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Human gamma delta T cells: evolution and ligand recognition

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Abstract

The $\gamma\delta$ T cell lineage in humans remains much of an enigma due to the low number of defined antigens, the non-canonical ways in which these cells respond to their environment and difficulty in tracking this population *in vivo*. In this review, we survey a comparative evolutionary analysis of the primate V, D and J gene segments and contrast these findings with recent progress in T cells in humans. Signatures of both defining antigen recognition by different populations of $\gamma\delta$ purifying and diversifying selection at the V δ and V γ gene loci are placed into context of V δ 1+ $\gamma\delta$ T cell recognition of CD1d presenting different lipids, and V γ 9V δ 2 T cell modulation by pyrophosphate-based phosphoantigens through the butyrophilins BTN3A. From this comparison, it is clear that co-evolution between $\gamma\delta$ TCRs and these ligands is likely occurring, but the diversity inherent in these recombined receptors is an important feature in ligand surveillance.

Keywords

Vdelta1; CD1d; Vgamma9Vdelta2; phosphoantigens; T cells; T cell receptor; butyrophilin; B30.2

There are three main lineages of lymphocytes in jawed vertebrates that use genetically recombined receptors to survey their environment and mediate host defense against disease: B cells, $\alpha\beta$ T cells and $\gamma\delta$ T cells. Similar, analogous lineages have been found in jawless vertebrates that use an entirely different family of receptors for surveillance [1]. The best studied of the T cell lineages are those expressing an $\alpha\beta$ TCR; within this lineage we know most about those $\alpha\beta$ T cells that are often described as “conventional”; those that express either CD4 or CD8 co-receptors and interact with the classical Major Histocompatibility Complex (MHC) molecules presenting peptides (MHCp). These T cells are generally categorized as either “helper CD4+ T cells”, secreting cytokines to modulate other immune cells, or are “killer CD8+ T cells”, exhibiting cytotoxicity towards the target cell(s). Less

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well understood are the specialized populations of $\alpha\beta$ T cells that recognize non-peptide presenting MHC molecules and are often found at high frequencies in particular tissues or organs. Many of these $\alpha\beta$ T cells express T cell receptors that have low variation, using only a small sampling of the Variable gene segments available during somatic rearrangement. These semi-invariant populations include invariant Natural Killer T (iNKT) cells and Mucosal Associated Invariant T (MAIT) cells.

Even more enigmatic are the cells that express a $\gamma\delta$ TCR; despite decades of research we still have little information regarding how many defined $\gamma\delta$ populations exist, what antigens/ligands they respond to and what roles they play in host defense and homeostasis. It is unclear whether we can extrapolate what we know from the study of $\alpha\beta$ T cell populations to that of the $\gamma\delta$ lineage; are there “classical” $\alpha\beta$ T cell equivalents that recognize diverse antigens in polymorphic antigen-presenting molecules? Are there semi-invariant $\gamma\delta$ T cell populations restricted to non-polymorphic antigen-presenting molecules? Do $\gamma\delta$ T cells use their recombined receptors to recognize antigen in an antibody-like fashion? This review focuses on the evolutionary pressures that have shaped the diversity of the γ and δ gene segments in primates (in relation to humans) and extrapolates this to the limited information we have on the molecular recognition of V δ 1+ and V δ 2+ human $\gamma\delta$ T cells.

In humans, $\gamma\delta$ T cells have a small repertoire of V gene segments to select from when undergoing chain rearrangement in comparison with those available for V α (43–45 [2]), V β (40–48 [3]), Ig light (IgV κ 34–38 [4], IgV λ 29–33 [5]), or Ig heavy (38–46 [6]) chain rearrangement. Three main V δ gene segments, V δ 1, V δ 2 and V δ 3, are most frequently used in rearrangement of the δ chain; less commonly used are the five V segments that have both V δ and V α designation (V δ 4/TRAV14, V δ 5/TRAV29, V δ 6/TRAV23, V δ 7/TRAV36 and V δ 8/TRAV38) [7]). This designation is in part due to the location of the δ locus within the α locus on chromosome 14 in humans. Seven functional V γ gene segments, V γ 2, V γ 3, V γ 4, V γ 5, V γ 8, V γ 9 and V γ 11, located within the γ locus on chromosome 7 in humans, are used for rearrangement of the γ chain. Several V γ pseudogenes are also found in the γ locus in humans and are not used in productively arranged $\gamma\delta$ TCRs: V γ 1, V γ 5P, V γ 6, V γ 7 and V γ 10. As discussed in more detail below, these do not all appear to be pseudogenes in other higher primates and at least one (V γ 10) has been found in productively rearranged γ chain transcripts in the chimpanzee [8]. The restricted repertoire of V δ and V γ gene segments available for rearrangement has led to speculation that these TCRs recognize conserved self-proteins of low variability [9]. This is further supported by the observation of particular V δ and V γ pairing requirements in the mouse [10] although pairing biases have not been experimentally observed in humans. This idea of limited $\gamma\delta$ TCR diversity was confounded by the discovery that δ chain rearrangement allows for the incorporation of multiple D δ segments (in forward and reverse direction) [11, 12] such that the loop encoded by this rearrangement, the CDR3 δ , is theoretically the most diverse, in sequence and in length, CDR3 loop of all the rearranged receptors [13]. Indeed, more recent discussion has compared this feature of the CDR3 δ loop to antibodies [14], along with the demonstration of “antibody-like” recognition of foreign proteins by some $\gamma\delta$ TCRs [15].

