Heteromeric interactions regulate butyrophilin (BTN) and BTN-like molecules governing γδ T cell biology

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The long-held view that gamma delta (γδ) T cells in mice and humans are fundamentally dissimilar, as are γδ cells in blood and peripheral tissues, has been challenged by emerging evidence of the cells’ regulation by butyrophilin (BTN) and butyrophilin-like (BTN) molecules. Thus, murine Btnl1 and the related gene, Skint1, mediate T cell receptor (TCR)-dependent selection of murine intraepithelial γδ T cell repertoires in gut and skin, respectively; BTN3L and BTN3L8 are TCR-dependent regulators of human gut γδ cells; and BTN3A1 is essential for TCR-dependent activation of human peripheral blood Vγ9Vδ2+ T cells. However, some observations concerning BTN/Btnl molecules continue to question the extent of mechanistic conservation. In particular, murine and human gut γδ cell regulation depends on pairings of Btnl1 and Btnl6 and BTN3L and BTN3L8, respectively, whereas blood γδ cells are reported to be regulated by BTN3A1 independent of other BTNs. Addressing this paradox, we show that BTN3A2 regulates the subcellular localization of BTN3A1, including functionally important associations with the endoplasmic reticulum (ER), and is specifically required for optimal BTN3A1-mediated activation of Vγ9Vδ2+ T cells. Evidence that BTN3L3/BTN3L8 and Btnl1/Btnl6 likewise associate with the ER reinforces the prospect of broadly conserved mechanisms underpinning the selection and activation of γδ cells in mice and humans, and in blood and extralymphoid sites.

gamma delta T cells | butyrophilins | endoplasmic reticulum | zolendronic acid | evolutionary conservation

Ever since their unanticipated discovery (1, 2), the T cell receptor (TCR) γ and δ chains and the cells that express them have offered insights into immunology. For example, rather than focusing on discrete, pathogen-specific epitopes (e.g., peptides), γδ TCRs collectively respond to highly diverse molecules, ranging from self-encoded moieties induced by cellular dysregulation through lipids presented by CD1 to microbial molecular patterns (3, 4). Conspicuous among these is the response of most human peripheral blood Vγ9Vδ2+ T cells to low-molecular-mass “phosphoantigens” (PAs), including hydroxymethyl but-2-ethyl pyrophosphate (HMBPP), an intermediate in the deoxyxylulose phosphate pathway in many bacteria and protozoa, and isopentenylpyrophosphate, an intermediate in the mevalonate pathway employed from bacteria to vertebrates and commonly overexpressed by virus-infected cells or cells transformed by p53 inactivation (5, 6). How such charged, low-molecular-mass molecules activate signaling from large, cell-surface TCR complexes is unclear, fueling the search for a presenting element, akin to CD1.

In that regard, PAg activation of Vγ9Vδ2+ T cells requires BTN3A1 (7), a member of the butyrophilin (BTN) and BTN-like (BTNL) family that in humans comprises 11 genes related to those encoding the B7-superfamily members, including PD-L1. The BTN3A1 gene lies close to BTN3A2 and BTN3A3, and all three encode type 1 transmembrane proteins comprising an Ig variable (V) and Ig constant (C) region linked via a single pass transmembrane (TM) domain to an intracellular region that in BTN3A1 and BTN3A3 includes a B30.2 domain, akin to that found in many BTN(L) and TRIM proteins (8).

BTN3A1 forms homodimers (9) and was reported to bind PAg via its extracellular IgV domain, thereby presenting it directly to the Vγ9Vδ2 TCR (10). However, this mechanism for PAg-dependent, TCR-mediated γδ cell activation failed to explain why BTN3A2 seemingly could not present PAg, despite its identical IgV domain. Moreover, others have reported PAg binding to the intracellular B30.2 domain, within which histidine 381 appeared particularly important (11–13). Sensing of intracellular PAg levels by BTN3A1 was shown to alter the conformation of its B30.2 domain (14, 15), although how this might trigger Vγ9Vδ2+ T cell activation remains unclear, as the PAg site can also bind biologically inactive negatively charged molecules (15).

