Author/s:
Fulford, TS;Soliman, C;Castle, RG;Rigau, M;Ruan, Z;Dolezal, O;Seneviratna, R;Brown, HG;Hanssen, E;Hammet, A;Li, S;Redmond, SJ;Chung, A;Gorman, MA;Parker, MW;Patel, O;Peat, TS;Newman, J;Behren, A;Gherardin, NA;Godfrey, DI;Uldrich, AP

Title:
Vγ9Vδ2 T cells recognize butyrophilin 2A1 and 3A1 heteromers

Date:
2024-08-01

Citation:
Fulford, T. S., Soliman, C., Castle, R. G., Rigau, M., Ruan, Z., Dolezal, O., Seneviratna, R., Brown, H. G., Hanssen, E., Hammet, A., Li, S., Redmond, S. J., Chung, A., Gorman, M. A., Parker, M. W., Patel, O., Peat, T. S., Newman, J., Behren, A. ,... Uldrich, A. P. (2024). Vγ9Vδ2 T cells recognize butyrophilin 2A1 and 3A1 heteromers. Nature Immunology, 25 (8), pp.1355-1366. https://doi.org/10.1038/s41590-024-01892-z.

Vγ9Vδ2 T cells recognize butyrophilin 2A1 and 3A1 heteromers.

Thomas S. Fulford[1], Caroline Soliman[1], Rebecca G. Castle[1], Marc Rigau[1,2], Zheng Ruan[1], Olan Dolezal[3], Rebecca Seneviratna[1], Hamish G. Brown[4], Eric Hanssen[4,5,6], Andrew Hammet[7], Shihan Li[1], Samuel J. Redmond[1], Amy Chung[1], Michael A. Gorman[5], Michael W. Parker[5,8], Onisha Patel[9,10], Thomas S. Peat[3], Janet Newman[3], Andreas Behren[11,12], Nicholas A. Gherardin[1], Dale I. Godfrey[1*], Adam P. Uldrich[1,13,14*]

[1]Department of Microbiology & Immunology at the Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Victoria, 3010, Australia.
[2]Institute of Molecular Medicine and Experimental Immunology, Rheinische Friedrichs-Wilhelms University of Bonn, D-53127 Bonn, Germany.
[3]Biomedical Manufacturing, Commonwealth Scientific and Industrial Research Organisation, 343 Royal Parade, Parkville, Victoria, 3052, Australia.
[4]Ian Holmes Imaging Centre, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, 3052, Australia.
[5]Department of Biochemistry and Pharmacology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, 3052, Australia.
[6]ARC Industrial Transformation Training Centre for Cryo-electron Microscopy of Membrane Proteins, University of Melbourne, Parkville, Victoria, 3052, Australia.
[7]CSL Limited, Bio21 Molecular Science and Biotechnology Institute, Parkville, Victoria 3052, Australia.
[8]St. Vincent's Institute of Medical Research, Fitzroy, Victoria, 3065, Australia.
[9]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, 3052, Australia.
[10]Department of Medical Biology, University of Melbourne, Parkville, Victoria, 3052, Australia.
[11]Olivia Newton-John Cancer Research Institute, and School of Cancer Medicine, La Trobe University, Heidelberg, Victoria, 3084, Australia.
[12]Department of Medicine, University of Melbourne, Parkville, Victoria, 3010, Australia
[13]Cancer Immunology Program, Peter MacCallum Cancer Centre, Melbourne, Australia.
[14]Lead contact

*Correspondence to: auldrich@unimelb.edu.au, godfrey@unimelb.edu.au

Abstract

Butyrophilin (BTN) molecules are emerging as key regulators of T cell immunity, however, how they trigger cell-mediated responses is poorly understood. Here, the crystal structure of a gamma-delta T cell receptor (γδTCR) in complex with BTN member 2A1 (BTN2A1) revealed that BTN2A1 engages the side of the γδTCR, leaving the apical TCR surface bioavailable. We reveal that a second γδTCR ligand co-engages γδTCR via binding to this accessible apical surface in a BTN3A1-dependent manner. BTN2A1 and BTN3A1 also directly interact with each other in cis, and structural analysis revealed formation of W-shaped heteromeric multimers. This BTN2A1–BTN3A1 interaction involved the same epitopes that BTN2A1 and BTN3A1 each use to mediate the γδTCR interaction; indeed locking BTN2A1 and BTN3A1 together abrogated their interaction with γδTCR, supporting a model wherein the two γδTCR ligand-binding sites depend on accessibility to cryptic BTN epitopes. Our findings reveal a new paradigm in immune activation, whereby γδTCRs sense dual epitopes on BTN complexes.

Main text

T cell receptor (TCR) recognition of antigen is a central event in immunity. Alpha-beta (αβ) T cells become activated following recognition of peptide fragments in complex with major histocompatibility complex molecules (pMHC), which are sensed by somatically rearranged T cell receptors (αβTCRs) derived from the TCRα (*TRA*) and TCRβ (*TRB*) gene loci. By contrast, gamma-delta (γδ) T cells represent a separate lineage of MHC-unrestricted T cells that express rearranged antigen (Ag) receptors derived from the TCRγ (*TRG*) and TCRδ (*TRD*) gene loci. γδ T cells play a key role in the priming and effector phases of immunity to infectious diseases as well as in tissue surveillance [1]. In humans, the majority of circulating γδ T cells express a semi-invariant Vγ9Vδ2$^+$ (TRGV9–TRGV2) γδTCR that confers reactivity to a distinct class of non-peptide Ag, termed phosphoantigens (pAg), which are metabolic intermediates in the biosynthesis of isoprenoids [2,3]. There are two classes of pAg; those derived from the non-mevalonate pathway such as (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP), found in bacteria and apicomplexan parasites, and those derived from either the mevalonate or non-mevalonate pathways, such as isopentenyl pyrophosphate (IPP), found in all classes of life including vertebrates. Both 'foreign' HBMPP and ubiquitous IPP pAgs are stimulatory for γδ T cells to differing degrees, and facilitate potent anti-microbial and anti-tumour immunity, respectively [4,5].

Butyrophilin (BTN) and butyrophilin-like (BTNL) molecules are a family of surface-expressed transmembrane proteins that are typically comprised of extracellular immunoglobulin-superfamily variable (IgV)- and constant (IgC)-like domains, as well as an intracellular B30.2 domain. In certain combinatorial pairs, BTN and BTNL molecules support the activation of discrete γδ T cell subsets. For instance, BTNL3 and BTNL8 are expressed by gut epithelia and cooperate to facilitate the activation of Vγ4$^+$ γδ T cells [6]. Likewise, in mice, Btnl1 and Btnl6 facilitate the activation of gut-resident Vγ7$^+$ γδ T cells [6,7], and the Btnl family members Skint1 and Skint2 are important for the development and function of skin-resident Vγ5Vδ1$^+$ dendritic epidermal T cells (DETCs) [8,9]. Unlike all other modes of T cell antigen recognition in which antigens are presented on the cell surface for recognition via the TCR, BTN member 3A1 (BTN3A1) sequesters pAg via a positively charged pocket within its intracellular B30.2 domain, which is an essential step in the initiation of Vγ9Vδ2$^+$ T cell activation [10]. Together, BTN member 2A1 (BTN2A1) and BTN3A1 mediate γδ T cell responses to pAg [11,12]. Thus, BTN molecules have emerged as important regulators of γδ T cell-mediated immunity and do so as heteromeric pairs.

Recently, reports have shown that BTN molecules can directly bind the γδTCR, including BTNL3, which interacts with human Vγ4, and mouse Btnl1 which interacts with Vγ7 [7,13]. Likewise, BTN2A1 is a binding partner for human Vγ9 domain [11,12]. However, whilst there appears to be an evolutionarily conserved mode of recognition between these systems based on overlapping regions of importance within Vγ domains [7,9,11-13], the molecular mechanism that governs BTN ligand recognition by γδTCR heterodimers is poorly understood, and structural information is lacking. Furthermore, in the case of BTN2A1, the mode of ligand recognition is unclear since one study found a dependence on the side of the Vγ9 domain [11], whereas other studies have additionally implicated the hypervariable-4 (HV4) loop [12,13] or the complementarity-determining region (CDR) 3δ loop [14], both located on the apical surface of the γδTCR.

In addition to Vγ9Vδ2$^+$ TCR binding to BTN2A1, mutational analysis revealed the presence of a second ligand-binding region that was distinct from the BTN2A1 binding site, which is also essential for immune reactivity to pAg [11,15]. In one model, BTN3A1 could represent the second ligand, however, a direct BTN3A1–γδTCR interaction has not been established. An alternative model proposes that an undefined ligand binds γδTCR, and BTN3A1 engages another undefined receptor expressed by Vγ9Vδ2$^+$ T cells, rather than the γδTCR itself [14]. Finally, although BTN2A1 and BTN3A1 are constitutively expressed by immune cells, γδ T cell activation only occurs following pAg challenge, or cross-linking with agonist anti-BTN3A antibodies such as clone 20.1 [16]. Thus, a molecular switch that involves either a conformational change and/or remodelling of BTN complexes on the cell surface of APCs appears to initiate γδ T cell responses. However, the nature of these regulatory mechanisms is unclear.

Here, we report the crystal structure of the BTN2A1–γδTCR complex, which represents the first structural example of a TCR engaging a ligand outside of an MHC/MHC-like family member, wherein BTN2A1 binds to the side of Vγ9. Furthermore, we reveal that binding of a second ligand to the apical surface of the Vγ9Vδ2$^+$ TCR is dependent on BTN3A1 expression. We also report the structure of a heteromeric BTN complex, namely BTN2A1–BTN3A1, in which the interface between the disparate BTN molecules involves the same epitopes that are required for γδTCR binding. Indeed, locking them together abrogated this interaction. Thus, the association between BTN2A1 and BTN3A1 in cis might serve as a regulatory mechanism by burying the TCR-binding determinants via sequestration with each other. Accordingly, we provide evidence for a two-ligand sensing system that, upon triggering, acquires affinity for

γδTCR through the exposure of cryptic epitopes, and coupled with pAg signalling via BTN3A1, can then co-bind the Vγ9Vδ2^{+} TCR.