Comparisons between the δ and γ V, D and J gene segments within the primate species has revealed an interesting pattern of how these loci have evolved [16] (Figure 1, upper left

panel). The gene segments of the δ locus have not changed substantially in the primate lineage from humans to marmosets; the gene order, overall, is conserved and most of the genes remain functional with few duplications or deletions. This holds true for the δ locus V gene segments as well as the D and J gene segments, which are located between the V δ 2 and V δ 3 gene segments. This can be further visualized through dot-plot comparisons of the entire locus; where the full length genomic sequences are compared across species and black dots indicate regions of high sequence identity (>90%) and grey, slightly lower (>80%) and displayed on a dot-matrix [16]. For the δ locus, the dot matrix reveals a prominent diagonal line, indicating high correlation between the gene organization and sequence from human to marmoset (Figure 1, lower panel). Regions of deletion or insertion are visualized as small breaks or gaps in the diagonal line.

In contrast, the pattern of gene organization across primate species is quite different at the γ locus. Three V γ gene segments, V γ 9, V γ 10 and V γ 11 (these were previously designated as groups II, III and IV, respectively [17]) are found in highly conserved positions in each of the primate genomic sequences examined, with the exception of V γ 11, which is a pseudogene in macaque and is missing from the marmoset genome [16]. The remaining group 1 V γ genes (V γ 1–V γ 8) cluster together. Surprisingly, the positioning and sequence homology of this group of genes quickly diverge in the genomic sequences of even the most closely related species to humans, the great apes (Figure 1, upper right panel). For example, the V γ 5P pseudogene is only present in humans and, in the orangutan, it is difficult to assign homology to human V γ 5, V γ 3, V γ 4 and V γ 2 (they are thus designated V γ 3/5, V γ 5/3 and V γ 4/2). Dot plot analysis reveals the close sequence homology between the group 1 V γ sequences, which is the product of the gene duplications, deletions and/or genetic exchange between them that has occurred recently in primate evolution [16] (Figure 1, lower panel). Phylogenetic analysis of the sequences of the V gene segments [16] reflect the conclusions derived from the dot plot analysis: the V δ gene segments group together with long branch lengths (reflecting evolutionary distance) and strong statistical support (bootstrapping analysis), in some cases including homologues from mouse (Figure 2, left panel). The phylogenetic tree of the V γ gene segments (Figure 2, right panel) reflect the dichotomy observed in the genomic organization across species; the V γ 9, V γ 10 and V γ 11 sequences group together with well-supported, long branch lengths similar to those of V δ , whereas the group 1 V γ sequences form a bush-like structural grouping, containing subgroups within consisting of the V γ 1, V γ 2/4, V γ 3/5, V γ 6, V γ 7 and V γ 8 sequences with very short branch lengths.

Evolutionary comparisons such as these provide insight into the selective pressures that shape genes or gene loci. Gene duplication, early on, was recognized as an ideal form of adaptive evolution [18] and has been widely observed in genes that participate in an organism's adaptation to a quickly changing environment. The highly polymorphic class I genes of the human MHC, HLA-A, -B and -C [19] have also been the product of frequent duplication and deletion, such that conservation of these genes is lost, similar to that of the group 1 V γ gene segments, the further out in primate evolution one explores [20]. The question that arises, then, is what has driven the rapid evolution of these group 1 gene segments during primate evolution? Why is this region so dynamic, where as the V γ 9, V γ 10 and V γ 11 gene segments, located only ~10 kilobases away, and the V δ gene segments have

remained so static? These intriguing patterns of evolution are most relevant when placed in the context of antigen recognition. While we are making progress on defining antigens for $\gamma\delta$ T cells in humans (see [14] for a comprehensive review of known antigens), unfortunately only a few of these have been successfully explored at the structural level. Below we focus on two of the three major V δ domains, V δ 1 and V δ 2, and the progress that has been made thus far in understanding antigen recognition by the T cells that utilize these domains in their TCRs. First, we will focus on recognition of the MHC-like protein CD1d by V δ 1+ T cells (both $\gamma\delta$ and $\alpha\beta$ [21]) and then will turn to the recent progress on understanding the modulation of the V γ 9V δ 2 T cell population by small pyrophosphate containing organic molecules called phosphoantigens.

