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INVITED REVIEW

The distinct MHC-unrestricted immunobiology of innate-like and adaptive-like human γδ T cell subsets—Nature's CAR-T cells

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Abstract

Distinct innate-like and adaptive-like immunobiological paradigms are emerging for human γδ T cells, supported by a combination of immunophenotypic, T cell receptor (TCR) repertoire, functional, and transcriptomic data. Evidence of the γδ TCR/ligand recognition modalities that respective human subsets utilize is accumulating. Although many questions remain unanswered, one superantigen-like modality features interactions of germline-encoded regions of particular TCR Vγ regions with specific BTN/BTNL family members and apparently aligns with an innate-like biology, albeit with some scope for clonal amplification. A second involves CDR3-mediated γδ TCR interaction with diverse ligands and aligns with an adaptive-like biology. Importantly, these unconventional modalities provide γδ T cells with unique recognition capabilities relative to αβ T cells, B cells, and NK cells, allowing immunosurveillance for signatures of "altered self" on target cells, via a membrane-linked γδ TCR recognizing intact non-MHC proteins on the opposing cell surface. In doing so, they permit cellular responses in diverse situations including where MHC expression is compromised, or where conventional adaptive and/or NK cell-mediated immunity is suppressed. γδ T cells may therefore utilize their TCR like a cell-surface Fab repertoire, somewhat analogous to engineered chimeric antigen receptor T cells, but additionally integrating TCR signaling with parallel signals from other surface immunoreceptors, making them multimolecular sensors of cellular stress.

KEYWORDS
adaptive-like, butyrophilin, innate-like, ligand, T cell receptor (TCR), tissue-associated, γδ T cells

1 | INTRODUCTION

Lymphocytes of the vertebrate adaptive immune system exploit somatic recombination to generate diverse antigen receptor repertoires capable of recognizing the broad range of pathogens encountered during life. These lymphocytes, namely B cells, αβ T cells, and γδ T cells, have coevolved over ~500 million years of vertebrate evolution. Following the seminal discovery of Major Histocompatibility Complex (MHC)-restricted antigen recognition,[1,2] research on T cells has focussed predominantly on the αβ compartment. However, the subsequent ground-breaking studies that confirmed the existence of an αβ T cell antigen receptor (TCR) also unexpectedly revealed a separate T cell lineage, γδ T lymphocytes.[3,4] These cells are defined by expression of a distinct somatically recombined γδ TCR, and unlike αβ T cells, are not thought to be MHC-restricted. However, beyond this central tenet, the functions

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and antigen recognition requirements of the γδ T cell compartment have remained substantially unclear.

γδ T cells are proposed to perform diverse functions in immunity, including critical contributions to protection against bacteria and viruses, immunosurveillance against tumors, and immunoregulation and maintenance of epithelial surfaces.[5] However, a lack of understanding of both their fundamental immunobiology and the antigens they recognize through their γδ TCR has hampered our understanding of how γδ T cells perform these functions. Advances in a diverse range of techniques are now catalyzing discoveries in both of these areas. Such efforts should clarify our understanding of the niche γδ T cells occupy in the immune system and potentially explain why they have been retained throughout vertebrate evolution. A granular understanding of functional heterogeneity of the γδ T cell compartment, and increased knowledge of both γδ TCR ligands and mechanisms of antigen recognition, is also likely to be important in facilitating therapeutic exploitation of γδ T cells, a focus of increasing interest.

In this review, we will summarize some of our own and others' recent research findings in this context, which have shed light on fundamental immunobiological paradigms and novel γδ TCR ligands. Our focus will largely be on human γδ T cells but we include reference to work on other species where relevant. The picture that emerges is one of a compartment comprising functionally distinct subsets, collectively providing immunosurveillance capabilities that are distinct from αβ T cells, B cells, and Natural Killer (NK) cells, mediated by the γδ TCR, enabling fragment antigen binding (Fab)-like recognition of intact, non-MHC antigens on the surface of target cells.

2 | PARADIGMS

In recent years, there has been a drive to understand the paradigms that best apply to human γδ T cells, and consequently their niche in the immune system relative to other lymphocyte compartments. A major focus of discussion has been whether γδ T cells align best with innate or adaptive biology. Although γδ T cells have often been viewed or analyzed *en masse* as a homogenous unconventional lymphocyte compartment, and typically referred to as either "innate-like T cells" or "at the interface between innate and adaptive immunity," recent studies clearly demonstrating considerable intra-compartmental functional and phenotypic heterogeneity[6-10] reveal this assessment is out of date. In fact, multiple immunobiological paradigms may likely be required to describe the compartment, such is this heterogeneity, particularly when different γδ T cell subsets but also different species are taken into account. This would mirror the αβ T cell compartment, which contains both innate-like[11] and conventional adaptive subsets.

The recurring question of innate versus adaptive immunobiology remains highly relevant to paradigms invoked for human γδ T cell subsets. However, since these terms are used in different senses by different researchers, it is important to define our own usage of these terms here. We employ "adaptive-like" and "innate-like" to refer to a collection of features. Hence, adaptive-like immunobiology typically involves (a) diversity within an initial immune receptor repertoire, which provides potential for subsequent antigen-driven clonotypic focussing; notably αβ T cell and B cell antigen receptor diversity enables a diversity of targets to be recognized; (b) lymphocytes adopt an initial naïve phenotypic status prior to a given challenge, but transition upon challenge to effector status, and have the potential to impart altered homing capability; (c) this transition to effector status can occur throughout life, whenever antigen is encountered; and (d) adaptive differentiation can result in long-lived responses and is likely to generate adaptive immune protection. In contrast, innate-like unconventional T cell immunobiology can involve (a) transition to effector phenotype in early life, (b) expression of semi-invariant TCR repertoires, frequently featuring conserved TCR chain usages, (c) recognition of non-polymorphic ligands, and (d) can be activated independently of TCR triggering, that is by cytokines and/or NK receptors.

Although such features may not all apply to a given unconventional T cell subset, as outlined below, specific γδ T cell subsets do appear substantially polarized toward either innate-like or adaptive-like biology. Of note, the ability to delineate such distinct immunobiologies is ideally addressed by a multimodal approach, enabling parallel characterization of immunophenotype, TCR repertoire, cellular function, and potentially transcriptional profile. New advances in single-cell methodologies mean it is possible to assess some such features in parallel. Therefore, the distinction between innate-like and adaptive-like paradigms remains an important and useful one, is increasingly feasible to assess, and provides a useful framework through which to investigate the contribution of such functionally distinct subsets to host defense, and ultimately to consider therapeutic avenues.

2.1 | Fundamental immunobiology of the human peripheral γδ T cell compartment

2.1.1 | Innate-like Vγ9Vδ2 T cells

Vγ9Vδ2 T cells, which are the dominant γδ T cell population in human peripheral blood, currently represent the strongest candidate for an innate-like human γδ T cell subset. Although rare and phenotypically naïve (CD27+CD45RA+) in cord blood, they expand in number and transition to a CD45RO+ phenotype in early childhood,[6,12] upregulating expression of granzymes and perforin[6] to become fully mature effector cells relatively early in life,[13] most probably driven by microbial exposure.[14,15] Crucially, mature effector Vγ9Vδ2 T cells universally respond in a TCR-dependent fashion to host and pathogen-derived phosphoantigens (pAgs), which include the highly potent microbially derived compound (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP).[16] An intermediate in the non-mevalonate pathway of isoprenoid synthesis absent in mammals,[16] HMBPP is an essential metabolite for many pathogenic bacteria and could be arguably regarded as a pathogen-associated molecular pattern (PAMP). Recognition of pAg-exposed

target cells can result in potent cytokine secretion and cytotoxicity. The Vγ9Vδ2 T cell repertoire in the peripheral blood of most adults consists of semi-invariant Vγ9 chains with relatively minimal non-templated (N)/pallindromic (P) region nucleotide addition, paired with relatively diverse Vδ2 chains.[6,7] In some cases, the same Vγ9 rearrangement is detected paired to multiple Vδ2 sequences, suggesting several common Vγ9 chain sequences can be recombined multiple times independently.[6,15] The fact that Vγ9Vδ2 T cells respond universally to pAgs and that both Vγ9 and Vδ2 chains are essential for this TCR-dependent reactivity, strongly suggests recognition of non-polymorphic ligands, one of which has recently been established as BTN2A1.[17,18] Along with their semi-invariant TCR repertoire, Vγ9Vδ2 T cells exhibit transcriptional similarities to invariant Natural Killer T cells (iNKT) and mucosal associated invariant T cells (MAIT) cells, such as expression of the transcription factor PLZF (encoded by the *ZBTB16* gene),[10,19] a hallmark of innate-like lymphocytes.[20] Notably, Vγ9Vδ2 T cells can also be activated in a TCR-independent manner by IL-12/IL-18 stimulation,[6] another hallmark of innate-like lymphocytes; notably, this ability is not shared by human adaptive-like γδ T cells.[6,7]

Although the features outlined above illustrate that Vγ9Vδ2 T cells adopt a broadly innate-like biology featuring Vγ9-JγP rearrangements and hydrophobic CDR3δ residues that are essential for pAg recognition, it is clear that their semi-invariant TCR repertoire allows the potential for clonal expansions and phenotypic differences in some individuals.[6] These are currently poorly understood but conceivably may relate to differences in CDR3-mediated antigen discrimination (see below) and are worthy of further investigation. Such clonal expansions may relate to phenotypic heterogeneity observed[10] in Vγ9Vδ2 T cells across donors and are also consistent with the findings that both CDR3γ and the highly diverse CDR3δ region of the Vγ9Vδ2 TCR are critical for pAg recognition. Moreover, phenotypic and clonotypic changes are likely to relate to either the current or past infection state, and consistent with this, Vγ9Vδ2 T cells have been shown to exhibit long-lived expansions in response to several pathogens.[16] These observations emphasize that the immunobiology of Vγ9Vδ2 T cells is best described as "innate-*like*" rather than "innate" and may in reality reflect a blend of a limited number of characteristics more typically associated with adaptive immunity, with numerous innate features. Irrespective of which terminology is applied to this subset, it is clear the immunobiology of the Vγ9Vδ2 T cell subset is fundamentally different to those of "adaptive-like" subsets outlined below.

2.1.2 | Adaptive-like subsets

Vδ2[neg] γδ T cells represent a minority (normally around 20%) of γδ T cells in peripheral blood, but are enriched relative to Vγ9Vδ2 T cells in solid tissues. In both settings, Vδ1^{+} T cells typically comprise the dominant proportion. Previous studies have highlighted the involvement of the Vδ2[neg] subset in response to several viral infections, including cytomegalovirus (CMV),[21] human immunodeficiency virus (HIV),[22] hepatitis C virus (HCV),[23] and Epstein-Barr virus (EBV).[24,25]

In the context of CMV, studies have highlighted CMV-driven expansions,[26,27] both in immunocompromised settings and healthy individuals.[28] In immunosuppressed patients, Vδ2[neg] T cell expansions were concomitant with the start of resolution of infection,[29] and ex vivo analyses revealed Vδ2[neg] cytokine and cytotoxicity in response to CMV-infected target cells, and suppression of viral infectivity in vitro.[27] Despite this extensive body of work, until recently the fundamental immunobiology of Vδ2^{neg} T cells remained substantially unclear. However, compelling evidence now suggests that human Vδ2^{neg} T cells, at least in peripheral blood, display an adaptive-like immunobiology.[30]

Recent studies on Vδ2^{neg} T cells have shed considerable light on both their TCR repertoire, phenotype, and how these link with cellular function. Based on analyses of cord blood γδ T cells, studies have shown that at the start of life Vδ2[neg] TCR repertoire is initially highly diverse and features numerous Vγ chains, unlike pAg responsive Vδ2 T cells, which uniformly express Vγ9 chains with limited sequence diversity.[6,7] There is extremely high diversity within Vδ2[neg] TCR CDR3 sequences, in both TCRγ and TCRδ chains, owing to a high level of exonuclease activity, a large number of N/P nucleotide additions, and for the Vδ chain, multiple Dδ-segment usage.[7] The resultant Vδ2[neg] TCR repertoire incorporates Vγ and Vδ CDR3 loops that are highly diverse in length and sequence, but is also overwhelmingly private,[7] in stark contrast to the semi-invariant repertoire of Vγ9Vδ2 T cells, which has restricted CDR3 lengths and incorporates a large proportion of public Vγ9 clonotypes.[31]

Importantly, by adulthood, the peripheral blood Vδ2[neg] TCR repertoire frequently contains dominant clonal expansions. While these can occur in response to CMV infection following SCT,[32] they are also detected in healthy CMV-seropositive and CMV-seronegative individuals,[7] suggesting multiple immune stimuli, most probably other infectious challenges, can induce clonal expansion within the compartment. While the full range of infections that can drive such clonal expansions is unclear, available data suggest these may also include viral infections such as EBV,[24,25] as well as parasitic infections such as *P. falciparum*.[33] Importantly, Vermijlen and colleagues have shown fetal responses to congenital CMV infection *in utero* were also heavily clonotypically focussed.[34] However, substantial differences have emerged in the underlying mechanisms in the fetal setting, as the dominant Vγ8Vδ1 TCRs exploited near-germline-encoded CDR3 regions, in contrast to postnatal responses. Subsequent work has suggested this highly TCR focussed prenatal response reflects low fetal thymic expression of terminal deoxynucleotidyl transferase (TdT), resulting in a low diversity TCR repertoire, and unlike postnatal Vδ1 responses involved intrathymic acquisition of effector function.[35]

Assessment of Vδ2[neg] subset phenotype also provides evidence of adaptive features. The dominant Vδ1 pool displays a naïve phenotype in cord blood alongside its unfocused TCR repertoire.[7] By adulthood, there are clearly distinct T$_{naive}$ and T$_{effector}$ states evident in the Vδ1 compartment, with considerable inter-individual variation in the relative dominance of each.[7] This contrasts with the Vγ9Vδ2

T cell compartment, which is effector like from an early age.[13] Phenotypically $V\delta1$ T_{naive} and $T_{effector}$ states appear to align relatively closely with CD8 T_{naive} and T_{emra} subtypes, respectively.[7,30] T_{naive} cells display high levels of CD27/CD28, relatively high levels of central homing markers (CD62L, CCR7), and low levels of effector markers such as granzymes and perforin. Conversely, $V\delta1$ $T_{effector}$ cells, like CD8 T_{emra} cells, exhibit decreased CD27/CD28 staining, a shift from central to peripheral homing marker expression (decreased CD62L/CCR7, increased CX_3CR1), and clear upregulation of granzymes and perforin. Crucially, $V\delta1$ T_{naive} and $T_{effector}$ status aligns closely with focussing of the $V\delta1$ TCR repertoire, with $V\delta1$ T_{naive} cells invariably consisting of diverse clonotypes, whereas clonally amplified $V\delta1$ TCR sequences reside exclusively within the $T_{effector}$ pool.[7,30] Finally, unlike $V\gamma9V\delta2$ T cells, $V\delta1$ T cells exhibited minimal changes in activation markers following IL-12/IL-18 exposure in the absence of CD3 agonist antibody, suggesting $V\delta1$ TCR stimulation may more stringently govern their activation.

