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Title:
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Date:
2020-11-01

Citation:
Uldrich, A. P., Rigau, M. & Godfrey, D. I. (2020). Immune recognition of phosphoantigen-butyrophilin molecular complexes by γδ T cells. Immunological Reviews, 298 (1), pp.74-83. https://doi.org/10.1111/imr.12923.

Persistent Link:
https://hdl.handle.net/11343/276406

Immune recognition of phosphoantigen–butyrophilin molecular complexes by γδ T cells

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Running title:
Role of butyrophilins in pAg recognition

Summary :
Gamma-delta (γδ) T cells are an important component of the immune system. They are often enriched in non-lymphoid tissues and exhibit diverse functional attributes including rapid activation, cytokine production, proliferation and acquisition of cytotoxicity following both TCR-dependent and -independent stimulation, but poor capacity for immunological memory. They can detect a broad range of antigens, although typically not peptide-MHC complexes in contrast to alpha-beta (αβ) T cells. In humans, a prominent population of γδ T cells, defined as Vγ9Vδ2^{+} cells, react to small phosphorylated non-peptide 'phosphoantigens' (pAgs). The molecular mechanism underpinning this recognition is poorly defined, but is known to involve butyrophilin family members and appears to involve indirect pAg recognition via alterations to butyrophilin molecular complexes. In this Review we discuss recent advances in our understanding of pAg recognition by γδ T cells including the role of butyrophilins and in particular, a newly described role for butyrophilin 2A1.

Keywords:

phosphoantigens, butyrophilins, γδ T cells, unconventional T cells, innate immunity

Introduction

The three main classes of lymphocytes bearing rearranged antigen receptors are B cells, αβ T cells and γδ T cells. In humans, γδ T cells represent about 1 to 10% of circulating PBMCs[1,2] and are named as such because their antigen receptors are a product of recombination-activating gene (RAG)-mediated somatic gene rearrangement of variable (V), diversity (D) and joining (J) gene segments that are derived from the gamma and delta T cell receptor (TCR) gene loci. Akin to αβTCR gene rearrangement during αβ T cell development, the resulting rearranged gamma and delta chains heterodimerize to form a surface-expressed γδTCR, and combinatorial diversity along with terminal deoxynucleotidyl transferase (TdT)-mediated non-template nucleotide additions/deletions at the V(D)J junctions results in diverse γδTCR repertoires. Structurally, the γδTCR is homologous to both the αβTCR and B cell receptor, where each chain contains three apical loops, termed complementarity-determining regions (CDRs), which are primarily responsible for Ag recognition. Here, the CDR1 and CDR2 loops are encoded by the V gene segments, whereas the CDR3 loops are encoded by the recombined V(D)J reading frames, and thus exhibit the most extensive diversity of the CDRs both in terms of length and amino acid composition.

In humans, γδ T cells are often divided into subsets based on Vδ (also referred to as TRDV) gene usage, with $V\delta1^+$ and $V\delta2^+$ γδ T cells being the most prominent, although others ($V\delta3^+$ to $V\delta8^+$) also exist. In peripheral blood, $V\delta2^+$ cells are abundant, often representing about 60% of γδ T cells[2], although this is highly variable and differs between geographic locations, age and ethnicity[1,3,4]. The vast majority of $V\delta2^+$ γδ T cells co-express a semi-invariant $V\gamma9–J\gamma P^+$ TCR γ-chain[5] and are hence usually referred to as TRGV9–$TRDV2^+$, or $V\gamma9V\delta2^+$ cells. They are normally $CD4^-$ $CD8^-$ and commonly express CD161 along with memory and effector cell markers[6,7]. $V\gamma9V\delta2^+$ cells are believed to arise at an early stage in the fetal liver and then fetal thymus[8], and accumulate numerically in the periphery through adolescence, and then gradually decline with age[2,4].

Notably, $V\gamma9V\delta2^+$ cells can respond to TCR-dependent or TCR-independent stimuli such as IL-18[9] and toll-like receptors (TLRs)[10,11], and IL-2 can augment their response[12]. They have a strong propensity for rapid IFN-γ and TNF production, which is consistent with their constitutive expression of the transcription factor Tbet[4] and a cytotoxic type 1 profile, and also produce chemokines such as MIP-1α, MIP-1β, RANTES and XCL1/lymphotactin[13]. Under certain culture conditions they can produce other cytokines such as IL-4[14], IL-5[15], IL-6[15], IL-9[15], IL-13[15], IL-17[16,17] and IL-22[17]. $V\gamma9V\delta2^+$ cells typically proliferate and acquire potent cytotoxic effector potential, including expression of granzymes, surface CD107a[6], granulysin[18,19], along with NKG2D and other NK cell

receptors[20,21], and they can also induce bystander activation of other immune cell types such as monocytes, dendritic cells, B cells, and αβ T cells[22-24]. For these reasons, Vγ9Vδ2$^+$ cells are likely to play an important role in both the priming and effector phases of immunity. Consistent with constitutive expression of promyelocytic leukemia zinc finger (PLZF)[25], they exhibit an innate-like transcriptome[26] that can be distinguished from other cell types including Vδ1$^+$ γδ T cells at the single-cell level[27]. As such they are grouped into the 'unconventional T cell' family, which also includes αβTCR$^+$ MAIT cells and NKT cells, along with other Vδ2$^-$ γδ T cells that all share common features[28].

