Sensing of pyrophosphate metabolites by $V_\gamma 9V_\delta 2$ T cells

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The predominant population of $\gamma\delta$ T cells in human blood express a T cell receptor (TCR) composed of a $V_\gamma 9$ (V$\gamma 2$ in an alternate nomenclature) and V$\delta 2$ domains. These cells came into the limelight when it was discovered they can respond to certain microbial infections and tumorigenic cells through the detection of small, pyrophosphate containing organic molecules collectively called “phosphoantigens” or “pAgs.” These molecules are intermediates in both eukaryotic and prokaryotic metabolic pathways. Chemical variants of these intermediates have been used in the clinic to treat a range of different cancers, however, directed optimization of these molecules requires a full understanding of their mechanism of action on target cells. We and others have identified a subclass of butyrophilin-related molecules (BTN3A1-3) that are directly involved in pAg sensing in the target cell, leading to engagement and activation of the T cell through the TCR. Our data and that of others support the pAg binding site to be the intracellular B30.2 domain of BTN3A1, which is the only isoform capable of mediating pAg-dependent stimulation of V$\gamma 9V_\delta 2$ T cells. Here, we review the data demonstrating pAg binding to the B30.2 domain and our studies of the structural conformations of the BTN3A extracellular domains. Finally, we synthesize a model linking binding of pAg to the intracellular domain with T cell detection via the extracellular domains in an “inside-out” signaling mechanism of the type characterized first for integrin molecule signaling. We also explore the role of V$\gamma 9V_\delta 2$ TCR variability in the CDR3 $\gamma$ and $\delta$ loops and how this may modulate V$\gamma 9V_\delta 2$ cells as a population in surveillance of human health and disease.

Keywords: V$\gamma 9V_\delta 2$, phosphoantigens, T cells, T cell receptor, butyrophilins, B30.2

Gamma delta T cells represent a conundrum when trying to understand the mechanisms of T cell ligand recognition that results in T cell activation. T cells expressing $\alpha\beta$ T cell receptors (TCRs) that develop normally in the thymus recognize all antigens with the requirement of an antigen-presenting molecule belonging to the MHC superfamily. This MHC requirement encompasses conventional $\alpha\beta$ T cell recognition of classical class I and class II MHC molecules as well as innate-like or semi-invariant T cell recognition of non-classical or MHC-like molecules. Examples include Type I invariant Natural Killer T (iNKT) and Type 2 NKT cell recognition of CD1d, Mucosal Associated Invariant T (MAIT) cell recognition of MR1, and non-conventional $\alpha\beta$ T cell recognition of the human Group 1 CD1s (1). While these different T cell types recognize their various MHC ligands with diverse footprints, the fact remains that they are all “restricted” to recognizing antigens in the context of their respective MHCs.