Recently, human $V\gamma9^{neg}V\delta2$ T cells have emerged as an additional adaptive-like subset.[6,31] Unable to respond to pAg, $V\gamma9^{neg}V\delta2$ T cells are generally low in frequency in peripheral blood and usually adopt a T_{naive} phenotype closely aligned with $V\delta1$ T_{naive} populations (high expression of CD27/CD28/CD62L/CCR7/IL7Rα), alongside an unfocussed TCR repertoire that incorporates a diverse range of $V\gamma$ chains. Moreover, when in rare individuals they become expanded, Davey et al[6] noted a phenotype closely aligned with $V\delta1$ $T_{effector}$ cells,[7] including analogous changes in homing receptor expression and upregulation of cytotoxic markers, aligned to a TCR repertoire denoting highly focussed clonal expansion. As for $V\delta1$ T cells, IL-12/IL-18 exposure resulted in only minimal activation marker expression in the absence of CD3 stimulation, and notably $T_{effector}$ populations retained sensitivity to CD3 stimulation. Collectively, these observations suggest that both peripheral blood $V\delta1$ and $V\gamma9^{neg}V\delta2$ T cells differ radically from $V\gamma9V\delta2$ T cells, and surprisingly, their biology aligns substantially with the conventional adaptive CD8 T cell compartment.

Another hallmark of adaptive T cell immunity is that the transition from naïve to effector status, which is typically associated with clonal expansion, occurs in response to antigen challenge, such as pathogen infection. There is now clear evidence that viral infection can drive such changes in both the peripheral blood $V\delta1$ and $V\gamma9^{neg}V\delta2$ T cell subsets. Building on previous data reporting increases in the proportion of effector memory-like $V\delta2^{neg}$ cells following CMV infection both in healthy individuals and following renal transplantation,[28] Ravens et al demonstrated that CMV drives clonal expansion within the $V\delta1$ T cell subset.[32] Moreover, Davey et al highlighted parallel clonal expansion and phenotypic transition from T_{naive} to $T_{effector}$ status that occurred selectively in $V\gamma9^{neg}V\delta2$ T cells[6] following solid organ transplant patients that had undergone acute CMV infection. Entirely consistent with this, Kaminski et al subsequently showed that $V\gamma9^{neg}V\delta2$ T cells can respond to CMV-infected cells, and longitudinal monitoring of their phenotype during CMV infection confirmed transition to a differentiated effector phenotype.[36] In addition, available evidence suggests that expanded clonotypes present within the $T_{effector}$ pool are relatively long-lived and can persist for years.[6,7,32]

The considerations above indicate remarkable parallels between adaptive-like human $\gamma\delta$ T cell populations and CD8 T cell biology, and are suggestive of an analogous but distinct branch of unconventional adaptive immunobiology. Clearly, the transition from T_{naive} to $T_{effector}$ status in such subsets is not inevitable, but likely dependent upon environmental exposure to specific challenges including infection, as is well established for conventional adaptive $\alpha\beta$ and B cell compartments. Further studies are required to explore the range of infectious and non-microbial stimuli that might drive such responses. Of strong current relevance, a recent study of COVID-19 patients determined higher levels of $CD27^{neg}$ $V\delta1^+$ T cells in peripheral blood, suggestive of an adaptive $\gamma\delta$ T cell response to SARS-CoV2.[37] Based on homing marker expression, it is highly likely that T_{naive} cells can recirculate between blood and secondary lymphoid organs, whereas $T_{effector}$ populations are likely equipped to migrate into tissues[30] (see below). Also, the long-term persistence of expanded adaptive-like $\gamma\delta$ TCR clonotypes, combined with retention of sensitivity to CD3 stimulation in peripheral blood, strong upregulation of cytotoxicity marker expression, and cytokine production capability, suggests the probability that $T_{effector}$ populations make long-term contributions to antigen-specific immunosurveillance against recurrently encountered or chronically persistent pathogens.[21,30,31] While in general the alignment between such subsets and mouse $\gamma\delta$ T cell subsets is unclear, of note, CMV challenge in mice led to expansion of $\gamma\delta$ T cells that populated a range of peripheral tissues and protected from disease pathology.[38,39] Although the processes governing adaptive differentiation from T_{naive} to $T_{effector}$ status in $\gamma\delta$ T cells are poorly understood, the strong link between clonal expansion and transition to $T_{effector}$ state suggests that like CD8 T cells, TCR triggering most likely plays a key role. As outlined below, this has implications for $\gamma\delta$ TCR ligand identification approaches. Additionally, the transcriptional pathways that regulate this unconventional adaptive differentiation process are poorly understood and must be addressed by future studies.

In summary, recent studies on human peripheral blood $\gamma\delta$ T cells have indicated remarkable parallels between $V\delta1^+$ and $V\gamma9^{neg}V\delta2$ subsets and conventional adaptive CD8 T cell populations. Important data are now emerging that suggest how human tissue-associated $\gamma\delta$ T cells may integrate with such populations.

2.2 | Tissue-associated human $\gamma\delta$ T cells

2.2.1 | Toward a holistic understanding of the human $\gamma\delta$ T cell compartment

While $V\gamma9V\delta2$ T cells are the predominant $\gamma\delta$ T cell subset in peripheral blood, in contrast $V\delta2^{neg}$ T cells, and especially $V\delta1^+$ T cells, are dominant in solid tissues.[40] However, our

understanding of such human tissue-associated γδ T cells lags far behind that of peripheral blood populations. Also, our knowledge of the interconnection between (and reciprocally, the divergence of) γδ T cell functionality and responses in blood *versus* tissues is currently poor. Nevertheless, a number of studies have made important advances for several human solid tissues. One of the first tissue-associated γδ T cell subsets to be heavily studied were dendritic epidermal T cells (DETC), a murine γδ subset localized to epidermis.[41] Although absent in humans, they are important to consider briefly as a prototypic tissue-associated γδ T cell subset.

DETC are the first γδ T cells to develop in the mouse thymus during embryonic development, and subsequently home to epidermis.[41] DETC appear highly specialized for their local microenvironmental niche, and as their name suggests adopt a dendritic shape, allowing their many processes to contact nearby keratinocytes, thereby enabling immunosurveillance of the epithelium for signs of damage or infection[41] (Figure 1A). DETC are not only self-renewing, but are also potent effectors, which express cytokines and cytotoxic markers. These effector functions can be elicited by TCR-dependent triggering, but also by TCR-independent signals, the latter including ligation of NKG2D on their surface by target cell-expressed NKG2D ligands (NKG2DL).[42,43] They can kill infected and tumor cells, yet can also produce growth factors such as KGF and IGF-1[44,45] and other molecules to support epithelial maintenance and wound healing. A striking feature of DETC is their universal expression of an invariant Vγ5Vδ1 TCR[46] with no junctional diversity, which is likely to be critical for thymic selection, regulating skin homing, and cutaneous immunosurveillance. These functions are dependent on the B7-like molecule Skint1.[47] In summary, multiple features of DETC suggest they represent a canonical innate-like tissue-associated subset, against which γδ T cells in different sites, as discussed below, can be compared.

2.2.2 | Hepatic γδ T cells

A recent study focussed on the phenotype, TCR repertoire and function of human hepatic γδ T cells (Figure 1B), which were dominated by Vδ2^{neg} T cells, in relation to comparable peripheral blood subsets.[9] Although it was perhaps not surprising that there were considerable similarities between the two compartments given the strong link between the liver and the peripheral circulation, there were nevertheless some telling distinctions. Liver γδ T cells were dominated by Vδ1[+] and Vδ3[+] lymphocytes paired with diverse Vγ chains, and as for peripheral blood Vδ2^{neg} T cells the associated TCR repertoire was highly private, with evidence for extensive exonuclease activity, N/P nucleotide addition, and use of multiple Dδ segments.[9] However, unlike peripheral blood (PB) Vδ2^{neg} T cells, hepatic Vδ2[neg] subsets were invariably highly clonally expanded (irrespective of whether the liver was normal or diseased), with dominance of a relatively small number of prevalent clonotypes.[9] Moreover, CD27^{hi} T$_{naive}$ Vδ2^{neg} cells were largely absent in the liver, with the majority of Vδ2^{neg} cells adopting a CD45RA^{hi}/CD27[lo/neg] phenotype that matches that of peripheral blood Vδ2^{neg} T$_{effector}$ cells.[9] These observations suggest that such populations are probably adaptive-like T$_{effector}$ cells that were originally centrally primed and expanded from antigen-inexperienced T$_{naive}$ cells, with changes in homing marker expression following adaptive differentiation probably critical in enabling hepatic homing.[9] Strongly supporting this, not only did such CD45RA^{hi}/CD27[lo/neg] T cells express CX$_3$CR1 as well as granzymes and perforin, but intra-individual single cell TCR analyses indicated substantial overlap in expanded TCR clonotypes present in matched liver/blood samples.[9]
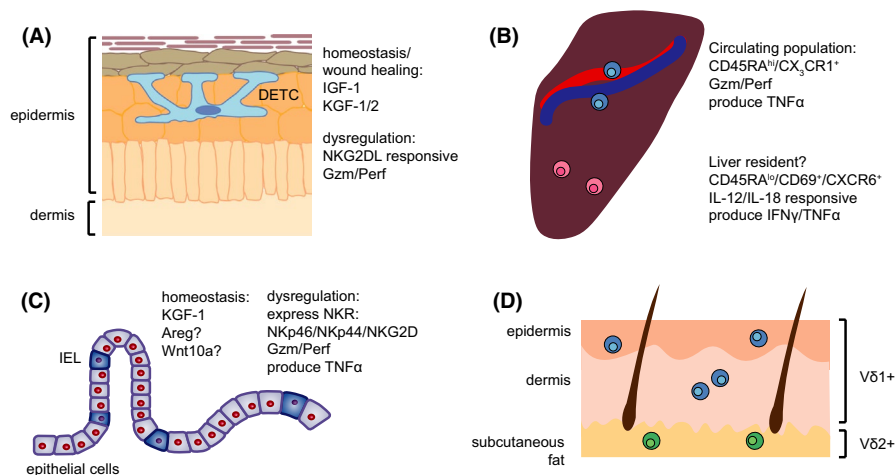
FIGURE 1 Tissue-associated γδ T cell subsets and their described functional capabilities in health and disease. A, Dendritic epidermal T cells (DETC) produce IGF-1 and KGF, thought to function in epithelial maintenance and in wound healing, but can also respond to increased expression of NKG2DL on keratinocytes. B, Two populations of Vδ2^{neg} γδ T cells can be found in the liver: a CD45RA^{+} circulating population similar to peripheral blood Vδ2^{neg} cells, and a CD45RA^{lo} potentially liver resident population. C, Vγ4Vδ1^{+} IELs in human may contribute to epithelial maintenance, while cytotoxicity and NKR-responsivity may be important in infection or tumorigenesis. D, Proposed γδ subsets in human skin compartments may express distinct TCR chains based on studies of normal γδ T cells and γδ cutaneous T cell lymphoma cells from dermis/epidermis and subcutaneous fat layers.