Vγ9Vδ2$^+$ cells in immunity

One intriguing aspect of Vγ9Vδ2$^+$ cell biology is the class of stimulatory Ag that they respond to. Unlike conventional αβ T cells, which recognize peptide Ag loaded onto MHC molecules via their TCR, the Vγ9Vδ2$^+$ TCR instead confers reactivity to small phosphorylated metabolite Ag, referred to as 'phosphoantigens' (pAg)[29]. Phosphoantigens are found in all cellular life forms, and are central intermediates in the synthesis of isoprenoids, which are a large family of compounds including cholesterol, vitamins and others that are vital for cellular function. Vγ9Vδ2$^+$ cells are the only known cell type that can respond to pAg, and are found in primates (and possibly some other mammals such as alpaca[30]) but not rodents. Bacteria and animals both synthesize isoprenoids but generally do so via distinct metabolic pathways. Many bacteria (but not all[24]) and apicomplexan parasites utilize the non-mevalonate pathway (also called the 2-C-methyl-D-erythritol 4-phosphate [MEP] pathway) for isoprenoid production, whereas vertebrates utilize the mevalonate pathway. Although both pathways yield isopentyl pyrophosphate (IPP) as a common product, the upstream chemical intermediates are distinct, and notably the non-mevalonate pathway yields (E)-1-hydroxy-2-methyl-but-2-enyl pyrophosphate (HMBPP)[31]. Although HMBPP, IPP and to a lesser extent its downstream isomer dimethylallyl pyrophosphate (DMAPP) are stimulatory pAg for Vγ9Vδ2$^+$ cells, HMBPP has ~10,000-fold higher stimulatory activity than IPP or DMAPP[32]. Indeed, HMBPP is one of the most potent natural Ags ever described for any immune cell, with a reported EC_{50} of less than 1 nM[33]. Thus, Vγ9Vδ2$^+$ cells are uniquely placed to respond to bacterial and apicomplexan pathogens via sensing of HMBPP pAg microbial signatures as a pathogen-associated molecular pattern (PAMP). Vγ9Vδ2$^+$ cells expand to up to 30-50% of circulating T cells in association with many bacterial infections, along with responses to eukaryotic pathogens including *Leishmania*, *Toxoplasma* and *Plasmodium*[34]. Transfer studies of human PBMC into immune-deficient mice suggest that Vγ9Vδ2$^+$ cells are important for anti-bacterial immunity, since in contrast to whole PBMC transfer, PBMC depleted of Vγ9Vδ2$^+$ cells fail to protect these mice[35]. Interestingly, a study testing a candidate *Plasmodium* vaccine in a human cohort also identified circulating Vγ9Vδ2$^+$ cell frequency as a

correlate of protective immunity[36], and likewise, an intra-venous *M. tuberculosis* vaccine in macaques was more effective than other administrative routes, and this correlated with a substantial but transient increase in Vγ9Vδ2$^+$ cell (and MAIT cell) proportion in bronchoalveolar lavage[37]. Thus, Vγ9Vδ2$^+$ cells likely play an important role in the generation of effective immunity against a wide variety of cellular pathogens. These cells may also have a role in viral immunity, including HIV[38], influenza[39,40] and potentially SARS-CoV-2[41,42].

Of note, whilst Vγ9Vδ2$^+$ cells do not spontaneously respond to endogenous host-derived IPP under steady-state conditions, they can respond to aberrant overexpression of IPP, such as often occurs in transformed cells. Consequently, Vγ9Vδ2$^+$ cells have attracted considerable interest due to their ability to directly recognize and kill a wide variety of tumor targets in a pAg-dependent fashion, and there have been numerous clinical trials attempting to harness their immunotherapeutic potential (summarized in [40,43]).

The enduring mystery of pAg recognition

A defining paradigm in T-cell mediated immunity is the concept of MHC-restriction, the Nobel Prize-winning discovery of Doherty and Zinkernagel[44,45]. This framed the concept that T-cells are restricted to interact with polymorphic MHC molecules derived from the host in which they develop, whereupon the TCR simultaneously co-recognises MHC and the antigen it presents to elicit a T cell signal. This holds true for MHC presentation of peptides to conventional CD4$^+$ and CD8$^+$ αβ T cells, and a similar mechanism applies for CD1d presentation of lipids to NKT cells and MHC class I-related (MR1) presentation of vitamin B metabolites to MAIT cells, although CD1 and MR1 are not polymorphic. The identification of pAg as the stimulatory Ag for Vγ9Vδ2$^+$ cells by Tanaka and colleagues in 1995[29] represented a seminal study, and clearly highlighted their unique functional capability compared with the aforementioned αβ T cell subsets. Unlike the Ag-recognition systems by αβ T cells, pAg reactivity appeared to be MHC- and β2m-independent[46], and instead depended on elements only found in human APCs, since APCs derived from other species such as mouse[47] and hamster[48] were unable to 'present' pAg to human Vγ9Vδ2$^+$ cells. Furthermore, activation by pAg required cell-cell contact with Ag-presenting cells[49], suggesting that a surface-expressed ligand, rather than direct recognition, was controlling pAg recognition. On the other hand, pAg-reactivity could be conferred onto reporter T cell lines of either human[50] or mouse[51] origin by artificially expressing a rearranged Vγ9Vδ2$^+$ γδTCR (plus, in the case of mouse reporter cells, a rat-mouse hybrid of CD28), and HMBPP signalling mimics the transcriptional events downstream of TCR ligation[52], indicating that pAg reactivity is dictated by the γδTCR.