This same MHC requirement does not appear to hold true for $\gamma\delta$ T cells. While defining ubiquitous antigens for this lineage of T cells has been challenging, a clearer perspective has started to emerge with recent breakthroughs in antigen definition for these cells. While a comprehensive survey of the results is not the focus of this review and has been discussed elsewhere (2, 3), what has emerged from these studies is that $\gamma\delta$ T cell are specific for both MHC and non-MHC proteins. To first understand this conundrum it is important to emphasize that $\gamma\delta$ T cells cannot be grouped together as a whole. Instead, $\gamma\delta$ T cells are divided into many different populations with different antigen reactivities, effector functions, and tissue residence (4, 5). Another important point is that there is little, if any, homology between $\gamma\delta$ T cell populations in mice with those in humans, suggesting that these cells have rapidly adapted to different antigenic stimuli or immunological environments in the two different hosts. This is supported by the observed rapid evolution of many of the V$\gamma$ genes within the primate lineage (6, 7). Recent work from our laboratory has focused on two very different $\gamma\delta$ T cell populations in humans: first, those that do recognize antigens in the context of CD1 molecules, which has been reviewed elsewhere (8); and secondly a population that appears to be MHC-independent and instead responds to small pyrophosphate antigens called “phosphoantigens (pAgs)”. This second population, called “V$\gamma 9V_\delta 2$”, “V$\gamma 2V_\delta 2$”, or “$\gamma 2S 2$” by different groups, called V$\gamma 9V_\delta 2$ here, is the topic of this review. Recent breakthroughs by several groups have started to reveal the complex mechanism behind pAg regulation of this cell population. These findings have led to a shift in the paradigm of what specificities regulate T cell activity and a better understanding of the molecular mechanisms behind regulation of this important T cell population in humans.
Vγ9Vδ2 T cells are the major subset of γδ T cells found in human blood, comprising up to 5% of the T cells in healthy individuals and expanding to 20–50% during infection or disease (9). These cells play important roles in mediating immunity against microbial pathogens, including *Mycobacterium tuberculosis* and *Mycobacterium leprae* [the causative agents of tuberculosis and leprosy, respectively, reviewed in Ref. (10)], and can respond potently against certain types of tumor cells (11, 12). No homologous pAg-reactive Vγ9Vδ2 T cell population has been identified in rodents or lagomorphs, however, genes homologous to both Vδ2 and Vγ9 have been identified in other placental mammalian species including sloth, armadillo, lemur, aye aye, bottlenose dolphin, killer whales, and horse (13). Furthermore, expression of Vγ9Vδ2 TCRs was demonstrated in alpacas. This suggests that Vγ9Vδ2 T cells are present in species outside the primate lineage and likely predate the split of the placental mammals. The lack of Vγ9Vδ2 T cells in rodents and lagomorphs demonstrate that this lineage has been lost in some species, perhaps compensated by selection for alternative T cell subtypes.

As mentioned above, Vγ9Vδ2 T cells represent an important departure from the classical T cell recognition paradigm, in that no MHC or MHC-like molecules have been implicated in their activation (14). Instead, the aforementioned pAgs (Figure 1), which are pyrophosphate containing metabolites, are the key trigger (15–18). Amongst these, isopentenyl pyrophosphate (IPP) (16, 19) is generated from the endogenous mevalonate (MVA) pathway (HMG-CoA, cholesterol biosynthesis) and accumulates intracellularly during dysregulated metabolism in many types of tumor cells. Addition of aminobisphosphonates like zoledronate (NBP) or alkylamines also causes intracellular IPP accumulation through inhibition of farnesyl pyrophosphate synthase (12, 20, 21); this strategy is used frequently in studies of Vγ9Vδ2 T cell stimulation. A much more potent set of pAgs (i.e., HDMAPP/HMBPP: hydroxy-methyl-butyl-pyrophosphate) are microbial metabolites from the isoprenoid pathway (17) and represents “non-self” pathogen signals. A synthetic pAg, bromohydrin pyrophosphate (BrHPP) also strongly activates Vγ9Vδ2 T cells and is often used in *in vitro* functional experiments (22). The pAg-induced recognition of target cells is TCR dependent, as Vγ9Vδ2 TCR transduced Jurkat cells become activated by pAgs (23). While no direct interaction has been detected between pAgs and the γδ TCR, cell-to-cell contact is necessary in pAg-induced γδ T cell activation (14, 24), indicating that molecules expressed on the cell-surface of target cells or γδ T cells are required for activation.