Results

BTN2A1 engages the side of γδTCR

To understand the molecular mode of BTN engagement by γδTCR, we solved the crystal structure of BTN2A1 ectodomain in complex with Vγ9Vδ2^{+} γδTCR, which diffracted to 2.1 Å resolution in space group $C222_1$ (**Extended Data Table 1**). The unbiased electron density was clear for both the γδTCR and BTN2A1 components of the structure (**Extended Data Fig. 1A**). The asymmetric unit of the complex contained two copies of BTN2A1, arranged as a V-shaped homodimer ('V-dimer'). One of the BTN2A1 copies liganded to γδTCR, and the other one was unliganded (**Fig. 1A**). BTN2A1 engaged the side of the γ-chain, binding to the Vγ9-encoded IgV domain, jutting out at an angle of ~54°, which starkly contrasted αβTCR engagement of pMHC, or γδTCR recognition of CD1d (**Fig. 1B**). The outer face of the Vγ9 germline-encoded β-sheet formed by the A, B, D and E β-strands (ABED face) mediated binding to the β-sheet encoded by the C, F and G β-strands (CFG face) of the BTN2A1 IgV domain (**Fig. 1C**), with interactions by all these strands (**Fig. 1D**). The γδTCR buried 470 Å^2 upon ligation, and BTN2A1 buried 480 Å^2, together forming a total interface buried surface area (BSA) of 950 Å^2) (**Fig. 1D)**. This is approximately half of a typical αβTCR-pMHC complex, with the molecules anchored together by fourteen H-bonds or salt bridges **(Extended Data Table 2**). On the γδTCR, the B-, D- and E-strands of Vγ9 contributed 57%, 17% and 11% of the BSA, respectively, whereas the CC′-loop, F- and G-strands of BTN2A1 contributed 35%, 15%, and 44%, respectively. Previous studies implicated the hypervariable region 4 (HV4) loop, also known as the DE loop, of Vγ9, as well as the CDR2γ and CDR3δ loops, in binding BTN molecules [12-14]. The crystal structure revealed that the BTN2A1 binding site on Vγ9 was distal to both the HV4γ and CDR2γ loops (>7 Å separation; **Fig. 1E** and **F**), and instead left the entire apical surface of the γδTCR solvent exposed (**Fig. 1A**). Within the BTN2A1 interface, the aromatic residues Phe43, Tyr98 and Tyr105 interacted with Vγ9Vδ2 γδTCR, as did the acidic residues Asp106 and Glu107. Ser41, Gln42, Phe43 and Ser44 formed the CC′ loop of BTN2A1 (**Fig. 1C**), and their involvement is consistent with the over-representation of this loop in other IgV-mediated interfaces [17].

Of note, the aromatic side chain of Phe43 sat planar to the guanidinium moiety of the Arg20γ side chain (**Fig. 1G**), facilitating a cation-π interaction with a predicted electrostatic binding energy of -4.6 kcal/mol. Arg20γ also formed a water-mediated H-bond with Gln100 of BTN2A1, along with main chain-mediated H-bonds to the Tyr105 side chain hydroxyl group (**Fig. 1G**), providing a structural basis for the importance of Arg20γ in binding to BTN2A1 [11]. Likewise, we have shown previously that mutations to Glu70γ and His85γ abrogate BTN2A1

Commented [DG1]: Your figure suggests >8A, not >7 or >10.

reactivity [11], and these residues were interconnected by a H-bond, and also bound BTN2A1 (**Fig. 1H**). Here, Glu70γ H-bonded to the Phe43 and Ser44 main chains, and His85γ made Van der Waal (VDW) contacts with Ser41, Gln42 and Phe43 on BTN2A1 (**Fig. 1H**). Further contacts were made by Lys13γ within the A-strand of Vγ9, which H-bonded to Tyr105, and Lys17γ within the B-strand of Vγ9, forming a salt bridge with Asp106 (**Fig. 1I**). The adjacent Thr18γ H-bonded with Glu107, and Ser16γ H-bonded to the Arg96 side chain (**Fig. 1J**). Accordingly, BTN engagement by γδTCR represents a fundamentally unique mode of ligand recognition by the immune system.

BTN2A1 forms V-shaped and head-to-tail homodimers

BTN2A1 is reported to exist on the cell surface predominantly as a homodimer, which is stabilized by a membrane-proximal interchain disulfide bond [12]. Our BTN2A1 ectodomain construct lacked the C-terminal Cys residue responsible for this disulfide bond and consequently behaved as a monomer in solution, based on elution profiles from size exclusion chromatography (**Extended Data Fig. 1B**). Nonetheless, the arrangement of two copies of BTN2A1 as a V-shaped homodimer resulted in co-localization of their IgC domains, in a mode that appeared to be compatible with a C-terminal interchain disulfide bond (**Fig. 2A**). This BTN2A1 V-dimer is broadly reminiscent of the BTN3A1 V-dimer [18], although the BTN2A1 V-dimer formed at an angle of 59°, which is significantly wider than BTN3A1 V-dimer (49°), and the BTN2A1 V-dimer was also twisted by 25° relative to BTN3A1 V-dimer [18] (**Fig. 2A** and **2B**). The V-dimer was characterized by a small interface with a buried surface area (BSA) of ~430 Å^2 per molecule (**Fig. 2C**). This involved π-π interactions mediated by Phe212, and cation-π interactions mediated by Arg126 and Phe207 (**Fig. 2D)**, along with limited polar interactions mediated by Ser211 and His128 (**Fig. 2E** and **Extended Data Table 3**). A head-to-tail dimer of BTN2A1 was also observed within the BTN2A1–γδTCR complex (**Fig. 2A** and **Extended Data Table 4**). This involved only the unliganded copy of BTN2A1 pairing with another unliganded copy via crystallographic symmetry, since the head-to-tail dimer interface overlapped with the γδTCR binding site (**Fig. 1D** and **Fig. 2C**). The head-to-tail dimer involved a mutually exclusive set of contacts and had a larger BSA of ~1180 Å^2 per molecule compared to the V-dimer (**Fig. 2C**), and could potentially form via a cis or a trans interaction on the cell surface, akin to the purported BTN3A1 head-to-tail homodimer (**Fig. 2A**) [18]. The head-to-tail dimer interface incorporated a hydrophobic core, with the side-chain of Tyr105 mediating Van der Waals interactions with Met174, Val164 and Thr176 (**Fig. 2F**), and the Met166 side-chain interacting with the aromatic side-chains of Tyr98, Phe43 and Phe39 (**Fig.**

2G). Furthermore, Glu136 formed a salt bridge with Arg103 (**Fig. 2H**) and the main-chains of Asp168 and Ala169 contacted the main-chain of Phe43 and the side-chain of Gln42, respectively (**Fig. 2I**). The N-glycans adjoined to Asn92 also appeared to interact (**Extended Data Table 4**).

To establish the generality of the BTN2A1 V-dimer and head-to-tail dimers, we also solved the crystal structure of BTN2A1 in unliganded state to 3.6 Å (anisotropic range 3.6 Å – 4.3 Å) resolution (**Extended Data Table 1**). Despite the lower resolution, the structure was refined to an R_{work} and R_{free} of 25.5% and 26.8%, respectively, and the unbiased electron density omit map was unambiguous (**Extended Data Table 1** and **Extended Data Fig. 1C**). The apo form of BTN2A1 contained five copies in the asymmetric unit in space group *C*2 (**Fig. 2J**). Despite the different crystal lattice and space group compared to the complex, all five copies of BTN2A1 each formed both a V-dimer and a head-to-tail dimer. The V-dimer conformation observed within the apo-BTN2A1 structure was very similar to that observed within the BTN2A1–Vγ9Vδ2$^+$ γδTCR complex structure, with only a subtle (11°) shift in the dimer angle (**Extended Data Fig. 1D**). Similarly, the head-to-tail dimer docking mode was essentially identical between the two crystal structures (**Extended Data Fig. 1E**). Thus, BTN2A1 ectodomain can form distinct dimers, which may impact the molecule's function.

BTN3A1 modulates Vγ9Vδ2$^+$ TCR tetramer interaction

Given the high bioavailability of the apical surface of the Vγ9Vδ2 TCR when liganded to BTN2A1, we hypothesized that Vγ9Vδ2 TCR co-binds a second ligand. Since BTN3A1 intracellular domain binds pAg [10], we first examined whether soluble BTN3A1 ectodomain could directly bind γδTCR by probing Vγ9Vδ2 TCR-transfected *BTN2A^{KO}.BTN3A^{KO}* HEK293T cells, which lack endogenous *BTN2A1*, *2A2*, *2A3p*, *3A1*, *3A2* and *3A3*, with BTN3A1 ectodomain tetramers. Consistent with an earlier report [10], we did not detect any staining (**Extended Data Fig. 2A**). We next tested whether mouse NIH-3T3 fibroblasts, which lack human BTN or BTNL molecules and are inherently incapable of mediating Vγ9Vδ2$^+$ T cell activation by pAg, could bind Vγ9Vδ2$^+$ TCR tetramer. These cells were transfected with either full-length human BTN2A1 or BTN3A1. Compared to BTN2A1$^+$ NIH-3T3 cells, which bound all Vγ9Vδ2$^+$ TCR tetramers (clones TCR3, TCR6, TCR7 and G115; **Fig. 3A**), BTN3A1$^+$ cells showed little, if any, staining (**Fig. 3B**, **empty bars; Extended Date Fig. 2B, black dots**). Previous studies showed that crosslinking of BTN3A1 on the surface of APCs with anti-BTN3A antibody (mAb clone 20.1) converts BTN3A1 into a stimulatory form that can activate

Vγ9Vδ2$^+$ T cells in a way that mimics pAg challenge [16,18]. Conversely, a separate anti-BTN3A mAb (clone 103.2) is a potent antagonist of Vγ9Vδ2$^+$ T cell reactivity to pAg [16,18]. Strikingly, cross-linking of BTN3A1$^+$ cells with agonistic mAb clone 20.1 induced clear staining with Vγ9Vδ2$^+$ TCR tetramers, particularly clones TCR3, TCR7 and G115 (**Fig. 3B, half-filled bars; Extended Data Fig. 2B, red plots**). This contrasted with the antagonist anti-BTN3A mAb clone 103.2, which did not induce any Vγ9Vδ2$^+$ TCR tetramer staining, nor did mAb 20.1 treatment of un-transfected or BTNL3-transfected cells (**Fig. 3B, 20%-filled bars; Extended Data Fig. 2B, blue plots**). We obtained a similar pattern of mAb 20.1-induced BTN3A1-dependent Vγ9Vδ2$^+$ TCR staining using BTN3A1-transfected human *BTN2A*^{KO}.*BTN3A*^{KO} HEK293T cells (**Extended Data Fig. 3C**). Furthermore, chimeric TCR tetramers comprised of a pAg-reactive Vγ9$^+$ γ-chain paired with an irrelevant Vδ1$^+$ δ-chain (**Extended Data Fig. 2D**) retained binding to BTN2A1$^+$ cells, but not to mAb 20.1-cross-linked BTN3A1$^+$ cells, indicating that unlike BTN2A1 protein interactions, the BTN3A1 association depends on Vδ2 and/or the CDR3δ loops (**Fig. 3C** and **D; Extended Data Fig. 2E**). Thus, mAb 20.1 pre-treatment of BTN3A1-transfected cells induces an interaction with Vγ9Vδ2$^+$ TCR via recognition of a second ligand, herein termed 'ligand-two'. Ligand-two association could be induced upon mAb 20.1 cross-linking of BTN3A1 in both human and mouse cell lines, and unlike the BTN2A1 interaction, this binding was dependent on the Vδ2 domain and/or the CDR3 loops, hereafter referred to as 'epitope two' (**Fig. 3F, cartoon inset**).

Lys53δ regulates the interaction with ligand-two

Since the ABED β-sheet of Vγ9 mediates binding to BTN2A1 (**Fig. 1C**), we tested whether the symmetrically equivalent ABED β-sheet of Vδ2 is also important in sensing pAg. Jurkat cells expressing Ala mutants within the Vδ2-encoded ABED β-sheet did not impair reactivity to zoledronate (Zol; an aminobisphosphonate that increases intracellular IPP pAg), suggesting there is no Vδ2-encoded equivalent ABED binding interface to the BTN2A1-binding domain on Vγ9 (**Extended Data Fig. 3A**–**C**). Since pAg-mediated γδ T cell responses depend on Arg51δ and Glu52δ, both located within the CDR2δ loop ([11,15] and **Extended Data Fig. 3C, red residues**), we screened two additional mutations within this loop: Lys53δ-Ala and Asp54δ-Ala. Whilst Asp54δ-Ala did not appreciably affect reactivity to zoledronate, surprisingly, Jurkat cells expressing G115 Vγ9Vδ2$^+$ TCR with a Lys53δ-Ala mutation (G115$^{Lys53δ\text{-}Ala}$) exhibited spontaneous activation, indicating that this residue may have a role in dampening γδ T cell reactivity to TCR stimuli (**Extended Data Fig. 3A**–**C**).