Perhaps in part due to the lack of any small animal model to investigate pAg biology, the identity of key pAg-sensing element(s) eluded researchers for many years. Finally, in 2012, Harly and colleagues[51] identified an otherwise poorly described surface ligand belonging to the butyrophilin (BTN) family – BTN3A1 – as a critically important element in the recognition of pAg. The BTN family are cell surface-expressed type I transmembrane proteins belonging to the Ig-superfamily, and in humans consist of six functional members (BTN1A1, BTN2A1, BTN2A2, BTN3A1, BTN3A2 and BTN3A3). They are often expressed on immune cells and their ectodomains share structural similarity to related co-receptors such as CD80 and PD-L1, which are all comprised of IgV- and IgC-like domains. The cytoplasmic domain of BTNs is distinct, and in some cases contains a juxtamembrane region with a sequence consistent with a coiled-coil, along with a B30.2 (PRYSPRY) domain (the only exception being BTN3A2), and in some instances a C-terminal tail[53]. Of note, BTN1A1 may have important functions outside of the immune system as it appears to regulate milk fat micelle formation, and there are also generalized associations between BTN molecules and immune regulation in a number of settings[54]. Harly *et al.*[51] revealed that without BTN3A1 expression on APCs, Vγ9Vδ2$^+$ cells were unable to respond to pAg, and furthermore, different clones of mAb raised against BTN3A molecules could either activate Vγ9Vδ2$^+$ cells in the absence of pAg, or antagonize their responses to pAg.

A subsequent study by Vavassori *et al.*[55] confirmed the importance of BTN3A1 in Vγ9Vδ2$^+$ cell response to pAg, and based on X-ray crystallographic structural models, proposed a mechanism whereby pAg is sequestered by the IgV domain of BTN3A1 and presented to γδTCR in a manner that loosely mimicked MHC presentation of peptides. However, this model of pAg presentation was rapidly disputed by Sandstrom *et al.*[47], who instead proposed a model where pAg bound to the intracellular B30.2 domain of BTN3A1 rather than the extracellular domain, which induced a form of inside-out signalling to elicit Vγ9Vδ2$^+$ cell activation. This was consistent with the initial study by Harly *et al.*, which also identified a key role for the intracellular domain of BTN3A1[51]. Follow-up studies confirmed this interaction between HMBPP and IPP analogues of pAg with BTN3A1 B30.2 domain, with approximate affinity (dissociation constant, K_D) values of 1 μM and 1,000 μM, respectively[33,47,56-60], which is in line with the markedly higher sensitivity of Vγ9Vδ2$^+$ cells to bacterial-derived HMBPP. A separate line of evidence for extracellular binding of pAg to BTN3A1 was also reported in the Vavassori study[55], showing a direct interaction between recombinant BTN3A1 ectodomain and pAg using surface plasmon resonance (SPR)[55]. However, others found no observable interaction using either isothermal titration calorimetry (ITC)[47] or nuclear magnetic resonance (NMR)-based chemical shift assays[61]. Since the original observation by Sandstrom *et al.* that pAg interacts with the intracellular B30.2 domain has now been supported by numerous groups

using ITC[33,47,56-60], NMR[60,61], X-ray crystallography[47,57,58] and fluorescence polarization[62], this is now the generally accepted model.

A new key player – BTN2A1

Despite the clear role of BTN3A1 in pAg-mediated immunity, a confounding issue has been that there was little evidence to support a direct interaction between BTN3A1 and the Vγ9Vδ2$^+$ γδTCR. This raised the possibility that BTN3A1 may not directly bind to the γδTCR, but instead is part of a larger complex in which other components are responsible for part or all of the binding to the γδTCR. Further experimental support of this hypothesis is that transfection of a mouse neuroblastoma cell line with BTN3A1 failed to support recognition of pAg by Vγ9Vδ2$^+$ cells, nor could these cells respond to agonist anti-BTN3A mAb in this system[47]. A separate study found that a hamster cell line transfected with BTN3A1 was also unable to mediate pAg responses but could, on the other hand, promote agonist anti-BTN3A activity[48]. Importantly, this latter study also revealed that CHO cells containing the full human chromosome 6 could support pAg responses by Vγ9Vδ2$^+$ cells, suggesting that a missing factor(s) was contained within this chromosome.

Given that human chromosome 6 contains more than 1,000 functional genes, it was a potentially daunting task to identify which ones were responsible for promoting pAg responses. We investigated this question using a separate approach, by producing Vγ9Vδ2$^+$ TCR ectodomain tetramers to use as probes for ligand discovery. By employing a whole-genome CRISPR/Cas9-mediated knockdown library screen of a human melanoma cell line (LM-MEL-62) that stained brightly for Vγ9Vδ2$^+$ TCR tetramers, we identified another member of the BTN family, BTN2A1, as a direct ligand for Vγ9Vδ2$^+$ TCR[59]. We also generated BTN2A1 ectodomain tetramers, which selectively stained only Vγ9$^+$ γδ T cells (including Vδ2$^+$ and Vδ2$^-$ cells) amongst PBMC. Given that Vγ9$^+$ γδ T cells in PBMC are polyclonal both in terms of their Vδ-pairing and CDR3 composition, the fact that BTN2A1 tetramers bound to essentially all of these cells suggested they were binding to a germline-encoded epitope within the Vγ9 domain. Biophysical assays further supported this hypothesis, revealing that Vγ9$^+$ γδTCR bound to full-length BTN2A1 (IgV–IgC$^+$) ectodomain with an affinity of K_D = 40 to 50 μM, irrespective of whether Vγ9 was paired with a Vδ1$^+$ or Vδ2$^+$ TCR δ-chain[59]. A subsequent study by another group used radiation hybrids between CHO-chromosome 6 cells and BTN3A1-transduced rodent fusion partners independently concluded that BTN2A1 IgV domain interacts with Vγ9$^+$ γδTCR with an affinity of 45 to 50 μM[63]. These findings appear to contrast with an earlier study from Vavassori *et al.*[55] which had excluded a role for BTN2A1 using small interfering RNA knockdown that did not prevent pAg recognition by Vγ9Vδ2$^+$ cells. However,

