M, Roberts GW, Meuter S, Williams JD, Topley N, Moser B. A Rapid Crosstalk of Human γδ T Cells and Monocytes Drives the Acute Inflammation in Bacterial Infections. *PloS Pathogens* (2009) 5(2): e1000308. doi: 10.1371/journal.ppat.1000308
77. Kalyan S, Chow AW. Linking Innate and Adaptive Immunity: Human V 9v 2 T Cells Enhance CD40 Expression and HMGB-1 Secretion. *Mediators Inflamm* (2009) 2009. doi: 10.1155/2009/819408
78. Davey MS, Lin C-Y, Roberts GW, Heuston S, Brown AC, Chess JA, et al. Human Neutrophil Clearance of Bacterial Pathogens Triggers Anti-Microbial γδ T Cell Responses in Early Infection. *PloS Pathogens* (2011) 7 (5):e1002040. doi: 10.1371/journal.ppat.1002040
79. Dieli F, Troye-Blomberg M, Ivanyi J, Fournié JJ, Bonneville M, Peyrat MA, et al. Vγ9/Vδ2 T Lymphocytes Reduce the Viability of Intracellular Mycobacterium Tuberculosis. *Eur J Immunol* (2000) 30(5):1512–9. doi: 10.1002/(SICI)1521-4141(200005)30:5<1512::AID-IMMU1512>3.0.CO;2-3
80. Ferrero E, Biswas P, Vettoretto K, Ferrarini M, Uguccioni M, Piali L, et al. Macrophages Exposed to Mycobacterium Tuberculosis Release Chemokines Able to Recruit Selected Leucocyte Subpopulations: Focus on γδ Cells. *Immunology* (2003) 108(3):365–74. doi: 10.1046/j.1365-2567.2003.01600.x
81. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan P-L, et al. Phosphoantigen-Expanded Human γδ T Cells Display Potent Cytotoxicity Against Monocyte-Derived Macrophages Infected With Human and Avian Influenza Viruses. *J Infect Dis* (2009) 200(6):858–65. doi: 10.1086/605413
82. Spencer CT, Abate G, Sakala IG, Xia M, Truscott SM, Eickhoff CS, et al. Granzyme A Produced by γ9δ2 T Cells Induces Human Macrophages to Inhibit Growth of an Intracellular Pathogen. *PloS Pathogens* (2013) 9(1): e1003119. doi: 10.1371/journal.ppat.1003119
83. Agrati C, Cimini E, Sacchi A, Bordoni V, Gioia C, Casetti R, et al. Activated Vγ9vδ2 T Cells Trigger Granulocyte Functions *via* MCP-2 Release. *J Immunol* (2009) 182(1):522–9. doi: 10.4049/jimmunol.182.1.522
84. Fazio J, Kalyan S, Wesch D, Kabelitz D. Inhibition of Human γδ T Cell Proliferation and Effector Functions by Neutrophil Serine Proteases. *Scand J Immunol* (2014) 80(6):381–9. doi: 10.1111/sji.12221
85. Kalyan S, Chandrasekaran V, Quabius ES, Lindhorst TK, Kabelitz D. Neutrophil Uptake of Nitrogen-Bisphosphonates Leads to the Suppression of Human Peripheral Blood γδ T Cells. *Cell Mol Life Sci* (2014) 71(12):2335–46. doi: 10.1007/s00018-013-1495-x
86. Sabbione F, Gabelloni ML, Ernst G, Gori MS, Salamone G, Oleastro M, et al. Neutrophils Suppress γδ T-Cell Function. *Eur J Immunol* (2014) 44(3):819–30. doi: 10.1002/eji.201343664
87. Ismaili J, Olislagers V, Poupot R, Fournié J-J, Goldman M. Human γδ T Cells Induce Dendritic Cell Maturation. *Clin Immunol* (2002) 103(3):296–302. doi: 10.1006/clim.2002.5218
88. Devilder M-C, Maillet S, Bouyge-Moreau I, Donnadieu E, Bonneville M, Scotet E. Potentiation of Antigen-Stimulated Vγ9vδ2 T Cell Cytokine Production by Immature Dendritic Cells (DC) and Reciprocal Effect on DC Maturation. *J Immunol* (2006) 176(3):1386–93. doi: 10.4049/jimmunol.176.3.1386
89. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, Morita CT, et al. CD1-Mediated γ/δ T Cell Maturation of Dendritic Cells. *J Exp Med* (2002) 196 (12):1575–84. doi: 10.1084/jem.20021515
90. Eberl M, Jomaa H, Hayday AC. Integrated Immune Responses to Infection–Cross-Talk Between Human γδ T Cells and Dendritic Cells. *Immunology* (2004) 112(3):364. doi: 10.1111/j.1365-2567.2004.01921.x
91. Münz C, Steinman RM, Fujii S-I. Dendritic Cell Maturation by Innate Lymphocytes: Coordinated Stimulation of Innate and Adaptive Immunity. *J Exp Med* (2005) 202(2):203–7. doi: 10.1084/jem.20050810
92. Dunne MR, Madrigal-Estebas L, Tobin LM, Doherty DG. (E)-4-Hydroxy-3-Methyl-But-2 Enyl Pyrophosphate-Stimulated Vγ9vδ2 T Cells Possess T Helper Type 1-Promoting Adjuvant Activity for Human Monocyte-Derived Dendritic Cells. *Cancer Immunol Immunother* (2010) 59(7):1109–20. doi: 10.1007/s00262-010-0839-8
93. van Beek JJ, Wimmers F, Hato SV, de Vries IJM, Skold AE. Dendritic Cell Cross Talk With Innate and Innate-Like Effector Cells in Antitumor

Immunity: Implications for DC Vaccination. *Crit Reviews™ Immunol* (2014) 34(6):517–36. doi: 10.1615/CritRevImmunol.2014012204
94. Pechhold K, Wesch D, Schondelmaier S, Kabelitz D. Primary Activation of V Gamma 9-Expressing Gamma Delta T Cells by Mycobacterium Tuberculosis. Requirement for Th1-Type CD4 T Cell Help and Inhibition by IL-10. *J Immunol* (1994) 152(10):4984–92.
95. Boullier S, Poquet Y, Debord T, Fournie JJ, Gougeon ML. Regulation by Cytokines (IL-12, IL-15, IL-4 and IL-10) of the Vγ9vδ2 T Cell Response to Mycobacterial Phosphoantigens in Responder and Anergic HIV-Infected Persons. *Eur J Immunol* (1999) 29(1):90–9. doi: 10.1002/(SICI)1521-4141(199901)29:01<90::AID-IMMU90>3.0.CO;2-1
96. Eberl M, Altincicek B, Kollas AK, Sanderbrand S, Bahr U, Reichenberg A, et al. Accumulation of a Potent γδ T-Cell Stimulator After Deletion of the lytB Gene in Escherichia Coli. *Immunology* (2002) 106(2):200–11. doi: 10.1046/j.1365-2567.2002.01414.x
97. Zhu Y, Wang H, Xu Y, Hu Y, Chen H, Cui L, et al. Human γδ T Cells Augment Antigen Presentation in Listeria Monocytogenes Infection. *Mol Med* (2016) 22(1):737–46. doi: 10.2119/molmed.2015.00214
98. Wu Y, Wu W, Wong WM, Ward E, Thrasher AJ, Goldblatt D, et al. Human γδ T Cells: A Lymphoid Lineage Cell Capable of Professional Phagocytosis. *J Immunol* (2009) 183(9):5622–9. doi: 10.4049/jimmunol.0901772
99. Mao C, Mou X, Zhou Y, Yuan G, Xu C, Liu H, et al. Tumor-Activated T Cells From Gastric Cancer Patients Induce the Antitumor Immune Response of T Cells via Their Antigen-Presenting Cell-Like Effects. *J Immunol Res* (2014) 2014. doi: 10.1155/2014/593562
100. Cairo C, Surendran N, Harris KM, Mazan-Mamczarz K, Sakoda Y, Diaz-Mendez F, et al. Vγ2vδ2 T-Cell Co-Stimulation Increases Natural Killer Cell Killing of Monocyte-Derived Dendritic Cells. *Immunology* (2015) 144 (3):422–30. doi: 10.1111/imm.12386
101. Pauza CD, Liou M-L, Lahusen T, Xiao L, Lapidus RG, Cairo C, et al. Gamma Delta T Cell Therapy for Cancer: It is Good to be Local. *Front Immunol* (2018) 9:1305. doi: 10.3389/fimmu.2018.01305
102. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang R-F. Tumor-Infiltrating γδ T Cells Suppress T and Dendritic Cell Function *via* Mechanisms Controlled by a Unique Toll-Like Receptor Signaling Pathway. *Immunity* (2007) 27(2):334–48. doi: 10.1016/j.immuni.2007.05.020
103. Chen Y, Lin P, Qiu S, Peng X-X, Looi K, Farquhar MG, et al. Autoantibodies to Ca2+ Binding Protein Calnuc is a Potential Marker in Colon Cancer Detection. *Int J Oncol* (2007) 30(5):1137–44. doi: 10.3892/ijo.30.5.1137
104. Merims S, Dokouhaki P, Joe B, Zhang L. Human Vδ1-T Cells Regulate Immune Responses by Targeting Autologous Immature Dendritic Cells. *Hum Immunol* (2011) 72(1):32–6. doi: 10.1016/j.humimm.2010.10.011
105. Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Wevers E, et al. Tumor-Infiltrating γδ T Lymphocytes Predict Clinical Outcome in Human Breast Cancer. *J Immunol* (2012) 189(10):5029–36. doi: 10.4049/jimmunol.1201892
106. Ye J, Ma C, Hsueh EC, Eickhoff CS, Zhang Y, Varvares MA, et al. Tumor-Derived γδ Regulatory T Cells Suppress Innate and Adaptive Immunity Through the Induction of Immunosenescence. *J Immunol* (2013) 190 (5):2403–14. doi: 10.4049/jimmunol.1202369
107. Ye J, Ma C, Wang F, Hsueh EC, Toth K, Huang Y, et al. Specific Recruitment of γδ Regulatory T Cells in Human Breast Cancer. *Cancer Res* (2013) 73 (20):6137–48. doi: 10.1158/0008-5472.CAN-13-0348
108. Muto M, Baghdadi M, Maekawa R, Wada H, Seino K. Myeloid Molecular Characteristics of Human γδ T Cells Support Their Acquisition of Tumor Antigen-Presenting Capacity. *Cancer Immunol Immunother* (2015) 64 (8):941–9. doi: 10.1007/s00262-015-1700-x
109. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, O'Shea D, et al. Cutting Edge: CD1d Restriction and Th1/Th2/Th17 Cytokine Secretion by Human Vδ3 T Cells. *J Immunol* (2013) 191(1):30–4. doi: 10.4049/jimmunol.1300121
110. Roy S, Ly D, Castro CD, Li NS, Hawk AJ, Altman JD, et al. Molecular Analysis of Lipid-Reactive Vdelta1 Gammadelta T Cells Identified by CD1c Tetramers. *J Immunol* (2016) 196(4):1933–42. doi: 10.4049/jimmunol.1502202
111. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, McPherson KG, Lim RT, et al. CD1d-Lipid Antigen Recognition by the γδ TCR. *Nat Immunol* (2013) 14(11):1137–45. doi: 10.1038/ni.2713
112. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The Majority of CD1d-Sulfatide-Specific T Cells in Human Blood Use a Semiinvariant Vδ1 TCR. *Eur J Immunol* (2012) 42(9):2505–10. doi: 10.1002/eji.201242531
113. Luoma AM, Castro CD, Mayassi T, Bembinster LA, Bai L, Picard D, et al. Crystal Structure of Vδ1 T Cell Receptor in Complex With CD1d-Sulfatide Shows MHC-Like Recognition of a Self-Lipid by Human γδ T Cells. *Immunity* (2013) 39(6):1032–42. doi: 10.1016/j.immuni.2013.11.001
114. Zeng X, Wei Y-L, Huang J, Newell EW, Yu H, Kidd BA, et al. γδ T Cells Recognize a Microbial Encoded B Cell Antigen to Initiate a Rapid Antigen-Specific Interleukin-17 Response. *Immunity* (2012) 37(3):524–34. doi: 10.1016/j.immuni.2012.06.011
115. Silva-Santos B, Schamel WW, Fisch P, Eberl M. Gamma-Delta T-Cell Conference 2012: Close Encounters for the Fifth Time. *Eur J Immunol* (2012) 42(12):3101–5. doi: 10.1002/eji.201270101
116. Le Nours J, Gherardin NA, Ramarathinam SH, Awad W, Wiede F, Gully BS, et al. A Class of γδ T Cell Receptors Recognize the Underside of the Antigen-Presenting Molecule MR1. *Science* (2019) 366(6472):1522–7. doi: 10.1126/science.aav3900
117. Godder K, Henslee-Downey P, Mehta J, Park B, Chiang K, Abhyankar S, et al. Long Term Disease-Free Survival in Acute Leukemia Patients Recovering With Increased γδ T Cells After Partially Mismatched Related Donor Bone Marrow Transplantation. *Bone Marrow Transplant* (2007) 39 (12):751–7. doi: 10.1038/sj.bmt.1705650
118. Siegers GM, Dhamko H, Wang X-H, Mathieson AM, Kosaka Y, Felizardo TC, et al. Human Vδ1 γδ T Cells Expanded From Peripheral Blood Exhibit Specific Cytotoxicity Against B-Cell Chronic Lymphocytic Leukemia-Derived Cells. *Cytotherapy* (2011) 13(6):753–64. doi: 10.3109/14653249.2011.553595
119. Donia M, Ellebaek E, Andersen MH, Straten PT, Svane IM. Analysis of Vδ1 T Cells in Clinical Grade Melanoma-Infiltrating Lymphocytes. *Oncoimmunology* (2012) 1(8):1297–304. doi: 10.4161/onci.21659
120. Knight A, Mackinnon S, Lowdell MW. Human Vdelta1 Gamma-Delta T Cells Exert Potent Specific Cytotoxicity Against Primary Multiple Myeloma Cells. *Cytotherapy* (2012) 14(9):1110–8. doi: 10.3109/14653249.2012.700766
121. Scheper W, van Dorp S, Kersting S, Pietersma F, Lindemans C, Hol S, et al. γδt Cells Elicited by CMV Reactivation After Allo-SCT Cross-Recognize CMV and Leukemia. *Leukemia* (2013) 27(6):1328–38. doi: 10.1038/leu.2012.374
122. Di Lorenzo B, Simões AE, Caiado F, Tieppo P, Correia DV, Carvalho T, et al. Broad Cytotoxic Targeting of Acute Myeloid Leukemia by Polyclonal Delta One T Cells. *Cancer Immunol Res* (2019) 7(4):552–8. doi: 10.1158/2326-6066.CIR-18-0647
123. Mikulak J, Oriolo F, Bruni E, Roberto A, Colombo FS, Villa A, et al. NKp46-Expressing Human Gut-Resident Intraepithelial Vδ1 T Cell Subpopulation Exhibits High Antitumor Activity Against Colorectal Cancer. *JCI Insight* (2019) 4(24):e125884. doi: 10.1172/jci.insight.125884
124. Wu Y, Kyle-Cezar F, Woolf RT, Naceur-Lombardelli C, Owen J, Biswas D, et al. An Innate-Like Vδ1+ γδ T Cell Compartment in the Human Breast is Associated With Remission in Triple-Negative Breast Cancer. *Sci Trans Med* (2019) 11(513):eaax9364. doi: 10.1126/scitranslmed.aax9364
125. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK Cells and T Cells by NKG2D, a Receptor for Stress-Inducible MICA. *science* (1999) 285(5428):727–9. doi: 10.1126/science.285.5428.727
126. Almeida AR, Correia DV, Fernandes-Platzgummer A, da Silva CL, da Silva MG, Anjos DR, et al. Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept. *Clin Cancer Res* (2016) 22(23):5795–804. doi: 10.1158/1078-0432.CCR-16-0597
127. Marlin R, Pappalardo A, Kaminski H, Willcox CR, Pitard V, Netzer S, et al. Sensing of Cell Stress by Human γδ TCR-Dependent Recognition of Annexin A2. *Proc Natl Acad Sci* (2017) 114(12):3163–8. doi: 10.1073/pnas.1621052114
128. Davey MS, Willcox CR, Baker AT, Hunter S, Willcox BE. Recasting Human Vδ1 Lymphocytes in an Adaptive Role. *Trends Immunol* (2018) 39(6):446–59. doi: 10.1016/j.it.2018.03.003

129. Bridgeman JS, Sewell AK, Miles JJ, Price DA, Cole DK. Structural and Biophysical Determinants of αβ T-Cell Antigen Recognition. *Immunology* (2012) 135(1):9–18. doi: 10.1111/j.1365-2567.2011.03515.x
130. Chan KF, Gully BS, Gras S, Beringer DX, Kjer-Nielsen L, Cebon J, et al. Divergent T-Cell Receptor Recognition Modes of a HLA-I Restricted Extended Tumour-Associated Peptide. *Nat Commun* (2018) 9(1):1026. doi: 10.1038/s41467-018-03321-w
131. Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T Cell Antigen Receptor Recognition of Antigen-Presenting Molecules. *Annu Rev Immunol* (2015) 33:169–200. doi: 10.1146/annurev-immunol-032414-112334
132. Imbert C, Olive D. γδ T Cells in Tumor Microenvironment. *Adv Exp Med Biol* (2020) 1273:91–104. doi: 10.1007/978-3-030-49270-0_5
133. Orsini DL, van Gils M, Kooy YM, Struyk L, Klein G, Elsen PVD, et al. Functional and Molecular Characterization of B Cell-Responsive Vδ1+ γδ T Cells. *Eur J Immunol* (1994) 24(12):3199–204. doi: 10.1002/eji.1830241243
134. Häcker G, Adam S, Wagner H. Interaction Between Gamma Delta T Cells and B Cells Regulating IgG Production. *Immunology* (1995) 84(1):105.