To reconcile these observations with mAb 20.1-induced Vγ9Vδ2$^+$ TCR tetramer staining of BTN3A1$^+$ cells, we produced G115 Vγ9Vδ2$^+$ TCR tetramer (hereafter referred to as G115 tetramer) with these corresponding Ala substitutions. As expected, wild-type G115 tetramer interacted with BTN2A1$^+$ NIH-3T3 fibroblasts, and also with mAb 20.1-pretreated BTN3A1$^+$ cells (**Fig. 3E** and **F; Extended Data Fig. 4A**). G115 tetramers with mutations at the BTN2A1 binding site ('epitope one'), notably G115$^{His85γ\text{-}Ala}$ and an G115$^{Arg20γ\text{-}Ala/Glu70γ\text{-}Ala/His85γ\text{-}Ala}$ 'triple-mutant', were unable to stain BTN2A1$^+$ cells, but still retained the ability to interact with mAb 20.1-pretreated BTN3A1$^+$ cells (**Fig. 3E** and **F; Extended Data Fig. 4A**). Conversely, TCR tetramers with 'epitope two' mutations, G115$^{Arg51δ\text{-}Ala}$ or G115$^{Glu52δ\text{-}Ala}$, readily stained BTN2A1$^+$ cells, but lost their ability to interact with mAb 20.1-pretreated BTN3A1$^+$ cells (**Fig. 3E** and **F; Extended Data Fig. 4A**). G115$^{Lys108γ\text{-}Ala}$, located within the CDR3γ and near the CDR2δ (5–8 Å distance), also exhibited a reduced association with mAb 20.1-pretreated BTN3A1$^+$ cells, but not to BTN2A1 (**Fig. 3E** and **F; Extended Data Fig. 4A**). Strikingly, G115$^{Lys53δ\text{-}Ala}$ tetramers, which was the mutant that resulted in autoactivation in functional assays (**Extended Data Fig. 3A** and **B**), did not affect the interaction with BTN2A1$^+$ cells, but stained BTN3A1$^+$ cells essentially independently of mAb 20.1 cross-linking (**Fig. 3E** and **F; Extended Data Fig. 4A**). The strong interaction of G115$^{Lys53δ\text{-}Ala}$ γδTCR tetramers with BTN3A1$^+$ cells also held true for other Vγ9Vδ2$^+$ TCR clones tested with the same substitution (**Extended Data Fig. 4B**), indicating that the Lys53δ-Ala mutation enhances Vγ9Vδ2$^+$ TCR binding potential irrespective of CDR3 sequence heterogeneity. As predicted, tetramers with combined His85γ-Ala (in epitope one) and Glu52δ-Ala (in epitope two) mutations, G115$^{His85γ\text{-}Ala/Glu52δ\text{-}Ala}$, lost the ability to interact with both BTN2A1$^+$ and mAb 20.1-pretreated BTN3A1$^+$ cells (**Fig. 3E** and **F; Extended Data Fig. 4A**).

In contrast to mAb 20.1, the antagonistic anti-BTN3A mAb clone 103.2 completely prevented the binding of G115$^{Lys53δ\text{-}Ala}$ γδTCR tetramers to BTN3A1$^+$ cells, supporting the notion that Vγ9Vδ2 TCR binding to ligand two is intimately associated with BTN3A1 (**Extended Data Fig. 4C**). In further support of the notion that Vγ9Vδ2$^+$ TCR closely associates with BTN3A1 following anti-BTN3A mAb 20.1-pretreatment, we co-stained BTN3A1- or BTN2A1-expressing cells with control SAv-PE or Vγ9Vδ2 TCR-PE tetramer along with isotype control-AF647 (MOPC21) or anti-BTN3A-AF647 (20.1) mAb (**Extended Data Fig. 4D**). Förster resonance energy transfer (FRET) was observed when BTN3A1$^+$ cells were co-stained with Vγ9Vδ2 TCR-PE tetramer and anti-BTN3A-AF647 Ab, suggesting close proximity (<10 nm) when co-bound to BTN3A1-transfected cells.

A recent study by Thomas Herrmann and colleagues established a role for the BTN3A isoforms, BTN3A2 and BTN3A3, in the pAg-reactive BTN3A heterodimer [26], and previous studies have suggested that the BTN3A isoforms stabilise BTN3A1 expression [25,27]. In agreement with the notion that other BTN3A isoforms can facilitate ligand-two engagement of Vγ9Vδ2 TCR, we showed a CDR2δ-mediated interaction of Vγ9Vδ2 TCR tetramers with cells expressing BTN3A2 and BTN3A3, suggesting that Vγ9Vδ2 TCR sensing by BTN3A isoforms is not limited to BTN3A1 (**Extended Data Fig. 13C**). Collectively, these data suggest 'ligand-two', being either BTN3A isoforms themselves or a closely associated molecule, binds to Vγ9Vδ2$^+$ TCR via 'epitope two', located on the apical surface of the Vγ9Vδ2$^+$ TCR and incorporating residues within the CDR2δ and CDR3γ loops. Within epitope two, Lys53δ appears to act as a gatekeeper residue for ligand-two accessibility, suggesting that upon cross-linking of BTN3A1 with agonist mAb 20.1, a conformational change to ligand-two occurs that partly circumvents this steric barrier.

BTN3A1 IgV domain interacts with both BTN2A1 and Vγ9Vδ2$^+$ TCR

BTN2A1 and BTN3A1 are located within 10 nm of each other in cis on the cell surface [11], however, whether they directly interact is unclear. To test this, we used surface plasmon resonance to measure the affinity of recombinant BTN2A1 and BTN3A1 ectodomains. Soluble BTN3A1 ectodomain (IgV–IgC) bound immobilized disulfide-linked BTN2A1 homodimer with an affinity of K_D = 680 μM, but not immobilized BTN3A1 homodimer. Conversely, soluble BTN2A1 ectodomain weakly bound immobilised BTN3A1 homodimer (K_D ~2,600 μM), but not immobilized BTN2A1 homodimer (**Fig. 4A**), indicating that BTN2A1 and BTN3A1 ectodomains are capable of directly interacting, albeit with a low affinity. Since BTN3A1 ectodomain exists as a homodimer and may therefore exhibit enhanced binding in SPR assays due to increased avidity, we also tested monomeric BTN3A1 IgV domain, which retained specific binding to BTN2A1 (K_D = 1,700 μM; **Fig. 4A**). In a separate experiment, irrelevant protein controls including anti-SARS-CoV-2 RBD nanobody (clone E1), human angiotensin-converting enzyme 2 (ACE2), and human BTN1A1 ectodomain, did not bind specifically to immobilised BTN2A1 or BTN3A1 homodimers, compared to BTN2A1 and BTN3A1 ectodomain/IgV domains (**Extended Data Fig. 6**). This suggests that, whilst low affinity, the interaction between BTN2A1 and BTN3A1 ectodomains is dependent on their respective IgV domains and is specific.

To understand the molecular nature of this interaction, we produced soluble BTN3A1–BTN2A1 ectodomain heteromeric complexes (**Extended Data Fig. 5A**–**C**), which were

tethered together with C-terminal leucine zippers. The BTN2A1–BTN3A1 heteromer complex retained staining with anti-BTN2A1 and anti-BTN3A1 mAb by ELISA (**Extended Data Fig. 5D**) and was comprised of two chains after purification (BTN2A1 and BTN3A1; **Extended Data Fig. 5B** and **C**). We crystallized the BTN2A1–BTN3A1–zipper complex ectodomains, and SDS-PAGE analysis of re-dissolved crystals indicated that they contained both BTN2A1-acidic zipper and BTN3A1-basic zipper (**Extended Data Fig. 5E**), suggestive of an intact conformation. The crystals diffracted weakly (anisotropic diffraction range 5.6 Å – 8.9 Å resolution), which nonetheless yielded a single solution in space group *F*222, wherein the asymmetric unit contained a single copy of BTN2A1 and BTN3A1, that interfaced via their IgV domains at a docking angle of ~29° (**Fig. 4B** and **Extended Data Table 1**). In this model, there was a 40 Å break in crystal packing along the C-axis. Given the proximity of this disordered layer to the C-termini of BTN2A1 and BTN3A1, it is likely that this layer contained the C-terminal zipper domains, which were 65 and 81 residues long on BTN2A1 and BTN3A1, respectively. The lack of clear electron density in this region implies that the zippers were disordered or had distinct overlaying conformations that could not be resolved at a low resolution, and they were thus not modelled. Regardless, an unbiased electron density omit map enabled clear identification of the IgV and IgC domains (**Extended Data Fig. 7A**). Distinct V-shaped homodimers of both BTN2A1 and BTN3A1 were also recapitulated via crystallographic symmetry (**Fig. 4B**), which were structurally similar to those found in the apo crystal structures, lending further confidence to the BTN2A1–BTN3A1 complex structural model (**Extended Data Fig. 7B**). The BTN2A1 and BTN3A1 V-dimers buried 620 $Å^2$ and 640 $Å^2$, respectively, for a combined BSA of ~1,300 $Å^2$ (**Extended Data Fig. 7C**). The BTN2A1 and BTN3A1 V-dimers came together at a planar angle of ~80° to form a distorted W-shaped heterotetramer (**Fig. 4B**), which could be even further expanded through crystallographic symmetry to yield a linear polymer of the composition $[BTN2A1_{homodimer}–BTN3A1_{homodimer}]_n$ (**Fig. 4C**).

We next tested which BTN3A1 residues are responsible for engaging BTN2A1 in cis on the cell surface [11]. Of a panel of forty-five BTN3A1 Ala ectodomain mutants, including residues within both the IgV and IgC domains, forty retained expression on the cell surface and retained staining by anti-BTN3A mAb clone 103.2 (**Extended Data Fig. 8A** and **B**). Mutations to five residues: $BTN3A1^{Arg44\text{-}Ala}$, $BTN3A1^{Leu96\text{-}Ala}$, $BTN3A1^{Tyr98\text{-}Ala}$ (and additionally $BTN3A1^{Tyr98\text{-}Phe}$), $BTN3A1^{Tyr105\text{-}Ala}$ and $BTN3A1^{Glu106\text{-}Ala}$ abrogated FRET between anti-BTN2A and anti-BTN3A mAbs (**Fig. 4D** and **Extended Data Fig. 8C**). Independently, soluble BTN3A1 bearing the null-FRET mutation $BTN3A1^{Tyr105\text{-}Ala}$ did not bind to immobilised

BTN2A1 homodimers by SPR, confirming the importance of this residue in interacting with BTN2A1 (**Extended Data Fig. 6**). Thus, mutations to these residues disrupt the association between BTN2A1 and BTN3A1 on the cell surface. These residues mapped to the CFG face of BTN3A1 and correlated closely with the crystallographic interface between BTN2A1 and BTN3A1. Accordingly, BTN2A1 and BTN3A1 interact via the CFG faces of their IgV domains and form W-shaped heterodimers and/or hetero-oligomers.