**FOCUS ON BUTYROPHILINS AS KEY PLAYERS IN THE Vγ9Vδ2 T CELL RESPONSE TO pAgs**

A major breakthrough in our understanding of Vγ9Vδ2 T cell activation came with the identification of the butyrophilin-3 (BTN3) protein family as a key mediator in this process (25). BTN3 proteins, also known as CD277, are type I membrane proteins with two immunoglobulin (Ig)-like extracellular domains (IgV and IgC) (26, 27) (Figure 2A) with close structural homology to the B7 superfamily of proteins. BTN3A molecules are members of a much larger butyrophilin superfamily with diverse roles in host homeostasis (28, 29). A key factor in the initial discovery of the role of BTN3A in Vγ9Vδ2 activation was the serendipitous discovery that a mouse antibody (clone 20.1), raised against human BTN3A molecules, caused a surprising proliferation and expansion of γδ T cells in IL-2 supplemented peripheral blood mononuclear cell (PBMC) cultures (25). The presence of this antibody elicited production of IFN-γ, TNF-α, and upregulation of the activation marker CD69 and has recently been shown to elicit very similar intracellular signaling in Vγ9Vδ2 T cells as pAgs (30). This phenomenon was restricted to the Vγ9Vδ2 population in PBMCs, with no effect on αβ T cells or those γδ not expressing a Vγ9Vδ2 TCR. Consistent with the lack of MHC requirement for stimulation of this γδ T cell population, addition of the 20.1 antibody to a panel of human tumor/transformed cell lines, some of which lack MHC surface expression, induced potent activation of responding Vγ9Vδ2 T cells (25). To rule out an effect of BTN3A expressed on Vγ9Vδ2 T cells, murine Vγ9Vδ2 TCR transductants, which do not express BTN3A molecules, were used as effector cells and shown to also respond to these 20.1 treated target cells. This experiment also confirms the requirement for the Vγ9Vδ2 TCR, supporting previous studies in Vγ9Vδ2 Jurkat transfectants (23). Similar results with the 20.1 Ab were found by another group (31). Other approaches also confirmed the role of BTN3A molecules in Vγ9Vδ2 T cell activation; taking a genetic approach, Vavassori et al. mapped genetic elements required for Vγ9Vδ2 T cell activation to the 3- to 27.4-Mb interval of human chromosome 6 and further refined their candidates by screening only those coding regions that had a predicted transmembrane element (32). Included within these candidates were the BTN3A molecules.
Three isoforms of BTN3A are present in humans, BTN3A1, BTN3A2, and BTN3A3, each encoded by a separate gene (26). The extracellular domains of the BTN3A molecules are highly sequence and structurally homologous, with only minor variations observed in the hinge angle between the IgV and IgC domains of their crystal structures when the three extracellular domain structures are superimposed (27) (Figure 2B). All three BTN3A isoforms are recognized by the 20.1 antibody and can mediate 20.1 mAb-induced activation of Vγ9Vδ2 T cells (25,27), suggesting that a shared epitope on BTN3A molecules is involved in the process of Vγ9Vδ2 stimulation. Curiously, a different BTN3A specific antibody, 103.2, had an antagonistic effect on pAg-mediated Vγ9Vδ2 stimulation after addition to target cells, suggesting that it either blocks an epitope on the BTN3A extracellular domain or induces or stabilizes a non-stimulatory conformation of BTN3A on the cell-surface (25).

In the crystal structures of the BTN3A extracellular domains, two dimeric interfaces were observed (27), one that would generate a symmetric V-shaped homodimer positioning the C-terminal transmembrane domains close together (Dimer 1, Figure 3) and the other a head-to-tail homodimer with an asymmetric dimer interface, requiring the BTN3A molecules to lay flat, parallel to the cell-surface (Dimer 2, Figure 3). Both dimer interfaces were of appreciable size, Dimer 1 buried ~1520 Å² whereas Dimer 2 buried ~1080 Å². Both dimer interfaces were also highly conserved between the three BTN3A isoforms; only 2 out of the 18 interface residues in Dimer 2 differed between the BTN3A isoforms. However, the Dimer 2 interface was observed in the crystal structures of all three BTN3A isoforms indicating these differences were tolerated. Residues involved in the Dimer 1 interface differed at three positions across the three BTN3A isoforms although examination of the contacts in this interface revealed that these interactions involved only main chain atoms, thus tolerating variation in the composition of the side chain residues. This suggests that these extracellular domains can form heterodimers adopting both dimeric conformations when co-expressed on the cell-surface. Using soluble extracellular domains, we were able to establish that BTN3A molecules exist as stable homodimers in solution and, using a FRET approach, that the dimer conformation in solution was Dimer 1 (27). This does not, however, rule out the possibility that both dimers can exist on the cell-surface, perhaps stabilized through the transmembrane or intracellular domains not present in the soluble molecules.