Provocatively, the implication of BTN3A1 in Vγ9Vδ2 T cell activation followed the earlier demonstration that the murine Btnl gene, Skint1, drives the selective development of murine intraepithelial Vγ9Vδ1+ T cells (16–18). Furthermore, we recently showed that murine Btnl1/Btnl6 drive the selective development of murine intestinal Vγ7+ intraepithelial T cells, whereas human BTN3L3/BTNL8 selectively regulate human gut Vγ4+ T cells (19). Thus, a general role is emerging for BTN/Btnl in γδ cell biology.

However, the notion of broadly conserved mechanisms of γδ cell regulation is challenged by the fact that TCR-dependent mouse and human gut γδ cell regulation requires pairings of proteins encoded by Btnl1/Btnl6 and BTN3L3/BTN3L8, respectively, whereas there is conflicting evidence as to whether Vγ9Vδ2+ T cell regulation by BTN3A1 also requires BTN3A2 and/or BTN3A3 (7, 10, 13). Addressing this paradox, our study shows that BTN3A2 regulates BTN3A1-dependent responses to metabolites (“phosphoantigens”) not seen by rodent γδ cells, whereas some rodent, γδ-rich compartments, notably in the skin, lack obvious human counterparts. Recently, however, mouse and human intraepithelial gut γδ cells were found to be regulated by pairings of BTN-like genes. This study now shows that BTN3A1 also functions as a pairing, with its subcellular trafficking and optimal activity both regulated by BTN3A2. Hence, seemingly diverse γδ cell biologies across species and tissues are underpinned by conserved mechanisms.

Significance

Although gamma delta (γδ) T cells compose an evolutionarily conserved third lineage of diversified lymphocytes, alongside αβ T cells and B cells, they can seem overly different across species and tissues. Thus, human blood γδ cells show butyrophilin (BTN)3A1-dependent responses to metabolites (“phosphoantigens”) not seen by rodent γδ cells, whereas some rodent, γδ-rich compartments, notably in the skin, lack obvious human counterparts. Recently, however, mouse and human intraepithelial gut γδ cells were found to be regulated by pairings of BTN-like genes. This study now shows that BTN3A1 also functions as a pairing, with its subcellular trafficking and optimal activity both regulated by BTN3A2. Hence, seemingly diverse γδ cell biologies across species and tissues are underpinned by conserved mechanisms.


The authors declare no conflict of interest.

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the subcellular localization of BTN3A1, including association with the ER, and is essential for the optimal activation of $\gamma V\delta 2^+$ T cells. Likewise, we find that BTN3/BTNL8 and Bn1/Bn6 are ER-associated. Hence, conserved molecular mechanisms seemingly underpin the regulation of major $\gamma \delta$ cell subsets across mice and humans, and across blood and extralymphoid tissues.

**Results**

**$\gamma V\delta 2^+$ T Cell Activation Requires BTN3A1 and BTN3A2.** Human HEK293T (293T) embryonic kidney epithelial cells readily stimulated $\gamma V\delta 2^+$ T cells when pulsed with either HMBPP or zoledronate (Zol), a clinically used aminobisphosphonate that induces isopentenylpyrophosphate accumulation by inhibiting farnesyl pyrophosphate synthase (20). $\gamma V\delta 2^+$ T cell stimulation is routinely assayed by increased surface expression of CD107a (LAMP-1), reflecting exocytotic degranulation of cytolytic lysosomes (Fig. S1A). Consistent with expression by many cell types, RNAs for all BTN3A genes were expressed by 293T cells (Fig. S1B), and pan anti-BTN3A antibody (monoclonal antibody 20.1), which cannot discriminate among the three proteins, detected cell surface BTN3A3, as it did on peripheral blood cell subtypes of three healthy donors (Fig. S1 C and D).