Using this same panel of BTN3A1 Ala mutants, we investigated which residues were involved in mediating the BTN3A1/mAb 20.1-dependent Vγ9Vδ2$^+$ TCR interaction. Thirty-four of the panel of forty-five mutants retained binding to anti-BTN3A mAb clone 20.1 **(Extended Data Fig. 8A**–**B** and **9A)**. Of these, six completely abrogated G115 tetramer staining of mAb 20.1-pretreated BTN3A1$^+$ cells: BTN3A1^{Val39}, BTN3A1^{Arg44}, BTN3A1^{His85}, BTN3A1^{Tyr98}, BTN3A1^{Phe104} and BTN3A1^{Tyr105}, plus a further four residues that reduced G115 tetramer staining by >90%: BTN3A1^{Phe26}, BTN3A1^{Lys37}, BTN3A1^{Ser42} and BTN3A1^{Leu96} (**Fig. 5A** and **Extended Data Fig. 9A**). The panel of BTN3A1 Ala mutants were next co-expressed with BTN2A1 (WT) in NIH-3T3 cells and used to activate Vδ2$^+$ T cells in the presence of zoledronate. All six BTN3A1 Ala mutants that abrogated G115 tetramer interaction – BTN3A1^{Val39}, BTN3A1^{Arg44}, BTN3A1^{His85}, BTN3A1^{Tyr98}, BTN3A1^{Phe104} and BTN3A1^{Tyr105} – also abrogated Vδ2$^+$ T cell activation, as did BTN3A1^{Leu96} (**Fig. 5B** and **Extended Data Fig. 9B**). Except for BTN3A1^{His85}, which mapped to the ABED face, all other residues mapped to the CFG face. These data extend upon an earlier report that the CFG face of BTN3A1 IgV domain is functionally important [13], and moreover, attribute a role for these residues in modulating ligand two binding to Vγ9Vδ2$^+$ TCR.

BTN2A1 and BTN3A1 utilize the same epitopes to bind each other and Vγ9Vδ2$^+$ TCR

Paradoxically, four of the seven BTN3A1 Ala mutants (BTN3A1^{Arg44}, BTN3A1^{Leu96}, BTN3A1^{Tyr98} and BTN3A1^{Tyr105}) that were important for ligand-two binding to Vγ9Vδ2$^+$ TCR were also critical for binding to BTN2A1 (**Fig. 4D, 5A,** and **Extended Data Fig. 8D**). Likewise, many of the residues within the BTN2A1 IgV domain that contacted γδTCR also mediated binding to BTN3A1, including BTN2A1^{Phe43} and BTN2A1^{Ser44} (**Extended Data Tables 2; Extended Data Fig. 9C**). Since the regions of BTN3A1 that engage BTN2A1 and those that are required for γδTCR–ligand two binding overlap, and conversely, the regions of BTN2A1 that engage BTN3A1 and γδTCR are also overlapping, it was challenging to reconcile how BTN2A1 and BTN3A1 can co-bind to each other along with Vγ9Vδ2$^+$ TCR.

Indeed, a superimposition of the BTN2A1–γδTCR and BTN2A1–BTN3A1 crystal structures identified major steric clashes between BTN3A1 and the γδTCR, suggesting that co-binding as a ternary complex in this manner is unlikely (**Extended Data Fig. 9D** and **Extended Data Movie 1**). This implies that whilst both BTN2A1 and BTN3A1 are ligands for each other, they must disengage or undergo major conformational changes to enable co-binding of Vγ9Vδ2^{+} TCR to BTN2A1 and ligand-two.

Collectively, these data suggest that BTN2A1 and BTN3A1 epitopes are tethered to each other in cis on the cell surface, which impairs the Vγ9Vδ2^{+} TCR from efficiently engaging to either BTN2A1 or to ligand-two. To further test this hypothesis, we reasoned that locking BTN2A1-IgV and BTN3A1-IgV domains together would abrogate their interaction with Vγ9Vδ2^{+} TCR. For this, we introduced cysteine (Cys) residues in the BTN2A1 and BTN3A1 IgV domain CFG faces that, based on the BTN2A1–BTN3A1 crystal structure, were optimally positioned for formation of an interchain disulfide bond. We identified two separate Cys mutant pairs: BTN2A1$^{\text{Gly102-Cys}}$ plus BTN3A1$^{\text{Asp103-Cys}}$, and BTN2A1$^{\text{Ser44-Cys}}$ plus BTN3A1$^{\text{Ser41-Cys}}$ (**Extended Data Fig. 10A**). Cells co-transfected with BTN2A1$^{\text{Ser44-Cys}}$ plus BTN3A1$^{\text{Ser41-Cys}}$, or BTN2A1$^{\text{Gly102-Cys}}$ plus BTN3A1$^{\text{Asp103-Cys}}$, exhibited a major reduction or total loss of G115 tetramer binding, respectively (**Fig. 6A**). In control samples where the Cys mutants were co-expressed with a corresponding WT molecule (for example, BTN2A1$^{\text{Gly102-Cys}}$ plus BTN3A1$^{\text{WT}}$, or vice versa), their binding to Vγ9Vδ2^{+} TCR was retained (**Fig. 6A**). Treatment of BTN2A1$^{\text{Cys+}}$ BTN3A1$^{\text{Cys+}}$ cells with graded doses of the reducing agent dithiothreitol (DTT) partly restored the ability of G115 tetramer to stain these cells, indicating that an interchain disulfide bond was responsible for the loss of interaction with Vγ9Vδ2^{+} TCR (**Fig. 6B** and **C**).

Based on the BTN2A1–BTN3A1 crystal structure, we predicted that soluble BTN2A1$^{\text{Gly102-Cys}}$–BTN3A1$^{\text{Asp103-Cys}}$ ectodomain complex would adopt a tetrameric conformation comprised of a central BTN3A1 V-dimer and two outer copies of BTN2A1, each linked to BTN3A1 via a disulfide bond (**Extended Data Fig. 10B**). In support of this model, 2D class averages of negatively stained electron micrographs of soluble BTN2A1$^{\text{Gly102-Cys}}$–BTN3A1$^{\text{Asp103-Cys}}$ complex revealed the presence of W-shaped particles (**Extended Data Fig. 10C**). Thus, the lack of staining observed on cells co-expressing BTN2A1$^{\text{Gly102-Cys}}$ and BTN3A1$^{\text{Asp103-Cys}}$ is likely due to Vγ9Vδ2^{+} TCR tetramers being unable to engage the BTN epitopes located within the IgV domains of BTN2A1 and BTN3A1 when they are tethered to each other.

The cytoplasmic tail of BTN2A1 mediates the BTN3A1–pAg–BTN2A1 IC interaction

The intracellular domains of BTN2A1 and BTN3A1 are both required for pAg-induced activation of Vγ9Vδ2$^+$ T cells [11,19], and it has recently been found that pAg induces an association between the BTN3A1 and BTN2A1 intracellular domains [20,21]. In further support of these studies, we identified three residues within the BTN2A1 intracellular domain – two in the C-terminal cytoplasmic tail (BTN2A1^{Thr482} and BTN2A1^{Leu488}) and one in the B30.2 domain (BTN2A1^{Arg449}) – that are critical for the activation of Vγ9Vδ2$^+$ T cells (**Fig. 7A** and **Extended Data Fig. 11A–B**). Using biolayer interferometry, we found that the B30.2 domain of BTN3A1 and the BTN2A1 full-length intracellular domain indeed interacted following pAg treatment (K_D ~200 – 800 nM) and that mutations of BTN2A1$^{Arg449\text{-}Ala}$ and BTN2A1$^{Thr482\text{-}Ala}$ abrogate this pAg-induced association (**Fig. 7B**). Thus, we propose that sequestration of pAg by the intracellular domains of BTN2A1 plus BTN3A1 facilitates allosteric changes to the ectodomains of BTN2A1–BTN3A1 complex, converting them from an inactive state into an active state. The Vγ9Vδ2$^+$ TCR is subsequently able to co-engage BTN2A1 plus ligand-two, with ligand-two being either BTN3A1 itself or a closely associated molecule (**Extended Data Fig. 12**).

Discussion

Akin to pMHC recognition by the αβTCR, BTN molecules have emerged as critical γδTCR ligands, however, their molecular mode of recognition is poorly defined. Furthermore, the precise mechanism by which Vγ9Vδ2$^+$ T cells recognise pAg remains unclear. Here we report the first structure of a TCR engaging an endogenous non-MHC or MHC-like ligand, namely BTN2A1, revealing that BTN2A1 engages the side of Vγ9, leaving the apical face of the Vγ9Vδ2$^+$ TCR exposed. We also demonstrate that a second ligand binds the apical Vγ9Vδ2$^+$ TCR surface in a manner that is dependent on BTN3A1, clarifying the mechanism behind pAg detection by Vγ9Vδ2$^+$ T cells. Finally, we establish the molecular basis of the BTN2A1–BTN3A1 complex and show that the TCR-binding determinants on the BTN molecules overlap with the BTN heterodimer interface.

The ability of Vγ9Vδ2$^+$ TCR to co-bind two ligands contrasts the recognition of MHC and MHC-like molecules by αβ T cells, which bind with one-to-one stoichiometry. Thus, the γδTCR appears to be capable of discriminating between a dual and a single ligand-binding event. Since Vγ9 is often incorporated into non-pAg-reactive Vγ9Vδ1$^+$ TCRs, other non-BTN γδ T cell ligands such as MICA, CD1 or MR1 might also co-bind in conjunction with BTN2A1. Likewise, there are numerous parallels between the BTN2A1–BTN3A1 axis and BTN-like proteins, e.g. human BTNL3/BTNL8 and mouse Btnl1/Btnl6. It is intriguing that the BTN

heterodimers appear to be associating via the same general molecular mechanism. Moreover, the similarity between the BTNL3-Vγ4 and Btnl1-Vγ7 interfaces is compelling, and it will also be interesting to see if the mouse Skint1-Skint2-DETC axis hold similarities [7,9,13]. However, no role for Ag has been described in those systems, nor have two-ligand binding sites been mapped onto the TCRs. Thus, whilst there is clearly a level of similarity, future studies will need to inform just how homologous those other BTN recognition systems are to that which we define here.

We previously identified three residues in the ABED face of Vγ9 that are important for mediating the interaction with BTN2A1 [11] which we confirm are important in our crystal structure. By contrast, the HV4 and CDR2γ loops, which were proposed by Karunakaran et al. (2020) [12] to be involved, do not form part of the interface with BTN2A1. Nonetheless, the binding mode between BTN2A1 and Vγ9Vδ2 TCR is consistent with the notion that germline-encoded sequence variability within the ABED sheet (also termed HV4γ region in some studies) is associated with a spatially distinct epitope that engages BTN molecules [7]. Based on the structurally determined mode of Vγ9Vδ2 TCR binding to BTN2A1, it needs to be considered whether this mode of binding can be accommodated by the BTN2A1 V-dimer in the context of a cell-cell trans interaction. While our structural model suggests that the unliganded copy of BTN2A1 might be pushed towards, and possibly clash with, the cell membrane, this may not be a problem in antigen-presenting cells that can have more fluidity within their membranes. It is also possible that the binding mode may involve conformational plasticity of one or both Vγ9Vδ2 TCR or BTN2A1, via flexibility in their membrane-proximal hinge regions. It will be important in future studies to determine whether covalently-linked BTN2A1 V-dimer can engage Vγ9Vδ2 TCR in the context of the cell membrane, and whether this binding mode impacts signal transduction.