135. Petrasca A, Melo AM, Breen EP, Doherty DG. Human Vδ3+ γδ T Cells Induce Maturation and IgM Secretion by B Cells. *Immunol Lett* (2018) 196:126–34. doi: 10.1016/j.imlet.2018.02.002
136. Scheper W, Sebestyen Z, Kuball J. Cancer Immunotherapy Using γδt Cells: Dealing With Diversity. *Front Immunol* (2014) 5:601. doi: 10.3389/fimmu.2014.00601
137. McCarthy NE, Eberl M. Human γδ T-Cell Control of Mucosal Immunity and Inflammation. *Front Immunol* (2018) 9:985. doi: 10.3389/fimmu.2018.00985
138. Hudspeth K, Fogli M, Correia DV, Mikulak J, Roberto A, Della Bella S, et al. Engagement of NKp30 on Vδ1 T Cells Induces the Production of CCL3, CCL4, and CCL5 and Suppresses HIV-1 Replication. *Blood J Am Soc Hematol* (2012) 119(17):4013–6. doi: 10.1182/blood-2011-11-390153
139. Mensurado S, Rei M, Lança T, Ioannou M, Goncalves-Sousa N, Kubo H, et al. Tumor-Associated Neutrophils Suppress Pro-Tumoral IL-17+ γδ T Cells Through Induction of Oxidative Stress. *PloS Biol* (2018) 16(5):e2004990. doi: 10.1371/journal.pbio.2004990
140. Paul S, Lal G. Regulatory and Effector Functions of Gamma–Delta (γδ) T Cells and Their Therapeutic Potential in Adoptive Cellular Therapy for Cancer. *Int J Cancer* (2016) 139(5):976–85. doi: 10.1002/ijc.30109
141. Lança T, Costa MF, Gonçalves-Sousa N, Rei M, Grosso AR, Penido C, et al. Protective Role of the Inflammatory CCR2/CCL2 Chemokine Pathway Through Recruitment of Type 1 Cytotoxic γδ T Lymphocytes to Tumor Beds. *J Immunol* (2013) 190(12):6673–80. doi: 10.4049/jimmunol.1300434
142. Glatzel A, Wesch D, Schiemann F, Brandt E, Janssen O, Kabelitz D. Patterns of Chemokine Receptor Expression on Peripheral Blood γδ T Lymphocytes: Strong Expression of CCR5 is a Selective Feature of Vδ2/Vγ9 γδ T Cells. *J Immunol* (2002) 168(10):4920–9. doi: 10.4049/jimmunol.168.10.4920
143. Tawfik D, Groth C, Gundlach J-P, Peipp M, Kabelitz D, Becker T, et al. TRAIL-Receptor 4 Modulates γδ T Cell-Cytotoxicity Toward Cancer Cells. *Front Immunol* (2019) 2044. doi: 10.3389/fimmu.2019.02044
144. Li Z, Xu Q, Peng H, Cheng R, Sun Z, Ye Z. IFN-γ Enhances HOS and U2OS Cell Lines Susceptibility to γδ T Cell-Mediated Killing Through the Fas/Fas Ligand Pathway. *Int Immunopharmacol* (2011) 11(4):496–503. doi: 10.1016/j.intimp.2011.01.001
145. Tokuyama H, Hagi T, Mattarollo SR, Morley J, Wang Q, Fai-So H, et al. Vγ9vδ2 T Cell Cytotoxicity Against Tumor Cells is Enhanced by Monoclonal Antibody Drugs—Rituximab and Trastuzumab. *Int J Cancer* (2008) 122 (11):2526–34. doi: 10.1002/ijc.23365
146. Couzi L, Pitard V, Sicard X, Garrigue I, Hawchar O, Merville P, et al. Antibody-Dependent Anti-Cytomegalovirus Activity of Human γδ T Cells Expressing CD16 (Fcγriiia). *Blood J Am Soc Hematol* (2012) 119(6):1418–27. doi: 10.1182/blood-2011-06-363655
147. Wesch D, Kabelitz D, Oberg HH. Tumor Resistance Mechanisms and Their Consequences on γδ T Cell Activation. *Immunol Rev* (2020) 298(1):84–98. doi: 10.1111/imr.12925
148. Wesch D, Glatzel A, Kabelitz D. Differentiation of Resting Human Peripheral Blood γδ T Cells Toward Th1-Or Th2-Phenotype. *Cell Immunol* (2001) 212 (2):110–7. doi: 10.1006/cimm.2001.1850
149. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, et al. Differentiation, Phenotype, and Function of Interleukin-17–Producing Human Vγ9vδ2 T Cells. *Blood J Am Soc Hematol* (2011) 118(1):129–38. doi: 10.1182/blood-2011-01-331298
150. Lafont V, Sanchez F, Laprevotte E, Michaud H-A, Gros L, Eliaou J-F, et al. Plasticity of Gamma Delta T Cells: Impact on the Anti-Tumor Response. *Front Immunol* (2014) 5:622. doi: 10.3389/fimmu.2014.00622
151. Peters C, Kabelitz D, Wesch D. Regulatory Functions of γδ T Cells. *Cell Mol Life Sci* (2018) 75(12):2125–35. doi: 10.1007/s00018-018-2788-x
152. Papotto PH, Yilmaz B, Silva-Santos B. Crosstalk Between γδ T Cells and the Microbiota. *Nat Microbiol* (2021); 6(9):1110–7. doi: 10.1038/s41564-021-00948-2
153. Zarobkiewicz MK, Wawryk-Gawda E, Kowalska W, Janiszewska M, Bojarska-Junak A. γδ T Lymphocytes in Asthma: A Complicated Picture. *Archivum Immunol Ther Experimentalis* (2021) 69(1):1–15. doi: 10.1007/s00005-021-00608-7
154. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. Regulation of Cutaneous Malignancy by γδ T Cells. *Science* (2001) 294 (5542):605–9. doi: 10.1126/science.1063916
155. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, et al. γδ T Cells Provide an Early Source of Interferon γ in Tumor Immunity. *J Exp Med* (2003) 198(3):433–42. doi: 10.1084/jem.20030584
156. Cordova A, Toia F, La Mendola C, Orlando V, Meraviglia S, Rinaldi G, et al. Characterization of Human γδ T Lymphocytes Infiltrating Primary Malignant Melanomas. *PloS One* (2012) 7(11):e49878. doi: 10.1371/journal.pone.0049878
157. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, et al. γδ T Cells for Immune Therapy of Patients With Lymphoid Malignancies. *Blood* (2003) 102(1):200–6. doi: 10.1182/blood-2002-12-3665
158. Street SE, Hayakawa Y, Zhan Y, Lew AM, MacGregor D, Jamieson AM, et al. Innate Immune Surveillance of Spontaneous B Cell Lymphomas by Natural Killer Cells and γδ T Cells. *J Exp Med* (2004) 199(6):879–84. doi: 10.1084/jem.20031981
159. Zumwalde NA, Sharma A, Xu X, Ma S, Schneider CL, Romero-Masters JC, et al. Adoptively Transferred Vγ9vδ2 T Cells Show Potent Antitumor Effects in a Preclinical B Cell Lymphomagenesis Model. *JCI Insight* (2017) 2(13):e93179. doi: 10.1172/jci.insight.93179
160. Perko R, Kang G, Sunkara A, Leung W, Thomas PG, Dallas MH. Gamma Delta T Cell Reconstitution is Associated With Fewer Infections and Improved Event-Free Survival After Hematopoietic Stem Cell Transplantation for Pediatric Leukemia. *Biol Blood Marrow Transplant* (2015) 21(1):130–6. doi: 10.1016/j.bbmt.2014.09.027
161. Handgretinger R, Schilbach K. The Potential Role of γδ T Cells After Allogeneic HCT for Leukemia. *Blood J Am Soc Hematol* (2018) 131 (10):1063–72. doi: 10.1182/blood-2017-08-752162
162. Wang J, Lin C, Li H, Li R, Wu Y, Liu H, et al. Tumor-Infiltrating γδt Cells Predict Prognosis and Adjuvant Chemotherapeutic Benefit in Patients With Gastric Cancer. *Oncoimmunology* (2017) 6(11):e1353858. doi: 10.1080/2162402X.2017.1353858
163. Todaro M, D'Asaro M, Caccamo N, Iovino F, Francipane MG, Meraviglia S, et al. Efficient Killing of Human Colon Cancer Stem Cells by γδ T Lymphocytes. *J Immunol* (2009) 182(11):7287–96. doi: 10.4049/jimmunol.0804288
164. Meraviglia S, Lo Presti E, Tosolini M, La Mendola C, Orlando V, Todaro M, et al. Distinctive Features of Tumor-Infiltrating γδ T Lymphocytes in Human Colorectal Cancer. *Oncoimmunology* (2017) 6(10):e1347742. doi: 10.1080/2162402X.2017.1347742
165. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting Human γδ T Cells With Zoledronate and Interleukin-2 for Immunotherapy of Hormone-Refractory Prostate Cancer. *Cancer Res* (2007) 67(15):7450–7. doi: 10.1158/0008-5472.CAN-07-0199
166. Liu Z, Eltoum I-EA, Guo B, Beck BH, Cloud GA, Lopez RD. Protective Immunosurveillance and Therapeutic Antitumor Activity of γδ T Cells Demonstrated in a Mouse Model of Prostate Cancer. *J Immunol* (2008) 180(9):6044–53. doi: 10.4049/jimmunol.180.9.6044
167. Zhao RY, Mifsud NA, Xiao K, Chan K-F, Oveissi S, Jackson HM, et al. A Novel HLA-B18 Restricted CD8+ T Cell Epitope is Efficiently Cross-Presented by Dendritic Cells From Soluble Tumor Antigen. *PloS One* (2012) 7(9):e44707. doi: 10.1371/journal.pone.0044707