To assess the contributions of BTN3A1, BTN3A2, and/or BTN3A3 with $\gamma V\delta 2^+$ T cell stimulation (Fig. 1A), we generated 293T lines with disruptions of BTN3A1 (CRA1), BTN3A2 (CRA2), BTN3A3 (CRA3), all three genes (CRA123), and BTN3A1+BTN3A2+BTN3A3 (CRA23; Fig. S2A), which we validated by failure to amplify full-length products of the relevant BTN3A genes (Fig. 1B) and diminished anti-BTN3A antibody staining (Fig. S2B). Consistent with deleting the BTN3A cluster, CRA123 cells also showed disrupted BTN2A2 expression (Fig. 1B). Control cells (CREV) were transfected with CRISPR (CR)/Cas9 lacking BTN targeting sequences.

The cell lines were pulsed with a dose-range of Zol and assessed for their activation of $\gamma V\delta 2^+$ T cells. CREV showed a potency ($E_{50}$ of $\sim 70\%$ of $\gamma V\delta 2^+$ T cells) in those cells transfected with, for example, less than $1\mu M$ Zol (Fig. 1C and Fig. S2C). As expected, this capacity was completely lost from cells with disrupted BTN3A1, either alone (CRA1) or along with BTN3A2 and BTN3A3 (CRA123) (Fig. 1C and Fig. S2C). Hence, BTN3A1 is, as described, strictly required for $\gamma V\delta 2^+$ T cell activation (7, 10, 11, 13). Curiously, CRA23 cells that express endogenous BTN3A1 but lack BTN3A2 also showed impaired activation of $\gamma V\delta 2^+$ T cells (Fig. 1C and Fig. S2C). Although the $E_{50}$ for CRA2 cells was also $\sim 10\%$ Zol, potency was reduced by $\sim 50\%$. Moreover, CRA23 cells that also express BTN3A1 but neither BTN3A2 nor BTN3A3 did not activate $\gamma V\delta 2^+$ T cells (Fig. 1C and Fig. S2C). Conversely, CRA3 cells lacking only BTN3A3 functioned comparably to CREV cells. In sum, the full functional activity of BTN3A1 required BTN3A2, with some capacity of BTN3A3 to substitute for BTN3A2. This perspective was reinforced by experiments in which the functional rescue of CRA123 cells by singular BTN3A genes and combinations thereof was attempted across a range of Zol concentrations. CRA123 cells transfected with BTN3A2, BTN3A3, or BTN3A2+BTN3A3 showed residual functional capacity. Conversely, CRA123 cells rescued with BTN3A1+3A2 or BTN3A1+3A3 showed $E_{50}$ and $E_{max}$ comparable to control cells (CREV), whereas cells transfected with only BTN3A1 showed greatly increased $E_{50}$ (Fig. 1D and Fig. S2D). Thus, BTN3A1 is necessary but insufficient to promote optimal, Zol-dependent, $\gamma V\delta 2^+$ T cell activation.