Commented [DG2]: not sure if you want to talk about cell membrane ruffles and undulations here?

Unlike αβTCRs and BCRs, which directly sense foreign Ag, pAg-reactive γδ TCRs are activated by inside-out signalling via BTN conformational changes. As such, additional regulatory mechanisms are likely required to maintain γδ T cell self-tolerance. To this end we identified two important molecular checkpoints, namely Lys53 in the CDR2δ loop of Vγ9Vδ2$^+$ TCR, which suppresses 'ligand-two' binding, and a second mechanism whereby the epitopes of BTN2A1 and BTN3A1 that mediate engagement with Vγ9Vδ2$^+$ TCR are partnered to each other in cis on the cell surface of APCs in a non-activating conformation. The ability of the Lys53δ-Ala mutant TCR to induce Vγ9Vδ2$^+$ T cell autoactivation, and stronger ligand-two binding, suggests that this residue may have a key role in negatively regulating Vγ9Vδ2$^+$ T cell activation. Upon pAg-induced conformational change of the BTN–complex, the Lys53δ side

chain steric barrier may be circumvented by the Vγ9Vδ2$^+$ TCR, enabling ligand-two to engage the Vγ9Vδ2$^+$ TCR via an epitope encompassing Arg51δ, Glu52δ and/or Lys108γ. These residues have been implicated in previous screens as being important for pAg-responsiveness of Vγ9Vδ2 T cells [11,15], and moreover, are conserved amongst pAg-reactive human Vγ9Vδ2 TCRs and orthologues of *TRDV2* identified in higher order primates (ensembl.org; **Extended Data Fig. 13A**), as well as pAg-reactive T cells isolated from Rhesus macaque [22]. We do not exclude a role for other residues identified as being important for pAg-responses based on conservation across pAg-reactive TCRs, such as the strong enrichment of isoleucine, leucine or valine at residue 97 in the adjacent CDR3δ ([23,24] and **Extended Data Fig. 13B**), although we do note that modification of residue Leu97 of G115 δ-chain did not heavily impact upon pAg-mediated activation in our previous study [11].

Whilst the affinity of BTN2A1 and BTN3A1 ectodomains is very low (K_D in the mM range), this is nonetheless sufficient to enable their association in cis on the cell surface. This weak affinity may ensure both ligands remain in an off-state and proximal to one another, yet able to dissociate in order to enable Vγ9Vδ2$^+$ TCR engagement of the BTN complex. Upon pAg encounter, the association of the BTN2A1 and BTN3A1 intracellular domains rapidly facilitates conversion of the BTN complex into a stimulatory form. Whilst the significance of the BTN2A1 V- and head-to-tail dimers remains to be tested, they are reminiscent of the reported BTN3A1 V- and head-to-tail dimers [18,25]. It has previously been suggested the head-to-tail dimer of BTN3A1 may support a regulatory function, although subsequent studies testing this hypothesis have been inconclusive [25].

Our data reveal that BTN3A1 plays a key role in driving ligand-two binding to the Vγ9Vδ2$^+$ TCR. The simplest interpretation of our findings is that BTN3A1 itself is ligand-two and binds directly to Vγ9Vδ2$^+$ TCR. However, an alternative interpretation is that instead of BTN3A1, another molecule is responsible for binding to the apical surface of Vγ9Vδ2$^+$ TCR, and BTN3A1 instead plays an indirect role. This latter interpretation would demand that ligand-two is expressed at relatively high levels on mouse NIH-3T3 fibroblasts, and can closely associate with BTN3A1. Accordingly, while we consider the former, simpler, interpretation as most likely, it is important to emphasize that our observations do not preclude the involvement of other proteins in facilitating or enhancing pAg-dependent activation of Vγ9Vδ2$^+$ T cells. Collectively, our findings reveal that a fundamentally unique mode of Ag-driven immune activation underpins γδ T cell immunity compared to αβ T cells.

References

1 Ribot, J. C., Lopes, N. & Silva-Santos, B. γδ T cells in tissue physiology and surveillance. *Nature reviews. Immunology* **21**, 221-232 (2021). https://doi.org:10.1038/s41577-020-00452-4

2 Constant, P. *et al.* Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science* **264**, 267-270 (1994). https://doi.org:10.1126/science.8146660

3 Tanaka, Y. *et al.* Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* **375**, 155-158 (1995). https://doi.org:10.1038/375155a0

4 Tyler, C. J., Doherty, D. G., Moser, B. & Eberl, M. Human Vγ9/Vδ2 T cells: Innate adaptors of the immune system. *Cell Immunol* **296**, 10-21 (2015). https://doi.org:10.1016/j.cellimm.2015.01.008

5 Hoeres, T., Smetak, M., Pretscher, D. & Wilhelm, M. Improving the Efficiency of Vγ9Vδ2 T-Cell Immunotherapy in Cancer. *Front Immunol* **9**, 800 (2018). https://doi.org:10.3389/fimmu.2018.00800

6 Di Marco Barros, R. *et al.* Epithelia Use Butyrophilin-like Molecules to Shape Organ-Specific γδ T Cell Compartments. *Cell* **167**, 203-218 e217 (2016). https://doi.org:10.1016/j.cell.2016.08.030

7 Melandri, D. *et al.* The γδ combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness. *Nat Immunol* **19**, 1352-1365 (2018). https://doi.org:10.1038/s41590-018-0253-5

8 Boyden, L. M. *et al.* Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal gammadelta T cells. *Nat Genet* **40**, 656-662 (2008). https://doi.org:10.1038/ng.108

9 Jandke, A. *et al.* Butyrophilin-like proteins display combinatorial diversity in selecting and maintaining signature intraepithelial γδ T cell compartments. *Nat Commun* **11**, 3769 (2020). https://doi.org:10.1038/s41467-020-17557-y

10 Sandstrom, A. *et al.* The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2 T cells. *Immunity* **40**, 490-500 (2014). https://doi.org:10.1016/j.immuni.2014.03.003

11 Rigau, M. *et al.* Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells. *Science* **367**, eaay5516 (2020). https://doi.org:10.1126/science.aay5516

12 Karunakaran, M. M. *et al.* Butyrophilin-2A1 Directly Binds Germline-Encoded Regions of the Vγ9Vδ2 TCR and Is Essential for Phosphoantigen Sensing. *Immunity* **52**, 487-498 e486 (2020). https://doi.org:10.1016/j.immuni.2020.02.014

13 Willcox, C. R. *et al.* Butyrophilin-like 3 Directly Binds a Human $V\gamma4^{+}$ T Cell Receptor Using a Modality Distinct from Clonally-Restricted Antigen. *Immunity* **51**, 813-825 e814 (2019). https://doi.org:10.1016/j.immuni.2019.09.006

14 Vyborova, A. *et al.* γ9δ2T cell diversity and the receptor interface with tumor cells. *J Clin Invest* **130**, 4637-4651 (2020). https://doi.org:10.1172/JCI132489

15 Wang, H., Fang, Z. & Morita, C. T. Vγ2Vδ2 T Cell Receptor recognition of prenyl pyrophosphates is dependent on all CDRs. *J Immunol* **184**, 6209-6222 (2010). https://doi.org:10.4049/jimmunol.1000231

16 Harly, C. *et al.* Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset. *Blood* **120**, 2269-2279 (2012). https://doi.org:10.1182/blood-2012-05-430470

17 Kundapura, S. V. & Ramagopal, U. A. The CC' loop of IgV domains of the immune checkpoint receptors, plays a key role in receptor:ligand affinity modulation. *Sci Rep* **9**, 19191 (2019). https://doi.org:10.1038/s41598-019-54623-y

18 Palakodeti, A. *et al.* The molecular basis for modulation of human Vγ9Vδ2 T cell responses by CD277/butyrophilin-3 (BTN3A)-specific antibodies. *J Biol Chem* **287**, 32780-32790 (2012). https://doi.org:10.1074/jbc.M112.384354

19 Cano, C. E. *et al.* BTN2A1, an immune checkpoint targeting Vγ9Vδ2 T cell cytotoxicity against malignant cells. *Cell Rep* **36**, 109359 (2021). https://doi.org:10.1016/j.celrep.2021.109359

20 Hsiao, C. C., Nguyen, K., Jin, Y., Vinogradova, O. & Wiemer, A. J. Ligand-induced interactions between butyrophilin 2A1 and 3A1 internal domains in the HMBPP receptor complex. *Cell Chem Biol* **29**, 985-995 e985 (2022). https://doi.org:10.1016/j.chembiol.2022.01.004

21 Yuan, L. *et al.* Phosphoantigens are Molecular Glues that Promote Butyrophilin 3A1/2A1 Association Leading to Vγ9Vδ2 T Cell Activation. *bioRxiv*, 2022.2001.2002.474068 (2022). https://doi.org:10.1101/2022.01.02.474068

22 Wang, H. *et al.* Conservation of Nonpeptide Antigen Recognition by Rhesus Monkey Vγ2Vδ2 T Cells. *The Journal of Immunology* **170**, 3696-3706 (2003). https://doi.org:10.4049/jimmunol.170.7.3696 PMID - 12646635

23 Davodeau, F. *et al.* Peripheral selection of antigen receptor junctional features in a major human γδ subset. *European Journal of Immunology* **23**, 804-808 (1993). https://doi.org:10.1002/eji.1830230405 PMID - 8384559

24 Yamashita, S., Tanaka, Y., Harazaki, M., Mikami, B. & Minato, N. Recognition mechanism of non-peptide antigens by human γδ T cells. *International Immunology* **15**, 1301-1307 (2003). https://doi.org:10.1093/intimm/dxg129 PMID - 14565928

25 Gu, S. *et al.* Phosphoantigen-induced conformational change of butyrophilin 3A1 (BTN3A1) and its implication on Vgamma9Vdelta2 T cell activation. *Proc Natl Acad Sci U S A* **114**, E7311-E7320 (2017). https://doi.org:10.1073/pnas.1707547114

26 Karunakaran, M. M. *et al.* A distinct topology of BTN3A IgV and B30.2 domains controlled by juxtamembrane regions favors optimal human γδ T cell phosphoantigen sensing. *Nature Communications* **14**, 7617 (2023). https://doi.org:10.1038/s41467-023-41938-8

27 Vantourout, P. *et al.* Heteromeric interactions regulate butyrophilin (BTN) and BTN-like molecules governing γδ T cell biology. *Proceedings of the National Academy of Sciences* **115**, 1039-1044 (2018). https://doi.org:10.1073/pnas.1701237115

28 Sebestyen, Z. *et al.* RhoB Mediates Phosphoantigen Recognition by Vγ9Vδ2 T Cell Receptor. *Cell Reports* **15**, 1973-1985 (2016). https://doi.org:10.1016/j.celrep.2016.04.081

29 Yang, Y. *et al.* A Structural Change in Butyrophilin upon Phosphoantigen Binding Underlies Phosphoantigen-Mediated Vγ9Vδ2 T Cell Activation. *Immunity* **50**, 1043-1053.e1045 (2019). https://doi.org:10.1016/j.immuni.2019.02.016

30 Ding, Y. H. *et al.* Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity* **8**, 403-411 (1998). https://doi.org:10.1016/s1074-7613(00)80546-4

31 Uldrich, A. P. *et al.* CD1d-lipid antigen recognition by the gammadelta TCR. *Nat Immunol* **14**, 1137-1145 (2013). https://doi.org:10.1038/ni.2713

Figure 1. BTN2A1 engages the side of Vγ9. (**A**) Surface and cartoon representation of the BTN2A1–Vγ9Vδ2^{+} TCR clone G115 crystal structure. G115 TCRδ, pink; G115 TCRγ, orange; liganded BTN2A1, green; unliganded BTN2A1, gray. (**B**) Comparison with a representative pHLA Class I–αβTCR (left, PDB code 1QSE [30]) and CD1d–α-GalCer–clone 9C2 Vγ5Vδ1^{+} TCR (right, PDB code 4LHU [31]). TCRα, dark pink; TCRβ, red; HLA-I, light blue; β2-microglobulin, dark blue; 9C2 TCRδ, yellow; 9C2 TCRγ, pale blue, CD1d, purple. (**C**) Cartoon of BTN2A1 depicting the CFG face of the IgV domain in gold and the ABED face of the IgV domain in blue. (**D**) Surface representation of BTN2A1 (green) and G115 Vγ9Vδ2 TCR (γ-chain, orange; δ-chain, light pink) depicting the interfaces (red) from the γδTCR-BTN2A1 complex. Molecular contacts between the Vγ9Vδ2^{+} clone G115 TCR (orange) and BTN2A1 (green) ectodomains showing the (**E**) HV4γ- and (**F**) CDR2γ- loops, (**G**) Arg20γ, (**H**) Glu70γ and His85γ, (**I**) Lys13γ and Lys17γ, (**J**) Ser16γ and Thr18γ side and/or main chains and their BTN2A1 contacts as sticks. H-bonds and salt-bridges, red; VDW and cation-π, black; 2mFo-DFc electron density composite omit map contoured at 1σ, blue mesh.