the knockdown was only partial - the APCs retained 19% of BTN2A1 mRNA levels, which may explain why no impact of BTN2A1 was observed in that study.

In addition to binding to Vγ9$^+$ γδTCR, BTN2A1 was also a critically important mediator of pAg responses by Vγ9Vδ2$^+$ cells in every functional setting that we tested. This included: (a) primary Vγ9Vδ2$^+$ cell activation and cytokine production in PBMC cultures following zoledronate (a bisphosphonate drug that acts as an inhibitor of the mevalonate pathway, leading to intracellular accumulation of IPP to levels above a threshold that can activate Vγ9Vδ2$^+$ cells) or HMBPP challenge; (b) responses by purified pre-expanded Vγ9Vδ2$^+$ cells in refined co-cultures with APCs, both in terms of activation and proliferation; (c) activation and cytokine production of purified pre-expanded Vγ9Vδ2$^+$ cells following HMBPP challenge in the absence of any APC; (d) pAg-mediated killing of LM-MEL-62 tumor cells; and (e) responses of Jurkat T cell lines expressing a Vγ9Vδ2$^+$ γδTCR to IPP, HMBPP and zoledronate[59]. Thus, akin to BTN3A1, BTN2A1 is critically important for immune responses to pAg by Vγ9Vδ2$^+$ cells. We also showed that BTN2A1 transfection could confer onto mouse- and hamster-origin cells the ability to support zoledronate-mediated pAg responses by Vγ9Vδ2$^+$ cells, but only when co-transfected with BTN3A1[59]. This indicates that, notwithstanding the potential importance of additional endogenous proteins that are functionally conserved between humans and rodents (for example RhoB[64]), the added combination of BTN2A1 plus BTN3A1 is necessary and sufficient for enabling rodent cells to initiate pAg responses by Vγ9Vδ2$^+$ cells. While this excluded a critical role for BTN3A2, which is also absent in rodents, inclusion of this butyrophilin molecule in our transfected cells moderately enhanced the response of Vγ9Vδ2$^+$ cells to pAg[59]. This aligns with an earlier study that indicated an important role for BTN3A2 in pAg-mediated Vγ9Vδ2$^+$ T cell activation[65]. Karunakaran *et al.* also confirmed these findings about the role of BTN2A1 by measuring activation of Vγ9Vδ2$^+$ reporter cells following challenge with HMBPP in cultures containing mouse- or hamster- origin cells co-transduced with BTN2A1 and BTN3A1[63].

What is the nature of the BTN2A1–BTN3A1 complex?

In steady state, BTN3A1 likely exists as a homodimer on the cell surface, and/or a heterodimer with other members of the BTN3A family such as BTN3A2[60,65]. Whilst being unable to directly bind pAg due to a lack of a B30.2 domain, BTN3A2 nonetheless appears to be important, possibly by facilitating migration of BTN3A1 from the endoplasmic reticulum to the cell surface[65]. X-ray crystallography-based structural analysis of the BTN3A1 ectodomain[66], along with cryo-electron microscopy 2D class averages of full-length BTN3A1–BTN3A2 heterodimer[60], indicate that a V-shaped homodimer is favoured in steady-state, although other dimer conformations could

conceivably exist. A potentially relevant head-to-tail dimer was identified within the crystal lattice of BTN3A1 ectodomain, leading to the hypothesis that this may represent an inactive conformation of BTN3A1 by allowing it to lie flat against the cell membrane and thus be inaccessible to the γδTCR[60], or, alternatively, that it may instead be an active conformation[58].

The functional cooperation between BTN2A1 and BTN3A1 raised the possibility that these ligands may be physically associated with each other. This was supported by transfection experiments where BTN2A1 and BTN3A1 needed to be co-expressed on the same APC (in cis), versus mixed co-cultures using APC that separately expressed BTN2A1 and BTN3A1 (in trans), which failed to elicit any response from Vγ9Vδ2$^+$ cells to pAg[59]. Using two independent Förster resonance energy transfer (FRET)-based approaches that measured FRET between either the ectodomains or intracellular domains, we showed that BTN2A1 and BTN3A1 are closely associated (within 5 to 10 nm) on the cell surface. Furthermore, a panel of anti-BTN2A1 mAb that exhibited antagonist activity on Vγ9Vδ2$^+$ cell responses to pAg also all strongly disrupted the association between BTN2A1 and BTN3A1, implying that this may be an important mode by which the mAb exert their antagonistic effects[59]. Karunakaran *et al.* also provided evidence of a direct association between BTN2A1 and BTN3A1 using pull-down assays and by identifying chemical shift perturbations of some residues within BTN3A1 IgV domain following exposure to soluble BTN2A1 IgV domain[63].