A second study also highlighted the presence of Vγ9^{neg}Vδ2 T cells in the liver,[6] which like hepatic Vδ1$^+$ T cells also incorporate a diverse range of Vγ chains. In stark contrast to the comparable Vγ9^{neg}Vδ2 subset in the blood of most healthy individuals, hepatic Vγ9^{neg}Vδ2 lymphocytes adopted a T$_{effector}$-like phenotype[6] that resembles both the majority of hepatic Vδ1$^+$ T cells,[7] and T$_{effector}$ Vγ9^{neg}Vδ2 populations that arise after viral infection.[6]

In addition to the populations outlined above, Hunter et al presented evidence for a clonotypically distinct, tissue-resident CD45RA^{lo}, CD69$^+$, CXCR6$^+$ Vδ1$^+$ hepatic subset,[9] which in contrast to CD45RA^{hi} cells could be activated by IL-12/18 alone. Interestingly, upon stimulation, this CD45RA^{lo} population produced IFNγ and TNFα but not Granzymes, whereas the CD45RA^{hi} population expressed cytotoxic markers and TNFα, but did not produce IFNγ.[9]

Collectively, these studies support a strong interconnection between adaptive-like γδ T cell biology in blood and liver, a picture quite different from the distinct, tissue-restricted biology of the DETC subset. They suggest that following appropriate stimulation, most likely including infectious challenge, circulating adaptive-like γδ T cells can give rise to T$_{effector}$ populations that home to or traffic through at least some peripheral tissues, including the liver.[30,31] In support of this, studies on mice have highlighted the potential of CMV challenge to drive expansions of γδ T cells that subsequently home to a diverse range of peripheral tissues, including the liver.[39] The finding that the prevalence of Vδ1$^+$ T cells in liver was correlated with CMV seropositivity[9] suggests that CMV might drive a similar population of γδ T cells to peripheral tissues in humans. Of note, Hunter et al, focussed on HCV and HBV-negative livers,[9] and others have also highlighted the potential of HCV to boost the frequency of hepatic γδ T cells, which may contribute to chronic inflammatory pathology.[48] The long-term nature of adaptive γδ expansions and strong effector capacity of such populations suggests long-term contributions to tissue immunosurveillance. Importantly, the relative contribution of such peripheral blood-linked adaptive-like responses to the γδ T cell compartment in different tissues, including those with a greater degree of anatomical separation from peripheral circulation than liver, is an important issue to be addressed by separate studies. In addition, the CD45RA^{lo} population of hepatic Vδ1$^+$ T cells reported by Hunter et al,[9] which may represent a new liver resident subset, also emphasizes the potential for distinct tissue-associated γδ T cell immunobiology to emerge, even in tissues with strong links to the peripheral circulation. While this subset appears to be significantly distinct from CD45RA^{hi} counterparts, both clonotypically, in its more cytokine-oriented effector profile, and in its potential for extra-TCR stimulation, many questions remain concerning its origin and role in hepatic immunosurveillance and immunoregulation.

2.2.3 | Cutaneous γδ T cells

Human epidermis lacks a γδ T cell population analogous to murine DETC; however, Vδ1$^+$ T cells have been described in the human dermis and epidermis and may contribute to wound healing.[49,50] A recent study of primary γδ cutaneous T cell lymphomas (CTCLs) and

γδ T cells in corresponding normal tissue reported that γδ CTCLs derived from the dermis and epidermis express diverse Vδ1 TCRs, while panniculitic γδ CTCLs found in the subcutaneous fat layer, express Vγ9^{neg}Vδ2 TCRs[51] (Figure 1C). The authors hypothesize that γδ CTCL subtypes develop in situ from the tissue-associated γδ T cell subsets normally present, a possibility currently under investigation. While phenotypic similarities between γδ CTCL populations and equivalent normal cutaneous γδ T cell populations are unclear, the former often expressed cytotoxic markers such as perforin and granzymes, and the transcriptional phenotype of the Vγ9^{neg}Vδ2 panniculitic CTCL indicated they expressed IL-12R/IL-18R, suggesting differences from adaptive-like Vγ9^{neg}Vδ2 equivalents in peripheral blood, including after acute CMV.[31] In summary, this study raises the strong possibility that as in mouse, human skin-associated γδ T cell subsets may exhibit TCR-linked differences in tissue localization under normal conditions. They also highlight the presence of Vγ9^{neg}Vδ2 T cells, like Vδ1$^+$ T cells, in multiple solid tissues.[31,51] However, further studies on the function, phenotype, and TCR repertoire of cutaneous Vδ1$^+$ and Vγ9^{neg}Vδ2 T cells is required. This should resolve the immunobiology of these skin-associated subsets in normal physiology and in γδ CTCL, clarify the degree of interconnection with or functional distinction from equivalent peripheral blood populations, and also establish the degree of functional similarity to the mouse DETC subset.

2.2.4 | Intestinal γδ T cells

The existence of an intestinal intraepithelial lymphocyte (IEL) compartment has long been known in mouse, and this is enriched for Vγ7$^+$ cells.[8] In contrast, the human IEL counterpart has only recently been reported to contain a conserved Vγ4$^+$Vδ1$^+$ subset[8] present in all healthy donors to varying degrees. The establishment of this population in early life (at least in mice),[8] its conserved TCR chain usage, reported expression of PLZF (*ZBTB16*),[52] and Vγ-mediated recognition of a non-polymorphic butyrophilin-like ligand[53,54] (see below) suggest this subset may align significantly with an with innate-like biology. However, many features of these cells, including their functional role in the intestine, remain a subject of ongoing investigation. Human Vγ4$^+$ and mouse Vγ7$^+$ IELs may play a dual role in the intestine, similar to DETC in the epidermis. Consistent with a role in immunosurveillance, Shires et al reported expression of NK-like receptors and granzymes in mouse IELs[55]; similarly, human Vγ4$^+$ IELs express the activating NK receptors NKG2D, NKp44, and NKp46,[56] and also granzymes A and B (Figure 1D), although interestingly they were reported not to secrete IFNγ or TNFα upon in vitro stimulation.[52] However, conversely, mouse Vγ7$^+$ IELs also express molecules involved in tissue homeostasis such as Keratinocyte Growth Factor (KGF)[44]; similarly, human Vγ4$^+$ IELs were reported to express the EGF family member Amphiregulin (Areg), Wnt10a, and Jagged1.[52]

The discovery of a human butyrophilin family-reactive tissue-associated subset that appears to align closely with an

analogous population in mouse is a significant step forward. However, much is still left unresolved regarding the human intestinal γδ T cell compartment, including firstly how the Vγ4⁺Vδ1⁺ subset is functionally regulated by BTNL3.8, both in steady state conditions and in disease states, and secondly regarding the relationship between Vγ4⁺ Vδ1⁺ T cells and Vγ4^{neg}Vδ1⁺ intestinal populations. Of relevance, it is notable that during celiac disease, intestinal Vγ4⁺ cells are outnumbered by an influx of Vδ1⁺ T cells expressing diverse Vγ chains, which are hypothesized to be important in driving disease pathogenesis,[52] which may be adaptive-like in nature. This switch may be influenced by down-regulation of BTNL3.8 expression (as noted for BTNL8 in ulcerative colitis[57]), which could potentially lead to a downstream loss of Vγ4-mediated immunoregulatory function.

2.2.5 | Breast-associated γδ T cells

Vδ1⁺ T cells have recently been described in normal human breast tissue and breast tumors.[58] Vδ1⁺ T cells in healthy breast tissue displayed an effector phenotype, featuring NKG2D expression, production of IFNγ and TNFα (but not IL-17, as opposed to murine breast γδ T cells[59]), and cytotoxic functionality. In addition, as observed for hepatic Vδ1⁺ T cells,[9] the TCR repertoire was relatively clonal, consistent with a T$_{effector}$ phenotype, and included diverse Vγ chains.[58] Some differences in TCR clonality were reported between tumors and healthy breast tissue, with the latter harboring less clonally focussed Vδ1⁺ populations for reasons that are unclear. Interestingly, Vδ1⁺ breast T cells were activated by IL-12/IL-18, and unlike CD8 T cells from the breast, in vitro assays indicated a minority of Vδ1⁺ T cells could be stimulated by NKG2DL alone, raising authors to suggest an innate-like biology.[58] In our view, some caution should be exercised in this assumption. Several features mirror those of hepatic Vδ1⁺ T cells, which themselves appear to be closely aligned with equivalent adaptive-like populations in the blood.[9] Such features include T$_{effector}$ status, private clonal expansions, and IL-12/IL-18-mediated activation, which was also observed for CD45RA^{lo} hepatic Vδ1⁺ populations.[9] Moreover, given the high level of expression of NKG2D on breast Vδ1⁺ T cells, it was striking that only a fraction of Vδ1⁺ T cells responded to NKG2DL alone and notable that anti-CD3/NKG2DL combination resulted in an additive effect on activation.[58] Although one possibility is that the Vδ1⁺ compartment has "dual functionality," operating as an adaptive subset in some scenarios and in an innate-like fashion in others, an alternative interpretation is that such breast Vδ1⁺ T cells might have arisen in the circulation by adaptive processes and subsequently undergone functional changes in breast tissue, resulting in a population with altered IL-12/IL-18 responsiveness relative to equivalent circulating subsets, and the ability to be controlled independently either by TCR or (in some cases) NKG2D. A similar mechanism has been proposed to explain differentiation of hepatic CD45RA^{lo} Vδ1⁺ T cells, which bear several features of tissue-resident T cell populations, based partly on analyses of matched liver/

blood samples.[9] Similar analyses on breast Vδ1⁺ T cells may help to resolve these issues.

3 | LIGAND RECOGNITION

How γδ T cell antigen recognition occurs in molecular detail has remained one of the more impenetrable aspects of γδ T cell immunobiology, and until the last 10 years, the γδ TCR could be regarded as an "orphan" receptor.[60] However in recent years, substantial progress has been made. This includes the central questions of the identity of direct ligands recognized by the γδ TCR, and the molecular basis of such interactions. Our aim here is not to provide a comprehensive overview of proposed ligands, particularly given previous reviews on this topic.[60,61] Instead, we will highlight those exemplars we believe are likely to be most instructive, their molecular features, and how these most likely relate to the underlying physiological biological paradigms (Table 1). For adaptive-like, TCR-diverse populations, one important consideration is how to distinguish cross-reactivities from physiological reactivities. For innate-like populations, several candidate ligands have emerged, and a key challenge has been to establish robust biophysical datasets that support their interaction with the γδ TCR. In both cases, it will be of paramount importance to align ligand-focussed studies with parallel studies on the TCR repertoire, immunophenotype, and responsiveness to immune stimuli of respective subsets, in order to understand ligand recognition in full physiological context.

3.1 | Innate-like reactivities and candidate ligands

3.1.1 | An emerging picture of B7-homology ligands

The overall functional and molecular concordance between the γδ T cell compartments of mice and humans has long been considered relatively poor. Despite this, studies on mouse γδ T cells have not only revealed latent parallels with the human γδ T cell compartment, but potentially provide a unique window into γδ TCR ligands emerging for human innate-like γδ subsets.[8,53,54] Importantly, several candidate innate-like γδ T cell populations exist in mice, typically characterized by conserved chain usage, and in some cases highly restricted CDR3 regions.[62] Furthermore, it has long been established that mouse γδ T cell subsets with distinct V region usage are typically restricted to different anatomical sites. Based on these observations, it has been proposed that these distinct mouse γδ T cell subsets engage different sets of host-encoded ligands, perhaps restricted in expression to specific tissues.[63]

Based on findings spanning the last 15 years, Skint family molecules, and members of the butyrophilin (BTN) and butyrophilin-like (BTNL/Btnl) families, which share homology with B7, have emerged as compelling candidate γδ TCR ligands for such populations. As outlined below, studies on Skint1 and Btnl1 have confirmed their strong potential to mediate selection of anatomically

TABLE 1 Immunobiology of innate-like and adaptive γδ T cell subsets

	Innate-like				Adaptive-like
Species	Mouse	Human	Human/mouse	Human	Human
Tissue	Skin (DETC)	Peripheral blood	Intestine (IEL)	Peripheral blood/liver/skin other tissues?	Peripheral blood/liver/skin other tissues?
γδ chain usage	Vγ5Vδ1	Vγ9Vδ2	Vγ4Vδ1 (h) Vγ7Vδ4/5/6/7 (m)	Vδ2^{neg} Vγ2,3,4,5,8,9 Vδ1/3/5/8	Vγ9^{neg} Vδ2 Vγ2,3,4,5,8 Vδ2
V(D)J diversity	Invariant CDR3γ and CDR3δ	Limited CDR3γ diversity; diverse CDR3δ	Limited CDR3γ diversity; diverse CDR3δ	Diverse CDR3γ and CDR3δ	Diverse CDR3γ and CDR3δ
Clonality	Clonal/ pseudoclonal	Typically intermediate clonality	unknown	T_{naive}: polyclonal $T_{effector}$: highly clonal	T_{naive}: polyclonal $T_{effector}$: highly clonal
Development of effector functions	Thymic selection	Selection in periphery in early life	Selection in neonatal intestine	After Ag challenge	After Ag challenge
Activation in absence of TCR signals?	NKG2DL	IL-12/18	unknown	PB: no Tissues: NKG2DL and/ or IL-12/18?	PB: no Tissues: unknown
CDR2/HV4 ligand	Skint1?	BTN2A1	BTNL3 (h)/Btnl6 (m)	unknown	unknown
CDR3 ligand	unknown	Hypothesized: (conserved mouse/ human)	unknown	Diverse ligands	Diverse ligands

Note: Various γδ T cell subsets align with an innate-like biology, due to their conserved Vγ or Vδ chain usage, semi-invariant TCR repertoire, development of effector functions early in life, and recognition of BTN-family ligands via the CDR2/HV4 loops. Other populations align more with adaptive biology, with expression of diverse γδ TCRs, development of effector functions only after specific antigen challenge, recognition of diverse MHC-like and non-MHC-like ligands, and no known link to BTN-like molecules.