Figure 2. BTN2A1 forms V-shaped and head-to-tail homodimers. (**A**) The BTN2A1 V-dimer (left), and cis (middle) or trans (right) interpretation of the head-to-tail homodimer. (**B**) Overlay of BTN2A1 V-dimer from apo structure (blue) and BTN3A1 V-dimer (pink) structures (PDB code 4F80). (**C**) Surface representation of BTN2A1 (green) depicting the head-to-tail dimer interface in red and the V-dimer interface in blue. Glycans depicted as sticks. Molecular contacts at the BTN2A1 ectodomain V-dimer interface between two copies of BTN2A1 (green and gray) showing (**D**) Arg126, Phe 207 and Phe212, and (**E**) His128 and Ser211. Molecular contacts at the BTN2A1 ectodomain head-to-tail dimer interface between two copies of BTN2A1 (green and orange) showing (**F**) Tyr105 contacts with Met174, Val164 and Thr176 (**G**) Met166 contacts with Tyr98, Phe43 and Phe39 (**H**) Arg103 contacts with Glu136 (**I**) Asp168 and Ala169 main chain contacts with Phe43 main chain and Gln42 side chain. (**J**) Surface and cartoon representation of the apo-BTN2A1 crystal structure with BTN2A1 copies shown in orange, grey, green, blue and light pink. H-bonds and salt-bridges, red; VDW and π interactions, black; 2mFo-DFc electron density composite omit map contoured at 1σ, blue mesh.

Figure 3. BTN3A1 supports binding to the apical surface of the Vγ9Vδ2$^+$ γδTCR . MFI of γδTCR tetramer staining of (**A**) gated GFP$^+$ BTN2A1-transfected or (**B**) GFP$^+$ BTN3A1-transfected NIH-3T3 cells treated with isotype control, anti-BTN3A (clone 20.1) or anti-BTN3A (clone 103.2) antibodies. Graphs are presented as mean ± SEM. N ≥ 3, where each point represents an independent experiment. MFI of chimeric γδTCR tetramer staining of (**C**) gated GFP$^+$ BTN2A1-transfected or (**D**) GFP$^+$ BTN3A1-transfected NIH-3T3 cells treated with anti-BTN3A (clone 20.1). Inset plots depict parent cell gating. Graphs are presented as mean ± SEM. N ≥ 3, where each point represents an independent experiment. Mutated G115 γδTCR tetramer staining of (**E**) GFP$^+$ BTN2A1-transfected or (**F**) GFP$^+$ BTN3A1-transfected NIH-3T3 cells treated with anti-BTN3A (clone 20.1). Cartoon inset depicts the locations of BTN2A1-epitope (red star) and the ligand-two epitope (green star). Graphs depict mean ± SEM. N ≥ 3, where each point represents an independent experiment.

Figure 4. BTN2A1 and BTN3A1 directly associate and form heteromers. (**A**) Sensorgrams (left) and saturation plots (right) depicting binding of soluble monomeric BTN2A1 ectodomain (top row, 890–28 μM), homodimeric BTN3A1 ectodomain (middle row, 1520–24 μM), or monomeric BTN3A1 IgV domain (bottom row, 1590–25 μM) to immobilised BTN2A1 ectodomain homodimer (red) or BTN3A1 ectodomain homodimer (blue), as measured by surface plasmon resonance. Insert graphs depict Scatchard plots. K_D, dissociation constant calculated at equilibrium ± SEM, derived from the mean of n=3 independent experiments each shown separately on the saturation plots. (**B**) The BTN2A1–BTN3A1 ectodomain complex crystal structure, showing the asymmetric unit as a surface and the V-dimers as a cartoon. BTN3A1, blue; BTN2A1, green. (**C**) Surface and cartoon representation of the BTN2A1 V-dimer–BTN3A1 V-dimer repeating unit within the crystal structure. Color scheme as in (B). (**D**) Association between BTN2A1 and BTN3A1 ectodomains on the cell surface of mouse NIH-3T3 fibroblasts co-expressing wild-type BTN2A1 and individual BTN3A1 mutants, as determined by mean percentage ± SEM of $FRET^+$ cells between anti-BTN2A1-AF647 (clone 259) and anti-BTN3A-PE (clone 103.2). Controls (right) depict FRET between transiently expressed CD80 and BTN3A1, or BTN2A1 and PD-L2. n = 6 where each point represents an individual experiment, except for controls where n = 3. NA, data were excluded if BTN3A1 mutant protein levels were > 2-fold lower than BTN3A1 WT as determined by anti-BTN3A (clone 103.2) mAb staining.

Figure 5. BTN3A1 IgV domain is critical for ligand-two interacting with Vγ9Vδ2$^+$ TCR. (**A**) G115 Vγ9Vδ2$^+$ TCR tetramer-PE staining of mouse NIH-3T3 fibroblasts transfected with either wild-type BTN3A1 or the indicated mutants, following pre-treatment with anti-BTN3A1-AF647 (clone 20.1) antibody. SAv, streptavidin-PE control staining of wild-type BTN3A1$^+$ cells. Bar graphs depict median fluorescence intensity (MFI) ± SEM. Dotted lines represents 90–98% reduction (orange) and >98% reduction (red) in MFI. Inset: surface representation of BTN3A1 V-dimer with mutations to residues that led to an abrogation of the anti-BTN3A antibody (20.1)-dependent G115 tetramer interaction coloured in red (> 98% reduction), orange (90–98% reduction), or gray (< 90% reduction). n = 3, where each point represents an independent experiment. NA, not applicable since BTN3A surface expression was too low to measure G115 tetramer staining. (**B**) Change in CD25 expression (normalized to unstimulated control for each sample) on purified in vitro-expanded Vδ2$^+$ γδ T cells co-cultured for 24 h with 5 µM zoledronate and mouse NIH-3T3 fibroblast APCs transfected with wild-type *BTN2A1* and individual *BTN3A1* mutants. Bar graphs depict mean ± SEM. Red dotted line represents >50% reduction in activation compared to BTN2A1–BTN3A1 WT. NA = data not available since BTN3A1 levels were too low to induce zoledronate-dependent activation of γδ T cells. Data are from 2-3 independent experiments each with n=1-2 different donors each. ** P < 0.01, *** P < 0.001, **** P < 0.0001, by two-way ANOVA with Šidák multiple comparison correction. Inset: surface representation of BTN3A1 V-dimer with mutations to residues that led to an abrogation of zoledronate-dependent Vδ2$^+$ γδ T cell activation shown in red, or did not impact Vδ2$^+$ γδ T cell activation shown in gray.

Figure 6. BTN2A1 and BTN3A1 must disengage in order to bind Vγ9Vδ2$^+$ TCR. (**A**) G115 Vγ9Vδ2$^+$ TCR tetramer-PE, or control streptavidin-PE (SAv) staining of mouse NIH-3T3 fibroblasts co-transfected with BTN2A1 and BTN3A1 wild-type or cysteine mutants in the depicted combinations. Representative of one of two independent experiments. (**B**) G115 Vγ9Vδ2+ TCR tetramer-PE, or control streptavidin-PE (SAv) staining of mouse NIH-3T3 fibroblasts co-transfected with the indicated BTN2A1 and BTN3A1 cysteine mutant pairs, or control BTNL3 plus BTNL8, following pre-treatment of cells with graded concentrations of dithiothreitol (DTT). Inset – MFI of PE parameter. Representative of one of four independent experiments. (**C**) G115 tetramer-PE staining of NIH-3T3 fibroblasts co-transfected with either WT or Cys-mutant BTN2A1 plus BTN3A1, or control BTNL3 plus BTNL8, following pre-incubation of the cells with DTT at indicated concentrations. Graphs depict mean ± SEM. Data pooled from 3-4 separate experiments.

Figure 7. The intracellular tail of BTN2A1 mediates a pAg-dependent interaction with the BTN3A1 B30.2 domain. (**A**) mean ± SEM of CD25-PE expression on purified pre-expanded Vδ2+ γδ T cells following co-culture with HEK293T BTN2AKO.BTN3AKO cells that were co-transfected with BTN3A1 plus the indicated BTN2A1 mutant, or alternatively, control BTNL3 alone or BTN3A1 alone, ± zoledronate (5 µM) for 24 h. Replicates with low transfection efficiency (< 10% GFP+) were excluded from analysis. Data are from three independent experiments each with n=2 different donors. ** $P < 0.01$ by two-way ANOVA with Šidák multiple comparison correction. Insert, molecular model of the BTN2A1 B30.2 intracellular domain generated by AlphaFold v2 with functionally important residues shown in red. (**B**) The B30.2 domain of wild-type (WT) BTN3A1 was used as ligand to probe the interaction with WT or mutant BTN2A1 full length intracellular domain (BFI), in the absence or presence of pAg (20 µM HMBPP) by biolayer interferometry (BLI).

Methods

Human samples

Healthy donor blood derived human peripheral blood cells (PBMCs) from male and female donors were obtained from the Australian Red Cross Blood Service under ethics approval 17-08VIC-16 or 16-12VIC-03, with ethics approval from University of Melbourne Human Ethics Sub-Committee (1035100) and isolated via density gradient centrifugation (Ficoll-Paque PLUS GE Health care) and red blood cell lysis (ACK buffer, produced in-house).

Cell lines

Jurkat (JR3-T3.5), LM-MEL-75, HEK293T and NIH-3T3 cells were existing tools in the lab and were maintained in RPMI-1640 (Invitrogen) supplemented with 10% (v/v) FCS (JRH Biosciences), penicillin (100 U/ml), streptomycin (100 µg/ml), Glutamax (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM) and HEPES buffer (15 mM), pH 7.2–7.5 (all from Invitrogen Life Technologies), plus 50 µM 2-mercaptoethanol (Sigma-Aldrich) (complete RMPI). Expi293F cells were purchased from ThermoFisher (Cat. No. A14527) and maintained in Expi293 Expression Medium (ThermoFisher, A1435101).