Insight into the binding sites and mode of action of the 20.1 and 103.2 antibodies was revealed with the complex crystal structures of single-chain versions of these antibodies (containing just the antigen-binding V domains) in complex with BTN3A1 (27).
These complex structures demonstrated that these two antibodies bind to separate epitopes on the BTN3A surface (Figure 4), a result confirmed by competition-binding assays performed by Surface Plasmon Resonance (SPR). Curiously, the 20.1 antibody binding site positions the antibody such that it cannot bind bivalently to one BTN3A dimer as the two binding sites are too distant. For both 20.1 antibody binding sites to be occupied in the Dimer 1 conformation would require engagement of two separate BTN3A homodimers. Thus, binding of the 20.1 antibody could effectively cross-link these molecules on the cell-surface. Also interesting was the finding that the 20.1 binding site overlaps with that of the Dimer 2 interface, suggesting that binding of the 20.1 antibody would compete with the Dimer 2 conformation (Figure 4) and instead select for, and stabilize, the Dimer 1 conformation. The 103.2 epitope is accessible in both Dimer 1 and Dimer 2 conformations; in contrast to the 20.1 antibody, 103.2 would likely bind with both binding sites to one BTN3A Dimer 1, but would have to cross-link BTN3A molecules in the Dimer 2 conformation. These results lead us to propose a model whereby these two dimeric states are related to the stimulatory potential of the cell upon which they are expressed. In normal, non-stimulatory conditions, BTN3A molecules would exist in the Dimer 2 state (head-to-tail) and thus not be in a state to provide a stimulatory signal to surveying Vγ9Vδ2 T cells. Upon addition of the 20.1 antibody, BTN3A molecules in the Dimer 2 conformation would be converted to Dimer 1; these would be cross-linked on the cell-surface via binding of one 20.1 antibody to two BTN3A dimers, and thus be converted into a “stimulatory” conformation permissible to stimulate Vγ9Vδ2 cells (Figure 4). The potential ability of the 20.1 antibody to cross-link BTN3A molecules in this model is consistent with the observed immobilization of BTN3A molecules via Fluorescence Recovery after Photobleaching (FRAP) that occurs during conversion of a cell from a non-stimulatory to stimulatory state (25). This model also proposes that addition of 103.2 antibody could either block a site on BTN3A required for Vγ9Vδ2 cells activation or stabilize the Dimer 2 conformation on the cell-surface (Figure 4), thus leading to the inhibitory activity observed when this antibody is added in conjunction with pAg.

But what is the role of pAg in this process? Failed efforts to show a direct interaction between the Vγ9Vδ2 TCR and pAg early on suggested additional players were involved in this process; the requirement of cell–cell contact for Vγ9Vδ2 T cell stimulation
also supported this hypothesis (14). Based on recent published results, two general models have been proposed to explain how pAg functions to stimulate Vγ9Vδ2 T cells. The first model is tantalizingly simple; it describes the extracellular domain of BTN3A molecules as “antigen-presenting” whereby BTN3A molecules associate with pAg and “present” it directly to the Vγ9Vδ2 TCR (32). While this model would fit well with the requirement of an antigen-presenting molecule for αβ T cell recognition of antigen, this model has met with controversy and is not supported by data generated from several groups and discussed further below. Model 2 is based on the finding that only one of the three BTN3A isoforms (BTN3A1) can support pAg-mediated Vγ9Vδ2 activation. This was demonstrated through siRNA knock-down experiments and reintroduction of individual BTN3A1, BTN3A2, or BTN3A3 isoforms; BTN3A1 alone was found to be pAg-reactive (25). This suggests that there is a unique element to this isoform that alone can initiate stimulation in a pAg specific way. Domain deletion and swapping experiments gave the first indication of the identity of this unique element: BTN3A1 lacking its intracellular domain failed to mediate pAg-mediated Vγ9Vδ2 stimulation but was highly stimulatory upon addition of the 20.1 antibody. BTN3A3, which cannot support pAg-mediated stimulation of Vγ9Vδ2 T cells, was made pAg stimulatory by swapping of its intracellular domain with that of A1 (31,33). These data strongly support a pivotal role of the intracellular domain of BTN3A1 as the pAg sensor.