168. Vantourout P, Hayday A. Six-Of-the-Best: Unique Contributions of γδ T Cells to Immunology. *Nat Rev Immunol* (2013) 13(2):88–100. doi: 10.1038/nri3384
169. Khan MWA, Curbishley SM, Chen H-C, Thomas AD, Pircher H, Mavilio D, et al. Expanded Human Blood-Derived γδt Cells Display Potent Antigen-Presentation Functions. *Front Immunol* (2014) 5:344. doi: 10.3389/fimmu.2014.00344
170. Tyler CJ, Doherty DG, Moser B, Eberl M. Human Vγ9/Vδ2 T Cells: Innate Adaptors of the Immune System. *Cell Immunol* (2015) 296(1):10–21. doi: 10.1016/j.cellimm.2015.01.008
171. Poupot M, Pont F, Fournié J-J. Profiling Blood Lymphocyte Interactions With Cancer Cells Uncovers the Innate Reactivity of Human γδ T Cells to Anaplastic Large Cell Lymphoma. *J Immunol* (2005) 174(3):1717–22. doi: 10.4049/jimmunol.174.3.1717
172. Schneiders FL, Prodöhl J, Ruben JM, O'Toole T, Scheper RJ, Bonneville M, et al. CD1d-Restricted Antigen Presentation by Vγ9vδ2-T Cells Requires Trogocytosis. *Cancer Immunol Res* (2014) 2(8):732–40. doi: 10.1158/2326-6066.CIR-13-0167
173. Albert ML, Pearce SFA, Francisco LM, Sauter B, Roy P, Silverstein RL, et al. Immature Dendritic Cells Phagocytose Apoptotic Cells *via* αvβ5 and CD36, and Cross-Present Antigens to Cytotoxic T Lymphocytes. *J Exp Med* (1998) 188(7):1359–68. doi: 10.1084/jem.188.7.1359
174. Greenberg ME, Sun M, Zhang R, Febbraio M, Silverstein R, Hazen SL. Oxidized Phosphatidylserine–CD36 Interactions Play an Essential Role in Macrophage-Dependent Phagocytosis of Apoptotic Cells. *J Exp Med* (2006) 203(12):2613–25. doi: 10.1084/jem.20060370
175. Capsomidis A, Benthall G, Van Acker HH, Fisher J, Kramer AM, Abeln Z, et al. Chimeric Antigen Receptor-Engineered Human Gamma Delta T Cells: Enhanced Cytotoxicity With Retention of Cross Presentation. *Mol Ther* (2018) 26(2):354–65. doi: 10.1016/j.ymthe.2017.12.001
176. Korenkov DA, Laurie KL, Reading PC, Carolan LA, Chan KF, Isakova SII, et al. Safety, Immunogenicity and Protection of A(H3N2) Live Attenuated Influenza Vaccines Containing Wild-Type Nucleoprotein in a Ferret Model. *Infect Genet Evol* (2018) 64:95–104. doi: 10.1016/j.meegid.2018.06.019
177. Grant E, Wu C, Chan K-F, Eckle S, Bharadwaj M, Zou QM, et al. Nucleoprotein of Influenza A Virus is a Major Target of Immunodominant $CD8^+$ T-Cell Responses. *Immunol Cell Biol* (2013) 91(2):184–94. doi: 10.1038/icb.2012.78
178. Laurie KL, Horman W, Carolan LA, Chan KF, Layton D, Bean A, et al. Evidence for Viral Interference and Cross-Reactive Protective Immunity Between Influenza B Virus Lineages. *J Infect Dis* (2018) 217(4):548–59. doi: 10.1093/infdis/jix509
179. Buonaguro L, Cerullo V. Pathogens: Our Allies Against Cancer? *Mol Ther* (2021) 29(1):10–2. doi: 10.1016/j.ymthe.2020.12.005
180. Bachelez H, Flageul B, Degos L, Boumsell L, Bensussan A. TCR γδ Bearing T Lymphocytes Infiltrating Human Primary Cutaneous Melanomas. *J Invest Dermatol* (1992) 98(3):369–74. doi: 10.1111/1523-1747.ep12499808
181. Lo Presti E, Pizzolato G, Corsale AM, Caccamo N, Sireci G, Dieli F, et al. γδ T Cells and Tumor Microenvironment: From Immunosurveillance to Tumor Evasion. *Front Immunol* (2018) 9:1395. doi: 10.3389/fimmu.2018.01395
182. Corsale AM, Di Simone M, Lo Presti E, Picone C, Dieli F, Meraviglia S. Metabolic Changes in Tumor Microenvironment: How Could They Affect γδ T Cells Functions? *Cells* (2021) 10(11):2896. doi: 10.3390/cells10112896
183. Donia M, Hansen M, Sendrup SL, Iversen TZ, Ellebæk E, Andersen MH, et al. Methods to Improve Adoptive T-Cell Therapy for Melanoma: IFN-γ Enhances Anticancer Responses of Cell Products for Infusion. *J Invest Dermatol* (2013) 133(2):545–52. doi: 10.1038/jid.2012.336
184. McCarthy NE, Bashir Z, Vossenkämper A, Hedin CR, Giles EM, Bhattacharjee S, et al. Proinflammatory Vδ2+ T Cells Populate the Human Intestinal Mucosa and Enhance IFN-γ Production by Colonic αβ T Cells. *J Immunol* (2013) 191(5):2752–63. doi: 10.4049/jimmunol.1202959
185. Riond J, Rodriguez S, Nicolau M-L, Saati T, Gairin JE. *In Vivo* Major Histocompatibility Complex Class I (MHCI) Expression on MHCIlow Tumor Cells is Regulated by γδ T and NK Cells During the Early Steps of Tumor Growth. *Cancer Immun Archive* (2009) 9(1):10. doi: 10.1158/1424-9634.DCL-10.9.1
186. Gocher AM, Workman CJ, Vignali DA. Interferon-γ: Teammate or Opponent in the Tumour Microenvironment? *Nat Rev Immunol* (2021) 22(3):158–72. doi: 10.1038/s41577-021-00566-3
187. Silva-Santos B, Serre K, Norell H. γδ T Cells in Cancer. *Nat Rev Immunol* (2015) 15(11):683–91. doi: 10.1038/nri3904
188. Gertner-Dardenne J, Bonnafous C, Bezombes C, Capietto A-H, Scaglione V, Ingoure S, et al. Bromohydrin Pyrophosphate Enhances Antibody-Dependent Cell-Mediated Cytotoxicity Induced by Therapeutic Antibodies. *Blood J Am Soc Hematol* (2009) 113(20):4875–84. doi: 10.1182/blood-2008-08-172296
189. Capietto A-H, Martinet L, Fournié J-J. Stimulated γδ T Cells Increase the *In Vivo* Efficacy of Trastuzumab in HER-2+ Breast Cancer. *J Immunol* (2011) 187(2):1031–8. doi: 10.4049/jimmunol.1100681
190. Hashimoto K. CD137 as an Attractive T Cell Co-Stimulatory Target in the TNFRSF for Immuno-Oncology Drug Development. *Cancers* (2021) 13(10):2288. doi: 10.3390/cancers13102288
191. Peters C, Oberg H-H, Kabelitz D, Wesch D. Phenotype and Regulation of Immunosuppressive Vδ2-Expressing γδ T Cells. *Cell Mol Life Sci* (2014) 71(10):1943–60. doi: 10.1007/s00018-013-1467-1
192. Schilbach K, Krickeberg N, Kaißer C, Mingram S, Kind J, Siegers GM, et al. Suppressive Activity of Vδ2+ γδ T Cells on αβ T Cells is Licensed by TCR Signaling and Correlates With Signal Strength. *Cancer Immunol Immunother* (2020) 69(4):593–610. doi: 10.1007/s00262-019-02469-8
193. Daley D, Zambirinis CP, Seifert L, Akkad N, Mohan N, Werba G, et al. γδ T Cells Support Pancreatic Oncogenesis by Restraining αβ T Cell Activation. *Cell* (2016) 166(6):1485–99.e15. doi: 10.1016/j.cell.2016.07.046
194. Bhagat G, Naiyer AJ, Shah JG, Harper J, Jabri B, Wang TC, et al. Small Intestinal CD8+ Tcrγδ+ NKG2A+ Intraepithelial Lymphocytes Have Attributes of Regulatory Cells in Patients With Celiac Disease. *J Clin Invest* (2008) 118(1):281–93. doi: 10.1172/JCI30989
195. Fisch P, Meuer E, Pende D, Rothenfußer S, Viale O, Kock S, et al. Control of B Cell Lymphoma Recognition *via* Natural Killer Inhibitory Receptors Implies a Role for Human Vγ9/Vδ2 T Cells in Tumor Immunity. *Eur J Immunol* (1997) 27(12):3368–79. doi: 10.1002/eji.1830271236
196. Halary F, Peyrat MA, Champagne E, Lopez-Botet M, Moretta A, Moretta L, et al. Control of Self-Reactive Cytotoxic T Lymphocytes Expressing γδ T Cell Receptors by Natural Killer Inhibitory Receptors. *Eur J Immunol* (1997) 27(11):2812–21. doi: 10.1002/eji.1830271111
197. Poccia F, Malkovsky M, Gougeon ML, Bonneville M, Lopez-Botet M, Fournié JJ, et al. γδ T Cell Activation or Anergy During Infections: The Role of Nonpeptidic TCR Ligands and HLA Class I Molecules. *J Leukocyte Biol* (1997) 62(3):287–91. doi: 10.1002/jlb.62.3.287
198. Poccia F, Cipriani B, Vendetti S, Colizzi V, Poquet Y, Battistini L, et al. CD94/NKG2 Inhibitory Receptor Complex Modulates Both Anti-Viral and Anti-Tumoral Responses of Polyclonal Phosphoantigen-Reactive V Gamma 9V Delta 2 T Lymphocytes. *J Immunol* (1997) 159(12):6009–17.