γδ T cell isolation and expansion

In some experiments γδ T cells were enriched by MACS using anti-γδTCR-PECy7 followed by anti-phycoerythrin–mediated magnetic bead purification. After enrichment $CD3^+$ $V\delta2^+$ γδ T cells were further purified by sorting using an Aria III (BD). Enriched γδ T cells were stimulated *in vitro* for 48 h with plate-bound anti-CD3ε (OKT3, 10 µg/ml, Bio-X-Cell), soluble anti-CD28 (CD28.2, 1 µg/ml, BD Pharmingen), phytohemagglutinin (0.5 µg/ml, Sigma) and recombinant human IL-2 (100 U/ml, PeproTech), followed by maintenance with IL-2 for 14–21 d. Cells were cultured in complete medium consisting of a 50:50 (v/v) mixture of AIM-V (Thermo Fisher) and RPMI-1640 supplemented with 10% (v/v) FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), Glutamax (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM) and HEPES buffer (15 mM), pH 7.2–7.5, plus 50 µM 2-mercaptoethanol.

Flow cytometry

To examine the capacity of γδTCR tetramers to bind to BTN molecules, NIH-3T3 cells were transfected with BTN2A1, BTN3A1 or control BTNL3 in pMIG (a gift from D. Vignali (Addgene plasmid # 52107) [32] using ViaFect® (Promega) in OptiMEM™ (Gibco, Thermo-Fisher). 48 h following transfection, cells were harvested with trypsin, filtered through a 30 or

70 µm cell strainer, and incubated with anti-BTN3A antibody (clone 20.1) or IgG1,κ isotype control (clone MOPC-21, BioLegend; or BM4-1, a gift from CSL Limited) at 5 µg/mL for 15 min at room temperature. Cells were then stained with PE-labelled γδTCR tetramers (produced in house, see below), or control PE-conjugated streptavidin, at 5 µg/mL for 30 min at room temperature. The median fluorescence intensity (MFI) of γδTCR tetramer interacting with BTN proteins was examined on gated GFP^+ cells by flow cytometry. For γδTCR tetramer staining, data were excluded if BTN3A1 mutant protein levels were > 2-fold lower than wild-type BTN3A1, as determined by anti-BTN3A mAb staining. To examine the capacity of BTN tetramers to bind to γδTCRs, HEK293T cells were co-transfected with γδTCR genes in pMIG using FuGENE® HD (Promega) in OptiMEM™ plus 2A-linked CD3εδγζ in pMIG [33]. 48 h following transfection, cells were collected by pipetting, filtered through a 30 or 70 µm cell strainer, and stained with anti-CD3ε antibody for 15 min at 4ºC. Cells were then stained with anti-γδTCR, anti-TCR Vδ2 as well as PE-labelled BTN tetramers (produced in house, see below), PE-labelled control mouse CD1d ectodomain tetramers (loaded with α-GalCer and produced in house, see below), or control PE-conjugated streptavidin (BD), for 30 min at 4°C. The MFI of BTN tetramer on gated $CD3^+GFP^+$ cells was measured by flow cytometry. In other assays, human peripheral blood-derived cells were stained with 7-aminoactinomycin D (7-AAD, Sigma) or LIVE/DEAD® viability markers (ThermoFisher) plus antibodies against: CD3ε, γδTCR , TCR Vδ2, CD45, CD25, CD69, and/or isotype controls (IgG1,κ clone MOPC-2) in various combinations (**Extended Data Table 5**). All data were acquired on an LSRFortessa™ II (BD) and analyzed with FACSDiva and FlowJo (BD) software. All samples were gated to exclude unstable events, doublets and dead cells using time, forward scatter area versus height, and viability dye parameters, respectively (**Extended Data Fig. 14**).

Generation of BTN2A.BTN3A-knockout cells

HEK293T cells were nucleofected with Cas9/RNP complexes and two guide RNAs, one targeting the intronic region directly upstream of BTN3A2 (5′-AACTTTCACCTACAAACCGC) and one downstream of BTN2A1 (5′-GAACCCTGACTGAAACGATC). Guides were designed using the Broad Institute CRISPick web tool [34]. After seven days in culture, $BTN2A^-$ $BTN3A^-$ cells were bulk-sorted (FACS Aria III) and after another round of culture were single cell-sorted based upon the same criteria. To verify excision of the BTN locus, genotyping of the expanded clones was performed using PCR primers targeting BTN3A2, BTN2A1 and the excised locus (**Extended Data Table 6**).

Jurkat assays

2.5×10^4 APCs (LM-MEL-75) cells were plated per well of a 96-well plate and incubated overnight, before 2×10^4 G115 mutant-expressing J.RT3-T3.5 (Jurkat) cells ± zoledronate (40 μM) were added for 20 h. CD69 expression was measured by flow cytometry on GFP^+ Jurkat cells. A panel of 15 single-residue alanine (Ala) mutants, each within in the Vδ2 domains of the Vγ9Vδ2$^+$ G115 TCR were generated by either site-directed mutagenesis using the primers listed in **Extended Data Table 6**, or by cloning of gene fragments (IDT). Primers (IDT) were phosphorylated (PNK, NEB) followed by 25 cycles of PCR using KAPA HiFi master mix (KAPA Biosystems) using G115 WT TCR in pMIG as template, and PCR product was digested with DpnI (NEB) and in some cases ligated with T4 DNA ligase (NEB). Construct sequences were verified by Sanger sequencing prior to use.

γδ T cell functional assays

For co-culture assays, NIH-3T3 cells were transfected with BTN2A1 in combination with wild-type or mutant BTN3A1, or separately with control BTNL3 and BTNL8 in pMIG with ViaFect® in OptiMEM™. 48 h following transfection, NIH-3T3 cells (3×10^4) were harvested, transferred to 96-well plates and incubated with purified in vitro-expanded Vδ2$^+$ γδ T cells (2×10^4) for 24 h ± zoledronate (5 μM). γδ T cell activation was determined by CD25 upregulation using flow cytometry. For γδ T cell functional assays, samples were excluded if transfection efficiency was less than 10%.

Detection of Förster Resonance Energy Transfer

Thirty-thousand NIH-3T3 cells were transfected with BTN2A1 in combination with wild-type or mutant BTN3A1, or control BTN2A1 transfected with PDL2 / BTN3A1 transfected with CD80, in pMIG with ViaFect® in OptiMEM™. 48 h following transfection, NIH-3T3 cells were harvested with trypsin, filtered through 30–70 μm cell strainers, and stained with anti-BTN2A1-AlexaFluor647 (clone 259) and BTN3A-PE (clone 103.2) or isotype controls (clones BM4-2a and MOPC-21, respectively) for 30 min at 4ºC. The frequency of cells identified as $FRET^+$ was examined on gated GFP^+AlexaFluor647$^+PE^+$ NIH-3T3 cells. For FRET experiments, data were excluded if BTN3A1 mutant protein levels were > 2-fold lower than BTN3A1 WT as determined by anti-BTN3A (clone 103.2) mAb staining.

Production of soluble proteins and tetramers

Soluble human BTN2A1–BTN3A1 ectodomains, or alternatively BTN2A1 ectodomains containing a C-terminal Cys (Cys247) and an acidic or basic leucine zipper [35], along with soluble γδTCRs, BTN1A1, BTN2A1 lacking Cys247, BTN3A1, BTN3A1 IgV domain, and mouse CD1d ectodomains were expressed by transient transfection of mammalian Expi293F or *MGAT1*^{null} (GNTI) HEK-293S cells using ExpiFectamine or PEI, respectively, with pHL-sec vector DNA encoding constructs with C-terminal biotin ligase (AviTag™) and His_6 tags [36]. Protein was purified from culture supernatant using immobilized metal affinity chromatography (IMAC) and gel filtration, and enzymatically biotinylated using BirA (produced in-house). Proteins were re-purified by size exclusion chromatography and stored at -80ºC. Biotinylated proteins were tetramerized with streptavidin-PE (BD) at a 4:1 molar ratio.

Structure determination

BTN2A1 and G115 γδTCR were mixed at a 1:1 molar ratio (15 mg/ml in Tris-buffered saline pH 8) and crystallized at 20°C in 20% polyethylene glycol (PEG) 3350/0.2 M sodium malonate/malonic acid pH 7.0; apo BTN2A1 (10 mg/ml in Tris-buffered saline pH 8) was crystallized at 20°C in 1.65 M ammonium sulfate/2% (v/v) PEG 400/0.1 M HEPES pH 8; and BTN2A1–BTN3A1–zippered complex (1 mg/ml in Tris-buffered saline pH 8) was crystallized at 20°C in 6% (w/v) PEG 6000/0.1 M magnesium sulfate/0.1 M HEPES pH 6 by sitting drop vapour diffusion (C3 facility, CSIRO, Australia). Crystals of BTN2A1–G115 γδTCR, apo BTN2A1 and BTN2A1–BTN3A1-zippered complex were flash frozen in mother liquor plus 27.5% (w/v) PEG/0.2 M sodium malonate, 1.8 M ammonium sulfate/2% (v/v) PEG 400/15% (v/v) glycerol, or in well solution plus 20% (v/v) glycerol, respectively. Data were collected at 100 K using the MX2 (3ID1) beamline at the Australian Synchrotron [37] with an Eiger detector operating at 100 Hz. Data were integrated using iMosflm version 7.3.0 [38] and, in the case of BTN2A1–G115 γδTCR, processed using the Aimless package in CCP4, or in the case of apo BTN2A1 and BTN2A1–BTN3A1-zippered complex, subjected to the STARANISO Server (Global Phasing Ltd.) (staraniso.globalphasing.org/cgi-bin/staraniso.cgi) to perform an anisotropic cut-off and to apply an anisotropic correction to the data. Apo BTN2A1 was solved by molecular replacement using the IgV and IgC domains of bovine BTN1A1 as separate search ensembles (PDB code 4HH8 [39]); BTN2A1–G115 γδTCR was solved by molecular replacement using G115 TCR (PDB code 1HXM [40]) and monomeric BTN2A1; BTN2A1–BTN3A1–zippered complex was solved by molecular replacement using monomeric BTN2A1, and BTN3A1 (from PDB code 4F80 [18]), with Phaser [41]. Refinement of BTN2A1–G115 γδTCR was performed by iterative rounds of model building into experimental maps in Coot and

refinement with Buster version 2.10.4 (Global Phasing), using non-crystallographic symmetry (NCS) restraints applied to BTN2A1, excluding residues at the TCR-binding interface [42]. Refinement of apo BTN2A1 and BTN2A1–BTN3A1-zippered complex were similarly restrained against the unliganded copy of BTN2A1 from BTN2A1– G115 γδTCR, or BTN3A1 from 4F80, excluding residues at the interfaces. The BTN2A1–G115 γδTCR, apo BTN2A1 ectodomain and BTN2A1–BTN3A1 ectodomain complex structural models were deposited in the Protein Data Bank under the accession codes 8DFW, 8DFY and 8DFX, respectively, and were analyzed with the CCP4 suite version 7.1 [43]. Molecular figures were generated with PyMOL (Schrödinger). Cation-π interactions were determined as described [43]. Angles were calculated between the center of masses of the Ig domains, or in some cases by the intersection of two planes, each defined by three points. Modelling in Extended Data Fig. 12 was performed using AlphaFold 2.0 [45]. In order to remove model bias, electron density 2mFo-DFc omit maps were generated as a composite of partial models that were refined with simulated annealing using Phenix.