**BTN3A1 Heteromers.** To assess how BTN3A proteins might collaborate, we focused on their potential to interact. Because BTN3A1 can homodimerize via its IgC domain (9), we considered that BTN3A proteins might similarly form heteromers. Indeed, heterodimers of BTN3A1 and BTN3A2 produced in high quantities in insect cells were recently visualized by electron microscopy, although they were not studied in detail (14). Thus, N-terminal FLAG-tagged BTN3A1 was expressed in CRA123 cells with or without N-terminal HA-tagged BTN3A2, or HA-3A2A1gC that lack the IgC domain. Anti-FLAG- and anti-HA-reactive bands of appropriate molecular weight were detected as input proteins by Western blotting lysates from singly- and doubly-transfected cells (Fig. 2A, Left; Hsp90, loading control). Importantly, anti-HA also detected HA-BTN3A2, but not HA-3A2A1gC, in anti-FLAG immunoprecipitates of doubly-transfected cells (Fig. 2A, Right). Thus, BTN3A2 complexed with BTN3A1 in an IgC-dependent manner. This was further investigated via mass spectrometry. By plotting protein intensity ratios (Welch Difference) versus log10 sum of intensities, BTN3A1 was, as expected, the most greatly enriched protein intensities, BTN3A1 was, as expected, the most greatly enriched
Motifs Within BTN3A1. CRA123 cells (lacking all BTN3A genes) were transfected with a set of BTN3A1 chimaeras (Fig. 3A) and assessed for surface expression and the corresponding capacity to stimulate Vγ9Vδ2+ T cells. Relative to BTN3A1, BTN3A3 possesses an extended C terminus beyond the B30.2 domain, and when this was added to BTN3A1 (3A1-3, Fig. 3A, purple), it improved its surface expression (Fig. 3B). Given that the C terminus can evidently modify the subcellular localization of BTN3A proteins, we generated a chimera fusing GFP to the C terminus of BTN3A1 (3A1-GFP), a strategy employed by others (7, 14). This too showed greatly increased BTN3A1 surface expression (Fig. 3A and B, green), suggesting caution in interpreting data wherein C-terminal BTN3A fusions are used interchangeably with wild-type increased surface BTN3A expression was also achieved by substituting the intracellular region (Gln272-Lys334) located between the TM and B30.2 domains of BTN3A1 with the corresponding region (Arg272-Ala334) of BTN3A2 (3A1-2; Fig. 3A and B, blue). When assayed for their functional potentials using a standard concentration of Zol (5 μM), greatly increased surface expression invariably decreased PAg-dependent Vγ9Vδ2+ T cell activation, the most extreme example being 3A1-2-1 (Fig. 3C and Fig. S6D). Hence, subcellular localization profoundly affected BTN3A1 function, as is further considered here.

Because the 3A1-2-1 construct includes the BTN3A1 ectodomain and the B30.2 domain, including His381, its failure to drive Vγ9Vδ2+ T cell activation implicates the region (Gln272-Lys334) as critical for function. Indeed, placing the entire intracellular region (Gln272-Ala334) of BTN3A1 downstream of the BTN3A2 ecto- and TM-domains (3A2/1) fully recapitulated the signature expression and function of BTN3A1 (Fig. 4 A–C), clearly demonstrating that the BTN3A1 and BTN3A2 extracellular domains are interchangeable. In contrast, a construct (3A2-1) in which just the BTN3A1 C terminus (Ala327-Ala334), including the B30.2 domain, was directly fused to the C terminus of BTN3A2 displayed increased surface expression relative to BTN3A1, but no function (Fig. 4 A–C), providing further evidence that BTN3A1 function depends on intracellular determinants lying between its TM and B30.2 domains, located between Gln272 and Lys334.

Motifs Within BTN3A2. To define BTN3A2 sequences required to functionally collaborate with endogenous BTN3A1, we employed CRA23 cells that express BTN3A1. Consistent with data presented earlier, functional rescue was achieved by reexpression of BTN3A2, but not BTN3A2ΔIgC, whose gene-product cannot associate with BTN3A1 (Fig. 4 D and E and Fig. S6B). Thereupon, we focused on the relatively short intracellular domain of BTN3A2, which lacks a B30.2 domain (Fig. 4I). Removal of the 20 most C-terminal amino acids (3A2Δ315–334) prevented BTN3A2 from functionally rescuing CRA23 cells, whereas truncation of the last 10 amino acids (3A2Δ325–334) did not (Fig. 4 D and E and Fig. S6B), thus establishing the importance of amino acid 334 of BTN3A2 for functional rescue.
ER Association of BTN3A1 and BTN3A2. We first assessed whether the intracellular domains of BTN3A1 and BTN3A2 did contribute to ER retention/retrieval. FLAG-tagged BTN3A1 introduced into CRA123 cells largely colocalized with the ER marker, protein disulphide isomerase (PDI), but not with a Golgi marker, Gm130 (Fig. 5A), quantitated by high bright detail similarity (BDS) of FLAG-PDI, but not of FLAG-Gm130 (Fig. 5A, Lower). Just as BTN3A2 enhanced BTN3A1 surface expression (Fig. 2D), BTN3A1 showed reduced ER colocalization in cells cotransfected with BTN3A2 (Fig. 5A, orange segregation from cyan; reduced FLAG-PDI BDS). Confocal microscopy likewise revealed that mCherry-tagged Sec61β, another ER-resident marker, colocalized with cotransfected BTN3A1 or BTN3A2 expressed singly, but less so when BTN3A1 and BTN3A2 were coexpressed (Fig. 5B).