The three BTN3A molecules differ substantially in their intracellular domains; A1 and A3 each contain a B30.2 domain (also known as PRY/SPRY domains) whereas A2 lacks this domain (Figure 2). The B30.2 domains found in A1 and A3 are highly homologous, with 87% amino acid identity between the two (Figure 5). The intracellular region of A3, however, has a unique 70 amino acid extension C-terminal to its B30.2 domain (Figures 2 and 3). B30.2 domains are classified as protein–protein interaction domains and are found in other butyrophilin family members as well as non-related proteins (over 50 genes in the human genome have predicted B30.2 domains). Many B30.2 domain-containing proteins have been reported to be important in immune function, including the TRIM and pyrin families (34), although in most cases the binding partners have not been characterized. The importance of the B30.2 domain in pAg sensing was first demonstrated through swapping of just this domain between the A1 (capable of pAg activation) and A3 (incapable of activation) isoforms (33). Introduction of the A1 B30.2 domain into the A3 isoform converted this isoform to stimulatory for Vγ9Vδ2 T cell in the presence of pAg, whereas, the reverse swap (A3-B30.2 into A1 isoform) abrogated its ability to stimulate Vγ9Vδ2 T cells in a pAg-dependent fashion.

**INTRACELLULAR B30.2 DOMAIN OF BTN3A1 AS THE pAg SENSOR**

Direct interactions between both endogenous and exogenous pAgs with the B30.2 domain of BTN3A1 were measured with a highly sensitive technique called Isothermal Titration Calorimetry (ITC), which measures the heat absorbed or lost during binding events (33,35). The affinities calculated from these techniques (KD = ∼1 µM for exogenous pAg, ∼1mM for endogenous pAg) also reflected the functional potency of these compounds
in mediating stimulation of Vγ9Vδ2 T cells. The endogenous IPP pAg is typically 1,000-fold weaker potency than that of the exogenous HMBPP (36). Association studies with the HMBPP pAg were also shown via chemical shift perturbations (CSP) via Nuclear Magnetic Resonance (NMR), an equally sensitive technique (35). Of note, no association of pAgs could be measured with the BTN3A3-B30.2 domain, or to the extracellular domains of BTN3A1, A2 or A3, with either of these techniques (33,35). The crystal structure of the B30.2 domain of BTN3A1 (Figure 6) was highly informative in deciphering the pAg binding site (33). The structure of BTN3A1 B30.2 domain was highly homologous to previously reported B30.2 domains, in particular the B30.2 domain of Trim21, an intracellular Fc receptor (37). Importantly, specific to the BTN3A1 B30.2 domain was a highly positively-charged (basic) pocket nestled in Sheet A of the structure (Figure 6). This pocket was lined with basic residues including arginines (R412, R418, and R469), histidines (H351 and H378) and a lysine (K393) (Figure 7). The charge complementarity between the B30.2 positively charged pocket and the negative charge of pAgs made this an excellent candidate for pAg binding.