Abbreviations: Ag, antigen; DETC, dendritic epidermal T cells; h, human; IEL, intestinal intraepithelial lymphocyte; m, mouse; NKG2DL, NKG2D ligands; PB, peripheral blood.

restricted γδ subsets in the skin and gut, respectively. Moreover, following seminal early work on Skint1, studies on humans, which lack functional Skint molecules, initially revealed the critical involvement of BTN3A1 in activation of the innate-like Vγ9Vδ2 T cell subset. Although obtaining robust biophysical evidence for direct γδ TCR interaction remains challenging, this has now been achieved for BTNL3 expressed in the gut,[54] and for BTN2A1,[17,18] a ligand for the human peripheral blood Vγ9Vδ2 T cell subset. An important consideration has been the molecular features of such interactions. A critical finding highlighted by recent publications is the conserved binding mode utilized in these interactions,[17,54] including exclusive dependence on germline-encoded regions of distinct Vγ chains. Future studies are required to resolve the role of CDR3 regions, the importance of which has been established for Vγ9Vδ2 TCRs.[64] In summary, while important differences clearly exist between functionally divergent subsets, the picture emerging for innate-like γδ T cell recognition appears to confirm the prediction of host-encoded conserved ligands associated with specific anatomical sites and that are linked to restricted TCR chain usage.[63]

3.1.2 | Skint1, a candidate ligand for the mouse DETC Vγ5Vδ1 TCR

Discovery of Skint1 was seminal in spawning interest in B7-homology molecules as candidate ligands for innate-like γδ populations. *skint1* was initially identified by genetic mapping in a mouse strain that specifically lacked DETC,[47,65] a γδ T cell subset implicated in skin homeostasis, immunosurveillance including in cancer, and wound repair.[41] Skint1 is the prototypic member of a family of 11 Skint molecules encoded on mouse chromosome 4. Skint proteins consist of an IgV (similar in fold to CD80 and PDL1) and IgC domain (Figure 2A) and are unusual in the Ig Superfamily for having multiple transmembrane domains. The mutation in *skint1* found in these mice results in premature truncation of the Skint1 protein in the second transmembrane domain.

Skint1 mRNA was shown to be expressed in thymus and skin.[47] The protein has not been detected on the surface of thymic epithelial cells and keratinocytes, despite the development of a pAb[66] and mAb,[67] so it is assumed to be expressed at very low levels on the cell surface. However, *Skint1* expression in
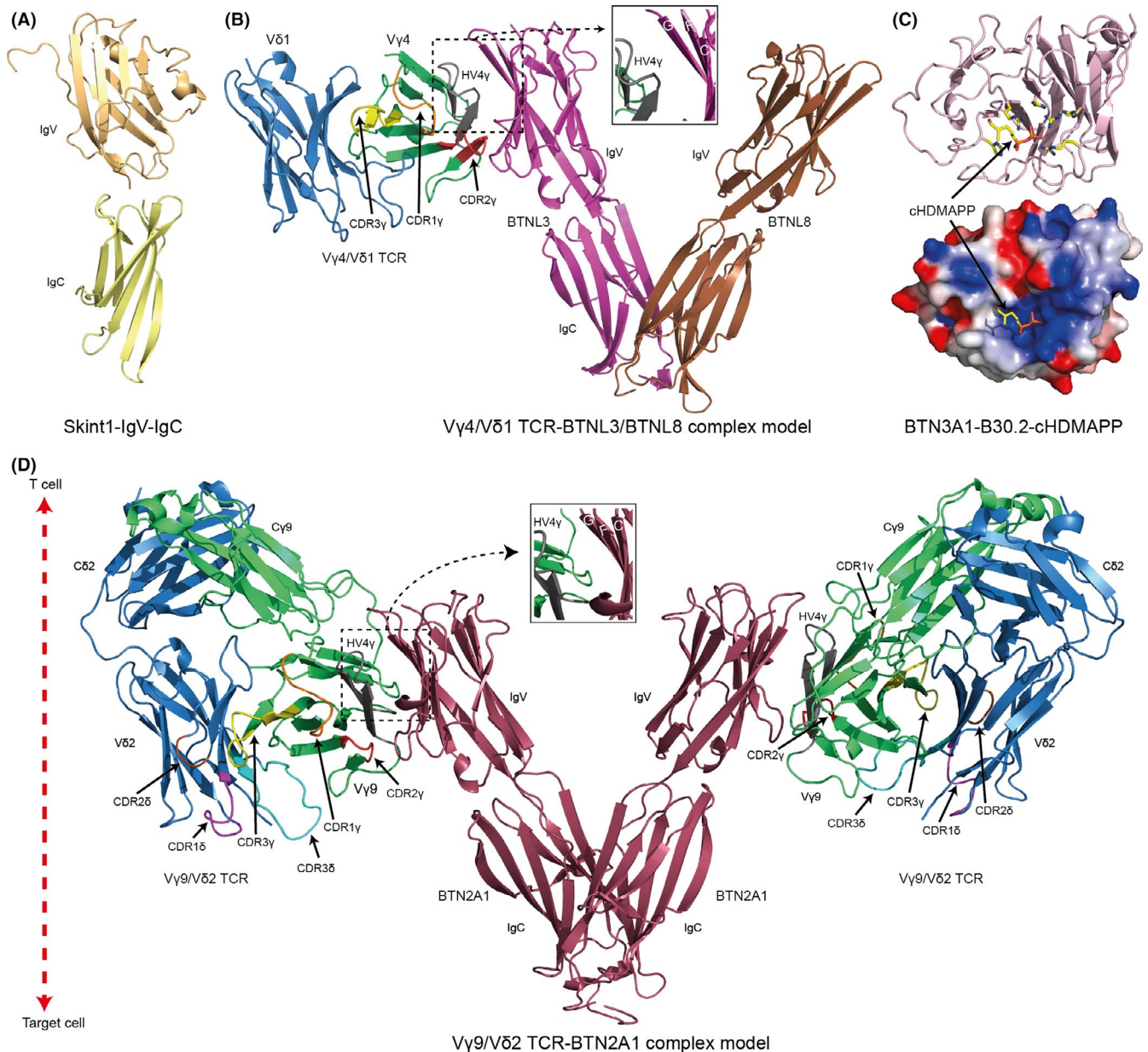
FIGURE 2 Candidate or confirmed γδ TCR ligands with B7 homology. A, Ribbon representation of the Skint1 IgV (PDB ID: 2N4I) and IgC domains (modeled with Phyre [Protein Homology/analogY Recognition Engine]). B, Best fit hypothetical model of the Vγ4Vδ1 TCR-BTNL3/BTNL8 complex generated using computational docking program SwarmDock. Germline-encoded CDR2 and HV4 regions from Vγ4 and the CFG face of BTNL3-IgV contribute to the interface. Boxed area shows close up view of the interface. BTNL3-IgV-IgC, light magenta; BTNL8-IgV-IgC, brown; Vγ4-chain, lime green; Vδ1-chain, sky-blue; CDR1γ, orange; CDR2γ, red; HV4γ, gray; CDR3γ, yellow. C, Ribbon representation of the BTN3A1-B30.2-cHDMAPP (PDB ID: 4N7U) complex (top). BTN3A1 residues that mediate interactions with cHDMAPP are highlighted (stick format). Electrostatic potential of the BTN3A1-B30.2-cHDMAPP complex calculated with DelPhi with the potential scale ranging from −7 (red) to +7 (blue) in units of kT/e (bottom). BTN3A1-B30.2 binds cHDMAPP through a positively charged surface pocket. D, Model of Vγ9Vδ2-BTN2A1 interaction incorporating BTN2A1 homodimer formation, and bilateral Vγ9Vδ2 interaction with BTN2A1 IgV domain. Germline-encoded CDR2 and HV4 regions from Vγ9 and the CFG face of BTN2A1-IgV contribute to the interface. Boxed area demonstrates close up view of the interface. BTN2A1-IgV-IgC, raspberry; Vγ9-chain, lime green; Vδ2-chain, sky-blue; CDR1γ, orange; CDR2γ, red; HV4γ, gray; CDR3γ, yellow; CDR1δ, magenta; CDR2δ, brown; CDR3δ, cyan. Abbreviations: cHDMAPP, hydroxy-methyl-butyl-pyrophosphate

thymic epithelial cells is critical for development and selection of mouse DETC subset, as Vγ5⁺Vδ1⁺ T cells that develop in the thymus of mice lacking functional skint1 fail to mature and home to the skin.[68]

DETC express high levels of NKG2D and can respond to over-expressed NKG2DL[43,69] seemingly in the absence of a TCR ligand. However, it is also conceivable that Skint1 or another TCR ligand is expressed on the surface of keratinocytes where DETC processes

contact keratinocytes. These processes contain sites known as phosphorylated-Tyrosine rich aggregates located on projections (PALPs), in which is concentrated both the TCR and constitutively phosphorylated CD3ζ and ZAP70, suggesting that TCR is constitutively engaging ligand and triggering at these sites.[70] It is therefore possible that the changes observed upon DETC activation in response to stress stimuli, including loss of PALPs, cell rounding,[71] and in some cases increased cytokine production,[41] might be initiated by diminished expression of such a TCR ligand. Therefore, it is conceivable that TCR ligand may have a role in "normality sensing" in the steady state.[72] Alternatively, it has been proposed that tissue damage leads to expression of a DETC TCR ligand, as probed by TCR tetramer staining.[73] This ligand may be distinct from the homeostatic ligand discussed above.

Although no direct DETC TCR/Skint1 binding data have been published, Skint1 and conceivably other family members remain plausible candidate ligands for the Vγ5Vδ1 TCR, a possibility that justifies further exploration.

3.1.3 | BTNL3 and Btnl6 interaction with human Vγ4 and murine Vγ7 TCRs

After the discovery that skint1 expression in the thymus is required for Vγ5+Vδ1+ DETC selection,[47] it was of interest to examine whether other butyrophilin family members regulated the development or maintenance of other tissue-restricted γδ T cell populations. One such population is the Vγ7+ intestinal intraepithelial cells (IELs) in the murine intestine.[8] Several butyrophilin-like (Btnl) molecules are expressed in the murine gut, including Btnl1, Btnl4, and Btnl6. *Btnl1* was targeted for genetic disruption, and in *Btnl1−/−* mice, Vγ7+ IELs were severely reduced.[8] Btnl1 re-expression in the intestine within the first 3 weeks of life, but not later, allowed Vγ7+ IELs to recover, suggesting Btnl1 functions in the establishment of the IEL compartment in early life.

Btnl1 and Btnl6 (Btnl1.6) co-transduced into target cells activated Vγ7+ IELs as well as T cell transductants expressing Vγ7+ TCRs. Activation of Vγ7 TCR transductants by Btnl1.6 complexes could be measured by CD25 and CD69 upregulation and TCR downmodulation.[8,53] Mutants of the Vγ7 TCR showed that CDR2 and HV4 regions of Vγ7 were important for Btnl1.6 responsiveness. Inspection of the γδ TCR repertoire from human intestine showed that Vγ4 was enriched in the IEL compartment, suggesting human Vγ4 IELs may be analogous to mouse Vγ7+ IELs.[8] Furthermore BTNL3 and 8 were found to be expressed in human intestine, and expression of BTNL3.8 in target cells caused TCR downregulation and activation of transductants expressing human Vγ4+ IEL TCRs.[8,53]

Using Surface Plasmon Resonance, BTNL3 was subsequently shown to bind directly to germline-encoded regions of Vγ4, specifically the CDR2 and HV4 loops[54] (Figure 2B), in a manner similar to superantigen binding to TCRβ chains. The CDR3 loops of Vγ4 and the TCRδ chain were not involved in BTNL3 binding, though they were essential for γδ TCR binding to a clonally restricted antigen

such as endothelial protein C receptor (EPCR).[54,74] Although direct binding of Btnl1 or Btnl6 to Vγ7 TCRs has not been demonstrated, Vγ7 TCR multimers bound to Btnl1.6 transductants, and mutations in Btnl6, analogous to those in BTNL3, disrupted binding,[54] suggesting that the binding mode is conserved across species.