199. Bakker AB, Phillips JH, Figdor CG, Lanier LL. Killer Cell Inhibitory Receptors for MHC Class I Molecules Regulate Lysis of Melanoma Cells Mediated by NK Cells, γδ T Cells, and Antigen-Specific CTL. *J Immunol* (1998) 160(11):5239–45.
200. Angelini DF, Zambello R, Galandrini R, Diamantini A, Placido R, Micucci F, et al. NKG2A Inhibits NKG2C Effector Functions of γδ T Cells: Implications in Health and Disease. *J Leukocyte Biol* (2011) 89(1):75–84. doi: 10.1189/jlb.0710413
201. Lesport E, Baudhuin J, Sousa S, LeMaoult J, Zamborlini A, Rouas-Freiss N, et al. Inhibition of Human Vγ9vδ2 T-Cell Antitumoral Activity Through HLA-G: Implications for Immunotherapy of Cancer. *Cell Mol Life Sci* (2011) 68(20):3385–99. doi: 10.1007/s00018-011-0632-7
202. Xiang Z, Tu W. Dual Face of Vγ9vδ2-T Cells in Tumor Immunology: Anti- Versus Pro-Tumoral Activities. *Front Immunol* (2017) 8:1041. doi: 10.3389/fimmu.2017.01041
203. Niu C, Li M, Zhu S, Chen Y, Zhou L, Xu D, et al. Decitabine Inhibits Gamma Delta T Cell Cytotoxicity by Promoting KIR2DL2/3 Expression. *Front Immunol* (2018) 9:617. doi: 10.3389/fimmu.2018.00617
204. Naji A, Durrbach A, Carosella ED, Rouas-Freiss N. Soluble HLA-G and HLA-G1 Expressing Antigen-Presenting Cells Inhibit T-Cell Alloproliferation Through ILT-2/ILT-4/FasL-Mediated Pathways. *Hum Immunol* (2007) 68(4):233–9. doi: 10.1016/j.humimm.2006.10.017

205. Pende D, Falco M, Vitale M, Cantoni C, Vitale C, Munari E, et al. Killer Ig-Like Receptors (KIRs): Their Role in NK Cell Modulation and Developments Leading to Their Clinical Exploitation. *Front Immunol* (2019) 1179. doi: 10.3389/fimmu.2019.01179
206. André P, Denis C, Soulas C, Bourbon-Caillet C, Lopez J, Arnoux T, et al. Anti-NKG2A mAb is a Checkpoint Inhibitor That Promotes Anti-Tumor Immunity by Unleashing Both T and NK Cells. *Cell* (2018) 175(7):1731–43.e13. doi: 10.1016/j.cell.2018.10.014
207. Mingari MC, Pietra G, Moretta L. Immune Checkpoint Inhibitors: Anti-NKG2A Antibodies on Board. *Trends Immunol* (2019) 40(2):83–5. doi: 10.1016/j.it.2018.12.009
208. Fisch P, Moris A, Rammensee H-G, Handgretinger R. Inhibitory MHC Class I Receptors on γδ T Cells in Tumour Immunity and Autoimmunity. *Immunol Today* (2000) 21(4):187–91. doi: 10.1016/S0167-5699(99)01576-5
209. Mingari MC, Ponte M, Bertone S, Schiavetti F, Vitale C, Bellomo R, et al. HLA Class I-Specific Inhibitory Receptors in Human T Lymphocytes: Interleukin 15-Induced Expression of CD94/NKG2A in Superantigen-or Alloantigen-Activated CD8+ T Cells. *Proc Natl Acad Sci* (1998) 95(3):1172–7. doi: 10.1073/pnas.95.3.1172
210. Bertone S, Schiavetti F, Bellomo R, Vitale C, Ponte M, Moretta L, et al. Transforming Growth Factor-β-Induced Expression of CD94/NKG2A Inhibitory Receptors in Human T Lymphocytes. *Eur J Immunol* (1999) 29 (1):23–9. doi: 10.1002/(SICI)1521-4141(199901)29:01<23::AID-IMMU23>3.0.CO;2-Y
211. Born WK, Huang Y, Reinhardt RL, Huang H, Sun D, O'Brien RL. γδ T Cells and B Cells. *Adv Immunol* (2017) 134:1–45. doi: 10.1016/bs.ai.2017.01.002
212. Rampoldi F, Ullrich L, Prinz I. Revisiting the Interaction of γδ T-Cells and B-Cells. *Cells* (2020) 9(3):743. doi: 10.3390/cells9030743
213. Komatsu N, Jackson HM, Chan K-F, Oveissi S, Cebon J, Itoh K, et al. Fine-Mapping Naturally Occurring NY-ESO-1 Antibody Epitopes in Melanoma Patients' Sera Using Short Overlapping Peptides and Full-Length Recombinant Protein. *Mol Immunol* (2013) 54(3):465–71. doi: 10.1016/j.molimm.2013.01.014
214. King HW, Orban N, Riches JC, Clear AJ, Warnes G, Teichmann SA, et al. Single-Cell Analysis of Human B Cell Maturation Predicts How Antibody Class Switching Shapes Selection Dynamics. *Sci Immunol* (2021) 6(56): eabe6291. doi: 10.1126/sciimmunol.abe6291
215. Ehl S, Schwarz K, Enders A, Duffner U, Pannicke U, Kühr J, et al. A Variant of SCID With Specific Immune Responses and Predominance of γδ T Cells. *J Clin Invest* (2005) 115(11):3140–8. doi: 10.1172/JCI25221
216. Gil J, Busto EM, Garcillán B, Chean C, García-Rodríguez MC, Díaz-Alderete A, et al. A Leaky Mutation in CD3D Differentially Affects αβ and γδ T Cells and Leads to a Tαβ–Tγδ+ B+ NK+ Human SCID. *J Clin Invest* (2011) 121 (10):3872–6. doi: 10.1172/JCI44254
217. Rezende RM, Da Cunha AP, Kuhn C, Rubino S, M'Hamdi H, Gabriely G, et al. Identification and Characterization of Latency-Associated Peptide-Expressing γδ T Cells. *Nat Commun* (2015) 6(1):1–12. doi: 10.1038/ncomms9726
218. Rhodes DA, Reith W, Trowsdale J. Regulation of Immunity by Butyrophilins. *Annu Rev Immunol* (2016) 34:151–72. doi: 10.1146/annurev-immunol-041015-055435
219. Barros RDM, Roberts NA, Dart RJ, Vantourout P, Jandke A, Nussbaumer O, et al. Epithelia Use Butyrophilin-Like Molecules to Shape Organ-Specific γδ T Cell Compartments. *Cell* (2016) 167(1):203–18.e17. doi: 10.1016/j.cell.2016.08.030
220. Wright A, Lee JE, Link MP, Smith SD, Carroll W, Levy R, et al. Cytotoxic T Lymphocytes Specific for Self Tumor Immunoglobulin Express T Cell Receptor Delta Chain. *J Exp Med* (1989) 169(5):1557–64. doi: 10.1084/jem.169.5.1557
221. Fisch P, Malkovsky M, Kovats S, Sturm E, Braakman E, Klein BS, et al. Recognition by Human Vγ9/Vδ2 T Cells of a GroEL Homolog on Daudi Burkitt's Lymphoma Cells. *Science* (1990) 250(4985):1269–73. doi: 10.1126/science.1978758
222. Fisch P, Malkovsky M, Braakman E, Sturm E, Bolhuis R, Prieve A, et al. Gamma/delta T Cell Clones and Natural Killer Cell Clones Mediate Distinct Patterns of non-Major Histocompatibility Complex-Restricted Cytolysis. *J Exp Med* (1990) 171(5):1567–79. doi: 10.1084/jem.171.5.1567
223. Fisch P, Oettel K, Fudim N, Surfus J, Malkovsky M, Sondel P. MHC-Unrestricted Cytotoxic and Proliferative Responses of Two Distinct Human Gamma/Delta T Cell Subsets to Daudi Cells. *J Immunol* (1992) 148(8):2315–23.
224. Bukowski JF, Morita CT, Tanaka Y, Bloom BR, Brenner MB, Band H. V Gamma 2V Delta 2 TCR-Dependent Recognition of non-Peptide Antigens and Daudi Cells Analyzed by TCR Gene Transfer. *J Immunol* (1995) 154 (3):998–1006.