Charge swapping mutagenesis studies, where the basic residues were mutated to acidic (negatively charged), completely abrogated pAg binding and reactivity in cell stimulation assays, providing compelling evidence that this indeed was the pAg binding pocket (33). However, these results did not explain entirely the differences of pAg binding to the A1 versus A3-B30.2 domains. Close examination of the amino acid differences between these isoforms revealed a single amino acid difference that lay within the binding pocket: position 351 was a histidine in A1 and an arginine in A3 (Figure 5). Swapping of this single amino acid difference between the domains (i.e., mutating the H to R in A1 and R to H in A3) transferred both pAg binding ability and functional ability to stimulate Vγ9Vδ2 T cells. Position 351 is quite buried within the pAg binding pocket; it is likely that the size and shape of the side chain difference from an H to an R changes the architecture of the binding pocket such the pAgs thus characterized do not bind to A3. This raises the possibility that there are other pyrophosphate compounds yet to be described that may preferentially bind to A3 over A1. It is unclear as to the role, if any, of the A2 and A3 isoforms in Vγ9Vδ2 T cell activation; their potential ability to form heterodimers with A1, assemble with alternative antigens via their B30.2 domain (in the case of A3) and modulate cell-surface assembly with other protein binding partners is an area of active investigation.

Additional insight into the mechanics of pAg binding to the B30.2 domain, pursued through crystallization and NMR experiments, have revealed evidence for a conformational change induced in the B30.2 domain upon pAg binding. The first insight into this was during our pursuit of a complex structure between the B30.2 domain and pAg where we attempted to “soak” pAg into already existing crystals (33). (This is a common approach for studying small molecule binding sites in proteins.) While protein crystals appear solid, they contain a substantial amount of liquid that forms solvent channels between the protein molecules. Thus, small molecules (such as pAgs) can move freely in the crystal lattice and bind to their appropriate binding site in the protein as it is locked in the crystal lattice. This methodology assumes
that binding of the small molecule does not induce changes in the conformation of the protein as this can disrupt the packing of the protein in the crystal lattice and cause the crystals to dissolve. Soaking of B30.2 domain crystals with pAgs did just this, causing the crystals to dissolve immediately upon addition. Locking of the protein–protein contacts within the crystal lattice via covalent crosslinking via glutaraldehyde preserved the crystal structure and allowed a complex structure of pAg and B30.2 to be resolved. Within this structure there is clear tetrahedral electron density for the beta- and alpha-phosphates of the pAg within the binding pocket, however, the organic portion of the pAg could not be resolved (33). More direct evidence for a conformational change is seen in CSP observed in the Heteronuclear single quantum coherence spectroscopy (HSQC) of apo (empty) B30.2 versus that with added HMBPP (35). Thus, it is clear that binding of pAg to the B30.2 domain induces structural rearrangements/conformational changes that we hypothesize is the first in a cascade of intracellular and extracellular events leading to target cell transmission of a stimulatory signal to the Vγ9Vβ2 TCR.