Although the studies outlined above establish BTNL3 and suggest Btnl6 as direct ligands for human and mouse Vγ4+ and Vγ7+ T cells, respectively, much is still unclear about the immunological significance of these interactions. Firstly, the functional consequences of γδ TCR engagement of epithelial BTNL/Btnl molecules on γδ IELs in physiological settings is uncertain, as is the impact of immune stimuli-induced changes in BTNL/Btnl levels on γδ IEL-mediated effector or homeostatic responses. Secondly, a number of studies have established that individual Vγ4/Vγ7 TCRs are in principle capable of both non-clonal recognition of BTNL/Btnl molecules and clonally restricted, CDR3-mediated recognition of "adaptive" ligands. These include Vγ4 TCRs shown to recognize either CD1c,[53] EPCR,[54] or CD1b,[75] and a mouse Vγ7 TCR reactive to the mouse non-classical class I MHC molecule T22.[53]

It is currently unclear whether such "dual reactivity" of individual γδ TCRs reflects any physiological interrelatedness between these distinct recognition modalities at a cellular level, or instead if they operate separately. Supporting the latter possibility, in some individuals Vγ4+ T cells can become clonally expanded within adaptive-like populations in both peripheral blood and liver,[7,9] which both lack BTNL3.8 expression.[8] Also, reporter lines transduced with "dually reactive" Vγ4 TCRs have demonstrated independent recognition of target cells expressing either BTNL3 or clonally restricted antigens (as assessed by TCR downregulation, activation marker expression, and signaling assays),[53,54,75] with a lack of requirement for both individual ligands to be co-expressed. Finally, although the Vγ4Vδ5 LES TCR used non-overlapping elements to directly bind EPCR and BTNL3, simultaneous binding of both ligands was not possible, most likely due to steric hindrance.[54] This argues against a universal requirement for co-engagement of BTNL3 and antigen ligands in TCR-mediated stimulation of Vγ4+ T cells. A model involving sequential use of the two modalities cannot be excluded, with selection and/or tonic engagement of BTN(L) molecules representing a prerequisite for Vγ4+ clonotypic antigen-specific responses. However, it appears equally possible that each modality is physiologically separate at a cell-intrinsic level, in which case dual reactivity may simply suggest the γδ TCR repertoire has evolved to exploit a number of "dual-use" Vγ genes that can both enable γδ T cells within particular tissue microenvironments to recognize specific BTN/BTNL/Btnl molecules in a non-clonal fashion, while alternatively and simultaneously contributing to CDR3-mediated responses focussed on clonotypically restricted ligands. Alongside Vγ4, the Vγ9 chain, which enables non-clonal recognition of BTN2A1 as an essential component of Vγ9Vδ2 pAg sensing[17,18] (see sections directly below), but also contributes to clonotypically restricted adaptive-like Vδ1+ responses,[7] would clearly also qualify as such a "dual-use" Vγ chain.

3.1.4 | BTN3A1—from direct Vγ9Vδ2 TCR ligand to pAg sensor

The Vγ9Vδ2 TCR is a strong candidate for interaction with host-encoded ligand(s), based on Vγ9Vδ2 T cells bearing many hallmarks of innate-like immunobiology, including a semi-invariant TCR repertoire, and dependence on both a specific Vγ and Vδ chain for functional reactivity to pAgs.

In a seminal study, Harly et al showed that the butyrophilin BTN3A1 was critical for pAg reactivity. A monoclonal antibody (mAb) against CD277 (BTN3 family) butyrophilins was found to stimulate Vγ9Vδ2T cell activation, similar to pAg stimulation.[76] Encoded on chromosome 6, there are three CD277/BTN3 family members in humans, *BTN3A1, BTN3A2, and BTN3A3*, with each protein comprising an IgV domain, IgC domain, transmembrane domain, and, in the case of BTN3A1 and BTN3A3, an intracellular B30.2 domain. Knockdown of all BTN3 isoforms in target cells abolished pAg reactivity, and re-expression of the BTN3A1 isoform alone reconstituted pAg reactivity, albeit to reduced levels compared to wild-type cells. While the form of BTN3A1 that is most active in stimulating pAg recognition is uncertain, a requirement for co-expression of BTN3A2 or BTN3A3 in order to form heteromers with BTN3A1 has been highlighted.[77]

The exact molecular role BTN3A1 plays in pAg recognition by Vγ9Vδ2 T cells is only now starting to become clear. BTN3A1 was initially suggested to act as both a presenting molecule for pAg and a target for direct TCR binding.[78] However, neither of these findings could be reproduced,[67,79] and crystal structures purporting to describe pAg bound to BTN3A1 IgV could be explained by the presence of crystallization components. Instead, the Adams laboratory used isothermal titration calorimetry (ITC) to convincingly demonstrate that pAgs actually bind to the intracellular B30.2 domain of BTN3A1.[79] This has been confirmed by several groups using ITC and NMR,[67,80] and moreover both mutagenesis studies[81] and crystal structures featuring pAg bound to BTN3A1 B30.2 domain[79,82] (Figure 2C), further support these findings. In addition, the fact that pAgs with improved intracellular targeting are considerably more potent[80,83] strongly support such an intracellular sensing mechanism. Although both X-ray crystallographic and Nuclear magnetic resonance-based data favor the idea that pAg binding to the B30.2 domain induces a conformational change in that domain or the juxtamembrane domain,[67,79,80,82,83] events subsequent to this have remained unclear. One suggestion has been that this conformational change is somehow transmitted to the extracellular domain through "inside-out" signalling,[84] leading to recognition of an altered conformation of the BTN3A1 IgV domain. However, not only is it unclear how this would occur, but there are few if any precedents for such mechanism involving an IgV domain. In addition, such a mechanism would still culminate in BTN3A1 binding to the Vγ9Vδ2 TCR, and to date no credible evidence for a direct interaction has been reported. Furthermore extensive mutagenesis of surface residues on the BTN3A1 IgV domain failed to identify a plausible interaction surface.[81]

Also, Riano et al found that pAg stimulation depended on both BTN3A1 and an additional gene(s) present on chromosome 6.[85] These led to the suggestion that pAg binding to BTN3A1 B30.2 domain may instead regulate association with a separate "Factor X" molecule,[86] which at the time was suggested to either affect pAg loading or BTN3A1 trafficking[85] or conceivably conformational changes in the BTN3A1 ectodomain.[81,84] In summary, while BTN3A1 is clearly critical for pAg recognition by Vγ9Vδ2 T cells, the available evidence strongly supports a molecular role as the non-redundant pAg sensor[79,81] in target cells, but not as a direct Vγ9Vδ2 TCR ligand, particularly in light of recent findings outlined below.

3.1.5 | BTN2A1: a direct Vγ9-focussed ligand for the Vγ9Vδ2 TCR

Two recent studies have identified another butyrophilin molecule, BTN2A1, as a direct ligand for the Vγ9Vδ2 TCR. Rigau *et al* used a Vγ9Vδ2TCR tetramer staining and CRISPR approach, and showed co-expression of BTN2A1 alongside BTN3A1 in target cells was critical for pAg sensing.[18] A parallel study by Karunakaran *et al* also identified BTN2A1 as a direct Vγ9Vδ2 TCR ligand,[17] building on their previous observation that rodent target cells are unable to support pAg sensing by Vγ9Vδ2T cells.[85] In these previous experiments, ectopic expression of BTN3A1 in rodent cells allowed 20.1 mAb-mediated activation of Vγ9Vδ2 T cells, but did not permit pAg sensing. However, introduction of human Chromosome 6 in rodent cells, alongside BTN3A1, did permit full pAg recognition,[85] suggesting Chromosome 6 encoded a separate "Factor X" protein that was an essential co-factor alongside BTN3A1 in pAg sensing. Genetic mapping of chromosome 6 genes in radiation hybrids combined with transfection experiments identified this gene as BTN2A1[17] and confirmed its functional synergy with BTN3A1 in pAg sensing. Furthermore, BTN2A1 binding was not only limited to direct interaction with germline-encoded regions of Vγ9,[17,18] but noted as similar to BTNL3 binding by Vγ4,[54] in that it was dependent on Vγ9 CDR2/HV4 residues, and involved residues on the CFG face of BTN2A1[17] (Figure 2D).

These highly concordant studies significantly advance our understanding of how Vγ9Vδ2 T cell recognition takes place and confirm BTN2A1 represents the elusive chromosome 6-encoded "Factor X." Nevertheless, at least two major issues remain resolved. The first relates to whether a direct interaction between BTN3A1, the pAg sensor, and BTN2A1, a direct ligand for the Vγ9 chain, is required for the sensing mechanism. However, Rigau *et al* used FRET to suggest proximity (≤10 nm) between both proteins at the cell surface,[18] and Karunakaran *et al* used a combination of chemical cross-linking and immunoprecipitation to establish close proximity (≤16 Å), and confirmed a direct interaction between BTN2A1 and BTN3A1 IgV domains by NMR.[17] Secondly, since the Vγ9Vδ2 interaction with BTN2A1 is limited to germline-encoded regions of the Vγ9 chain, they do not explain

the well-established importance of Vγ9 and Vδ2 CDR3 regions in pAg recognition,[81] changes to which did not affect BTN2A1 interaction. The observation that different Vγ9Vδ2 clonotypes confer variable functional avidity for tumor cells when expressed in αβ T cells[87] also confirms CDR3 importance. One possibility is that while Vγ9 directly contacts BTN2A1, the Vδ2 domain directly interacts with the BTN3A1 ectodomain following pAg stimulation.[18] This mechanism would presumably require an "inside-out" conformational change initiated in the intracellular B30.2 pAg-binding domain to be conferred to the BTN3A1 ectodomain and also predicts direct TCR/BTN3A1 interaction that has not been convincingly established to date. An alternative possibility suggested by Karunukaran et al is that following pAg exposure, BTN3A1 facilitates recruitment of a third component of a ligand complex, which is recognized in a CDR3-dependent manner alongside Vγ9-mediated BTN2A1 interaction, as part of a composite ligand.[17] In this model, the importance of BTN3A1/BTN2A1 association, which was observed experimentally both directly by NMR and indirectly by cell-surface cross-linking and mass spectroscopy, would be in orienting such a ligand precisely to allow optimal TCR interaction with both components of this complex. The identity of such a putative additional component is currently unclear. Vyborova, *et al* have subsequently proposed a third model, which incorporates a CDR3-mediated ligand as previously suggested by Karunakaran et al,[17] but instead predicts this ligand is constitutively present at the cell surface.[87] In this alternative model, pAg stimulation serves to increase BTN3A1 levels on the target cell surface, which allows interaction with an as-yet-unidentified ligand on the T cell, thereby helping to establish a stable immune synapse, in a process that involves RhoB.[88] Of note, other molecules have been identified as being involved in pAg recognition, such as periplakin[89] and ABC transporters,[90] but the role they play is currently unclear. Clearly, additional work will be required to clarify the molecular mechanisms involved.

In summary, recent studies firmly establish BTN2A1 as one critical component of an activator ligand complex for Vγ9Vδ2-mediated pAg recognition, but the full molecular nature of this complex is yet to be defined, although the involvement of an as-yet-unidentified CDR3-recognized ligand appears likely. Furthermore, the recent studies on Vγ9/BTN2A1[17,18] and Vγ4/BTNL3 (and Vγ7/Btnl6)[54] interactions highlight a striking evolutionary conservation in ligand binding modes for two anatomically and functionally distinct innate-like γδ T cell subsets, as well as unexpected concordance between mouse and human gut γδ T cells, and raise the question of whether other innate-like γδ T cell subsets will recognize alternative members of the BTN/BTNL family in a similar fashion. Furthermore, the involvement of a putative CDR3-recognized ligand for the peripheral blood BTN2A1-dependent Vγ9Vδ2 T cell subset suggests the possibility of additional CDR3 ligands for other innate-like BTN family-reactive subsets, such as those in intestine, and potentially mouse skin, and conceivably other unidentified populations.

3.2 | Adaptive-like reactivities and candidate ligands

3.2.1 | General considerations

The identity of physiologically relevant γδ TCR ligands for adaptive-like γδ T cell populations (eg the human Vδ2-negative T cell subset) and the immune recognition paradigms they reflect, including the relative importance of host-encoded versus foreign antigenic targets, are major questions that remain substantially unresolved.[60] However, advances in our understanding of the immunobiology of relevant subsets and of T cell recognition in general provide important clues that both help contextualize ligands reported for adaptive-like subsets and will shape future investigations.

From this perspective, four points are particularly pertinent and will inform our discussion of proposed ligands below. Firstly, studies on Vδ1+, Vδ1neg Vδ2neg, and Vγ9neg Vδ2+ subsets suggest they possess a very diverse TCR repertoire, which underlines the observation that γδ CDR3 loop lengths bear more similarity to antibodies than to αβ T cells.[91] The obvious implication of this is that there may be huge potential in the Tnaive compartment for recognition of diverse ligands. However, an important caveat is that since such high diversity repertoires likely have an inherently high capacity for recognition of diverse ligands, discriminating experimentally between spurious cross-reactivities and physiologically relevant targets remains a challenge for the field.[60] Although the number of ligands recognized in vivo is entirely unclear at present, the high diversity in the TCR repertoire of such adaptive-like subsets and the broad, structurally disparate range of ligands identified to date is consistent with a high diversity of ligands, including potential for recognition of both host-encoded and foreign antigens. Despite this, degenerate recognition of individual ligands by related or even unrelated TCR clonotypes cannot currently be excluded.

Secondly, evidence suggests that clonotypic expansion is linked to differentiation from Tnaive to Teffector status, and that long-lived clonotypes result, which are persistently sensitive to TCR triggering.[30,31] Mechanistically, this suggests TCR/ligand engagement may be critical both for initiating this adaptive differentiation, and for triggering recall responses as part of immunosurveillance linked to chronic or recurrent immune challenges, as is the case for conventional αβ adaptive responses. Determining the differentiation state (eg Tnaive, Teffector)[30,60] of a given clonotype will be important both in future ligand identification studies and also to understand the significance of current ligands. Specificities aligning with Tnaive clonotypes likely reflect the range of potential targets that can *in principle* be recognized, but not necessarily those that *actually are* targeted during physiological responses to *bona fide* immune challenges. An understanding of ligands recognized by in vivo effector responses may require Teffector-derived clonotypes to be defined during ligand identification studies, perhaps ideally in the context of a particular immune challenge.

A third point is that if indeed TCR/ligand interaction does drive such adaptive γδ T cell responses, then the highly clonally focussed nature of the responses suggests that the underlying TCR/ligand recognition events are likely to be CDR3 dependent.