225. Fayen J, Tykocinski M. The Expansion of Human γδ T Cells in Response to Daudi Cells Requires the Participation of CD4+ T Cells. *Immunology* (1999) 97(2):272. doi: 10.1046/j.1365-2567.1999.00761.x
226. Vantourout P, Mookerjee-Basu J, Rolland C, Pont F, Martin H, Davrinche C, et al. Specific Requirements for Vγ9vδ2 T Cell Stimulation by a Natural Adenylated Phosphoantigen. *J Immunol* (2009) 183(6):3848–57. doi: 10.4049/jimmunol.0901085
227. Le Floch A-C, Imbert C, De Gassart A, Orlanducci F, Le Roy A, Gorvel L, et al. Targeting BTN2A1 By a Unique Activating Mab Improves Vγ9vδ2 T Cell Cytotoxicity Against Primary Acute Lymphoblastic Blasts. *Blood* (2021) 138:2302. doi: 10.1182/blood-2021-152577
228. Hebbeler AM, Cairo C, Cummings JS, Pauza CD. Individual Vγ2-Jγ1. 2+ T Cells Respond to Both Isopentenyl Pyrophosphate and Daudi Cell Stimulation: Generating Tumor Effectors With Low Molecular Weight Phosphoantigens. *Cancer Immunol Immunother* (2007) 56(6):819–29. doi: 10.1007/s00262-006-0235-6
229. Pellicci DG, Koay H-F, Berzins SP. Thymic Development of Unconventional T Cells: How NKT Cells, MAIT Cells and γδ T Cells Emerge. *Nat Rev Immunol* (2020) 20(12):756–70. doi: 10.1038/s41577-020-0345-y
230. Vermijlen D, Gatti D, Kouzeli A, Rus T, Eberl M. γδ T Cell Responses: How Many Ligands Will it Take Till We Know? *Semin Cell Dev Biol* (2018) 84:75–86. doi: 10.1016/j.semcdb.2017.10.009
231. Bald T, Krummel MF, Smyth MJ, Barry KC. The NK Cell–Cancer Cycle: Advances and New Challenges in NK Cell–Based Immunotherapies. *Nat Immunol* (2020) 21(8):835–47. doi: 10.1038/s41590-020-0728-z
232. Sivori S, Pende D, Quatrini L, Pietra G, Della Chiesa M, Vacca P, et al. NK Cells and ILCs in Tumor Immunotherapy. *Mol Aspects Med* (2021) 80:100870. doi: 10.1016/j.mam.2020.100870
233. Guillerey C. NK Cells in the Tumor Microenvironment. *Tumor Microenviron* (2020) 1273:69–90. doi: 10.1007/978-3-030-49270-0_4
234. Melaiu O, Lucarini V, Cifaldi L, Fruci D. Influence of the Tumor Microenvironment on NK Cell Function in Solid Tumors. *Front Immunol* (2020) 10:3038. doi: 10.3389/fimmu.2019.03038
235. Chan KF, Carolan LA, Druce J, Chappell K, Watterson D, Young P, et al. Pathogenesis, Humoral Immune Responses, and Transmission Between Cohoused Animals in a Ferret Model of Human Respiratory Syncytial Virus Infection. *J Virol* (2018) 92(4):e01322–17. doi: 10.1128/JVI.01322-17
236. Chan KF, Carolan LA, Korenkov D, Druce J, McCaw J, Reading PC, et al. Investigating Viral Interference Between Influenza A Virus and Human Respiratory Syncytial Virus in a Ferret Model of Infection. *J Infect Dis* (2018) 218(3):406–17. doi: 10.1093/infdis/jiy184
237. Björkström NK, Strunz B, Ljunggren H-G. Natural Killer Cells in Antiviral Immunity. *Nat Rev Immunol* (2021) 22(2):112–23. doi: 10.1038/s41577-021-00558-3
238. Cózar B, Greppi M, Carpentier S, Narni-Mancinelli E, Chiossone L, Vivier E. Tumor-Infiltrating Natural Killer Cells. *Cancer Discov* (2021) 11(1):34–44. doi: 10.1158/2159-8290.CD-20-0655
239. Wu S-Y, Fu T, Jiang Y-Z, Shao Z-M. Natural Killer Cells in Cancer Biology and Therapy. *Mol Cancer* (2020) 19(1):1–26. doi: 10.1186/s12943-020-01238-x
240. Liu H, Wang S, Xin J, Wang J, Yao C, Zhang Z. Role of NKG2D and its Ligands in Cancer Immunotherapy. *Am J Cancer Res* (2019) 9(10):2064.
241. Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, et al. MICA Engagement by Human Vγ2vδ2 T Cells Enhances Their Antigen-Dependent Effector Function. *Immunity* (2001) 15(1):83–93. doi: 10.1016/S1074-7613(01)00168-6
242. Champsaur M, Lanier LL. Effect of NKG2D Ligand Expression on Host Immune Responses. *Immunol Rev* (2010) 235(1):267–85. doi: 10.1111/j.0105-2896.2010.00893.x
243. Correia DV, Lopes AC, Silva-Santos B. Tumor Cell Recognition by γδ T Lymphocytes: T-Cell Receptor vs. NK-Cell Receptors. *Oncoimmunology* (2013) 2(1):e22892. doi: 10.4161/onci.22892

244. Raulet DH, Gasser S, Gowen BG, Deng W, Jung H. Regulation of Ligands for the NKG2D Activating Receptor. *Annu Rev Immunol* (2013) 31:413–41. doi: 10.1146/annurev-immunol-032712-095951
245. Silva-Santos B, Strid J. Working in "NK Mode": Natural Killer Group 2 Member D and Natural Cytotoxicity Receptors in Stress-Surveillance by γδ T Cells. *Front Immunol* (2018) 9:851. doi: 10.3389/fimmu.2018.00851
246. Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier J-F, Scotet E, et al. Vγ9vδ2 T Cell Response to Colon Carcinoma Cells. *J Immunol* (2005) 175(8):5481–8. doi: 10.4049/jimmunol.175.8.5481
247. Girlanda S, Fortis C, Belloni D, Ferrero E, Ticozzi P, Sciorati C, et al. MICA Expressed by Multiple Myeloma and Monoclonal Gammopathy of Undetermined Significance Plasma Cells Costimulates Pamidronate-Activated γδ Lymphocytes. *Cancer Res* (2005) 65(16):7502–8. doi: 10.1158/0008-5472.CAN-05-0731
248. Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. Activation of Vγ9vδ2 T Cells by NKG2D. *J Immunol* (2005) 175(4):2144–51. doi: 10.4049/jimmunol.175.4.2144
249. Wrobel P, Shojaei H, Schittek B, Gieseler F, Wollenberg B, Kalthoff H, et al. Lysis of a Broad Range of Epithelial Tumour Cells by Human γδ T Cells: Involvement of NKG2D Ligands and T-Cell Receptor-Versus NKG2D-Dependent Recognition. *Scand J Immunol* (2007) 66(2-3):320–8. doi: 10.1111/j.1365-3083.2007.01963.x
250. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D Ligand ULBP4 Binds to Tcrγ9/δ2 and Induces Cytotoxicity to Tumor Cells Through Both Tcrγδ and NKG2D. *Blood J Am Soc Hematol* (2009) 114(2):310–7. doi: 10.1182/blood-2008-12-196287
251. Lawand M, Déchanet-Merville J, Dieu-Nosjean M-C. Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications. *Front Immunol* (2017) 8:761. doi: 10.3389/fimmu.2017.00761
252. Gomes AQ, Correia DV, Grosso AR, Lança T, Ferreira C, Lacerda JF, et al. Identification of a Panel of Ten Cell Surface Protein Antigens Associated With Immunotargeting of Leukemias and Lymphomas by Peripheral Blood γδ T Cells. *Haematologica* (2010) 95(8):1397. doi: 10.3324/haematol.2009.020602
253. Lanca T, Correia DV, Moita CF, Raquel H, Neves-Costa A, Ferreira C, et al. The MHC Class Ib Protein ULBP1 is a Nonredundant Determinant of Leukemia/Lymphoma Susceptibility to γδ T-Cell Cytotoxicity. *Blood J Am Soc Hematol* (2010) 115(12):2407–11. doi: 10.1182/blood-2009-08-237123
254. Poggi A, Venturino C, Catellani S, Clavio M, Miglino M, Gobbi M, et al. Vδ1 T Lymphocytes From B-CLL Patients Recognize ULBP3 Expressed on Leukemic B Cells and Up-Regulated by Trans-Retinoic Acid. *Cancer Res* (2004) 64(24):9172–9. doi: 10.1158/0008-5472.CAN-04-2417
255. Kamei R, Yoshimura K, Yoshino S, Inoue M, Asao T, Fuse M, et al. Expression Levels of UL16 Binding Protein 1 and Natural Killer Group 2 Member D Affect Overall Survival in Patients With Gastric Cancer Following Gastrectomy. *Oncol Lett* (2018) 15(1):747–54. doi: 10.3892/ol.2017.7354
256. Chitadze G, Lettau M, Luecke S, Wang T, Janssen O, Fürst D, et al. NKG2D- And T-Cell Receptor-Dependent Lysis of Malignant Glioma Cell Lines by Human γδ T Cells: Modulation by Temozolomide and A Disintegrin and Metalloproteases 10 and 17 Inhibitors. *Oncoimmunology* (2016) 5(4):e1093276. doi: 10.1080/2162402X.2015.1093276
257. Niu C, Jin H, Li M, Zhu S, Zhou L, Jin F, et al. Low-Dose Bortezomib Increases the Expression of NKG2D and DNAM-1 Ligands and Enhances Induced NK and γδ T Cell-Mediated Lysis in Multiple Myeloma. *Oncotarget* (2017) 8(4):5954. doi: 10.18632/oncotarget.13979
258. Garner H, de Visser KE. Immune Crosstalk in Cancer Progression and Metastatic Spread: A Complex Conversation. *Nat Rev Immunol* (2020) 20(8):483–97. doi: 10.1038/s41577-019-0271-z
259. Nersesian S, Schwartz SL, Grantham SR, MacLean LK, Lee SN, Pugh-Toole M, et al. NK Cell Infiltration is Associated With Improved Overall Survival in Solid Cancers: A Systematic Review and Meta-Analysis. *Trans Oncol* (2021) 14(1):100930. doi: 10.1016/j.tranon.2020.100930
260. Son Y-I, Dallal RM, Mailliard RB, Egawa S, Jonak ZL, Lotze MT. Interleukin-18 (IL-18) Synergizes With IL-2 to Enhance Cytotoxicity, Interferon-γ Production, and Expansion of Natural Killer Cells. *Cancer Res* (2001) 61(3):884–8.