These findings collectively raise several important mechanistic considerations for how BTN2A1 and BTN3A1 might be co-operating, particularly with respect to the stoichiometry of this complex. In the simplest model, a single copy of BTN3A1 associates with a single copy of BTN2A1, herein termed a 'one-plus-one' heterodimeric model (**Fig. 1**). This is possible, although if BTN3A1 is sequestered in the form of a BTN3A1–BTN3A dimer (i.e. BTN3A1 paired with either BTN3A1, BTN3A2 or BTN3A3) during steady-state, this model would require disruption of these existing dimers and reformation of active BTN3A1–BTN2A1 heterodimers. Karunakaran *et al.* also noted that BTN2A1 forms homodimers, and the interface incorporates a membrane-proximal interchain disulphide bond[63]. Interestingly, disruption of this disulphide bond had no effect on the functional ability of BTN2A1 to prime Vγ9Vδ2$^+$ cell responses to pAg, implying that either the BTN2A1 dimer is not functionally relevant, or that it is stabilised by other non-covalent interactions. Therefore, the one-plus-one model would additionally require disruption of this BTN2A1 homodimer interchain disulphide bond. Another model comprising a 'two-plus-two' stoichiometry might be compatible with the known steady-state dimer conformations of both BTN2A1 and BTN3A, i.e. BTN2A1 homodimers coupled with BTN3A1–BTN3A heterodimers (**Fig. 1**). In this scenario, BTN3A1 might homodimerise, or alternatively might instead heterodimerise with BTN3A2. In support of the latter possibility, the BTN3A1–BTN3A2 heterodimer is reportedly stable within lipid nanodiscs[60], and

BTN3A2 also facilitates enhanced surface expression of BTN3A1[65]. Willcox *et al.* reported that a mutant form of BTN3A1 containing either an Arg73-Glu or Lys136-Met mutation, located in the C′ and G β-strands, respectively, ('CFG face') of the IgV-like domain, rendered BTN3A1 unable to support pAg-mediated activation, indicating that the CFG face plays an important role in pAg responses. Interestingly, wildtype BTN3A2 co-expression overcame this defect, although the converse was not the case, since wildtype BTN3A1 co-transfected with mutated BTN3A2 was poor at pAg sensitization[67]. This suggests that even though BTN3A2 cannot directly bind pAg due to the lack of a B30.2 domain, it can pair with BTN3A1 and support ligand-binding. A chemical shift perturbation assay performed by Karunakaran *et al.* suggested that at least one of these same residues (Lys136), along with others on the CFG face of the BTN3A1 IgV-like domain, are responsible for interfacing with the BTN2A1 IgV-like domain[63].

A third model comprising a heterotrimeric 'two-plus-one' stoichiometry is possible, where a BTN2A1 homodimer pairs with a single copy of BTN3A1, or conversely a 'one-plus-two', in which a single BTN2A1 molecule pairs with a BTN3A1–BTN3A dimer. Both of these models would be subject to the same caveats with respect to existing dimer disruption as the one-plus-one model. Lastly, a higher order 'n-plus-n' multimer model, wherein BTN2A1 and BTN3A1 (and possibly BTN3A2 and BTN3A3) form an extended polymerization pattern on the cell surface upon pAg binding, could efficiently crosslink the Vγ9Vδ2$^+$ γδTCR (**Fig. 1**). It cannot be formally excluded that other membrane proteins such as the ATP-binding cassette transporter A1 (ABCA1)[68] might also be incorporated into the BTN2A1–BTN3A complex and play an important role in forming the core biological unit of pAg-detection. It will be important for future studies to address all of these key possibilities.

Is BTN2A1 involved in the proximal events following pAg binding to BTN3A1?

Earlier studies demonstrated that pAg binding to BTN3A1 induces a number of effects. Both IPP and HMBPP bind to the same positively charged pocket on the side of the B30.2 domain, and this appears to result in conformational changes to solvent-exposed residues on the B30.2 surface that are both proximal and distal to the pAg-binding pocket[60,61]. Site-directed mutagenesis of various residues within the B30.2 domain indicates that many of the residues surrounding the binding pocket are important for inducing Vγ9Vδ2$^+$ cell responses to pAg[69]. Notably, His351 and Arg469, which are located within the pAg-binding pocket, are critical[47,69]. Interestingly, an Arg351-His mutation within the B30.2 domain of BTN3A3, which is normally unable to mediate efficient pAg detection by Vγ9Vδ2$^+$ cells, resulted in a gain-of-function phenotype in terms of pAg binding and ability to elicit Vγ9Vδ2$^+$ cell responses[47]. Other residues such as Trp350 and Trp391 also appear to be essential in

pAg-mediated activation of Vγ9Vδ2$^+$ cells[69], despite the fact that mutations to these residues only had a moderate and minor effect, respectively, on the affinity for pAg[58]. This implies that they are important for mediating some other functional aspect of pAg-mediated signal transduction aside from directly binding pAg.