V δ 1+ T cell recognition of CD1d

$\gamma\delta$ T cells expressing a V δ 1 domain paired with various γ chains represent more than 50% of fetal blood $\gamma\delta$ T cells at birth [22]. In adults, V δ 1+ $\gamma\delta$ T cells constitute a minority of the blood $\gamma\delta$ T cell population and instead mainly populate epithelial tissues, to a large extent the intestine [23], and are also found responsive to epithelial tumors [24–26] and lymphomas [27, 28]. V δ 1+ $\gamma\delta$ T cells have been reported to recognize several different members of the MHC superfamily family [29], all of which are outside the classical MHC family and are deemed “MHC-like” [30]. Some present antigens, such as the CD1 family presenting lipids, while others are stress-induced and do not appear to present variable ligands. One of the CD1 family members, CD1c, was the first $\gamma\delta$ ligand identified in humans [31] and more recently CD1d has been shown to be a ligand for at least a subset of both V δ 1+ and V δ 3+ $\gamma\delta$ T cells [32–35]. Indeed, CD1d is the only shared ligand between $\gamma\delta$ T cells in mouse and humans described to date [36] suggesting an important, and conserved, role for CD1 molecules in $\gamma\delta$ T cell surveillance. Many of the CD1 reactive V δ 1+ $\gamma\delta$ T cells respond to CD1 molecules presenting endogenous lipids [35, 37, 38], suggesting that many V δ 1+ $\gamma\delta$ T cells are capable of autoreactivity.

Recent structural studies on CD1 recognition by V δ 1+ $\gamma\delta$ T cells derived from work initiated by tetramer-based, fishing strategies on human blood donated from healthy donors [39, 40]. These studies used either CD1d loaded with the lipid sulfatide [33], a lipid abundant in tissues such as the brain, kidney and gastrointestinal tract [41], or α -galactosylceramide (α GalCer) [40], a glycolipid generated at low levels endogenously [42] but originally identified as a potent agonist of the invariant Natural Killer T (iNKT) cell population [43]. Of the peripheral blood T cells that stained with the CD1d-sulfatide tetramer, on average 80% of these were V δ 1+ $\gamma\delta$ T cells; in contrast, the majority of CD1d- α GalCer tetramer-positive cells were iNKT cells, although small numbers of CD1d- α GalCer+ V δ 1+ T cells were identified in all individuals examined. It was later noted that a significant proportion of these V δ 1+ CD1d- α GalCer T cells were actually $\alpha\beta$ T cells using a V δ 1 gene fragment rearranged with J α -C α [21]; this population will be discussed more later. In each of these studies, single T cell clones were derived and were activated by their respective ligands when loaded in CD1d. Cloning of these T cells also enabled the isolation of the TCR sequences and thus further molecular and structural characterization of their interactions with CD1d and their lipid antigens.

The affinities of the TCRs for CD1d-ligand were derived through use of recombinantly expressed TCRs and CD1d protein loaded with their respective ligands. For CD1d-sulfatide, two separate TCRs were selected for study due to their use of different V γ chains, DP10.7 (V δ 1V γ 4) and AB18.1 (V δ 1V γ 5). The DP10.7 TCR had exquisite specificity for sulfatide (K_D of $\sim$ 5 μ M) as binding to CD1d loaded with other lipids; even β -galactosylceramide (structurally identical to sulfatide except lacking the sulfate) was not detectable [39]. In contrast, the AB18.1 TCR preferentially bound CD1d-sulfatide (K_D of $\sim$ 9 μ M) yet also bound CD1d-loaded with endogenous lipids (“unloaded”, K_D of $\sim$ 20 μ M), indicating a likely bias for the CD1d molecule and a “tolerance” for alternative lipids. For CD1d- α GalCer, one TCR clone was studied in detail, 9C2 (V δ 1V γ 5) [40], this preferentially bound CD1d- α GalCer (K_D of $\sim$ 16 μ M) but also bound CD1d-unloaded with lower affinity (K_D of $\sim$ 35 μ M), consistent with the cross-reactivity observed with the AB18.1 TCR that also contains a V γ 5 domain in its TCR. A thorough alanine-scanning mutagenesis strategy of the DP10.7-CD1d-sulfatide interaction failed to reveal any “hot-spot” residues (those that when mutated to alanine abrogate binding), suggesting that the binding energy of the DP10.7 TCR for CD1d was widely distributed over the assembly of contacts established in the complex structure.

To more fully understand the atomic interactions that mediate the interactions between these V δ 1+ γ δ TCRs and CD1d-sulfatide or CD1d- α GalCer, the structures of their complexes were determined at 3.0Å [39] and 2.9Å [40], respectively. While these two structures have several important differences between them, they overall have a similar global docking orientation that differs from that of other TCR/MHC interactions (Figure 3). Both V δ 1+ γ δ TCRs dock onto the CD1d surface over the A' tunnel, with an extensive bias towards use of the V δ 1 domain. Their overall diagonal docking orientation is reminiscent of that seen with conventional $\alpha\beta$ TCR/classical MHC-peptide structures (Figure 3, third panel from left), but is quite different than that seen for the iNKT TCR/CD1d- α GalCer complex [44] where the iNKT TCR is focused predominantly over the F' tunnel (Figure 3, fourth panel from left). Surprisingly, the V δ 1+ γ δ TCR structures are similar to that of the Type II NKT TCR structures with CD1d-sulfatide and lysosulfatide [45, 46], where the focus is again over the A' tunnel with a similar docking angle (Figure 3, far right panel). Both V δ 1+ γ δ TCRs have a clear bias towards use of their V δ 1 domains in contacting antigen. In fact, all contacts with CD1d-sulfatide by the DP10.7 TCR are mediated by the V δ 1 domain CDR loops [39] (Figure 4A, 4B left panel). In the interaction network between the 9C2 TCR with CD1d- α GalCer, the V δ 1 domain contributed 75% of the buried surface area (25% contributed by the γ domain) (Figure 4B, right panel). Furthermore, in both complexes there is a predominant usage of the CDR1 δ loop in engaging CD1d, in particular the residue Trp30, which is responsible for the majority of the footprint in both structures. The importance of the CDR1 δ loop in V δ 1 recognition of CD1d is further exemplified in the recent structure of the V δ 1/ $\alpha\beta$ TCR in complex with CD1d- α GalCer [21], discussed in more detail below. This correlates well with the relative conservation of the V δ 1 CDR1 loop in evolutionary comparisons [16]; when examining coding (nonsynonymous) versus silent (synonymous) changes across species in this region, the ratio between these was below 1, suggestive of purifying selection at this region. Curiously, this phenomenon did not extend to the CDR2 δ loop, located only $\sim$ 20 amino acids away, which exhibited extensive diversifying selection