Consistent with ER association being driven by each of the putative motifs in BTN3A1 and BTN3A2 (Fig. 4F), their nonconservative mutation (BTN3A1: RWR313>RGER322,325, AWA-AGEA, and BTN3A2: KRKKIQ312-318 > KRAAIQ) substantially increased the proteins’ cell surface expression, whereas conservative R/K substitutions (RWR-RGER > KWK-KGEK) did not, in one case exaggerating BTN3A2 retention (KRKKIQ > KRRRIQ) (Fig. 6A).

Given that subcellular localization is critical for function, we considered how the ER retention motifs might affect the proteins’ functional collaboration. If expressed with either wild-type BTN3A2 (KRKKIQ) or a KRAAIQ mutant, both of which strongly co-localize with the ER (Fig. 6A), nonexpression of regulated subcellular localization and ER associations for BTN3A1/BTN3A2 mutant pairings (e.g., 3A1-KWK-KGEK + 3A2-KRRRIQ) were functional despite decreased cell surface expression (Fig. 6B, Right and Fig. 6C and D).

Conserved ER Retention of BTN/L/Btn Proteins. Given the importance of regulated subcellular localization and ER associations for BTN3A1-BTN3A2 collaboration, we examined whether similar properties might underpin Bn nl1-Bnl6 and BTN3-L/Btn3L collaborations. After transfection of 293T cells that do not ordinarily express these four proteins, all showed strong PDI colocalization (Fig. 8A–D). Moreover, Bnl1/Bnl6 coexpression reduced PDI colocalization (Fig. S8A and B), as was observed for BTN3A2-BTN3A1 collaboration (Fig. 5A). Although this was a priori less apparent for BTN3L/BTN3L8 coexpression (Fig. S8C and D), the effect of coexpression became clearer when asking whether a protein sat within or outside of a “mask” defined by PDI expression: clearly BTN3L/BTN3L8 coexpression increased the fraction of protein outside the mask, albeit to a lesser extent than Bn nl1/Bnl6 coexpression (Fig. S9A). Consistent with this, BTN3L coexpression increased BTN3L8 surface expression (Fig. S9B; compare the green histograms). In sum, all BTN(L)/Bnl proteins implicated in specific y sub subset regulation displayed hallmarks of preferential ER association that were diminished by coexpression of their functionally relevant partners.

Further Functional BTN/BTN(L)/Bnl Conservation. Finally, another short subregion between the TM and B30.2 domains of BTN3A1 (YNEWKAKLFPA), recently found to be functionally important (23), is centered on an EWK motif that we also found to be functionally indispensable for Vγ9Vδ2+ cell activation (Fig. S9C). However, it was unaffected by mutation to DWR, which is conserved similarly close to the B30.2 domains of BTN3L, BTN8, Bn nl1, and Bnl6 (Fig. S9D), providing further indications of conservation of mechanism.