This model of intracellular sensing of pAgs is consistent with the fact that many of the physiologically relevant pAgs are first generated and accumulate inside target cells. Endogenous pAgs, such as IPP or DMAPP, are intermediates of the MVA pathway, which is conserved in eukaryotes and archaea for isoprenoid biosynthesis (38). It has been reported that these pAgs accumulate intracellularly during dysregulated metabolism in many types of human tumor cells. For example, overexpression of HMG-CoA reductase, the rate limiting enzyme of the MVA pathway, in the non-Hodgkin B cell lymphoma cell-line Daudi and mammary cancer cells such as breast adenocarcinoma cells, can cause an increased level of IPP that is then recognized by Vγ9Vβ2 T cells (12, 39). Manipulation of the MVA pathway by various synthetic drugs (such as statins and aminobisphosphates) or short hairpin RNAs targeted to enzymes either upstream or downstream of IPP production can trigger or suppress pAg-induced T cell activation, supporting the idea that Vγ9Vβ2 T cells can sense intracellular IPP levels (12, 20, 21, 40). Another set of pAgs derive from exogenous microbial sources and are also much more potent. These metabolites, such as HMBPP from the microbial methlyerythritol phosphate (MEP) pathway, exist in bacteria and several photosynthetic eukaryotes (38). Some microbes producing these pAgs are intracellular pathogens such as Mycobacterium tuberculosi and Listeria monocytogenes, which can enter and survive within the host cells (41, 42). Immune cells such as monocytes, macrophages and dendritic cells can also engulf these pathogens and elicit pAg-dependent T cell responses (43–46). Extracellular pathogens like Escherichia coli can also be phagocytosed by immune cells like neutrophils (47). The subsequent Vγ9Vβ2 T cell response is strictly dependent on the ability of the phagocytosed pathogens to produce HMBPP. A more recent study also demonstrated that after T cell priming, these pathogen-harboring neutrophils develop an antigen-presenting phenotype (48). These studies suggest that the presence of exogenous pAgs, intracellularly, is critical for Vγ9Vβ2 T cell activation and function. Admittedly, exogenous pAgs can also be secreted by some extracellular pathogens or immune cells like neutrophils, and administration of soluble pAgs in the presence of BTN3A-expressing antigen-presenting cells trigger Vγ9Vβ2 T cell activation, as was demonstrated in early studies of these T cells (24). It is unknown how these extracellular pAgs get internalized, giving that their negatively charged features render direct membrane permeability unlikely. Possible mechanisms for passing through the plasma and endocytic membranes may include specific membrane transporters or charge neutralization by ester formation.

**PHOSPHOANTIGEN STRUCTURE AND BIOACTIVITY**

The variable chemical structures of pAgs relate directly to their bioactivity. The pyrophosphate moiety is central since its strong negative charge can make electrostatic contacts with the positively charged binding pocket of the B30.2 domain. Indeed, monophosphate substituents have significantly reduced specific activity compared with their pyrophosphate counterparts (49). On the other hand, large chemical groups like AMP can be added without affecting the bioactivity of pAgs (49) and, in fact, some natural pAgs are nucleotic conjugates (50, 51) that may be processed by specific antigen-presenting cells. More intriguingly, it has been found that hydrolysis of the pyrophosphate moiety is associated with pAg bioactivity and non-hydrizable analogs of pAgs can even inhibit the T cell activation (52). These features suggest that either pre- or post-processing of pAgs can occur before or after association with the B30.2 domain and may have important implications for T cell activation.