with only coding substitutions noted. An additional similarity between the two structures is the use of the recombined, highly variable, CDR3 loops in contacting the lipid ligand. In the DP10.7 complex structure, contact with the lipid ligand is mediated by junctional residues encoded within the CDR3 δ loop and contact with α GalCer in the 9C2 structure is through the CDR3 γ loop. Use of these recombined loops in contacting the lipid ligand suggest that CDR3 loop variability is an important feature in V δ 1+ γ δ T cell surveillance of lipids in the context of CD1d molecules. Perhaps these complexes represent only a small proportion of the V δ 1+ γ δ T cell/CD1d interactions with others dependent on presentation of other, yet defined, lipids by CD1d.

Despite the similarities in domain bias, docking orientation, and CDR1 loop usage, the contacts between the two V δ 1+ TCRs and their respective CD1d-lipid complexes are quite different, resulting in shifted footprints of these TCRs on the CD1d-lipid surface (Figure 4A). Mentioned above and most notable is the differential use of the V γ chain in engaging CD1d; the DP10.7 TCR uses no V γ chain residues to engage its CD1d-sulfatide ligand whereas the 9C2 TCR uses both the CDR1 γ (contacting the CD1d α 1 helix) and CDR3 γ (contacting α GalCer) loops in ligand engagement. Two major factors may contribute to this difference between TCRs. First, the two TCRs use different V γ chains that derive from different families within the group 1 γ chain sequences (Figure 2); DP10.7 uses a V γ 4 domain whereas 9C2 uses V γ 5. It is possible that differences encoded in the CDR1 and CDR2 loops between these two family members may result in different specificities for CD1d. However, examination of the contact residues of the V γ 5 domain CDR1 γ loop with CD1d in 9C2 reveals that they are conserved within the CDR1 γ loop of the V γ 4 domain of DP10.7, therefore the absence of appropriate contact residues is not the explanation for the altered footprint between the two TCRs. The second, and more likely factor explaining the differences in footprint of these complexes is the different length of the two CDR3 loops, CDR3 δ and CDR3 γ . The DP10.7 TCR has a loop length of 14 amino acids, the median of CDR3 δ loop lengths [13] whereas the 9C2 TCR CDR3 δ loop lies in the shorter than median range at 11 amino acids. Indeed, the AB18.1 TCR, using the same V γ domain as 9C2, has a CDR3 δ length of 15 amino acids. Superposition of the DP10.7 and AB18.1 TCRs onto the 9C2 footprint show a clear clash of their long CDR3 δ loops with the CD1d surface [47], indicating either the need for a significant conformational change in the CDR3 δ loop to accommodate this footprint, or adoption of an alternative docking orientation such as that seen in the DP10.7 complex structure. There are also major differences in the length of the CDR3 γ loop between the DP10.7 and 9C2 TCR; DP10.7 has a loop length of 8 residues, close to the average length for human CDR3 γ loops [13], whereas the 9C2 TCR has an exceptionally long CDR3 γ loop with 13 amino acids. The 9C2 CDR3 γ loop is used to contact both CD1d and lipid, it is therefore likely to be a key contributing factor to the shift in docking mode observed for these TCRs.

The dominance of the V δ 1 chain in interactions with CD1d in both structures has led to speculation [39, 40] that this domain serves as the core restriction element for CD1d reactivity. Recent structural characterization of $\alpha\beta$ T cells that utilize the V δ 1 domain ($\delta/\alpha\beta$ T cells) further emphasizes the importance of this domain and the relatively conserved contacts with CD1d. $\delta/\alpha\beta$ T cells were first noted over 25 years ago and can incorporate both the V δ 1 and V δ 3 gene segments during rearrangement with J α -C α [48–50]. While the

proportion of CD1d- α GalCer V δ 1+ T cells in the blood are quite low, analysis of this population demonstrated that a considerable proportion of these were actually $\alpha\beta$ T cells using a V δ 1 domain [21]. A crystal structure of one of these $\delta/\alpha\beta$ TCRs (9B4) in complex with CD1d- α GalCer revealed a footprint similar to that observed with the V δ 1+ $\gamma\delta$ TCRs discussed previously; the V δ 1 domain dominated the contacts with CD1d (66% buried surface area). Remarkably, the contacts of the CDR1 δ loop were essentially identical between the 9B4 TCR ($\delta/\alpha\beta$) and the 9C2 TCR ($\gamma\delta$). This further demonstrates the importance of this loop as a restriction element for V δ 1 recognition of CD1d. Contact with the lipid ligand, α GalCer, was mediated entirely by the β chain, specifically the CDR1 β and CDR3 β loops, similar to the use of the CDR3 γ loop by the 9C2 TCR.