One hypothesis, consistent with these data, is that in addition to an ectodomain-mediated homodimer, the intracellular domain of BTN3A1 can also independently homodimerize. This notion is supported by X-ray crystallographic structures wherein two distinct and mutually exclusive dimer patterns have been observed. One exhibits two-fold symmetry with an interface that is distal to the pAg-binding pocket, and the other is asymmetric wherein a pAg-proximal domain of one B30.2 molecule interfaces with a pAg-distal domain of a second molecule[60]. Yang *et al.* provided evidence in support of the latter asymmetric dimer being functionally important by showing that bulky analogues of HMBPP had superior affinity for BTN3A1 B30.2 domain yet markedly lower biological activity, indicating that they were interfering with dimer formation via steric hindrance[58]. How these events propagate out to the extracellular environment is unclear, although additional motifs within the coiled-coil juxtamembrane domain of BTN3A1 also appear to be essential for driving activation of Vγ9Vδ2$^+$ cells[53,56].

Akin to BTN3A1, BTN2A1 also contains a B30.2 domain and a juxtamembrane motif, plus a cytoplasmic tail of about 20 residues in length. In the most simplistic sense, and based on the aforementioned observations, BTN2A1 could merely be acting as a coreceptor in stabilising the Vγ9$^+$ TCR interaction in order to enable it to bind some other ligand. To test if BTN2A1 has any role beyond binding to Vγ9$^+$ γδTCR, we generated a modified form of BTN2A1 in which the entire transmembrane and intracellular domains were exchanged with those from an irrelevant protein (mouse paired immunoglobulin-like type 2 receptor beta). This chimeric protein associated with BTN3A1 and bound to Vγ9$^+$ TCR, yet strikingly, failed to support pAg recognition by Vγ9Vδ2$^+$ cells[59]. This indicates that in addition to binding the Vγ9$^+$ TCR, BTN2A1 has additional important functions mediated through its transmembrane and/or intracellular domains, and therefore appears to be more than simply a coreceptor. The precise role of these domains within BTN2A1 has not been explored further, but at the very least recombinant BTN2A1 B30.2 domain did not appear to directly bind pAg[59], which is consistent with the fact that it does not possess a cationic pAg-binding pocket like BTN3A1 does[53]. This raises a critical question of what role does the BTN2A1 intracellular domain play in pAg biology? One possibility is that the BTN2A1 intracellular domain directly associates with, or dissociates from, its BTN3A1 counterpart upon pAg encounter, thereby forcing an intracellular conformational change that results in promotion of an active/stimulatory form, or removal of an inactive 'checkpoint' form. To this end, the aforementioned literature regarding

BTN3A1 B30.2 domain dimers should be reconciled with the possibility that BTN2A1 may serve as an additional binding partner. If and how BTN2A1 senses pAg is unclear, since we did not detect any alterations in the levels of FRET between the BTN2A1 and BTN3A1 intracellular domains upon pAg encounter[59], and Karunakaran *et al.* also did not observe any change in BTN2A1–BTN3A1 complex isolation efficiency in the presence or absence of zoledronate[63]. However, these observations should not necessarily be taken as absolute evidence against a model of pAg-induced BTN2A1–BTN3A1 dimer remodelling, since these assays may not be very sensitive to subtle alterations. Alternatively, BTN2A1 could facilitate intracellular signal transduction or recruit other important molecules. Notably, BTN3A1 appears to be capable of transmitting signals into the cells that express them upon cross-linking on the cell surface[70-72], and in general, B30.2 domains are commonly involved in protein-protein interactions[73]. Clearly there are some important studies to be carried out to explore these key questions.

How do BTN2A1–BTN3A1 complexes activate Vγ9Vδ2$^+$ cells?

The anti-BTN3A mAb that exerts agonist activity (clone 20.1) binds to the BTN3A1 IgV domain in such a way that it likely precludes both arms of the mAb from binding within a single copy of BTN3A1 homodimer[66]. Instead, this mAb probably bridges adjacent BTN3A1 homodimers, thus potentially cross-linking BTN3A1 on the cell surface. In support of this notion, Fab fragments as well as a single-chain variable fragment of 20.1 exhibit much reduced stimulatory capacity compared to full-length mAb. By contrast, the antagonist anti-BTN3A mAb (clone 103.2) binds to a separate epitope on the IgV domain in such a way that both arms of this mAb can likely interact with a single BTN3A1 homodimer. We found that Vγ9Vδ2$^+$ cell activation mediated by agonist anti-BTN3A mAb was dependent on BTN2A1, since BTN2A1^{null} APCs, or Vγ9Vδ2 TCR$^+$ cell lines with point mutations in the BTN2A1-binding domain (see below), abrogated responsiveness to agonist anti-BTN3A mAb[59]. Collectively, these observations suggest that Vγ9Vδ2$^+$ cell reactivity to pAg requires BTN2A1 expression *and* (a) cross-linking of BTN3A1 on the APC cell surface, *and/or* (b) a conformational change of the BTN3A1 ectodomain. In support of (a), the dynamics of BTN3A1 within the plasma cell membrane appear to change following pAg challenge and become less mobile[47,51,64]. Further, the GTPase activity of RhoB appears to be important, and this can bind the intracellular domain of BTN3A1 and potentially alter the distribution of BTN3A1 by cytoskeletal trapping[64]. An interaction between periplakin and the intracellular domain of BTN3A1 was also reported, which could further regulate cytoskeletal interactions, however knockdown of periplakin using small hairpin RNA did not significantly affect Vγ9Vδ2$^+$ cell responses to pAg, indicating that periplakin may not be essential for pAg responses[57].