Since the pyrophosphate moiety is essentially the same for every pAg, the potency of pAgs is largely dependent on their organic moieties. Changes in the length of the alkyl chain and positions of the double bond, even though very subtle, can lead to dramatic change in potency (49). Notably, the structural difference between endogenous ligand IPP and exogenous HMBPP only lies in the additional hydroxyl group on HMBPP, yet the potency and binding affinity of this strong ligand increases by about 1000-fold. One possible explanation that was explored early on was the chemical reactivity of pAgs. The polarizability of the C3 substituent correlates with bioactivity: an increased specific activity for T cells was observed upon addition of a halohydrin to the C3 position (52). This leads to an interesting speculation that a covalent reaction may occur between the pAg and its binding partner. More recently, some studies have pointed out that the chirality of the pAgs may also play a role in their bioactivity. It has been found that E-stereoisomers are much more potent antigens for T cell activation (53). Potential intramolecular hydrogen-bonding states of these isomers affect the overall shape of the ligand and may be important for binding to the B30.2 domain. Most of the studies regarding the structure of pAgs were conducted before the identification of the B30.2 domain of BTN3A1 as the binding partner; optimization of binding to this domain has already begun with exciting implications for pAg-based therapeutics (35).
Whether BTN3A is directly recognized by the Vγ9Vδ2 TCR is controversial; Vavassori and colleagues were able to measure an interaction between the IgV domain of BTN3A1 and a Vγ9Vδ2 TCR used in their studies (32) while we have not been able to do this in the intracellular pAg concentration has a similar effect except the dimer conversion is mediated by changes in the intracellular B30.2 domain, which (31, 33) whereby the binding of pAg intracellularly is translated extracellularly for detection by the Vγ9Vδ2 TCR. This could be through several means that work individually or in concert to initiate TCR recognition: (1) immobilization/clustering of BTN3A that increases the avidity for the TCR, (2) a conformational change of the BTN3A extracellular domains from non-stimulatory (Dimer 2) to stimulatory (Dimer 1), or (3) the two previous situations resulting in the recruitment of an additional factor that directly engages the Vγ9Vδ2 TCR. Number 1 and 2 invoke a direct interaction between Vγ9Vδ2 TCRs and the extracellular domains of BTN3A whereas version 3 involves an unknown accessory protein that is the true Vγ9Vδ2 TCR ligand (Figure 8). Whether BTN3A is directly recognized by the Vγ9Vδ2 TCR is controversial; Vavassori and colleagues were able to measure an interaction between the IgV domain of BTN3A1 and a Vγ9Vδ2 TCR used in their studies (32) while we have not been able to do the same with the G115 Vγ9Vδ2 TCR and the full-length extracellular domain of BTN3A1 (33). In addition, we have not been able to stimulate Vγ9Vδ2 T cells with murine transfected BTN3A1 either through 20.1 antibody addition or through pAg treatment (33) whereas others, using a different Vγ9Vδ2 TCR have observed stimulation via 20.1 Ab treatment of BTN3A1 transfected Chinese-hamster ovary (CHO) cells (54). pAg-mediated stimulation was only observed in CHO cells containing human Chromosome 6. These conflicting results represent probably the most important conundrum in the Vγ9Vδ2 T cell field at present. BTN3A molecules are necessary but are they sufficient for Vγ9Vδ2 recognition? Evidence for other molecules playing a role in Vγ9Vδ2 recognition and activation such as HSP-60 (55) and F1-ATPase with or without ApoA1 (56) suggest this system could be a complicated coordination of many molecular players. Further coordination through adhesion molecules, activating and inhibitory Natural Killer receptors, Toll-like receptors (TLRs), and Fc Receptors [reviewed in Ref. (57)] also fine-tune Vγ9Vδ2 activation thresholds and functional responses.

A feature of Vγ9Vδ2 TCRs that may be important in answering the question regarding a direct interaction between the TCR and BTN3A is that despite the fact that this population uniformly use a Vγ9 and a Vδ2 chains in their TCR, there exists considerable diversity within the Vγ-Jγ and Vδ-Dδ-Jδ rearrangement, which transcribes into significant amino acid diversity within the CDR3γ and CDR3δ loops of these TCRs (58–60). These loops have been shown via mutagenesis to be important for Vγ9Vδ2 T cell activation (60) and sequence variation of these loops between T cell clones (controlled for expression of Natural Killer receptors) translates into a range of different, graded, reactivity (61). It is thus possible that use of Vγ9Vδ2 TCRs with different sequences, and thus different affinities for BTN3A, may be playing a role in the differing abilities to measure interactions between the Vγ9Vδ2 TCR and BTN3A. Alternatively there may be a requirement for additional co-factors to be expressed on target cells in conjunction with BTN3A to fully engage the majority of the Vγ9Vδ2 T cell repertoire.
Clearly, we have just begun to pull the curtain back on the inner-workings of Vγ9Vδ2 T cell ligand recognition and activation; identification of the B30.2 domain as the pAg sensor has already resulted in compounds developed with higher potency in vitro (35). Identification of additional players in this process will lead to important therapeutic targets with implications both for the treatment of tumors and microbial infections, but also for minimizing immune-related side effects during the use of bisphosphonates for the treatment for osteoporosis and cancer-related bone fractures.

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