Together, these structures provide important insight into V δ 1+ $\gamma\delta$ T cell recognition of antigen. For those V δ 1+ $\gamma\delta$ T cells that do recognize CD1d, there is a clear bias towards use of the V δ 1 domain in their TCR, however the variability in docking observed is unlike that of the conserved docking of other “semi-invariant” T cell populations such as iNKT or MAIT [51, 52]. While the overall proportion of V δ 1+ $\gamma\delta$ T cells in the blood is quite low, they are abundant in mucosal tissues such as the gut [53, 54]. There are hints that some gut V δ 1+ $\gamma\delta$ T cells exhibit similar reactivities [39], but the extent of the lipid repertoire to which these cells respond remains unclear. The conservation of the V δ 1 domain throughout primate evolution, particularly the CDR1 δ loop which plays a pivotal role in all three V δ 1 complex structures solved to date, suggests CD1d surveillance by these cells predates divergence of the primate lineage. Variability intrinsic in the CDR3 δ loop and pairing with alternative V γ domains (e.g. V γ 4, V γ 5, which are members of the rapidly evolving group 1 V γ s, and V γ 9) may provide V δ 1+ $\gamma\delta$ T cells with the ability to recognize a range of lipid ligands presented by CD1d or even to recognize other CD1 molecules. Perhaps this pairing with the rapidly evolving V γ s provides an additional adaptive advantage to this recognition system. Indeed, CD1c, a member of the group 1 CD1 molecules in human, was the first $\gamma\delta$ ligand to be described [31]; clones responding to CD1c expressed TCRs with V δ 1/V γ 9 pairing. Further structural elucidation of complexes between V δ 1+ $\gamma\delta$ TCRs and these CD1 molecules presenting diverse lipids will further reveal the role of the V δ 1 domain in these interactions, and what role the diverse V γ domains may be playing in this recognition process.

V γ 9V δ 2 T cell activation by phosphoantigens

V γ 9V δ 2 T cells are characterized by the expression of a TCR comprised of a V γ 9 domain paired with a V δ 2 domain and reactivity to small, organic based pyrophosphate molecules [55–58] (Figure 5). They are the major subset of $\gamma\delta$ T cells in human blood and can comprise between 1–10% of total blood T cells in healthy humans [59]. These cells are also found at high frequency in the gut, liver and other mucosal tissues [60–63]. V γ 9V δ 2 T cells respond potently to certain tumor cells [64, 65] and to microbial pathogens such as *Mycobacterium tuberculosis* and *leprae* [66] and are thus a population of great interest for immunotherapeutic manipulation. Both the V δ 2 and V γ 9 gene segments are well conserved throughout primate evolution (Figures 1 and 2, [16]) and these cells have been studied functionally in other primate species [67, 68]. While this cell population has often been termed “primate-specific”, recent analysis of homologous sequences in other mammalian

species strongly suggest these cells exist in species outside the primate lineage and likely predate the divergence of mammals [69], although they are not found in rodents or lagomorphs and thus are likely to have been lost in these lineages.

The molecular basis for V γ 9V δ 2 T cell activation by the pyrophosphate-based “phosphoantigens” or “pAgs” has remained much of a mystery. It is clear that V γ 9V δ 2 T cells can become activated when in the presence of target cells that are incubated with lysates from certain microbial species that produce pAgs (i.e. hydroxymethyl-butyl-pyrophosphate: HDMAPP/HMBPP) through the alternative MEP (2-C-methyl-D-erythritol 4-phosphate) isoprenoid pathway [55, 56]; cells that have dysregulated metabolism and accumulate metabolites from the mevalonate pathway, such as isopentenyl-pyrophosphate (IPP) [58, 70]; or have been treated with inhibitors of the mevalonate pathway enzyme farnesyl pyrophosphate synthase [64, 71, 72], such as the aminobisphosphonate zoledronate (NBP), resulting in accumulation of intracellular IPP (Figure 5). Extracellular addition of pAgs, natural or synthetic [73], also lead to potent activation of V γ 9V δ 2 T cells. V γ 9V δ 2 T cell activation is dependent on expression of the V γ 9V δ 2 TCR as Jurkat cells transfected with this TCR are activated in a pAg dependent fashion [74]. Furthermore, while no direct contact between the V γ 9V δ 2 TCR and pAg have been reported, cell-to-cell contact is necessary to achieve V γ 9V δ 2 T cell activation [75, 76], suggestive of a cell-surface ligand on the target cell.