Finally, studies on αβ T cell recognition, including in the context of the kinetic segregation model,[92] have highlighted that TCR ligand size imposes constraints on ligand-driven activation, with smaller ligands generally more likely to be conducive to efficient TCR triggering.[93] It is entirely possible that similar constraints operate for γδ T cell recognition.

3.2.2 | MHC-like molecules

Several MHC-like molecules, including classical MHC and non-classical class I MHC molecules, have been confirmed as direct ligands for γδ TCRs.

Classical MHC molecules

γδ T cells develop in MHC class I, MHC class II, and β2m-deficient mice,[94] leading to the assumption, which is still generally accepted, that they are generally not MHC-restricted. Consistent with this, reactivity of most γδ T cell clones cannot be blocked by mAbs against conventional class I and class II MHC molecules. However, in a minority of cases, γδ T cells clones have been identified which recognize classical polymorphic MHC molecules. Early studies involving immunization of nude mice or repeated in vitro stimulation with allogeneic lymphocytes, identified γδ T cell clones which could recognize allogeneic molecules encoded in the MHC.[95-97] One clone, LBK5, was analyzed in detail. The LBK5 clone specifically recognized the class II molecule I-E^k,[96] but was not peptide-dependent, and recognition did not involve polymorphic α1α2 residues which were critical for αβ TCR recognition.[98] Instead, recognition was dependent on a several residues at one end of the α1α2 platform which did not affect αβ TCR recognition and may be influenced by a glycosylation site.[99]

Human γδ T cell clones that recognize classical MHC class I and class II molecules have also been described (reviewed by Haas[100]); these include an HLA-B58 reactive clone.[101] These cells were mostly peptide specific. Recently, HLA-A2 MART-1 reactive γδ T cell clones were generated by sorting HLA-A2 dextramer-positive cells from peripheral blood or in vitro selected cord blood hematopoietic stem/progenitor cells.[102] Mutagenesis and structural studies revealed that γδ TCRs can bind peptide-MHC in a similar manner to αβ TCRs (Figure 3A), but there is some variability in the binding mode of different γδ TCRs.

While it is clearly possible for particular γδ TCRs to recognize pMHC, MHC-reactive γδ T cells are rare ex vivo and are therefore highly unlikely to contribute to large Vδ1 clonal expansions observed in many individuals.[7,32] These clonotypes map to the most TCR-diverse human γδ T cell subsets, and it is at present unclear whether such γδ TCR/pMHC interactions reflect cognate reactivities. This provides an important note of caution when assessing the likely relevance of other candidate γδ TCR ligands.

Non-classical class I MHC molecules

Some of the early mouse γδ T cell clones generated by mixed lymphocyte reaction were not reactive to classical MHC molecules, but instead were focussed on polymorphic non-classical MHC molecules encoded within the H-2 locus, specifically H-2T10/T22.[103,104] H-2T molecules adopt an MHC class I fold and are β2m associated but do not present peptide. Although clones were generated against allogeneic T10/T22, use of T22 tetramers showed that up to ~0.4% of γδ T cell splenocytes and IELs bound autologous T22 in addition to foreign T22, and T22-reactive γδ T cells developed even in mice lacking the genes for T22.[105] Tetramer-positive γδ T cells were isolated and TCRs sequenced, revealing diverse Vγ and Vδ chains were used, but CDR3δ usually contained the W...(S)EGYEL motif.[106] Strikingly, the crystal structure of the G8 TCR bound to T22 showed that germline-encoded residues of CDR3δ made the majority of contacts with T22, while the CDR3 loop of the TCRγ chain made very few contacts[107] (Figure 3B). Notably the W...(S)EGYEL motif required for T22 binding is encoded by one reading frame of Dδ2, one of only two Dδ regions utilized in mouse, and it is not particularly dependent on N/P nucleotide additions. This rearrangement is not uncommon, which accounts for the relatively high percentage of γδ T cells that recognize T22. While no direct orthologs of T10 and T22 are known to exist in humans, these studies highlighted the potential of CDR3 regions to mediate recognition of both host-encoded and foreign MHC-like molecules. Nevertheless, the functional significance of T10/T22-specific γδ T cell populations in mice has remained somewhat unclear.

Because non-classical MHC molecules have commonly been regarded as attractive candidates for γδ TCR ligands,[63] a number of other studies have generated tetramers of other non-classical MHC molecules and used these to probe for γδ T cells reactive to them in the human and mouse. Of note, these have focussed on non-classical MHC molecules (CD1, MR1) that are now well validated TCR-mediated ligands for established, and likely physiologically relevant unconventional αβ T cell populations. CD1b, CD1c, CD1d, and MR1 have all been tested for γδ T cell reactivity via the tetramer staining approach, and in each case, tetramers identify a minority of γδ T cells from PBMC[75,108-111] which are derived exclusively from the Vδ1$^+$ peripheral blood subset (although cellular reactivity to CD1d has been reported for some Vδ3 TCRs[112]). Use of tetramer technology has permitted stained cells to be sorted and either expanded in vitro, or alternatively TCRs to be isolated by single cell PCR. Expression of the TCR in reporter cell lines has confirmed TCR-mediated recognition of CD1 and MR1. For CD1, although the tetramer used to identify the cells can be loaded with antigen, in some cases the γδ TCRs isolated do not discriminate antigen and/or can recognize empty molecules.[75,111] In structural studies of γδ TCR/CD1 interaction (Figure 3C,D), CDR3δ, by far the most sequence diverse of the two CDR3 regions, has often been shown to be involved and frequently appears to mediate the majority of contacts.[109,111] Although not yet supported by structural data, recent data on Vδ1$^+$ T cells specific for CD1b suggest that interaction is similarly dominated by the Vδ1 CDR3 loop.[75] For MR1, in addition to PBMC, a number of other tissues were tested for the presence of tetramer-reactive populations, and somewhat higher levels of staining were obtained in some
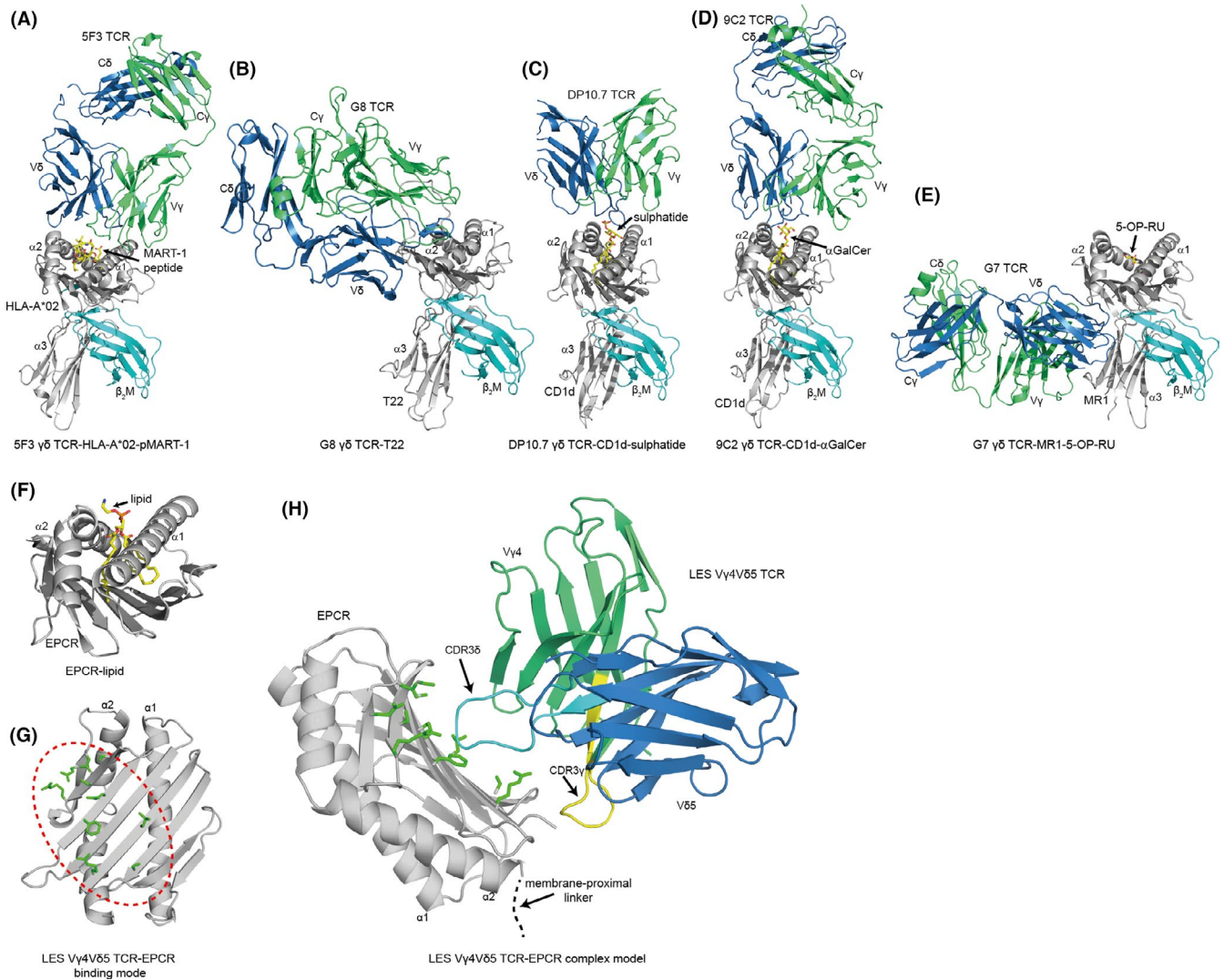
FIGURE 3 MHC and MHC-like molecules established as direct ligands for γδ TCRs. A-E, Cartoon representation of the 5F3 γδ TCR/HLA-A*02-pMART-1 (PDB ID: 6D7G), G8 γδ TCR/T22 (PDB ID: 1YPZ), DP10.7 γδ TCR/CD1d-sulfatide (PDB ID: 4MNG), DP10.7 γδ TCR/CD1d-α-GalCer (PDB ID: 4LHU), and G7 γδ TCR-MR1-5-OP-RU (PDB ID: 6MWR) complexes, all derived from x-ray crystallographic studies. HLA-A*02, T22, CD1d, and MR1 gray; β2-microglobulin (β2M), cyan; MART-1 peptide, sulfatide, α-GalCer and 5-OP-RU, stick format; γ-chain, lime green; δ-chain, sky-blue. F, Crystal structure of EPCR (gray) bound to lipid (PDB ID: 1L8J). G, Mapping of EPCR residues (green) on the β-sheet surface, opposite the lipid-binding face, that affects LES Vγ4Vδ5 TCR-EPCR affinity. Lipid moiety has been omitted for clarity. Putative docking mode is shown (red dashed line). H, Best fit hypothetical model of the LES Vγ4Vδ5 TCR-EPCR complex generated using computational docking program HADDOCK (High Ambiguity Driven protein-protein DOCKing). CDR3γ (yellow) and CDR3δ (cyan) of the LES Vγ4Vδ5 TCR both contribute to the interface. Membrane-proximal linker region is indicated (black dashed line). Abbreviations: MART1-1, Melanoma antigen recognized by T cells; α-GalCer, alpha-galactosylceramide; MR1, major histocompatibility complex related-1 protein; 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; EPCR, endothelial cell protein C receptor

cases.[108] Moreover, intriguingly, structural (Figure 3E) and mutagenesis studies revealed at least two potential modes of γδ TCR/MR1 interaction, one involving a highly unexpected interaction of the γδ TCR with the underside of the MR1 platform, which was mediated by the Vδ1 region, and as for Vδ1 recognition of CD1c/d recognition, heavily involved the Vδ1 CDR3 region. Of relevance, Reijneveld et al also recently characterized diverse Vδ1 CDR3-mediated TCR binding modes for CD1b,[75] with one hypothesized to involve the underside of the CD1b antigen-binding platform as for MR1.[108]

The studies above demonstrate unequivocally that some Vδ1+ (and in the case of CD1d, Vδ3+) T cells are able to interact with CD1 or MR1, and outline the molecular basis of this recognition.[108-112] However, the significance of these interactions remains unclear. Intriguingly, the γδ T cells involved invariably derive from the highly TCR-diverse Vδ2neg compartment, for which there is now substantial evidence for adaptive-like immunobiology, at least in peripheral blood, and they typically involve its most variable molecular feature, the CDR3δ region.[113] This contrasts with non-classical MHC recognition by at least some αβ T cell populations, including

iNKTs and MAITs. Unlike peripheral blood Vδ2^{neg} T cells, these are innate-like subsets that express semi-invariant TCR repertoires, allowing a majority of these entire subsets to recognize CD1d and MR1, respectively.[11,114] Moreover, CD1 and MR1 typically bind only very small percentages of Vδ2^{neg} cells from PBMC. While phenotypic information regarding CD1/MR1 tetramer-positive γδ T cells is either lacking or limited, these low percentages suggest that in many cases the populations involved may be drawn from the TCR-diverse T$_{naive}$ compartment, which is most likely antigen-inexperienced. What these rare γδ T cell populations could contribute to immunity in addition to the existing relatively large CD1d- and MR1-reactive iNKT and MAIT populations is currently unclear. However, one possibility is that following immune stimuli such as infection or inflammation, relevant CD1 or MR1 molecules could become loaded with as-yet-unknown antigens that drive expansions of responding Vδ2[neg] T cell clonotypes. In line with this, MR1 tetramer was shown to stain 41% in the γδ IELs of a single celiac disease patient.[108] Future investigations will be required to explore the potential immunological significance of such populations.