261. Caligiuri MA. Human Natural Killer Cells. *Blood J Am Soc Hematol* (2008) 112(3):461–9. doi: 10.1182/blood-2007-09-077438
262. Miyagawa F, Tanaka Y, Yamashita S, Minato N. Essential Requirement of Antigen Presentation by Monocyte Lineage Cells for the Activation of Primary Human γδ T Cells by Aminobisphosphonate Antigen. *J Immunol* (2001) 166(9):5508–14. doi: 10.4049/jimmunol.166.9.5508
263. Roelofs AJ, Jauhiainen M, Mönkkönen H, Rogers MJ, Mönkkönen J, Thompson K. Peripheral Blood Monocytes are Responsible for γδ T Cell Activation Induced by Zoledronic Acid Through Accumulation of IPP/DMAPP. *Br J Haematol* (2009) 144(2):245–50. doi: 10.1111/j.1365-2141.2008.07435.x
264. Constant P, Davodeau F, Peyrat M-A, Poquet Y, Puzo G, Bonneville M, et al. Stimulation of Human Gamma Delta T Cells by Nonpeptidic Mycobacterial Ligands. *Science* (1994) 264(5156):267–70. doi: 10.1126/science.8146660
265. Zhao X, Wei Y-Q, Kariya Y, Teshigawara K, Uchida A. Accumulation of γ/δ T Cells in Human Dysgerminoma and Seminoma: Roles in Autologous Tumor Killing and Granuloma Formation. *Immunol Investigat* (1995) 24(4):607–18. doi: 10.3109/08820139509066861
266. Katsuta M, Takigawa Y, Kimishima M, Inaoka M, Takahashi R, Shiohara T. NK Cells and γδ+ T Cells are Phenotypically and Functionally Defective Due to Preferential Apoptosis in Patients With Atopic Dermatitis. *J Immunol* (2006) 176(12):7736–44. doi: 10.4049/jimmunol.176.12.7736
267. Fowler DW, Copier J, Dalgleish AG, Bodman-Smith MD. Zoledronic Acid Causes γδ T Cells to Target Monocytes and Down-Modulate Inflammatory Homing. *Immunology* (2014) 143(4):539–49. doi: 10.1111/imm.12331
268. Welton JL, Morgan MP, Martí S, Stone MD, Moser B, Sewell AK, et al. Monocytes and γδ T Cells Control the Acute-Phase Response to Intravenous Zoledronate: Insights From a Phase IV Safety Trial. *J Bone Mineral Res* (2013) 28(3):464–71. doi: 10.1002/jbmr.1797
269. Ariasnegrete S, Keller K, Chadee K. Proinflammatory Cytokines Regulate Cyclooxygenase-2 mRNA Expression in Human Macrophages. *Biochem Biophys Res Commun* (1995) 208(2):582–9. doi: 10.1006/bbrc.1995.1378
270. Gonnermann D, Oberg H-H, Kellner C, Peipp M, Sebens S, Kabelitz D, et al. Resistance of Cyclooxygenase-2 Expressing Pancreatic Ductal Adenocarcinoma Cells Against γδ T Cell Cytotoxicity. *Oncoimmunology* (2015) 4(3):e988460. doi: 10.4161/2162402X.2014.988460
271. Tong D, Liu Q, Wang L, Xie Q, Pang J, Huang Y, et al. The Roles of the COX2/PGE2/EP Axis in Therapeutic Resistance. *Cancer Metastasis Rev* (2018) 37(2):355–68. doi: 10.1007/s10555-018-9752-y
272. Hashemi Goradel N, Najafi M, Salehi E, Farhood B, Mortezaee K. Cyclooxygenase-2 in Cancer: A Review. *J Cell Physiol* (2019) 234(5):5683–99. doi: 10.1002/jcp.27411
273. Daley D, Mani VR, Mohan N, Akkad N, Ochi A, Heindel DW, et al. Dectin 1 Activation on Macrophages by Galectin 9 Promotes Pancreatic Carcinoma and Peritumoral Immune Tolerance. *Nat Med* (2017) 23(5):556–67. doi: 10.1038/nm.4314
274. Seifert AM, Reiche C, Heiduk M, Tannert A, Meinecke A-C, Baier S, et al. Detection of Pancreatic Ductal Adenocarcinoma With Galectin-9 Serum Levels. *Oncogene* (2020) 39(15):3102–13. doi: 10.1038/s41388-020-1186-7
275. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vδ1+ γδ T Cells Control Early Infiltration of Neutrophils After Escherichia Coli Infection *via* IL-17 Production. *J Immunol* (2007) 178(7):4466–72. doi: 10.4049/jimmunol.178.7.4466
276. Nerdal PT, Peters C, Oberg H-H, Zlatev H, Lettau M, Quabius ES, et al. Butyrophilin 3a/CD277–dependent Activation of Human γδ T Cells: Accessory Cell Capacity of Distinct Leukocyte Populations. *J Immunol* (2016) 197(8):3059–68. doi: 10.4049/jimmunol.1600913
277. Uhlen M, Karlsson MJ, Zhong W, Tebani A, Pou C, Mikes J, et al. A Genome-Wide Transcriptomic Analysis of Protein-Coding Genes in Human Blood Cells. *Science* (2019) 366(6472):eaax9198. doi: 10.1126/science.aax9198
278. Monaco G, Lee B, Xu W, Mustafah S, Hwang YY, Carre C, et al. RNA-Seq Signatures Normalized by mRNA Abundance Allow Absolute Deconvolution of Human Immune Cell Types. *Cell Rep* (2019) 26(6):1627–40.e7. doi: 10.1016/j.celrep.2019.01.041
279. Oberg H-H, Wesch D, Kalyan S, Kabelitz D. Regulatory Interactions Between Neutrophils, Tumor Cells and T Cells. *Front Immunol* (2019) 10:1690. doi: 10.3389/fimmu.2019.01690

280. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β:"N1" Versus "N2" TAN. *Cancer Cell* (2009) 16(3):183–94. doi: 10.1016/j.ccr.2009.06.017
281. Valero C, Lee M, Hoen D, Weiss K, Kelly DW, Adusumilli PS, et al. Pretreatment Neutrophil-to-Lymphocyte Ratio and Mutational Burden as Biomarkers of Tumor Response to Immune Checkpoint Inhibitors. *Nat Commun* (2021) 12(1):1–9. doi: 10.1038/s41467-021-20935-9
282. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of Monocytes and Dendritic Cells in Blood. *Blood J Am Soc Hematol* (2010) 116(16):e74–80. doi: 10.1182/blood-2010-02-258558
283. Mildner A, Jung S. Development and Function of Dendritic Cell Subsets. *Immunity* (2014) 40(5):642–56. doi: 10.1016/j.immuni.2014.04.016
284. Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, et al. Reciprocal Activating Interaction Between Dendritic Cells and Pamidronate-Stimulated γδ T Cells: Role of CD86 and Inflammatory Cytokines. *J Immunol* (2005) 174(1):252–60. doi: 10.4049/jimmunol.174.1.252
285. Collins C, Wolfe J, Roessner K, Shi C, Sigal LH, Budd RC. Lyme Arthritis Synovial γδ T Cells Instruct Dendritic Cells *via* Fas Ligand. *J Immunol* (2005) 175(9):5656–65. doi: 10.4049/jimmunol.175.9.5656
286. Martino A, Casetti R, Poccia F. Enhancement of BCG-Induced Th1 Immune Response Through Vγ9vδ2 T Cell Activation With non-Peptidic Drugs. *Vaccine* (2007) 25(6):1023–9. doi: 10.1016/j.vaccine.2006.09.070
287. Devilder M-C, Allain S, Dousset C, Bonneville M, Scotet E. Early Triggering of Exclusive IFN-γ Responses of Human Vγ9vδ2 T Cells by TLR-Activated Myeloid and Plasmacytoid Dendritic Cells. *J Immunol* (2009) 183(6):3625–33. doi: 10.4049/jimmunol.0901571
288. Eberl M, Moser B. Monocytes and γδ T Cells: Close Encounters in Microbial Infection. *Trends Immunol* (2009) 30(12):562–8. doi: 10.1016/j.it.2009.09.001
289. Shrestha N, Ida JA, Lubinski AS, Pallin M, Kaplan G, Haslett PA. Regulation of Acquired Immunity by γδ T-Cell/Dendritic-Cell Interactions. *Ann New York Acad Sci* (2005) 1062(1):79–94. doi: 10.1196/annals.1358.011