The recent discovery of the central role that members of the butyrophilin family, BTN3A, play in V γ 9V δ 2 activation has been a major breakthrough towards unraveling the molecular steps that are taken during pAg detection and V γ 9V δ 2 T cell activation. The initial discovery of BTN3A proteins in this role was through the use of a mouse monoclonal antibody, 20.1, raised against human BTN3A molecules, which upon addition to peripheral blood mononuclear cells (PBMCs) caused proliferation and activation of the V γ 9V δ 2 subset in ways similar to that of pAg addition [77, 78]. The importance of BTN3A molecules was also confirmed later through a genetic approach [79]. The BTN3A proteins, also known as CD277, are members of a large butyrophilin family with diverse roles in host homeostasis [80, 81]. There are three BTN3A family members in humans, BTN3A1, BTN3A2 and BTN3A3 [82], each with an extracellular domain comprised of an IgV and an IgC domain [82, 83] (Figure 6A, B), structurally homologous to the B7 superfamily of proteins. The extracellular domains of the three BTN3A isoforms are structurally similar, with only minor angle differences between the IgV and IgC domains noted (Figure 6B) [83]. The composition of the intracellular domain varies across these three isoforms; BTN3A1 and A3 have an intracellular B30.2 domain (also known as PRY/SPRY) whereas A2 lacks this domain. BTN3A3 also has a unique 70 amino acid extension C terminal to its B30.2 domain (Figure 6A).

The precise role of BTN3A molecules in pAg induced activation of V γ 9V δ 2 T cells has been controversial; two general models have been proposed to explain how pAg and BTN3A function to stimulate V γ 9V δ 2 T cells. The first model proposes that the BTN3A molecules act as antigen-presenting molecules, capturing and presenting pAg on the cell surface to V γ 9V δ 2 T cells which recognize this complex directly through their TCR [79]. While attractive in its simplicity, this model has not been supported by work of others that

form the basis for the second model. In this model, the focus is on BTN3A1, which in one study was shown to be the only isoform that can mediate pAg-induced activation of V γ 9V δ 2 T cells [77]. Another study demonstrated the requirement of all three isoforms for V γ 9V δ 2 T cell activation [84]. (Of note, the 20.1 antibody can induce activation with all three BTN3A isoforms [77].) The importance of the BTN3A1 isoform was soon mapped to its intracellular B30.2 domain through swapping of the intracellular domains across the BTN3A isoforms [77]. The non-stimulatory BTN3A3, upon transfer of the B30.2 domain of BTN3A1, was made stimulatory and surpassed the ability of the wildtype BTN3A1 to stimulate V γ 9V δ 2 T cells, whereas the reverse swap (BTN3A3 B30.2 domain into BTN3A1) abrogated the stimulatory activity of BTN3A1. Thus attention turned to the intracellular B30.2 domain of BTN3A1 as the pAg sensor.

B30.2 domains are classified as protein-protein interaction domains and are found in many proteins, including other butyrophilin family members. Many of these B30.2 domain-containing proteins, particularly the TRIM and pyrin families [85] have been reported to have immunological function, although in many cases the interacting partners have not been characterized. For BTN3A1, direct binding between the B30.2 domain and pAg was measured, with affinities commensurate with functional potency. Microbial derived (exogenous in Figure 5) pAg is 1000 fold more functionally potent than their endogenous counterparts [86]; the affinity (K_D) of the B30.2 domain for these exogenous pAg was calculated to be $\sim 1\mu\text{M}$, whereas the K_D of the endogenous pAg was ~ 1000 fold weaker ($\sim 1\text{mM}$) [87]. Direct binding between HMBPP and the B30.2 domain was also shown via Nuclear Magnetic Resonance (NMR) studies [88]. Elucidation of the structure of the B30.2 domain from BTN3A1 was highly informative in defining a putative binding site for pAg [87]. This structure adopted the canonical B30.2 domain fold seen in other B30.2 structures, but contained a highly unique, positively-charged pocket located in Sheet A (Figure 7). This pocket was lined with basic residues including histidines (His351 and His378), arginines (Arg412, Arg418 and Arg469) and a lysine (Lys393) (Figure 7), providing a highly positive charged environment complementary to the negative charge of the pyrophosphate moiety of pAg. Mutagenesis of this pocket through converting the basic residues to acidic completely abrogated pAg binding and functional activity of the full length BTN3A1 [87]. Furthermore, close examination of the differences between the B30.2 domains of BTN3A1 and BTN3A3 (which does not bind pAg nor mediate pAg activation of V γ 9V δ 2 T cells), revealed a single amino acid difference within this binding pocket at position 351, histidine (BTN3A1) versus arginine (BTN3A3). Swapping of the histidine residue alone into BTN3A3 transferred the ability to both bind pAg and mediate V γ 9V δ 2 T cell activation whereas introduction of the Arg into BTN3A1 abrogated binding and pAg-induced activation [87].