α1α2 domain homologs

Endothelial protein C receptor. Building on several studies highlighting the potential of cytomegalovirus infection to stimulate Vδ2^{neg} T cells, Endothelial Protein C Receptor (EPCR) was identified as a ligand for the LES Vδ2[neg] clonotype.[74] EPCR is a type I transmembrane protein encoded on chromosome 20 and expressed on diverse endothelial cells that negatively regulates coagulation and suppresses endothelial apoptosis. Although it possesses an ectodomain with an α1α2 domain homologous to class I MHC (Figure 3F), EPCR has a radically different tertiary structure that is closer to ULBP/RAE molecules as it lacks both a membrane-proximal α3 domain and β2m association. Furthermore, unlike both classical and non-classical class I MHC molecules, the α1α2 domain of EPCR has evolved highly hydrophobic channels, enabling it to bind lipids in an analogous fashion to CD1 molecules. Direct Vγ4Vδ5 LES TCR/EPCR recognition was validated by surface plasmon resonance protein binding studies, indicating a low K_d of ~100 μM, allowing extensive mutagenesis that identified the TCR interaction site on the "underside" of the lipid-binding α1α2 platform (Figure 3G) and did not appear to be directly affected by the nature of the bound lipid present.[74]

Several features of LES TCR/EPCR recognition appear to align closely with characteristic features of the adaptive-like Vδ2[neg] compartment that have emerged in subsequent years. Firstly, the LES clonotype was heavily clonally expanded in the individual it was derived from, representing 50% of total T cells, its phenotype was CD28^{neg}CD45RO^{neg},[29] and it displayed TCR/ligand-dependent cytotoxicity to CMV-infected target cells,[27] suggesting strongly it belonged to the T$_{effector}$ subpopulation of Vδ2^{neg} T cells[7,30] and was antigen-experienced. Secondly, this clonal expansion occurred in the context of acute CMV infection, which has been shown to induce both clonal expansions[6,7,26,32] and accompanying shifts from T$_{naive}$ to T$_{effector}$ status in adaptive-like TCR subsets.[6,28] Finally, consistent

with the prediction that such clonotypically focussed responses are driven by CDR3-mediated TCR/ligand recognition events, mutation of either LES TCRγ or TCRδ CDR3 loops completely abrogates LES/EPCR interaction[54] (Figure 3H). As for other γδ TCR ligands, recognition was dependent on a folded conformation of EPCR.[74]

Given these features, LES/EPCR interaction may feasibly hold wider significance as a canonical example of adaptive T$_{effector}$ γδ TCR ligand recognition. In this context, it is noteworthy that EPCR is expressed on endothelial cells, a key site of CMV infection in vivo, and is also upregulated on certain tumor cell lines,[115] some of which can activate the LES clone and LES TCR-transfected reporter lines. Despite the low affinity of LES/EPCR interaction, it was essential for the LES clone's ability to specifically recognize CMV-infected target cells, which was potentiated by a CMV-induced multimolecular stress signature in target cells that included upregulation of adhesion/costimulatory ligands.[74] It was similarly essential for LES TCR-mediated recognition of appropriate EPCR[+] tumor target cells. The relatively short dimensions of the EPCR ectodomain, which are most likely similar to those of eg UL16 Binding Proteins (encoded by the *RAET1* genes) molecules, may ameliorate the impact of its low affinity for TCR, as this may aid retention of TCR/EPCR complexes within close contact zones favorable for TCR triggering. Interestingly, interaction of LES TCR with the "underside" of the EPCR platform again distances LES/EPCR recognition from classical αβ TCR/MHC interaction, and suggests it is more akin to a low affinity cell-surface Fab/ligand interaction (Figure 3H). Finally, the private nature of the LES TCR sequence is entirely in keeping with the high levels of sequence diversity observed in adaptive-like γδ TCR repertoires. This can be rationalized by that fact that recombination of the LES Vδ TCR chain required a high number of non-germline nucleotides (16), a number also characteristic of the Vδ1 TCR repertoire as a whole (average ~15.5 nucleotides/chain).[7] Privacy of the LES clonotype provides a potential explanation for the failure to detect EPCR reactivities in other patients,[74] although in principle one might expect other TCR clonotypes to also permit EPCR interaction and analogous CMV-specific γδ T cell responses. While technical challenges may underlie this failure, the extent to which the γδ T cell response relies on private ligands rather than commonly recognized targets is unclear.

3.2.3 | Non-MHC-like molecules

A disparate collection of non-MHC-like proteins have been proposed and in several cases confirmed as direct γδ TCR ligands (Figure 4).

Annexin A2 and other host-encoded ligands

Additional studies have shed light on a number of other host-encoded TCR ligands that potentially play a role either in the γδ T cell response to CMV, or to transformed cells. These include Annexin A2, which was found to be a ligand for the 73R9 Vγ8Vδ3 TCR[116] using a blocking antibody approach similar to that used for identification of EPCR.[74] A predominantly intracellular protein that binds phospholipids in a Ca^{2+}-dependent manner (Figure 4A), Annexin A2 can translocate to the cell
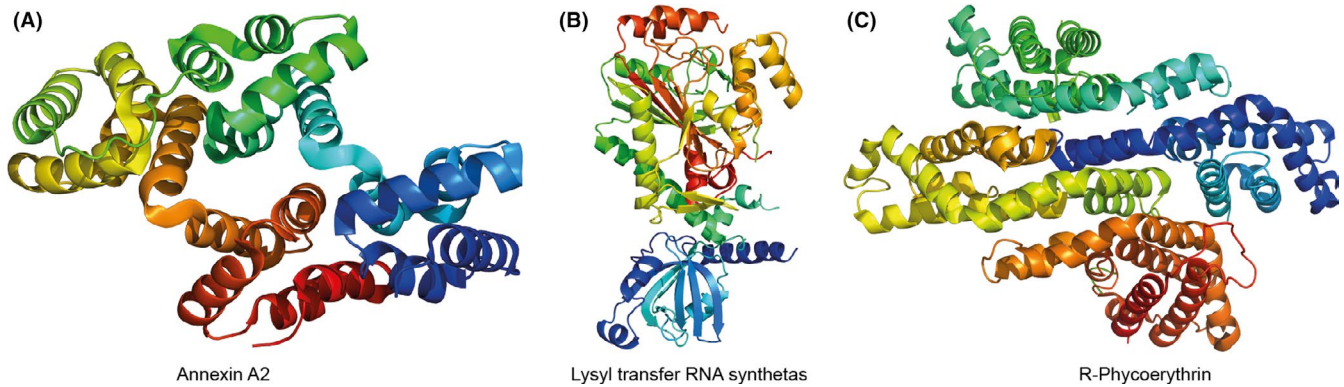
FIGURE 4 Non-MHC-like molecules that may serve as direct ligands for γδ TCRs. A-C, Cartoon representation of Annexin A2 (PDB ID: 2HYW), lysyl transfer RNA synthetase (PDB ID: 1BBW) and R-phycoerythrin (PDB ID: 1EYX)

surface as part of a heterotetrameric complex with S100A10. The 73R9 T cell clone from which this TCR derived was generated from healthy donors by mixed lymphocyte culture of PBL in the presence of Burkitt lymphoma cells. Transfection studies showed the 73R9 TCR could confer reactivity of reporter lines to specific target cells. This reactivity correlated with staining of target cells by a blocking antibody hypothesized to recognize the putative target ligand. Immunoprecipitation studies ultimately identified Annexin A2 as the putative ligand and direct TCR/Annexin A2 binding was confirmed using surface plasmon resonance. Interestingly, exposure of target cells to a number of stress stimuli, including hypoxia, heat shock, and high confluence growth, increased Annexin A2 translocation to the surface and consequent 73R9 reactivity, most probably partly via increases in oxidative stress linked to ROS. In addition, CMV infection of target cells also increased Annexin A2 cell-surface expression and 73R9 reactivity. These observations suggest that Annexin A2 translocation to the cell surface could represent a unified stress signal for the 73R9 TCR. Although two other Annexin A2-specific T cell clones were reported by Marlin et al,[116] and Annexin A2 was reported to induce proliferation of a small population of Vδ2^{neg} T cells, it is unclear whether these cells align to antigen-inexperienced T[naive] or antigen-experienced T[effector] subsets, or whether they reflect clonotypes expanded in vivo. Therefore, the full significance of Annexin A2 as a focus of adaptive γδ T cell responses, as opposed to a potential in vivo reactivity, is unclear. In addition to Annexin A2, Vδ2[neg] TCR reactivity to the receptor tyrosine kinase EphA2,[101] a well-established tumor associated antigen, as well as to abnormal forms of class I MHC molecules,[117] has also been proposed in the context of CMV and/or tumor cell recognition, however these specificities await full description.

Aminoacyl transfer RNA synthetases in autoimmune myositis

Autoimmunity has also been proposed as an alternative context in which Vδ2[neg] T cell ligand recognition might drive physiologically important immune responses. Studies on a single patient with polymyositis, a disease characterized by autoaggressive T cells that destroy muscle fibres, highlighted profound infiltration of γδ T cells surrounding and invading non-necrotic muscle fibers, suggesting a γδ-mediated pathology.[118] Subsequent work defined a single Vγ3-Jγ1 Vδ2-Jδ3 TCR, subsequently termed M88, which dominated the muscle-infiltrating T cells.[119]

Following these studies, Bruder et al[120] identified aminoacyl transfer RNA synthetases (AA-RS) (Figure 4B) as candidate ligands for this TCR, since purified immobilized forms of AA-RS were capable of stimulating M88-expressing T cell hybridomas. Interestingly, antigens recognized included human AA-RS for histidine and alanine, both of which are established autoantibody targets for polymyositis, but also bacterial AA-RS and a bacterial initiation factor, *E coli* IF1. Although TCR/ligand binding was not validated by direct binding assays, mutagenesis studies involving IF1 suggested recognition of a similar conformational rather than linear epitope in each case, which as for recognition of other adaptive-like TCR ligands, and was dependent on CDR3 regions of both the Vγ and Vδ chains of M88. These observations suggested γδ T cell-driven autoimmunity may have been triggered by responses to bacterial infection.

Although these studies focus on a single patient with γδ T cell-mediated myositis, they appear to align strongly with important features of adaptive-like γδ subsets. The TCR usage of the M88 clone matches that of the adaptive-like Vγ9[neg]Vδ2 population recently highlighted[6] in human peripheral blood. In addition, the clonal amplification and suggestion of cytotoxicity of these muscle-infiltrating cells strongly indicates a T[effector] phenotype. This conclusion is consistent with changes in homing markers from T[naive] to T[effector] state shown for both Vδ1 and Vγ9[neg]Vδ2 T cells, which involve downregulation of central homing markers (CD62L, CCR7), and upregulation of CX₃CR1, which could aid recruitment to endothelium and peripheral tissues.[6,7] Indeed Davey *et al* noted the presence of clonally expanded Vγ9[neg]Vδ2 T cells in liver,[6] and transition to a T[effector] phenotype concomitant with ligand-driven clonal expansion could explain both infiltration of muscle tissue and myocytotoxicity, and would be consistent with a CDR3 dependent antigen recognition event.

Phycoerythrin and other foreign antigens

Phycoerythrin (PE) is a red pigmented protein belonging to a family of light-harvesting proteins called phycobilins present in algal species. Exploited previously as a model foreign immunogen to study induction of antigen-specific B cell memory in mice,[121] Zeng et al used PE to study antigen-specific γδ T cell recognition[122] (Figure 4C). PE was found to stain γδ T cells in both naïve mice and human peripheral blood, at frequencies (0.04% in mice, ~0.025% in human T cells),

broadly comparable to B cells in naïve animals (~0.1%). PE-specific TCRs bound to the intact antigen, as T cell staining was blockable by preincubation by anti-PE Fab, indicating that as for B cells, no antigen processing and presentation was required.[122] Importantly, TCR interaction with PE was confirmed for murine TCRs in direct protein binding assays, with a K_d of 2.7 µM. However, the hexameric nature of PE enabled its use as a cell staining reagent, resulting in an apparent K_d of 0.3 nM for PE antigen-specific T cells.[122]

Of relevance, staining of human γδ T cells was exclusively to the adaptive-like Vδ1 T cell compartment, which exhibits high CDR3 diversity. The low frequency of the populations detected suggest they are unlikely to account for dominant clonal expansions typically observed in this compartment[7,32] and instead may derive from the T_{naive} compartment and reflect an antigen-inexperienced state. This conclusion is consistent with exploitation of PE as a model foreign antigen unlikely to be encountered naturally in humans. Consistent with this, most PE-specific T cells in unimmunized mice displayed a naïve-like phenotype,[122] and this study also highlighted a potential for immunization of mice to switch phenotype from a naïve-like one (CD62L+ CD44lo phenotype) to one associated with activation/Ag-exposure (CD62Llo, CD44+), and to induce changes in cytokine expression (IL-17) and cytokine receptor expression (IL-1, IL-23), allowing subsequent amplification of the IL-17 response. A further study used model hapten antigens Cyanine3 (Cy3) and 4-hydroxy-3-nitrophenylacetyl (NP) to identify similar percentages of reactive γδ T cells in humans and mice, which as for PE reactivity mapped to the Vδ1 T cell subset.[123]

The findings outlined above are important in highlighting the potential of the human peripheral blood adaptive-like compartment (but notably not the semi-invariant Vγ9Vδ2 subset, which is numerically dominant in peripheral blood) to contain specificities for foreign, microbially derived antigens. This parallels previous studies defining γδ T cell specificities capable of recognizing herpes simplex virus (HSV) glycoprotein I (gI).[124] In addition, the impact of immunization of mice with PE aligns with subsequent studies on human adaptive-like T cells, which also suggest the potential for an immune stimulus (eg CMV infection) to induce differentiation of antigen-specific effectors with increased potential for cytokine production and altered homing capabilities.[6,32] The findings also emphasize recurring themes of CDR3-dependent recognition, a hallmark of adaptive antigen-specific recognition, and, as for HSV gI and other γδ TCR ligands, recognition of intact antigen. While likely highly relevant to foreign antigen recognition, an important caveat of Zeng et al's results[122,123] is that they also highlight the potential to detect antigen-specific γδ T cell populations for ligands that are unlikely to ever be encountered physiologically, based on the extreme CDR3-focussed sequence diversity present within adaptive TCR repertoires.