Other studies have tested possibility (b) – a conformational change in the BTN3A1 ectodomain. Sebestyen *et al.*[64] measured FRET between fluorochromes within the cell membrane and those conjugated to anti-BTN3A mAb. Upon exposure to zoledronate, there was a decrease in FRET levels, suggesting an increase in the distance between the cell membrane and the BTN3A1 IgV domain[64]. Another study introduced an artificial disulfide bond at a position in the BTN3A1 IgC domain that, based on the crystal structure, would permit formation of the V-shaped homodimer of BTN3A1 but likely impair other conformational changes and also any re-pairing of BTN3A1 molecules with other proteins. This modification significantly reduced the ability of these BTN3A1$^+$ cells to support Vγ9Vδ2$^+$ cell responses to both pAg and anti-BTN3A agonist mAb, suggesting that conformational changes to the BTN3A1 ectodomain appear to be important, and/or that BTN3A1 dimers with other proteins such as BTN3A2 or BTN3A3 (or BTN2A1 as we would now expect) may shuffle upon pAg encounter[60]. Regardless, it appears that rather than direct Ag recognition, modulation of BTN complexes by pAg, and their subsequent recognition by Vγ9Vδ2$^+$ cells, falls into a new class of indirect altered-self recognition.

How are Vγ9Vδ2$^+$ γδ T cells sensing pAg?

Given that BTN2A1 binds all Vγ9$^+$ γδ T cells including Vγ9Vδ1$^+$ cells, yet only a subset of these – Vγ9Vδ2$^+$ cells – respond to pAg, an additional ligand or element must be responsible for restricting pAg responses to Vγ9Vδ2$^+$ cells, and in particular those with a permissive semi-invariant CDR3γ loop. Through site-directed mutagenesis of the Vγ9Vδ2$^+$ TCR, we identified three residues within the Vγ9 domain that were each important for binding to BTN2A1. The side chains of these residues – Arg20, Glu70 and His85 – formed a closely spaced triad that maps to the side of Vγ9 on the ABED β-sheet face, and well-removed from the CDR loops and apical surface of Vγ9[59]. This was also consistent with the absence of any role for CDR loops or the δ-chain in binding to BTN2A1 tetramer.

Another recent study proposed a role for the hypervariable region 4 (HV4) loop or region of the Vγ9 domain in BTN2A1 recognition, being the sequence on the apical surface of the TCR that connects the D and E strands of Vγ9[67]. Our observations do not necessarily exclude this hypothesis since we only tested the role of one residue within this loop, Glu76, which did not appreciably affect BTN2A1 binding or pAg responsiveness[59]. However, our data indicate that the BTN2A1 binding site encompasses multiple residues on the side of the TCR. Neither Glu70 nor His85 can really be included in any definition of the HV4 region since they are both distal (7 and 8 residues, respectively) to apex of this loop.

In addition to abrogating BTN2A1-binding, mutations to Arg20, Glu70 and His85 also ablated reactivity to zoledronate, lending further support to the importance BTN2A1. Consistent with

an earlier study[74], we found that mutations to two other residues – Arg51 within the Vδ2-encoded CDR2δ loop, and Lys108 within the CDR3γ loop – impaired reactivity to zoledronate. Yet, mutations to these same residues had no effect on BTN2A1 binding[59]. Therefore, aside from any convoluted allosteric interpretations, the most likely explanation is that these residues form part of a separate ligand-binding domain on the opposing side of the TCR, which is 30 to 40 Å distal to the BTN2A1-binding domain. This second binding domain lies atop the TCR near the Vδ2–Vγ9 interface, and potentially abuts but does not necessarily strongly interface with the typically polyclonal CDR3δ. As such this model would explain three fundamental features of the pAg-reactive Vγ9Vδ2$^+$ cell repertoire. Firstly, the strong requirement for Vδ2 gene usage could be explained by the key role of the CDR2δ motif in this second binding site. Secondly, the largely invariant sequence of the CDR3γ could be explained because, based on the Vγ9Vδ2$^+$ TCR structure, a Lys at or close to position 108 appears to be in close proximity to the CDR2δ[75], and therefore may contribute to this second ligand interface. Thirdly, the high degree of heterogeneity in the length and composition of the CDR3δ loop could be explained by the fact that this loop, whilst often bulky, is not extensively incorporated into the second ligand-binding domain since it sits in a slightly more centric position on the TCR surface.

What the identity of the ligand is for this second binding domain remains to be demonstrated, although BTN3A1 is strong candidate since it would inevitably be in close proximity to the TCR anyway due to its association with BTN2A1. Given that our findings showed that BTN2A1 and BTN3A1 must be expressed by the same APC, this suggests that the interaction with the TCR involves simultaneous binding of both molecules, although it's presently an assumption that both ligands can bind to the same TCR molecule, rather than adjacent TCR molecules. With this in mind, perhaps pAg-mediated inside-out signalling might induce an alteration in the relative positioning of the BTN2A1 and BTN3A1 molecules such that they can both straddle and interact with the TCR. It is also possible that the BTN2A1–BTN3A1 complex forms a composite epitope with another undefined molecule, or that another molecule independently binds to the second binding site on the TCR instead, however these models would demand that any such ligand be conserved between humans and rodents.