Based on the strong data that supports pAg detection via the intracellular B30.2 domain of BTN3A1, we and others [78, 87] have proposed an inside-out signaling model whereby binding of the pAg intracellularly is transitioned to the extracellular side of the membrane via two possible mechanisms. First, we postulate that binding of pAg to the B30.2 domain can induce a conformational change that modulates the structure of the BTN3A extracellular domains. Our structural studies of the extracellular domains [89] revealed two potential dimers that may exist in BTN3A1, thus interconversion of these dimers (see [90] for more in depth discussion) may be a key step in the engagement of the V γ 9V δ 2 TCR. It is

controversial as to whether BTN3A molecules are directly recognized by the V γ 9V δ 2 TCR; Vavassori and colleagues reported a interaction between the IgV domain of BTN3A1 and a multimerized V γ 9V δ 2 TCR used in their studies [79], while we have not been able to do the same with a similarly multimerized G115 V γ 9V δ 2 TCR and full length BTN3A1 [87]. Along a similar vein, murine cells transfected with full-length BTN3A1 do not support pAg or 20.1 induced V γ 9V δ 2 T cell activation [87], suggesting that BTN3A1 may not be the only player involved in TCR engagement. Yet others, using BTN3A1 transfected hamster cells (CHO) have shown these can support activation of V γ 9V δ 2 T cells when treated with the 20.1 antibody and, when the CHO cells contained human Chromosome 6, they also supported V γ 9V δ 2 T cell activation via pAg [91]. These data suggest an additional cell-surface molecule not present in rodents and present on human Chromosome 6 (where, incidentally, the MHC region is located) is required for pAg induced V γ 9V δ 2 T cell activation. Therefore our model also includes the potential recruitment of this “protein X” upon pAg binding; this may be related to, or independent of, a BTN3A dimer interconversion.

The discrepancies in interaction measurements between BTN3A molecules and V γ 9V δ 2 TCRs may also lie in the diversity intrinsic to this TCR. While V γ 9V δ 2 TCRs uniformly use V γ 9 and V δ 2 domains in their TCRs, there is considerable diversity with the junctional regions of the V γ -J γ and V δ -D δ -J δ domains, translating into amino acid variation at the CDR3 γ and CDR3 δ loops, respectively [74, 92, 93]. These loops have been shown via alanine-scanning mutagenesis to be involved in V γ 9V δ 2 T cell activation [93] and sequence variation amongst different V γ 9V δ 2 T cell clones produces a range of response potency [94]. Identification of other molecular players that are involved in some, but not all, V γ 9V δ 2 T cell clone activation such as HSP-60 [95] and F1-ATPase with or without ApoA1 [96] suggest that this system may be a complicated coordination of a cast of molecular actors. It is also curious that in evolutionary analysis of the CDR loops of the V γ 9 and V δ 2 gene fragments, that three of the four CDR loops have a coding/silent (dn/ds) ratio greater than one, suggestive of diversifying selection in these regions [16]; these loops were also shown to be involved in V γ 9V δ 2 activation through mutagenesis [93]. Overall this may indicate that selection is subtly adapting this population to a changing ligand environment (such as BTN3A molecules). This adaptation has not resulted in strict species-specific recognition as human/monkey chimeric V γ 9V δ 2 T cells maintain pAg reactivity [97] but not to the level of within species activation. It is therefore possible that BTN3A is directly recognized by the V γ 9V δ 2 TCR but that some V γ 9V δ 2 clones have higher affinities than others and can support an interaction with soluble, recombinant (albeit multimerized) forms of their TCRs with BTN3A1 molecules whereas others may need additional accessory molecules to mediate a robust interaction. The molecular steps that convert the surface of a target cell from non-stimulatory to stimulatory for V γ 9V δ 2 T cells remains unclear, although with the identification of BTN3A we can begin to propose testable models to explain this phenomenon. Further characterization of candidate intracellular players, such as the recently identified periplakin protein [84], will aid in assembling the molecular steps that are taken during the pAg-induced conversion of target cells from non-stimulatory to stimulatory.

The signals that regulate $\gamma\delta$ T cell activity in humans still remains mostly unclear however breakthroughs have come through the work of many groups in this field. We are beginning

to understand the true complexity of this population, that many subpopulations with different antigenic specificities exist even within the V δ 1+ T cell subset, and that these cells should not necessarily be grouped in with other T cell populations with “innate-like” phenotypes. The presence of these cells in areas of disease relevance (tissues prone to cancers and/or infection) makes them an obvious target for immunotherapy. Understanding the antigens to which they respond, and how they respond to them, will provide the first step in effective management of these cells in the clinic.

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- We review work on the evolution of primate V, D and J gene segments of $\gamma\delta$ T cells.
- We also review CD1d-lipid recognition of V δ 1+ $\gamma\delta$ and $\alpha\beta$ T cells.
- Lastly we review recent work on phosphoantigen modulation of V γ 9V δ 2 T cells.