Discussion

This study has provided many insights into how BTN and BTN(L) proteins regulate yδ cell biology. First, CR-mediated gene deletion...
profoundly impaired the cellular or for cell regulation may have unique BTN3A2 Skint1-10 cells. Conversely, although no broadly accepted ev- BTN3A1 intra- was expressed with cell stimula- genes, po- T cell activation. “BTN3A2 BTN3A3 and BTN3A3. cells are broadly dissimilar.

ER association of BTN3 is critical for its B Upper | denotes functional deficiency of cells whose BTN3A expression is shown in purple-boxed plot no. 5 SD. Representative of three independent experiments. P values are relative to the combination of WT BTN3A1+BTN3A2. Purple arrow in C denotes functional deficiency of cells whose BTN3A expression is shown in purple-boxed plot in B. (D) CRA123 cells were transfected with pCS1GW, either as an EV or encoding indicated BTN3 WT or ER motif mutants before a 12-h treatment with Zol (0.04-50 μM, 5x dilution steps) and assayed for their capacity to promote Vγ9Vδ2+ T cell degranulation. Data points are the mean of duplicate stimulations and representative of two independent experiments.

(rather than shRNA knock-down) provided unequivocal evidence that specifically disrupting BTN3A2 profoundly impaired the capacity to mediate PAg-dependent Vγ9Vδ2+ T cell activation. Moreover, BTN3A2 could be stoichiometrically communoprecipitated with BTN3A1, consistent with their forming heteromeric complexes. Although the requirement for BTN3A2 was not absolute, the like reflected partial compensation by BTN3A3, which could also be communoprecipitated with BTN3A1. Indeed, BTN3A1 cells lacking BTN3A2 and BTN3A3 showed no residual functional activity. Moreover, in experiments in which the functional potentials of BTN3A-null CRA123 cells were rescued with BTN3 genes, potencies (E_{\text{max}}) comparable to wild-type cells were achieved only when BTN3A1 was expressed with BTN3A2 and/or BTN3A3. Nonetheless, because BTN3A3 was not ordinarily required for Vγ9Vδ2+ T cell activation in cells expressing BTN3A1 and BTN3A2, this study has focused on BTN3A1 and BTN3A2 cooperation. Optimal Vγ9Vδ2+ T cell responses over a range of Zol concentrations depended on the BTN3A2-IgC domain required for BTN3A2 to interact with BTN3A1, thereby linking complex formation with function. Complex formation was also linked to in- creased BTN3A1 cell surface expression, although this alone could not explain functional collaboration, as unrestricted surface expres- sion habitually decreased function. Instead, functionally impor- tant domains were mapped to intracellular regions of BTN3A1 and BTN3A2 that regulated the proteins’ association with the ER. This may have been overlooked by studies employing GFP-fusion proteins because BTN3A1 proteins with altered C-termini can show atypical subcellular localization. In contrast, the importance of ER association was highlighted by a clear instance of functional complementation in which optimal Vγ9Vδ2+ T cell stimulation requires ER retention motifs in either BTN3A1 or BTN3A2 but not both. This is most readily explained by the bioactive unit being a physically associated BTN3A1-3A2 heteromer.

Thus, we propose a model whereby the BTN3A1 ectodomain and B30.2-domains are active entities in Vγ9Vδ2+ cell stimula- tion, with which BTN3A2 collaborates by regulating the appro- priate routing, kinetics, and/or stability of BTN3A1. This may include a critical “dwell time” in the ER, mediating a quality control step for multimer formation, and/or permitting interaction with other moieties (e.g., cargo). Of note, sterol metabolism, which BTN3A1 may monitor via isopentenylpyrophosphate binding, involves ER-resident enzymes of the mevalonate pathway that are regulated by sterol regulatory element-binding protein factors whose inactive forms also reside in the ER (24). Possibly, the BTN3A1-3A2 heteromers promote ER exit by masking the ER retention motifs, as has been reported in other systems (25). The partial rescue of CRA123 cells by BTN3A1 likely reflects some capacity of overexpressed homomeric BTN3A1 to exit the ER without BTN3A2/BTN3A3, but its suboptimal routing, ki- netics, and/or stability result in suboptimal EC_{max} and possibly reduced E_{\text{max}}. Likewise, the model predicts the observed func- tional deficits of constructs that accelerate surface expression at the expense of ER association. Of note, BTN3A1 lacks the extended C-terminal sequences of BTN3A3 that promote efficient cell surface BTN3A3 expression.