Another interesting question is why BTN2A1 does not elicit spontaneous activation of Vγ9$^+$ cells under steady-state conditions, given that it is constitutively expressed on most PBMCs[59]. One possible explanation might be that which it can readily bind to Vγ9, it cannot induce TCR-mediated signal transduction in isolation. Alternatively, the BTN2A1 complex with BTN3A1 plus other potential co-factors may normally reduce the binding affinity or sterically hinder BTN2A1 for Vγ9 in the absence of pAg. A recent study undertook a large-scale ligand discovery campaign for Ig superfamily proteins and identified a new candidate binding partner for BTN2A1, FAM187B, plus

eight new potential binding partners for BTN3A, so it will be very interesting to see if any of these molecules have a role in pAg or γδ T cell biology[76]. These are the next fundamental questions that must be answered in order to develop a better understanding of the pAg-sensing system, which is turning out to be highly distinct from all other characterised modes of Ag recognition.

Generality of γδ T cell–butyrophilin interactions

Aside from BTN2A1–BTN3A, there are growing extended links between the biology of the broader BTN family and γδ T cells. A seminal study by Di Marco Barros *et al.* in 2016 revealed that members of the closely-related family of butyrophilin-like (BTNL) proteins, of which there are five human and six mouse genes, also play an important role in γδ T cell immune regulation both in mice and humans[77]. In humans, BTNL3 and BTNL8, which are membrane proteins commonly expressed on gut epithelia, play an important role in the immune regulation of Vγ4$^+$ γδ T cells, and subsequent studies have underscored the importance of the Vγ4$^+$ γδTCR in this process, which is a direct molecular target of BTNL3[67,78]. Like BTN2A1 and BTN3A1, both BTNL3 and BTNL8 also consist of extracellular IgV and IgC domains and an intracellular B30.2 domain, although the function of their B30.2 domains is unclear. The BTNL3–BTNL8 and BTN2A1–BTN3A1 systems share common characteristics since in both cases potentially similar epitopes on the IgV domains within a heterodimeric BTN or BTNL complex serve as a direct ligand for a germline-encoded region within the Vγ domain of a γδTCR[59,63,67,78]. Furthermore, residues in Vγ4 that are in equivalent positions to Glu70 and His85 in Vγ9 (Asp69 and Arg83 in the mature protein sequence of Vγ4, respectively) might also be important for BTNL3 recognition, suggesting a related docking mode.

Further strengthening the ties between BTN and BTNL molecules and γδ T cells, Di Marco Barros *et al.* also found that in mice, Btnl1 and Btnl6 were important regulators of TCR-dependent responses by intestinal mouse Vγ7$^+$ γδ T cells[77]. Similar to above, domain swapping experiments suggest a conserved mode of Vγ7 recognition by Btnl6 compared with Vγ4 recognition by BTNL3[67]. Coupled with the association between and importance of the related *Skint1* and *Skint2* gene products in regulating Vγ5Vδ1$^+$ dendritic epidermal T cells (DETC) in mouse skin[79,80], it is becoming evident that BTN, BTNL and related molecules form heteromeric complexes and play a generalized role in regulating γδ T cell immunity.

Of additional interest will be examining the role of BTN2A2 in the γδ T cell-mediated pAg-sensing system. Despite 87% sequence homology with BTN2A1, we found a reduced binding capacity of BTN2A2-transfected cells for Vγ9Vδ2$^+$ tetramers, compared to BTN2A1-transfected cells. It is possible that the level of BTN2A2 on the cell surface of these transfected cells is lower, or that BTN2A2 has lower affinity for Vγ9Vδ2$^+$ tetramers[59]. This suggests that the role of BTN2A1 in

pAg-sensing is non-redundant with BTN2A2, however, Karunakaran *et al.* found a comparable binding affinity of BTN2A2 IgV domain for Vγ9Vδ2$^+$ TCR in a cell-free assay[63]. Unlike BTN2A1, or BTN3A family members, there is a functional murine homologue of BTN2A2 with a purported role in regulating T cell immunity[81]. Since mice lack pAg-reactive γδ T cells, it remains to be determined how BTN2A2 functions, and mice may serve as a suitable model to study this further. Whether there are any additional BTN or BTNL family members that also regulate other γδ T cell subsets beyond those mentioned here also remains an open and important question.

Concluding remarks

Recent breakthroughs that identified BTN2A1 and BTN3A1 as core mediators of pAg sensing by Vγ9Vδ2$^+$ cells, and underscores just how distinct this mode of Ag recognition is compared to the MHC recognition system that governs αβ T cell function. Despite these findings, there are still major gaps in our understanding of how pAg mediates inside-out signalling, and how BTN complexes are recognized by γδ T cells. A deeper understanding of this biology, how BTN molecules are regulated in health and disease, and how this impacts on γδ T cell function, is important if we are to develop more effective BTN/γδ T cell immunotherapy-based treatments for cancer and other diseases.

Acknowledgments

This work was supported by the Cancer Council of Victoria (#1126866), the Australian Research Council (ARC; CE140100011, DP170102471); the National Health and Medical Research Council of Australia (NHMRC; 1184906, 1165467, 1113293). APU was supported by an ARC Future Fellowship (FT140100278); MR is supported by the Deutsche Forschungsgemeinschaft (GRK2168) and the University of Melbourne through the International Research and Research Training Fund; DIG is supported by NHMRC Senior Principal Research Fellowship (1117766).

Competing interests: APU, MR and DIG are listed as inventors on patent applications related to the use of BTN2A1 to influence immune reactions.

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Figure 1. Models of the BTN-pAg signalling complex. Different models depicting the composition of the BTN-pAg biological unit are shown on the APC cell surface, which are recognised by the Vγ9Vδ2^{+} TCR.