Figure 1. Genomic organization of the V, D, and J gene segments comprising the primate $\gamma\delta$ TCR

Top: arrangement of the V δ , J δ and D δ (left) and V γ and J γ (right) gene segments from five representative species: human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), orangutan (*Pongo pygmaeus*), macaque (*Macaca mulatta*) and marmoset (*Callithrix jacchus*). Gene organization, from left to right, for the δ locus is shown centromeric to telomeric and for γ locus, telomeric to centromeric. J δ and D δ are shown in the inset, as they are located between the V δ 2 and V δ 3 gene segments. J γ gene segments are also shown in inset; they are located telomeric to V γ 11. The V and J gene segments are shown as arrows to indicate coding direction. D segments are shown as blocks as they can be read in both directions. Dashed borders indicate pseudogenes. V δ genes shown in grey are infrequently used in V δ domain rearrangement and also have V α designations, shown in parentheses. *Bottom:* Dot-plot analysis of the genomic regions encoding the δ and γ loci across representative species as in the top panel. The relative position of the V gene segments are shown with arrows; colored as they were in the top panel. Black dots on the plot correspond to regions with greater than 90% sequence identity whereas grey dots indicate greater than 80% sequence identity.

Figure 2. Phylogenetic relationships of primate Vδ and Vγ gene segments

Shown are neighbor-joining trees (left: Vδ, right: Vγ). Bootstrap confidence values are shown for most branches; well-supported groupings are shaded according to colors relevant to Figure 1. Bootstrap values less than 50% are shown as “*”. Branch length correlates to evolutionary distance (nucleotide substitutions per site) with scale shown at bottom of each tree.

Figure 3. Comparison of TCR-ligand co-complexes and docking footprints

Top: Side view of ribbon representations of, from left to right, complexes between the Vδ1+γδ TCRs (DP10.7 and 9C2) with CD1d presenting their respective lipids, sulfatide and α-galactosylceramide (αGalCer); a classical αβ TCR 2C with its MHC classical class I ligand Kb presenting the dEV8 peptide; an invariant Natural Killer T cell (iNKT) TCR in complex with CD1d presenting αGalCer; and a Type II NKT TCR in complex with CD1d presenting the lipid sulfatide. PDB accession codes, from left to right are: 4MNG, 4LHU, 2CKB, 2PO6 and 4EI5. The α1 and α2 platform domain of the MHC molecules is shown in grey (α3 domain and β2m are absent); MHC bound ligands are shown in yellow; and TCRs are shown as Variable domains only. *Bottom:* Cartoon representations of the MHC platform domain α1 and α2 helices from CD1d or Kb and their presented ligands; colored rectangles indicate the general docking footprint of the TCR onto these surfaces. Colors relate to the TCR chain coloring in the top panel, i.e. pink: Vδ1 domain; dashed outlines indicate chains that do not participate in docking (i.e. DP10.7 γ chain).

Figure 4. Shifted docking footprints between the V $\delta 1$ + DP10.7 and 9C2 TCRs on the CD1d surface

A) The positioning of the CDR loops from the DP10.7 TCR and 9C2 TCRs are shown superimposed on the surface of CD1d (shown in white molecular surface). The DP10.7 CDR loops are shown in shades of green (δ : dark green, γ : light green). The 9C2 CDR loops are shown in shades of orange (δ : bold orange, γ : light oranges). Loops shown as semi-transparent do not contact the CD1d-lipid surface. B) TCR contacts with the CD1d surface in the DP10.7 complex (left) and 9C2 complex (right). Loops are colored by chain, δ =dark pink, γ =dark blue; surface contact residues are colored according to the TCR chain contacts: δ chain contacts are shown in pink, γ chain contacts in blue and contacts made by both chains are colored green.

Figure 5. Structures of phosphoantigens (pAgs) that stimulate V γ 9V δ 2 T cells

PAs can derive from endogenous (mauve box) or exogenous (orange box) sources.

Endogenous sources include metabolites in the mevalonate pathway (endogenous IPP and the synthetic derivative EtPP) and exogenous sources include microbial metabolites from the isoprenoid pathway (HDMAPP or synthetic cHDMAPP). The common pyrophosphate motif is outlined in red; the chemically diverse organic moieties are shown as lines.

Figure 6. Domain organization of the butyrophilin-3A (BTN3A) proteins

A) The BTN3A extracellular domains belong to the B7-superfamily with an N-terminal IgV and C-terminal IgC domain. BTN3A proteins are single-pass transmembrane proteins and vary in their intracellular domains. BTN3A1 and BTN3A3 intracellular domains contain a B30.2 domain whereas BTN3A2 does not. B) Three-dimensional structures of the extracellular domains of BTN3A1 alone (left, colored cyan) and superimposed with BTN3A2 (gold) and BTN3A3 (pink). The structures are highly homologous, varying only in the hinge angles between the IgV and IgC domains.

Figure 7. Structure of the intracellular B30.2 domain of BTN3A1

Top: Ribbon diagram of the B30.2 domain dimer (monomers in yellow and green) identified in the crystal lattice. Dashed lines indicate where the N terminal linker would pass through the plasma membrane, shown in pink, and connect to the IgV-IgC extracellular domains.

Bottom: Electrostatic surface representation of one of the B30.2 monomers, showing the location of the positively charged, pAg binding pocket. Inset shows a close-up of the pocket with bound pAg. Basic residues lining this pocket are labeled with their single letter amino acid designation.