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Microtitre) were coated with 50 µl/well of 5 µg/ml NeutrAvidin (Thermo Fisher Scientific, #31000) for 1 h at room temperature, washed with 0.05% v/v Tween-20 in PBS (PBST), blocked with 1% w/v BSA in PBS for 1 h at room temperature, and biotinylated BTN proteins captured. Alternatively, BTN proteins were coated directly on to an ELISA plate, washed with PBST and blocked with 1% BSA in PBS for 1 h at room temperature. BTN proteins were then probed with 2 µg/ml anti-BTN2A1 (mAb clone 259), anti-BTN3A (mAb clones 103.2 or 20.1 as indicated) or appropriate isotype control mAb BM4-2a (IgG2a,κ; a gift from CSL Limited) or MOPC-21 (IgG1,κ) and detected with goat anti-mouse Ig-HRP (Thermo Fisher Scientific, #63-65-20). 50 µl/well of TMB (Thermo Fisher Scientific; 1-Step Ultra TMB-ELISA) was added and incubated for ~10 min, and subsequently quenched with 2.25 M HCl, followed by measuring absorbance at 450 nm on a ClariostarPlus (BMG LabTech). Data were analysed in GraphPad Prism.

Surface plasmon resonance

SPR experiments were conducted at 25°C on a Biacore T200 instrument (GE Healthcare) using 10 mM HEPES-HCl (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20 buffer. Biotinylated BTN ectodomains were immobilized to 1,500-2,300 resonance units (RU) on a Biacore sensor chip SA pre-immobilized with streptavidin. The assay depicted in

Supplementary figure 6 was conducted using a Biacore S200 instrument, and streptavidin was immobilized on a CM5 chip via amine-coupling to 10,000 RU, followed by immobilization of ligands as above. Soluble BTN molecules or G115 γδTCR were two-fold serially diluted and simultaneously injected over test and control surfaces at a rate of 30 µl/min. After subtraction of data from the control flow cell (BTN1A1) and blank injections, interactions were analyzed using Biacore evaluation software (GE Healthcare), Scrubber (Biologic) and Prism version 10 (GraphPad), and equilibrium dissociation constants were derived at equilibrium.

Biolayer Interferometry (BLI)

Affinity measurements for the BTN2A1-BTN3A1 interaction were determined by BLI on an Octet Red 96e instrument (Sartorius). Assays were performed in black 96 well flat-bottom plates at 25°C with agitation (1000 rpm), using sample buffer 1 (150 mM NaCl, 10 mM HEPES, 0.05% Tween-20, 3 mM EDTA, pH 7.4), sample buffer 2 (150 mM NaCl, 10 mM HEPES, 0.05% Tween-20, 3 mM EDTA, 1 mM DTT, pH 7.4) and regeneration buffer (1 M NaCl, 10 mM HEPES pH 7.4). First, Streptavidin Biosensors (Sartorius) were hydrated for 20 minutes at 100 rpm in sample buffer 1. Biotinylated BTN3A1-B30.2 was then loaded at 5 µg/ml in sample buffer 1 to a ligand coating density of 1.2 nm, and a baseline signal was established for 180 seconds in sample buffer 1. For series in the absence of phosphoantigen, a second baseline signal was established in sample buffer 2 before dipping sensors into 2-fold serial dilutions of BTN2A1-BFI (wildtype or mutant) in sample buffer 2 starting at 10 µM. A 360 second association phase was recorded, followed by 2 separate 600 second dissociation phases in different wells of buffer 2. Sensors were then regenerated by three cycles of immersion in regeneration buffer followed by buffer 2 for 90 seconds per step, and assay steps were repeated for remaining sample wells. For series in the presence of phosphoantigen (20 µM HMBPP), a baseline signal was established in wells containing sample buffer 2 with HMBPP ahead of ligand association in BTN2A1-BFI (wildtype or mutant) along with HMBPP. This was followed by a dissociation phase in buffer 2 with HMBPP, and then a further dissociation phase in buffer 2 without HMBPP. Reference sensor data from the BTN3A1-B30-coated sensor dipped in buffer 2 alone was subtracted to account for baseline drift, and the data were aligned to the y-axis at baseline. Kinetic constants were determined using the Octet Data Analysis HT software v12.0.2.59 (ForteBio). Curve fitting was performed using a 1:1 global fit biding model.

Electron microscopy

Soluble BTN2A1 Gly130-Cys–BTN3A1 Asp132-Cys complex was enzymatically digested with thrombin to remove C-terminal leucine zippers, repurified by size exclusion and anion exchange chromatography, and spotted onto glow-discharged 400 mesh thin carbon-coated copper grids at 380 μg/ml in TBS for 30 seconds, followed by negative staining with 2% w/v uranyl acetate. Grids were observed on a FEI Tecnai F30 (Eindhoven, NL) 300 kV transmission electron microscope at a nominal magnification of ×52,000. Seventeen micrographs were acquired on a CETA (Thermofisher, USA) camera with a 3.7 Å pixel size. Particles were picked using blob picking followed by 2D class averaging in cryoSPARC [46], with 10,238 particles contributing to the final set of 2D class averages.

Statistical analysis

γδ T cell functional assays were analysed by 2-way ANOVA with Šidák's correction when comparing γδ T cell activation ($CD25^+$) with and without treatment across various BTN mutants. Change in His85γ-Ala tetramer MFI staining with Zol treatment was analysed by Wilcoxon matched-pairs signed rank test. All independent datapoints are biological replicates.

Data and materials availability

Reagents used in this study are available upon request. The crystal structures are deposited in the Protein Data Bank under accession codes 8DFW, 8DFY and 8DFX.

References

32 Holst, J. *et al.* Generation of T-cell receptor retrogenic mice. *Nat Protoc* **1**, 406-417 (2006). https://doi.org:10.1038/nprot.2006.61

33 Le Nours, J. *et al.* Atypical natural killer T-cell receptor recognition of CD1d-lipid antigens. *Nat Commun* **7**, 10570 (2016). https://doi.org:10.1038/ncomms10570

34 Kim, H. K. *et al.* Deep learning improves prediction of CRISPR-Cpf1 guide RNA activity. *Nat Biotechnol* **36**, 239-241 (2018). https://doi.org:10.1038/nbt.4061

35 Moll, J. R., Ruvinov, S. B., Pastan, I. & Vinson, C. Designed heterodimerizing leucine zippers with a ranger of pIs and stabilities up to 10(-15) M. *Protein Sci* **10**, 649-655 (2001). https://doi.org:10.1110/ps.39401

36 Aricescu, A. R., Lu, W. & Jones, E. Y. A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr D Biol Crystallogr* **62**, 1243-1250 (2006). https://doi.org:10.1107/S0907444906029799

37 Aragao, D. *et al.* MX2: a high-flux undulator microfocus beamline serving both the chemical and macromolecular crystallography communities at the Australian Synchrotron. *J Synchrotron Radiat* **25**, 885-891 (2018). https://doi.org:10.1107/S1600577518003120

38 Battye, T. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallogr D Biol Crystallogr* **67**, 271-281 (2011). https://doi.org:10.1107/S0907444910048675

39 Eichinger, A., Neumaier, I. & Skerra, A. The extracellular region of bovine milk butyrophilin exhibits closer structural similarity to human myelin oligodendrocyte glycoprotein than to immunological BTN family receptors. *Biol Chem* **402**, 1187-1202 (2021). https://doi.org:10.1515/hsz-2021-0122

40 Allison, T. J., Winter, C. C., Fournie, J. J., Bonneville, M. & Garboczi, D. N. Structure of a human gammadelta T-cell antigen receptor. *Nature* **411**, 820-824 (2001). https://doi.org:10.1038/35081115

41 Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**, 213-221 (2010). https://doi.org:10.1107/S0907444909052925

42 Smart, O. S. *et al.* Exploiting structure similarity in refinement: automated NCS and target-structure restraints in BUSTER. *Acta Crystallogr D Biol Crystallogr* **68**, 368-380 (2012). https://doi.org:10.1107/S0907444911056058

43 Winn, M. D. *et al.* Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* **67**, 235-242 (2011). https://doi.org:10.1107/S0907444910045749

44 Gallivan, J. P. & Dougherty, D. A. Cation-pi interactions in structural biology. *Proc Natl Acad Sci U S A* **96**, 9459-9464 (1999). https://doi.org:10.1073/pnas.96.17.9459

45 Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-589 (2021). https://doi.org:10.1038/s41586-021-03819-2

46 Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods* **14**, 290-296 (2017). https://doi.org:10.1038/nmeth.4169

Acknowledgements

We thank Con Panousis, Geoffrey Matthews and staff at CSL Limited for providing anti-BTN2A1 mAb. We thank Meghan Hattarki from CSIRO for support with SPR. We thank staff at the C3 protein crystallisation facility (CSIRO), the Ian Holmes Imaging Centre (Bio21 Institute, University of Melbourne), the MX beamlines at the Australian Synchrotron, the Melbourne Cytometry Platform (University of Melbourne), and AGRF for DNA sequencing support. This research was undertaken in part using the MX2 beamline at the Australian Synchrotron, part of ANSTO, and made use of the ACRF detector. Infrastructure support from the NHMRC Independent Research Institutes Infrastructure Support Scheme and the Victorian State Government Operational Infrastructure Support Program are gratefully acknowledged. This work was supported by a Miller Foundation Research Accelerator fund (to APU), Cancer Council of Victoria (1126866), the National Health and Medical Research Council of Australia (NHMRC; 1184906, 1165467); the Australian Research Council (ARC; CE140100011). MR is supported by a Cancer Council Victoria Postdoctoral Research Fellowship. AB is supported by a fellowship from the Department of Health and Human Services acting through the Victorian Cancer Agency. MWP is supported by an NHMRC Investigator Fellowship (APP1194263). NAG is supported by an ARC Discovery Early Career Researcher Award (DE210100705). DIG was supported by NHMRC Senior Principal Research Fellowship (1117766) and subsequently by an NHMRC investigator grant (2008913).

Author Contributions

Conceptualization, APU, DIG, TSF, NAG, AB; methodology, TSF, CS, MR, OD, SL, AC, TSP, NAG, APU; investigation, TSF, CS, RGC, MR, OD, HGB, EH, APU; resources, ZR, RS, AH, SL, SJR, MAG, MWP, OP, JN, AB, NAG, DIG, APU; writing – original draft, APU, TSF; writing – reviewing & editing, APU, DIG, TSF, NAG, CS, EH, TSP, MR, MWP, OP, AH; supervision, APU, DIG, TSF; funding acquisition, APU, DIG, AB.

Competing interests

AH is an employee of CSL Limited and can partake in employee share schemes. AB and AH are inventors on a patent regarding the use of BTN2A1 to influence immune reactions (WO2015077844). TSF, MR, AH, AB, DIG and APU are inventors on a patent regarding methods of inhibiting or activating γδ T cells (WO2020257871). APU, DIG, TSF, NAG, MR are inventors on filed patents regarding BTN-mediated activation of γδ T cells. All other authors declare no competing interests.

Additional Information

Correspondence and requests for materials should be addressed to Dr Adam Uldrich [auldrich@unimelb.edu.au] or Prof Dale Godfrey [godfrey@unimelb.edu.au].