Our study also establishes parallels of BTN3A1/BTN3A2 bi- ology with Brl1/Bnl1 and BTN3L3/BTN3L8 that respectively reg- ulate murine and human gut γδ T cells. In each case, optimal function required pairs of proteins that intrinsically displayed ER association, thereby limiting cell surface expression until coex- pressed (19). Furthermore Skint1, on which murine Vγ5+ intra- epidermal T cells depend, also shows poor surface expression and is functionally ablated by constructs that drive it to the cell surface (26). Hence, our current study emphasizes conserved cellular and molecular mechanisms that may underpin γδ cell regulation across mouse and human, and across peripheral tissues and blood. This is a major departure from the long-held view that human lymphoid γδ T cells and murine tissue-resident γδ cells are broadly dissimilar.

Nonetheless, each subtype of γδ cell regulation may have unique signatures. In particular, no equivalent of PAgAs has been identified for BTN3L1/BnL1-dependent gut γδ cells or for Skint1-dependent murine skin γδ cells. Conversely, although no broadly accepted ev- idence currently exists that BTN3A1-3A2 present PAgs cargo to γδ T cells, our model for BTN3A1-3A2 function has possible parallels with ER dwell times required for CD1d, MHC-I, and MR1 proteins to acquire low-molecular-mass lipid, peptide, and vitamin B me- tabolite cargos, respectively. Indeed, those cargoes stabilize the proteins and regulate their cell-surface display to T cells (27–29). Moreover, the actions of different BTN3LNL gene products may also have parallels with antigen activation of human T cells by MHC class II DO and DR molecules, for which the closely related mol- ecules, DO and DM, are not absolutely required, but are important in regulating the quality of antigen presentation.

Materials and Methods

All reagents, cells, culture conditions, plasmids, and primers are listed in the SI Materials and Methods.

Generation of CR Lines, Cloning, and RT-PCR. Targeting sequences were cloned using short complementary oligos (Table S1). 293T CR lines were generated by transient transfection of plG2C and single-cell sorting on the basis of GFP expression at 72 h. Tagged constructs were generated by overlapping PCR except mCherry-Sec61p (gift from Professor Martin-Serrano, KCL). Murine FLAG-Btnl1 and HA-Btnl6 and human FLAG-BTNL3 and FLAG-BTNL8 were described (19). Wild-type BTN1A1, BTN2A1, BTN2A2, BTN3A1, BTN3A2, and BTN3A3 were cloned from 293T cells, and wild-type BTNL3, BTNL8S, and BTNL8 from Caco-2 cells (primers in Table S2). Gene expression was checked by RT-PCR, using the same primers.

Flow Cytometry, Confocal Microscopy, and Image Cytometry. 293T cells were seeded at 0.5 × 10^6 per well in 12-well plates and transfected the next day with 1 μg total plasmid DNA before expression assessment by flow, confocal imaging, and image cytometry.

Flow Cytometry. Cells were washed in FACS buffer (PB; PBS, 5% FCS), stained with the indicated antibodies (45 min, 4 °C), washed twice, and resuspended in PB. Data were acquired on a FACS Canto II (BD) and analyzed in FlowJo. For intracellular staining, see following.
Confluent Microscopy. Cells were transferred 24 h posttransfection onto 13-mm glass coverslips, left to adhere for 24 h (37 °C, 5% CO₂), washed with PBS and fixed with CellFix buffer (BD), permeabilized (or not) with permeabilization/wash buffer (PWB; eBioscience), stained in BF (surface) or PWB (intracellular) and washed twice in BF or PWB and then twice in BF. Coverslips were mounted using Prolong Gold (Thermo Fisher). Data were acquired on a TCS SP2 AOBS microscope (HCS PL APO CS 63.0x/1.4 oil objective; Leica) and analyzed in ImageJ.

Image Cytometry. Cells were fixed and permeabilized and stained in PWB supplemented with DAPI. Data were acquired on an ImageStreamX (Merck Millipore) and analyzed in IDEAS. Data were analyzed in IDEAS, and histograms were generated in R.

CD107α Assay. Briefly, 293T cells (24 h posttransfection when relevant) were pretreated or not for 12 h with HMIBP or Zol, washed twice in DMEM, coincubated for 5 h with Vγ9Vδ2 T cells (3:1 ratio) and PE anti-CD107a antibody (1 µg/ml) in RPMI complete media (37 °C, 5% CO₂), washed with BF and stained with AlexaFluor647 anti-CD3 and FITC anti-TCRVδ2, and washed twice and resuspended in BF for acquisition (30).

Comimmunoprecipitation and Western Blotting. 293T cells were seeded at 1 × 10⁶ per well in six-well plates, transfected the next day with 2 µg total plasmid DNA, washed in PBS 48 h posttransfection, and harvested in 0.5 mL lysis buffer [50 mM Tris HCl at pH 7.4, 150 mM KCl, 10 mM MgCl₂, 1 mM CaCl₂, 0.5% Nonidet P-40, 0.1% digitonin, 5% glycerol, Complete Protease inhibitor (Roche Diagnostic)]. Lysates were centrifuged at 12,000 × g (4 °C, 5 min), 50 µL mixed with Laemmli buffer, and boiled for input analysis. Remaining lysates were incubated for 4 h at 4 °C on a rotating wheel with 50 µL protein G Dynabeads (Thermo Fisher) preincubated with anti-FLAG antibody and washed three times in lysis buffer. Proteins were eluted in 30 µL Laemmli and analyzed by SDS/PAGE and Western blotting. Blots were visualized by ImageQuant using HRP-anti-HA (3F10, Roche) and HRP-anti-FLAG (M2; Sigma), and by Li-cor Odyssey imaging using rabbit anti-HSP90 (H-114; Santa Cruz Biotechnology) and IRDye800LT anti-rabbit antibodies (Li-cor).


Mass Spectrometry. Anti-FLAG immunoprecipitation (Comimunoprecipitation and Western Blotting) was performed on 5 × 10⁶ CRL123 cells transfected with an empty vector control (EV), FLAG-BTN3A1+HA-BTN3A2, and FLAG-BTNL3+HA-BTNL8. Eluted proteins were subjected to SDS/PAGE until the running front had migrated 1 cm into the gel. Proteins were in-gel digested using trypsin, and peptides were analyzed with an Orbitrap-Fusion Lumos mass spectrometer coupled to an Ultimate3000 HPLC equipped with an EASY-Spray nanosource (Thermo Fisher Scientific). Raw data for triplicate runs were processed using MaxQuant v1.6.0.1, using label-free quantification (MaxLFQ) selected as the quantification algorithm. The proteingroup.txt output table was imported into Perseus software for further processing. LFQ intensities were log₂ transformed, and the dataset was filtered for proteins having at least three values in at least one group (each group consisting of triplicate injections). The remaining missing values were imputed using default Perseus settings by drawing from a simulated noise distribution with a down shift of 1.8 and a width of 0.3 compared with the log₂ LFQ intensity distribution. Two-sample Welch t tests were performed with a permutation-based FDR set at 0.05.

Statistics. Data were analyzed using unpaired two-tailed Student’s t test. Reference datasets used to determine significance are indicated in each case.

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