4 | IMMUNOBIOLOGICAL AND EVOLUTIONARY "NICHE"

The retention of γδ T cells as distinct lineage alongside αβ T cells and B cells during 500 million years of vertebrate evolution suggests they provide important non-redundant contributions to vertebrate immune defense. A full appreciation of the niche(s) occupied by γδ T cells in vertebrate immunity will require a more complete picture of how they utilize their TCR to recognize target cells. Nevertheless, the current evidence regarding their immunobiology and recognition capabilities suggests some possibilities.

The distinct modes of antigen recognition they employ is an obvious but critical feature that distinguishes γδ T cells from not only αβ T cells, but also B cells and NK cells. Use of a unique somatically recombined γδ TCR is highly likely to provide the immune system with evolutionarily advantageous recognition capabilities. Like antibodies but unlike the αβ TCR, the γδ TCR enables MHC-independent recognition of unprocessed antigen. However like αβ T cells and unlike antibodies, γδ TCR-mediated interactions inherently occur in the context of cell-cell recognition. Therefore, from a simplistic extreme, the γδ TCR could arguably be regarded as arming γδ T cells with a cell-surface "Fab-like" repertoire, rendering γδ T cells arguably analogous to "Nature's CAR-Ts" (Figure 5).

The modes of TCR-mediated antigen recognition utilized by γδ T cells, particularly given diversification of the compartment to include both innate-like and adaptive subsets, appear highly relevant in a range of physiologically relevant scenarios. Innate-like paradigms involving recognition of tissue-specific BTN-family members are likely to populate specific sites in the body with γδ subsets employing distinct homeostatic and/or effector functions. Such tissue-specific subsets and their respective TCR/ligand recognition modes are most probably highly specialized to specific microenvironmental niches. Recruitment to such sites can occur from an early age[8,68] and could help pre-populate them with appropriate immunoprotective lymphocytes before adaptive responses have occurred that drive appropriate effector populations into tissues. Therefore these tissue-specific γδ populations may truly represent a first line of defense, but have also likely evolved roles in tissue homeostasis.

γδ T cell subsets where this paradigm most likely applies include mouse DETC, which are implicated in protection from cutaneous malignancy[125] and also cutaneous tissue repair processes,[71] but also intestinal Btnl/BTNL-reactive γδs (Vγ7 T cells in mice, Vγ4 in human),[8] and human Vγ9Vδ2 T cells that dominate peripheral blood.[15] Vγ9Vδ2 T cells are particularly apt, as they respond to the presence of key pyrophosphate metabolites (pAg) of many pathogenic bacteria, mycobacteria and even malaria.[16] The canonical foreign pAg HMBPP essentially represents a PAMP, and although there is still variability in the Vγ9Vδ2 TCR repertoire, its semi-invariant features enable the Vγ9Vδ2 TCR to act as a surrogate pattern recognition receptor.[15] This is a clear example of innate-like γδ T cell biology providing a unique recognition capability over and above that achieved by αβ, B cells and NKs, and its significance extends beyond peripheral blood as after activation, Vγ9Vδ2 T cells can traffic into solid tissues.[126]

Adaptive-like γδ T cell subsets have been linked to a number of different pathogen infections, including viruses such as CMV and EBV, and also to parasite infections such as malaria.[30,31,127] For a pathogen such as CMV that is able to potently suppress αβ T cell responses by inhibiting the MHC antigen presentation pathway at
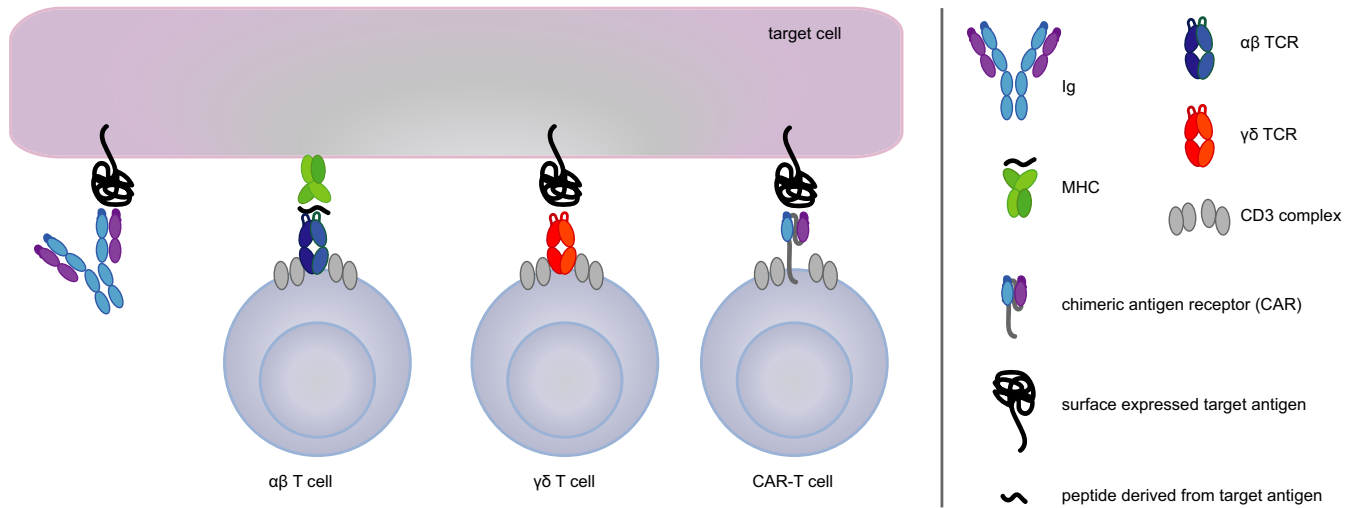
FIGURE 5 Distinct modes of antigen recognition by lymphocyte lineages bearing somatically recombined antigen receptors. Unlike αβ T cells, which recognize antigenic peptides presented by MHC molecules, γδ T cells are thought to recognize intact surface antigens. Antibodies can also recognize intact surface antigen, but are not linked to cell-mediated immune mechanisms. Similar to γδ T cells, T cells expressing engineered chimeric antigen receptors (CAR-T) link recognition of intact surface antigen to cell-mediated immunity

multiple points,[128] the availability of an alternative and non-MHC-restricted lymphocyte compartment capable of similarly potent cell-cell recognition, cytotoxicity, and cytokine production is likely to be physiologically important.[21] Consistent with this, in vivo mouse data show the γδ T cell compartment can provide as potent long-term protective responses to CMV as αβ T cells and B cells.[39] Moreover, MHC-independent T cell-target cell recognition is likely to be highly relevant in other infectious challenges, such as *P. falciparum*, where infected red blood cells, which lack MHC molecules, are potentially a critical immune target.[127] Consistent with this, a recent study reports clonal expansions and activation in adaptive-like γδ T cell subsets in response to *P. falciparum* infection/vaccination.[33]

While such arguments emphasize advantages relative to αβ T cells, what advantages would such adaptive-like T cell responses provide compared to NK cell-mediated responses, which appear well adapted to recognize dysregulated target cells including specifically those that downregulate MHC molecules[129]? A strong hint is again provided by studies on CMV. In this context, NK responses are, like MHC-restricted αβ T cells, a major focus of potent CMV immunosuppression, involving numerous virus gene products, many of which downregulate or sequester ligands for activatory NK receptors.[130] However, crucially, NK cells appear particularly susceptible to pathogen immune evasion, since they ultimately rely on germline-encoded receptors[131,132] to recognize virally infected target cells. In contrast, the highly diverse TCR repertoires of somatically recombined adaptive-like γδ T cell subsets are likely to prove substantially less susceptible to immune evasion, potentially enabling responses against either host-encoded or pathogen-encoded targets, including those which may display high variability.

In summary, the ability of γδ T cells to exploit a somatically recombined antigen receptor to initiate powerful cell-mediated effector responses in a way that is not dependent on target-expressed

MHC molecules is likely to provide a major evolutionary advantage when present in combination with αβ T cells, NK cells, and B cells. The unique antigen recognition modes and distinct immunobiologies of different γδ T cell subsets provide the vertebrate immune system with unique immunosurveillance and immunoregulation capabilities in the face of highly divergent immune challenges.

5 | FUTURE DIRECTIONS

Much progress has been made in the last 10 years in understanding both the fundamental immunobiology of γδ T cells, and how γδ T cell recognition takes place at a molecular level, including knowledge of some γδ TCR ligands.

These studies highlight the ability of γδ T cells to carry out cell-mediated recognition of intact antigens on target cells, using a repertoire of clonally expressed recombined Ig-like receptors that is analogous to a surface-expressed antibody Fab repertoire. Despite this, a comparison with CAR-T cells should not be overstated. Clearly CAR-T cells[133] are artificially engineered, and their targets rationally selected. In contrast, γδ T cells have arisen naturally in evolution, and the targets they recognize have arisen in this context. Moreover, some "innate-like" γδ subsets have evolved to exploit conserved elements of the TCR for interactions with BTN/BTNL molecules on target cells that mimic Fab/superantigen interactions.[17,18,54] To our knowledge, such recognition modes have yet to be exploited in current CAR-T design. In addition, for "adaptive-like" subsets, selective expansion of particular specificities from a diverse repertoire of γδ TCR clonotypes likely drives physiologically relevant responses to particular pathogens. This might feasibly involve direct recognition of a combination of pathogen-encoded targets and of host components dysregulated by infection. Analogous processes may result in

responses to non-microbial stimuli, for example via recognition of endogenous antigens altered or upregulated in disease states.[134] Although these likely occur via CDR3-mediated recognition of intact cell-surface antigens as for Fab/ligand interaction, they are orders of magnitude lower in affinity. Furthermore, for Vγ9Vδ2 T cells, it seems likely that the TCR can act as a "smart" receptor by engaging BTN2A1 alongside CDR3-mediated recognition of separate moieties as part of a composite ligand.[17,18] It comes as no surprise that evolution has resulted in a level of sophistication in the γδ TCR that exceeds current CAR approaches, which largely exploit single-chain fragment variable (scFv) specificities for single antigenic targets. Nevertheless, despite these differences, the analogy with CAR-T cells does emphasize how the defining feature of γδ T cells, namely their somatically recombined γδ TCR, provides them with a unique niche in the immune system relative to αβ T cells, B cells, and NK cells, and is a useful perspective from which to plan future studies.

For innate-like subsets, it will be crucial to establish to what extent Vγ-restricted recognition of specific BTN/BTNL/B7-like proteins extends beyond blood and gut.[8,17,18,54] Based on the predominant Vγ9 subset in the peripheral blood and Vγ4/Vγ7 population in gut, the prevalence of particular Vγ regions might provide clues as to the distinct innate-like subsets present in different sites. Furthermore, it will be important to assess if parallel innate-like and adaptive-like γδ arms operate in many tissues around the body, as appears to be the case in blood.[15,30] In addition, a major question going forward is if BTN/BTNL recognition by the TCR frequently occurs in isolation, or whether alternatively, and as hypothesized for the Vγ9Vδ2 T cell subset, BTN/BTNL proteins may provide a scaffold for simultaneous recruitment and recognition of CDR3-mediated ligands.[17] Aside from Vγ9Vδ2 T cells, reactivity of individual Vγ4/Vγ7 γδ TCRs to both tissue-specific BTNL molecules and exemplar clonotypically restricted antigens could conceivably reflect a fundamental functional and physiological link between these two recognition modalities. However, alternatively such dual reactivity may simply reflect simultaneous exploitation by the γδ TCR repertoire of certain Vγ regions for parallel but separate innate-like and adaptive arms of the γδ T cell response. Resolving such issues should also help to clarify whether individual γδ T cell subsets necessarily operate exclusively via either an innate-like or adaptive-like paradigm, or alternatively whether some possess the potential for "dual functionality," responding in an innate-like fashion in some situations and an adaptive-like mode in others. For adaptive-like subsets, a key question is the identity of relevant antigenic targets, which likely reflect low affinity Fab-like recognition of a range of targets on the surface of infected/dysregulated cell types. However, understanding how such ligands denote infected/dysregulated self during physiological responses is arguably as important a goal. Finally, while the field is at a relatively early stage in attempts to exploit γδ T cell biology therapeutically, the distinct immunobiology of this compartment, its lack of MHC restriction, and capacity for potent effector functionality suggest exciting developments lie ahead in the context of both infection and cancer.

CONFLICT OF INTEREST

I have no conflict of interest with regard to the topics discussed in this review manuscripts.

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