290. Martino A, Casetti R, D'Alessandri A, Sacchi A, Poccia F. Complementary Function of γδ T-Lymphocytes and Dendritic Cells in the Response to Isopentenyl-Pyrophosphate and Lipopolysaccharide Antigens. *J Clin Immunol* (2005) 25(3):230–7. doi: 10.1007/s10875-005-4080-8
291. Kunzmann V, Kretzschmar E, Herrmann T, Wilhelm M. Polyinosinic-Polycytidylic Acid-Mediated Stimulation of Human γδ T Cells *via* CD11c+ Dendritic Cell-Derived Type I Interferons. *Immunology* (2004) 112(3):369–77. doi: 10.1111/j.1365-2567.2004.01908.x
292. von Lilienfeld-Toal M, Sievers E, Bodemüller V, Mihailescu C, Märten A, Gorschlüter M, et al. Coculture With Dendritic Cells Promotes Proliferation But Not Cytotoxic Activity of γ/δ T Cells. *Immunol Lett* (2005) 99(1):103–8. doi: 10.1016/j.imlet.2005.02.001
293. Gruenbacher G, Gander H, Rahm A, Nussbaumer W, Romani N, Thurnher M. CD56+ Human Blood Dendritic Cells Effectively Promote TH1-Type γδ T-Cell Responses. *Blood J Am Soc Hematol* (2009) 114(20):4422–31. doi: 10.1182/blood-2009-06-227256
294. Li W, Kubo S, Okuda A, Yamamoto H, Ueda H, Tanaka T, et al. Effect of IL-18 on Expansion of γδ T Cells Stimulated by Zoledronate and IL-2. *J Immunother* (2010) 33(3):287–96. doi: 10.1097/CJI.0b013e3181c80ffa
295. Li H, Pauza CD. Interplay of T-Cell Receptor and Interleukin-2 Signalling in Vγ2vδ2 T-Cell Cytotoxicity. *Immunology* (2011) 132(1):96–103. doi: 10.1111/j.1365-2567.2010.03343.x
296. Fowler DW, Copier J, Wilson N, Dalgleish AG, Bodman-Smith MD. Mycobacteria Activate γδ T-Cell Anti-Tumour Responses *via* Cytokines From Type 1 Myeloid Dendritic Cells: A Mechanism of Action for Cancer Immunotherapy. *Cancer Immunol Immunother* (2012) 61(4):535–47. doi: 10.1007/s00262-011-1121-4
297. Cui Y, Kang L, Cui L, He W. Human γδ T Cell Recognition of Lipid A is Predominately Presented by CD1b or CD1c on Dendritic Cells. *Biol Direct* (2009) 4(1):1–12. doi: 10.1186/1745-6150-4-47
298. Van Acker HH, Anguille S, De Reu H, Berneman ZN, Smits EL, Van Tendeloo VF. Interleukin-15-Cultured Dendritic Cells Enhance Anti-Tumor Gamma Delta T Cell Functions Through IL-15 Secretion. *Front Immunol* (2018) 658. doi: 10.3389/fimmu.2018.00658
299. Fiore F, Castella B, Nuschak B, Bertieri R, Mariani S, Bruno B, et al. Enhanced Ability of Dendritic Cells to Stimulate Innate and Adaptive Immunity on Short-Term Incubation With Zoledronic Acid. *Blood J Am Soc Hematol* (2007) 110(3):921–7. doi: 10.1182/blood-2006-09-044321
300. Takahara M, Miyai M, Tomiyama M, Mutou M, Nicol AJ, Nieda M. Copulsing Tumor Antigen-Pulsed Dendritic Cells With Zoledronate Efficiently Enhance the Expansion of Tumor Antigen-Specific CD8+ T Cells via Vgamma9gammadelta T Cell Activation. *J Leukoc Biol* (2008) 83 (3):742–54. doi: 10.1189/jlb.0307185
301. Su X, Zhang L, Jin L, Ye J, Guan Z, Chen R. Coculturing Dendritic Cells With Zoledronate Acid Efficiently Enhance the Anti-Tumor Effects of Cytokine-Induced Killer Cells. *J Clin Immunol* (2010) 30(5):766–74. doi: 10.1007/s10875-010-9434-1
302. Cabillic F, Toutirais O, Lavoué V, de la Pintiere CT, Daniel P, Rioux-Leclerc N, et al. Aminobisphosphonate-Pretreated Dendritic Cells Trigger Successful Vγ9vδ2 T Cell Amplification for Immunotherapy in Advanced Cancer Patients. *Cancer Immunol Immunother* (2010) 59(11):1611–9. doi: 10.1007/s00262-010-0887-0
303. Castella B, Riganti C, Fiore F, Pantaleoni F, Canepari ME, Peola S, et al. Immune Modulation by Zoledronic Acid in Human Myeloma: An Advantageous Cross-Talk Between Vγ9vδ2 T Cells, αβ CD8+ T Cells, Regulatory T Cells, and Dendritic Cells. *J Immunol* (2011) 187(4):1578–90. doi: 10.4049/jimmunol.1002514
304. Scotet E, Nedellec S, Devilder M-C, Allain S, Bonneville M. Bridging Innate and Adaptive Immunity Through Gammadelta T-Dendritic Cell Crosstalk. *Front Biosci* (2008) 13(5):6872–85. doi: 10.2741/3195
305. Galati D, Zanotta S, Bocchino M, De Filippi R, Pinto A. The Subtle Interplay Between Gamma Delta T Lymphocytes and Dendritic Cells: Is There a Role for a Therapeutic Cancer Vaccine in the Era of Combinatorial Strategies? *Cancer Immunol Immunother* (2021) 70(7):1797–809. doi: 10.1007/s00262-020-02805-3
306. Moser B, Eberl M. γδ T-APCs: A Novel Tool for Immunotherapy? *Cell Mol Life Sci* (2011) 68(14):2443–52. doi: 10.1007/s00018-011-0706-6
307. Van Acker HH, Anguille S, Van Tendeloo VF, Lion E. Empowering Gamma Delta T Cells With Antitumor Immunity by Dendritic Cell-Based Immunotherapy. *Oncoimmunology* (2015) 4(8):e1021538. doi: 10.1080/2162402X.2015.1021538
308. Boltjes A, Van Wijk F. Human Dendritic Cell Functional Specialization in Steady-State and Inflammation. *Front Immunol* (2014) 5:131. doi: 10.3389/fimmu.2014.00131
309. Sugie T, Murata-Hirai K, Iwasaki M, Morita CT, Li W, Okamura H, et al. Zoledronic Acid-Induced Expansion of γδ T Cells From Early-Stage Breast Cancer Patients: Effect of IL-18 on Helper NK Cells. *Cancer Immunol Immunother* (2013) 62(4):677–87. doi: 10.1007/s00262-012-1368-4
310. Fournié J-J, Sicard H, Poupot M, Bezombes C, Blanc A, Romagné F, et al. What Lessons can be Learned From γδ T Cell-Based Cancer Immunotherapy Trials? *Cell Mol Immunol* (2013) 10(1):35–41. doi: 10.1038/cmi.2012.39
311. Deniger DC, Moyes JS, Cooper LJ. Clinical Applications of Gamma Delta T Cells With Multivalent Immunity. *Front Immunol* (2014) 5:636. doi: 10.3389/fimmu.2014.00636
312. Barros MS, de Araújo ND, Magalhães-Gama F, Pereira Ribeiro TL, Alves Hanna FS, Tarragó AM, et al. γδ T Cells for Leukemia Immunotherapy: New and Expanding Trends. *Front Immunol* (2021) 3770. doi: 10.3389/fimmu.2021.729085
313. Le Floch A-C, Orlanducci F, Briantais A, Le Roy A, Chretien A-S, Vey N, et al. Anti-BTN3A 20.1 Agonist Monoclonal Antibody Enhances Autologous Vγ9vδ2 T Cells Cytotoxicity Against Primary Acute Myeloid Blasts. *Blood* (2019) 134:5153. doi: 10.1182/blood-2019-125541
314. Herrmann T, Fichtner AS, Karunakaran MM. An Update on the Molecular Basis of Phosphoantigen Recognition by Vγ9vδ2 T Cells. *Cells* (2020) 9 (6):1433. doi: 10.3390/cells9061433
315. Pei Y, Wen K, Xiang Z, Huang C, Wang X, Mu X, et al. CD137 Costimulation Enhances the Antiviral Activity of Vγ9vδ2-T Cells Against Influenza Virus. *Signal Transduct Targeted Ther* (2020) 5(1):1–10. doi: 10.1038/s41392-020-0174-2

Conflict of Interest: AB declares research funding from CSL Ltd. AB and SO are inventors on a patent about mechanisms to activate γδ T cells.

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership