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Epithelia Use Butyrophilin-like Molecules to Shape Organ-Specific $\gamma\delta$ T Cell Compartments

**Highlights**

- Gut epithelial butyrophilin-like 1 ($Btnl1$) shapes the local $\gamma\delta$ T cell compartment
- Other organ-specific epithelial $Btnl$ genes select cognate $\gamma\delta$ cells in other sites
- $Btnl$ heteromers can specifically activate $\gamma\delta$ T cells with cognate T cell receptors
- Human $BTN$ genes reveal a conserved biology of epithelial T cell regulation

**In Brief**

Epithelial cells provide signals that instruct the development and function of their local $\gamma\delta$ T cell compartments so that these immune cells can support the non-immune functions of the different barrier tissues.

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**Data Resources**

GSE85422

**Graphical Abstract**
Epithelia Use Butyrophilin-like Molecules to Shape Organ-Specific $\gamma\delta$ T Cell Compartments

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SUMMARY

Many body surfaces harbor organ-specific $\gamma\delta$ T cell compartments that contribute to tissue integrity. Thus, murine dendritic epidermal T cells (DETCs) uniquely expressing T cell receptor (TCR)-V$\gamma$5 chains protect from cutaneous carcinogens. The DETC repertoire is shaped by Skint1, a butyrophilin-like (Btnl) gene expressed specifically by thymic epithelial cells and suprabasal keratinocytes. However, the generality of this mechanism has remained opaque, since neither Skint1 nor DETCs are evolutionarily conserved. Here, Btnl1 expressed by murine enterocytes is shown to shape the local TCR-V$\gamma$7* $\gamma\delta$ compartment. Uninfluenced by microbial or food antigens, this activity evokes the developmental selection of TCRx$\beta$* repertoires. Indeed, Btnl1 and Btnl6 jointly induce TCR-dependent responses specifically in intestinal V$\gamma$7* cells. Likewise, human gut epithelial cells express BTN3L and BTN8L that jointly induce selective TCR-dependent responses of human colonic V$\gamma$4* cells. Hence, a conserved mechanism emerges whereby epithelia use organ-specific BTNl genes to shape local T cell compartments.

INTRODUCTION

The specialized differentiation of body surface epithelia is most commonly viewed from the perspective of organ-specific physiological functions, such as nutrient absorption in the gut and prevention of trans-epidermal water loss. Likewise, differentiated epithelia provide physical and chemical barriers to pathogens and toxins (Janeway et al., 2001). However, it is now appreciated that body surfaces at steady state comprise diverse cell types, including many immune cells (Vantourout and Hayday, 2013). Among these, intraepithelial lymphocytes (IELs) expressing $\alpha\beta$ T cell receptors (TCRs) can mount rapid recall responses to pathogens, while other IELs, commonly expressing $\gamma\delta$ TCRs, contribute to the maintenance of body surface integrity that is key to metazoan viability. Thus, murine gut $\gamma\delta$ T cells regulate enterocyte differentiation and turnover (Komano et al., 1995) and prevent inflammatory damage (Hayday and Tigelaar, 2003; Hayday, 2000; Roberts et al., 1996), while TCR$\gamma\delta$* dendritic epidermal T cells (DETCs) (Kuziel et al., 1987; Stiingl et al., 1987a) limit inflammation, promote wound resolution, and increase cutaneous resistance to carcinogens (Girardi et al., 2001, 2002; Jameson et al., 2002; Strid et al., 2008). Moreover, the skin and gut of jawless fish harbor cells similar to TCR$\gamma\delta$* IELs, arguing that such compartments fulfill critical, evolutionarily conserved roles (Hirano et al., 2013).

In mice, large IEL compartments are defined by $\gamma\delta$ TCRs that match particular anatomical sites: V$\gamma$5 in skin, V$\gamma$7 in gut, and V$\gamma$6 in the uterus (Allison and Havran, 1991; Asarnow et al., 1988; Guy-Grand et al., 2013; Itohara et al., 1990; Kyes et al., 1989; Lefrancois and Goodman, 1989; Stiingl et al., 1987a, 1987b; Vantourout and Hayday, 2013). Conceivably, these alignments are determined by organ-specific products of epithelial differentiation, particularly since TCR$\gamma\delta$* IELs are largely unaffected by major histocompatibility complex (MHC) antigens that shape $\alpha\beta$ T cell repertoires. Likewise, whereas some $\gamma\delta$ TCRs bind CD1 and MR1, genetic studies have largely excluded these MHC-related molecules from the selection of murine $\gamma\delta$ cell repertoires (Bigby et al., 1993; Correa et al., 1992; Hayday and Vantourout, 2013; Kuziel et al., 1987; Lefrancois et al., 1990; Pereira et al., 1997).

One insight into how epithelia might shape local IEL repertoires was provided for the skin by the discovery of Skint1, the founding member of a novel multi-gene family specifically expressed by thymic epithelial cells and keratinocytes. Skint1 drives the selective maturation of V$\gamma$5* DETC progenitors, and DETCs are >90% ablated in Skint1 mutant mice, while all other T cells are unaffected (Barbee et al., 2011; Boyden et al., 2008; Turchinovich and Hayday, 2011). However, the generality of this mechanism for IEL selection was questioned, since neither DETCs nor Skint1 is broadly conserved and because Skint genes are only expressed in skin and thymus (Boyden et al., 2008).
Figure 1. Selective Maturation and Expansion of Intestinal IELs in Mice

(A) Gating strategy for small intestinal (SI) V\textsuperscript{γ7+} IELs in 12-week-old C57Bl/6 mice (n ≥ 12). Bottom right: V\textsuperscript{γ7+} IEL representation over time (n = 5, week 20–36; n ≥ 12, other time points).

(B) IEL composition assessed by confocal microscopy of proximal SI whole mounts (n = 3) and corresponding quantification (right).

(C) Top: surface phenotypes of V\textsuperscript{γ7+}, V\textsuperscript{γ7−/CD8−} (CD3+TCR\textsuperscript{β+}V\textsuperscript{γ7−/CD8−}) and ab IELs from 21– to 40-day-old mice (n ≥ 8). Bottom: gene expression in V\textsuperscript{γ7+} versus V\textsuperscript{γ7−/CD8−} IELs (n = 3).

(D) Surface phenotypes of V\textsuperscript{γ7+} IELs at days 14–17 versus days 21–40 (n ≥ 7).

(E) Top: surface phenotype of V\textsuperscript{γ7+} IELs at day 14 and day 28 (CD122 median fluorescence intensity [MFI]-colored text). Bottom: surface phenotype of CD122\textsuperscript{HI}Thy1+ versus CD122\textsuperscript{LO}Thy1+ IELs at days 14–17 (n ≥ 7).

(F) Heatmap of genes differentially expressed between V\textsuperscript{γ7+}CD122\textsuperscript{HI} and V\textsuperscript{γ7+}CD122\textsuperscript{LO} IELs from day 14–17 mice and between Skint1-selected and non-selected V\textsuperscript{γ7+} DETC progenitors (n = 4).

(legend continued on next page)
This notwithstanding, Skint genes sit within the Btnl family comprising six rodent and five human genes. Their poorly understood gene products are structurally similar to CD80 and PDL1 co-stimulatory and inhibitory molecules, which are themselves considered to be evolutionarily related to the MHC (Abelert-Dörner et al., 2012; Afrache et al., 2012; Barbee et al., 2011; Rhodes et al., 2001; Salim et al., 2016; Stammers et al., 2000). By definition, Btnl/BTNL genes are structurally similar to butyrophilin (Btm BTN) genes, of which mice have two and humans six. Butyrophilin genes derive their name (“butter-loving”) from the founding member, Btn1a1/BTN1A1, that encodes a milk fat micelle-associated protein (Franke et al., 1981). However, this function appears atypical in contrast to the recent implication, albeit largely imprecise, of several BTN/Btn/BTNL/Btnl gene products in immune regulation (Rhodes et al., 2016). Provocatively, human BTN3A1 facilitates peripheral blood γδ T cell responses to low-molecular-mass microbial and endogenous metabolites (so-called phosphoantigens), although it is not known whether this is mediated by direct TCR-BTN3A1 binding (Adams et al., 2015; Harly et al., 2012; Palakodeti et al., 2012; Vavassori et al., 2015; Harly et al., 2012; Palakodeti et al., 2012; Vavassori et al., 2013; Wang et al., 2013).

To explore whether Btnl genes might mediate epithelial regulation of local γδ T cells, we considered the mouse gut, the major site of Btn1, Btnl4, and Btnl6 expression (Bas et al., 2011). Here, we identify a time window early in the development of young mice in which Btn1 expressed by post-mitotic, small intestinal villus epithelial cells critically and selectively promotes the maturation and expansion of Vγ7+ T cells, thereby shaping the IEL compartment. Requiring neither microbial nor food antigens, this process evokes Skint1-mediated DETC selection and γδ T cell selection by the MHC. Indeed, we show that intestinal epithelial cells expressing Btnl1 jointly with Btnl6 can induce TCR-dependent stimulation uniquely of intestinal Vγ7+ T cells. γδ cells have often been viewed as species specific with few features conserved between mouse and humans (Kazen and Adams, 2011; Vantourout and Hayday, 2013). However, it is increasingly clear that human tissues too harbor large γδ cell compartments with TCRs distinct from those in peripheral blood (Landau et al., 1995; Vantourout and Hayday, 2013; our unpublished data). Furthermore, a large bioinformatics study of thousands of cancer patients presenting with a broad range of carcinomas established that the best correlate of overall survival was a tumor-associated γδ T cell gene signature (Gentles et al., 2015). Hence, there is a pressing need to define how human epithelia interact with tissue-resident γδ T cells.

Addressing this, we provide a refined description of human colonic γδ cells and show that a signature subset expressing TCRVγ4 is specifically regulated by human BTN1L3 and BTN1L8 expressed by human gut epithelium. Hence, the specialized differentiation of intestinal epithelial cells in mice and in humans includes the expression at steady state of site-specific regulators of local T cell compartments.

RESULTS

Intestinal Epithelial T Cell Selection

By flow cytometry of cells recovered from epithelium, and by confocal visualization of epithelial whole mounts, we found that the signature murine small intestinal Vγ7+ IEL compartment largely took shape at 2–3 weeks of age and remained stable for at least 9 months thereafter (Figures 1A and 1B). At day 21, Vγ7+ cells mostly phenocopied mature Skint1-selected DETCs, expressing uniformly high levels of CD122 (the IL-2R/IL-15Rβ chain), TIGIT (an inhibitory co-receptor), and the TCR (detected with anti-CD3 antibodies) and low levels of RNA for Rorcγ and Sox13, two transcription factors contributing to γδ T cell differentiation (Vantourout and Hayday, 2013) (Figure 1C). Vγ7- IELs (mostly Vγ1 or Vγ4) did not show this phenotype, and whereas both Vγ7+ and Vγ7- IEL subsets were mostly CD45RB+CD44++, and CCR9+, Vγ7+ IELs were distinct in being Lag3+, Thy1−, CD69−, CD5+, and CD8αα+ (Figures 1C and S1A).

Prior to day 21, however, Vγ7+ IELs phenocopied Vγ7- IELs of adult mice. Thus, by sequential gating and radar plots of surface protein co-expression, one could clearly distinguish mature Vγ7+ IELs (CD122hiMFI > 500, Thy1+, TIGIT-, Lag3-, CD8αα-, CD5-, CD24-, TCRαβ) from putative Vγ7- IEL progenitors (CD122loMFI < 200, Thy1+, TIGIT-, Lag3-, CD8αα-, CD5-, CD24+, TCRαβ) (Figures 1D, 1E, and S1B), with the latter also phenocopying DETC progenitors prior to Skint1 selection (Turchino and Hayday, 2011).

To further compare IELs with their putative progenitors, CD122hi Vγ7+ and CD122lo Vγ7+ IELs were purified from the same day 14–17 mice on four independent occasions and assessed by RNA sequencing (RNA-seq) (Figure S1C). Consistent with their distinct phenotypes, the cells showed significantly different expression of many genes for cell surface proteins (Figure S1C). Furthermore, many genes up- (e.g., Tnfrsf9 [4-1BB/CD137], Xcl1 [lymphotactin], Naspi) or downregulated (e.g., sox13, Bcl11b, Cx3cr1) in CD122hi versus CD122lo Vγ7+ cells were likewise regulated by Skint1 selection of DETC progenitors (Figures 1F and S1C).

Additionally, CD122hi Vγ7+ cells were enriched in cell-cycle genes, consistent with which ~100% of Vγ7+ IELs at day 21–24 were Ki67+ (i.e., outside of G0), compared to <40% of Vγ7- cells (p < 0.0001) (Figure 1G). Likewise, Vγ7+ IELs at day 28 phenocopied rapidly dividing thymocytes in that ~10% incorporated ethynyldeoxyuridine (EdU) (a labeled nucleotide) during a 3-hr pulse, compared to only 4% of Vγ7- IELs (Figure S1D).

In sum, these data are consistent with the gut supporting the selective maturation and expansion of CD122hi Thy1−, TIGIT-, Lag3−, CD8αα−, CD5−, CD24+, TCRαβ Vγ7+ cells that by weeks 3–4 dominate the γδ IEL compartment. After week 5, the fraction of cycling (Ki67+) Vγ7+ IELs at steady state declined to levels comparable to Vγ7- IELs (Figure 1G).

(G) Ki67 expression in Vγ7+ versus Vγ7- IELs directly ex vivo (n = 4, day 19; n = 8–27, other time points).

Data are representative of one (C, qPCR, B and F) or three or more (C, cytometry, D and E, top) independent experiments. Some panels present data pooled from three or more (E), more than ten (G), and >20 (A) independent experiments. D, day; W, week. All error bars represent mean ± SD. See also Figure S1.
A Gut Epithelial Selecting Element

Because Skin1 selects for signature Vγ7+ DETC progenitors in the thymus, DETCs are absent from athymic NU/NU mice. By contrast, intestinal IELs were present in NU/NU, and although there was some decrease in numbers (average of ~1.3 x 10^5 cells compared to ~2.0 x 10^5 cells in euthymic mice; see below), the compartment was again dominated by CD122^+ Vγ7^+ IELs. Moreover, ~25% of Vγ7^+ IELs in NU/NU and in euthymic mice reacted with antibody GL2 that detects Vδ4 (TRDV2-2 encoded) chains. Consistent with this, TRDV2-2 sequences accounted for ~25% of TCRβ chain RNAs expressed by purified Vγ7^+ IELs (Figures 2A and S2A). In sum, the shaping of the gut Vγ7^+ IEL compartment did not require a thymus.

Consistent with this, Vγ7^+ thymocytes were rare, comprising <10% of TCRγδ^+ cells in fetal and post-natal thymus across the first 8 weeks of life, the peak period of thymus function in mice (Figure S2B). Furthermore, most Vγ7^+ thymocytes were CD45RB^b, Thy1^+^, CD65^+, CD122^b, TCR^b, and CD8^xoa^, thus offering no evidence for intrathymic maturation (Figures S2C–S2E). Likewise, neither lymph nodes nor Peyer’s patches (PP) were required to shape the IEL compartment, since normal numbers of Vγ7^+ and Vγ7GL2^+ IELs with signature phenotypes were present in alabal (alymphoplasia) mice (Shinkura et al., 1999) that following surgery were confirmed to lack PP and peripheral mesenteric lymph nodes (MLN) (Figure S2F).

As intestinal driver(s) of IEL maturation in weanling mice, microbial and/or food antigens were logical candidates. However, C57Bl/6 mice bred into and maintained in a germ-free environment and/or on elemental, protein-antigen-free diet displayed Vγ7^+ and Vγ7GL2^+ IEL compartments comparable to conventionally housed counterparts (Figure 2B). The Vγ7^+ IELs were uniformly TCR^b, CD122^b, and absolute numbers were somewhat increased, partially compensating for the decline of TCRαβ IELs in germ-free and protein-antigen-free mice (Figure 2B).

Thus, the local T cell compartment is most likely shaped by an endogenous intestinal element(s).

In seeking that element(s), we focused on three genes, Btnl1, Btnl4, and Btnl6, that are closely related to Skint1 (Abeler-Dörner et al., 2012; Afrache et al., 2012; Bas et al., 2011). Btnl4 was expressed at low levels in proximal small intestine, commencing in the fetus. Btnl1 and Btnl6 RNAs were detected at day 6 post-partum, and Btnl1 levels further increased at around day 14 before the expression of all three Btnl genes stabilized (Figure 2C).

Expression was in post-mitotic villus enterocytes that are interspersed with IELs and was essentially absent from villus crypts that house replicating epithelial cell progenitors and lack IELs (Figures 2D, 2E, and S2G). Btnl1 expression peaked in proximal and medial small intestine (Figure S2H), where its expression was >10^4-fold higher than in the thymus (Figure S2I). These expression patterns could permit Btnl1, Btnl4, and/or Btnl6 to act locally upon Vγ7^+ IELs in weanling mice. To investigate this possibility, we obtained three independent strains of Btnl1^−/− mice and one of Btnl4^−/− mice, each generated by targeted mutagenesis of embryonic stem cells (ESCs), and a strain with an internally deleted Btnl1 locus generated by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 in mouse eggs (Figure S2J).

The strains were confirmed as gene knockouts by DNA analysis and loss of respective Btnl RNAs (Figures 2E and S2I–S2K).

Btnl1 Shapes the Intestinal IEL Compartment

The four Btnl1^−/− strains each displayed major, highly selective losses of Vγ7^+ IELs, assessed by flow cytometry or confocal microscopy (Figures 3A, 3B, and S3A). Vγ7^+ IEL numbers were depleted by ~90%, with Vγ7GL2^+ cells almost ablated. Because Vγ7^+ IEL numbers barely increased, the percentage representation of Vγ7^+ cells among γδ IELs was reduced only by ~3-fold relative to wild-type (WT) mice, but this was set against a background of dramatically reduced γδ IEL numbers (Figures 3A and S3A). By contrast, TCRβ^CD8^xa^ IEL numbers increased significantly in Btnl1^−/− mice (Figures 3A and 3B).

The specificity of Btnl1 for Vγ7^+ IELs was emphasized by comprehensive immune phenotyping of Btnl1^−/−, WT, and Btnl1^+/− mice that showed comparable splenic or MLN immune cell subsets (including γδ cell repertoires) and comparable representation and phenotypes of Vγ7^+ thymocytes from day 4 to week 8 (Figures S3B–S3E). Consistent with its expression pattern, Btnl1 acted extrathymically; thus, Btnl1 deficiency crossed onto NU/NU mice reduced the average number of Vγ7^+ IELs by ~90%, with almost total loss of Vγ7GL2^+ IELs (Figure 3C). Since NU/NU mice lack most αβ T cells, this result excludes the formal albeit unlikely possibility that Vγ7^+ IEL losses in euthymic Btnl1^−/− mice indirectly reflected expanded TCR αβ IELs. The specificity of Vγ7^+ IELs for Btnl1 was emphasized by the fact that Btnl4^−/− mice displayed no overt defects in any major IEL subset (Figure 3D).

Btnl1 Selects Vγ7^+ IEL In Trans

To determine how and when Btnl1 impacts IELs, we examined residual Vγ7^+ and Vγ7GL2^+ IELs in Btnl1^−/− mice. Relative to those in WT mice, significantly fewer Vγ7^+ IELs incorporated EdU at D28 [p < 0.0001] or expressed Ki67, and this did not change until week 7 when most Vγ7^+ IELs in WT mice moved out of cycling (Figures 4A and S4A). Thus, there was no selective expansion of Vγ7^+ IELs in Btnl1^−/− mice. Similarly, although their TCR levels were high, many residual Vγ7^+ and Vγ7GL2^+ IELs in week 3–5 Btnl1^−/− mice were CD122^b, Thy1^+, TIGIT^+, Lag3^+, CD8^xa^, CD5^+, CD24^+, thereby phenocopying immature Vγ7^+ IELs of day 14–17 WT mice (Figures 4B, 4C, and S4B).

To demonstrate that Btnl1 exerts its selective impact on Vγ7^+ T cells in trans, we established conditions in which the Vγ7^+ and Vγ7GL2^+ IEL compartments of week 3–5 WT mice were reconstituted within ~5 weeks of donor bone marrow (BM) transfer to irradiated 8- to 10-week, γδ T cell-deficient TCRδ^−/− mice (Figure S4C). BM from either WT or Btnl1^−/− mice proved equally effective at IEL reconstitution (Figure 4D). Not surprisingly, WT BM reconstitution of Vγ7^+ IELs in irradiated, congenic T cell-sufficient CD45.2^+ WT recipients was less effective than it was in TCRδ^−/− hosts (compare plots, top left in Figures 4D and 4E), but nonetheless, reconstitution of Btnl1^−/− hosts was much less effective, and the few Vγ7^+ and Vγ7GL2^+ IELs that developed in Btnl1^−/− recipients phenocopied residual Vγ7^+ cells in Btnl1^−/− mice and immature IELs in day 14–17 WT mice (Figure 4E). Complementing these findings, purified IELs from 4-week-old mice could reconstitute Vγ7^+ IELs in recipient TCRδ^−/− mice, albeit very inefficiently, and this too was greatly
impaired in Btn1<sup>−/−</sup>-TCR<sup>Δ/−</sup> hosts (Figure S4D). Thus, Btn1 acts in non-hematopoietic cells to support the selective expansion and maturation of V<sub>γ</sub>7<sup>+</sup> IEL.

To attempt to restore IEL selection, we rendered Btn1<sup>−/−</sup> mice transgenic for Btn1 expressed from a doxycycline (Dox)-inducible promoter (Figures S5A and S5B) and crossed them onto
Here, we restricted Bttnl1 induction to mature enterocytes by generating Bttnl1−/− mice transgenic for rtTA expressed from the villin promoter. Within 1–2 weeks of Dox-treatment of several W11 BiTg Bttnl1−/− mice, most Vγ7+ IELs had become Lag3+ (Figure 5A and 5B). Again, this phenotypic transition was Vγ7+ IEL specific, albeit there were sometimes Bttnl1-independent increases in K67+ TCRγδ+ IELs in sugar-water-treated mice (Figure 5B). Moreover, the numbers and representation of Vγ7+ IELs were unchanged, even in mice retained on Dox for 3–4 weeks (Figure 5C). This establishes that Bttnl1 can effect phenotypic conversion of immature Vγ7+ IELs rather than merely promote a selective outgrowth of mature Vγ7+ cells.

However, there was significantly increased representation of Vγ7+ and Vγ7GL2+ IEL when Bttnl1 expression was induced in Bttnl1−/− mice in early-life by commencing Dox-treatment of nursing females at D7 or of weanlings at D21 and then maintaining treatment for 2–5 weeks (Figure 5D). Moreover, the expanded Vγ7+ cells phenocopied IEL of W4-5 WT mice, and were significantly different from the IEL of Dox-treated StiTg mice and Bttnl1−/− mice (Figure 5E). Thus, acute expression of Bttnl1 purely in the gut epithelium induces the selective phenotypic maturation and expansion of Vγ7+ IEL, but these effects are separable, with selective expansion mostly confined to a developmental window within the first five weeks of life.
secreted cytokines (Figure 6E; compare blue with yellow bars in bottom panel). CD25 upregulation by IELs in contact with L1+6 cells showed dose-dependent inhibition by PP2, which inhibits signaling by src-family kinases, such as Lck and Fyn, but was not inhibited by PP3, an established control for PP2 specificity (Figure S6E).

Just as residual Vγ7+ IEL in Btnl1−/− mice responded to acute transgenic Btnl1 induction in vivo, they were comparable to WT Vγ7+ IELs in responding to Btnl1 plus Btnl6 ex vivo (Figure 6F).

Interestingly in the same experiments, WT Vγ7+ IELs showed relatively poor responses to anti-CD3, phenocopying the attenuated responsiveness imposed on Vγ5+ DETC progenitors by Skint1 (Wencker et al., 2014), whereas Vγ7+ IELs from Btnl1−/− mice, and TCRαβ+ and Vγ7− IELs from WT mice, none of which subsets had experienced prior Btnl1 selection in vivo, all showed strong responses to anti-CD3 (Figures 6F and 6G).

Finally, the supernatants of IEL co-cultures with L1+6 cells showed small but significant increases in interferon-γ (IFN-γ),
CCL4, and granulocyte macrophage colony-stimulating factor (GM-CSF) among 36 cytokines tested (Figure 6H). These are typical IEL effectors, and although it was technically challenging to attribute production to V<sup>g7+</sup> IELs, such increases were not seen in supernatants of L1+6 cells cultured with IELs from TCR<sup>d+/C0/C0</sup> mice. (Note the higher background cytokine expression in TCR<sup>d+/C0/C0</sup> mice may reflect spontaneous inflammation often associated with γδ deficiency; Hayday and Tigelaar, 2003.) In

Figure 5. Villin-Specific Btnf1 Induction Rescues V<sub>y7</sub>* IEL In Vivo
(A–D) Week 7–13 (adult) or day 7–21 (pups) mice of indicated genotypes on a Btnf1<sup>−/−</sup> background were administered Dox (1 mg/ml, 2% sucrose) or control water (2% sucrose) for times indicated, and IELs were analyzed by flow cytometry. n ≥ 5 (A and B); n ≥ 6 (C and D). (E) Comparative cell-surface phenotypes of V<sub>y7</sub>* IELs from week 4–5 WT mice and animals indicated (n = 4–8). (A) is representative of two or more independent experiments; (B)–(E) present data pooled from three or more experiments. Statistical significance in (C) was determined using the Holm-Sidak method. All error bars represent mean ± SD. See also Figure S5.
sum, a range of metrics attested to a highly specific and direct interaction ex vivo of Vγ7+ IELs with Btn1 and Btn6 co-expressed on gut epithelial cells.

**Signature Human Intestinal γδ T Cells**

Because of limited tissue access, human gut T cells are understudied. Nonetheless, there are gut-associated γδ T cells whose TCR usage differs markedly from Vγ2/Vδ2 cells that dominate the peripheral blood (Landau et al., 1995). To better characterize such cells, we submitted biopsy specimens from healthy ascending colon to a modified version of a protocol used to isolate human skin T cells (Clark et al., 2006). For 16 of 17 donors, the γδ T cells were enriched in Vδ1+ cells, although Vδ1/Vδ2- cells were also present; hence, the term “Vδ2-” is used to distinguish tissue-associated γδ T cells from Vδ2+ cells that could also be recovered from most gut samples, albeit in highly variable numbers (Figure 7A).

Of six functional human Vγ1 chain genes (Vγ12, 3, 4, 5, 8, and 9) (Arden et al., 1995), Vγ4 was reported to be the signature chain of intestinal Vδ2- cells (Landau et al., 1995). Indeed, for up to ten donors examined, most intestinal Vδ2- cells reacted with a Vγ2/3/4-specific antibody, but not a Vγ5/3/6-specific antibody (blue and red bars, Figure 7B; Table S1A), and TCR deep sequencing showed that Vγ4 sequences far outnumbered Vγ2 sequences (Figure S7A). Thus, despite individual variation, most gut γδ T cell compartments included a substantial Vγ4/Vδ2- subset, while some donors also displayed relatively high representation of Vγ8/Vδ1+ cells (Figure 7B; Table S1A).

**BTNL3 and BTNL8 Regulate Human Vγ4+ Cells**

There is no human equivalent of the Btnl2-proximal amplicon on mouse chromosome 17 that encodes Btn1, Btn4, and Btn6 (Abeler-Dörner et al., 2012; Afrache et al., 2012). However, adjacent to human BTNL9 is an amplicon that encodes BTNL3 and BTNL8 whose expression is highly enriched in gut, particularly EpCAM+ epithelial cells (Figures S7B–S7D). Interestingly, akin to the behavior of Btn1 and Btn6 described above, neither BTNL3 nor BTNL8 protein was efficiently expressed on cells transfected with their respective genes, unless both were co-expressed (Figure S7E, green line, top right histogram; blue line, bottom right histogram). Conversely, BTNL8S (a splice variant of BTNL8) failed to rescue surface BTNL3 expression (Figure S7E, red line, top right histogram).

Whereas we could not test for a developmental dependence of human gut γδ T cells on BTNL3 and BTNL8, we could assess whether BTNL3 and BTNL8 phenocopied Btn1 and Btn6 by specifically activating signature gut γδ T cells in a TCR-dependent fashion. Thus, we established short-term co-cultures of primary-gut-derived lymphocytes with HEK293T cells transduced with BTNL3 (L3), BTNL8 (L8), BTNL3 and BTNL8 (L3+8), or EV (Figure S7F). For the representative donor shown, some of the discrete subsets of Vδ1+ and Vδ1+ γδ T cells that were apparent in T cell co-cultures with control (EV, L3, and L8) cells showed marked TCR downregulation when co-cultured with L3+8 cells (red arrows Figure 7C). Emphasizing specificity, TCR downregulation occurred in response to L3+8 cells in 21 of 23 donors but was never seen in co-cultures with L3 or L8 cells, and was never shown by intestinal Vδ2- or TCRγδ+ cells, even in the same cultures as responding Vδ2- cells (Figure 7D). Although higher baseline CD25 expression reduced the sensitivity of this assay for human versus mouse gut T cell activation ex vivo, L3+8 cells induced significant CD25 upregulation vis-a-vis gut T cells co-cultured with control cells (Figure 7E), and CD25 upregulation was most evident on cells with downregulated TCRs (Figure 7G).

Not all Vδ2- cells responded to L3+8 (Figure 7C). Thus, we considered that TCRγ chains might determine BTNL responsiveness, as is true in mice. Indeed, human Vδ2- populations that downregulated TCRs in co-cultures with L3+8 cells were detected by the Vγ2/3/4-specific antibody, but not by antibodies to Vγ8, Vγ5/3, or Vγ9 (Figures 7F and 7G). Moreover, productively rearranged Vγ4 genes were prevalent when L3+8-responsive cells with downregulated TCRs were flow cytometry sorted from one donor (Figure S7H) and their Vγ chains amplified without bias and sequenced (red notation, Table S1B). By contrast, TCRγ transcripts from skin-derived TCRγδ+ cells (G234SK01) were biased toward Vγ3 (purple notation Table S1B). Interestingly, of two donors showing no substantial response to BTNL3+8, one proved a posteriori to have an atypical intestinal γδ T cell repertoire dominated by Vγ8+ cells (Figure S7I). Likewise, L3+8 cells induced no significant TCR down-modulation by primary γδ T cells from skin or blood among which Vγ4+ cells are rare (Figure 7H). Thus, epithelial BTNL genes regulate human-tissue-resident γδ T cells in an organ-specific, TCRγ-chain-specific manner.

**DISCUSSION**

This study shows that the unique composition of the murine intestinal intraepithelial T cell compartment arises from a selective maturation and expansion of Vγ7+ T cells driven by Btn1, and most likely Btn6, expressed by differentiated enterocytes.
Likewise, BTNL3 and BTNL8 co-expressed by human intestinal epithelial cells selectively regulate gut Vγ4+ T cells. Given that Skint1 expressed by thymic epithelial cells and keratinocytes selectively regulates intra-epidermal Vγ5+ T cells, tissue-specific Btnl genes may offer a generalizable means by which epithelia shape and regulate local γδ T cell compartments. This may reflect an even broader utilization of BTNL/BTN genes in γδ biology, since BTN3A1 is critical to human peripheral blood γδ cell activation (Harly et al., 2012; Palakodeti et al., 2012; Vavasori et al., 2013; Wang et al., 2013).

Figure 7. Regulation of Human Gut Vγ4+ Cells by BTNL3 and BTNL8

(A and B) Vδ (A, n = 17) and Vγ (B, n = 6–10) expression by human gut γδ cells.

(C) Surface TCRγδ/Vδ1 expression on human gut lymphocytes after 12-hr co-culture with BTNL3 (L3) or BTNL3 plus BTNL8 (L3+8)-transduced HEK293T cells. Red arrows denote shifts in TCR staining.

(D) Top: TCRγδ/CD3 expression on designated human gut T cells after 12-hr co-culture with denoted HEK293T transductants. Bottom: mean fluorescence intensities (MFIs) calculated relative to co-culture with HEK293T.EV (n > 22). For two donors, MFIs for Vδ2+ cells remained unchanged (dots within the ellipse).

(E) Percentage of CD25hi cells among TCRγδ+ T cells after co-culture with denoted cells (n = 5). Statistical analysis was performed by paired t test.

(F) Surface Vγ2/3/4 and Vδ1 expression on Vδ2+ γδ T cells after co-culture with denoted cells.

(G) TCRγδ expression on indicated subsets after co-culture with denoted cells.

(H) TCRγδ expression on γδ cells from peripheral blood mononuclear cells (PBMCs) or skin after co-culture with denoted cells.

All error bars represent mean ± SD. See also Figure S7 and Table S1.
 Btnl1 and Skint1 effect many of the same changes in Vγ7+ and Vγ5+ IEL progenitors, respectively. These include upregulation of the receptor for IL15, a growth factor expressed by epithelial cells essential for γδ IEL maintenance (De Creus et al., 2002; Lai et al., 2008; Lodolce et al., 1998); suppression of sox13, rogc, and bcl11b that are associated with γδ cells producing IL-17, which is not a property of IELs (Jensen et al., 2008; Turchinovich and Hayday, 2011); and attenuation of TCR responsiveness, consistent with IELs adopting an innate-like, rapidly responsive, tissue surveillance role (Wencker et al., 2014).

Being members of the B7 superfamily, Btnl/BTNLSkint gene products may act as co-stimulators for IEL receptors yet to be identified. In this case, they will be the first co-stimulators specific for cells with particular TCRs; e.g., Vγ7+, but not Vγ7−, IELs from mouse gut; and Vγ4+, but not Vγ8+, T cells from human gut. Alternatively, their exquisite specificities may reflect interactions of Btnl/ BTNLSkint with cognate TCRs, possibly via unique Vγ-CDR1/2 regions. Likewise, human BTN3A1 appears to mediate its effects via the Vγ9Vδ2 TCR, although there are no clear direct binding data. This may reflect a highly complex interaction that includes critical co-factors. Of note, BTN3A1, BTN3A2, Btnl1, and Btnl6 each contain B30.2 domains related to the phosphoantigen-binding domain of BTN3A1, raising the possibility that low-molecular-weight metabolite(s) might have a broad role in γδ cell regulation by Btnl/BTNLSkint genes (Adams et al., 2015). A requirement for co-factors might also explain the developmental time window during which Btnl1 could drive the maturation and expansion of Vγ7+ IELs.

Peptide-MHC complexes have different impacts on γδ T-lineage cells, including positive and negative selection of thymocytes, T-regulatory cell differentiation, and activation or anergy of mature peripheral T cells (Burkly et al., 1989; Fink and Bevan, 1978; Jenkins and Schwartz, 1987; Jordan et al., 2000). These outcomes are dictated by the state of the T cell and/or the biological context. In so far as parallels may be drawn, our study offers genetic evidence that Btnl1 drives Vγ7+ IEL selection and cell biological evidence that Btnl1 and Btnl6 can promote weak activation of Vγ7+ IELs, which was likewise true for human BTN3L3 plus BTN5L8 interactions with mature gut γδ cells. These different contexts might explain a seeming paradox that CD122 does not induce γδ T cell development, dysregulation, and/or repair might epithelial Btnl/BTNLSkint gene products and/or co-factors communicate a need for IEL activation? Such insight can inform genome-wide association study (GWAS) data implicating BTNLSkint polymorphisms in numerous immunopathologies (Prescott et al., 2015; Rhodes et al., 2016; Valenforye et al., 2005) and may reveal why epithelia use organ-specific, as opposed to pan-epithelial, Btnl gene products to regulate local T cells. In fact, different γδ TCRs offer IELs a means by which to discriminate organ-specific epithelia that is not obviously available to innate lymphoid cells.

Our and others’ studies have by now implicated Btnl1, Btnl6, Skint1, BTN3A1, BTN3A2 in γδ cell regulation. Nonetheless, they may be pleiotropic. For example, the long cytoplasmic tails of BTN3L3, BTN5L8, Btnl1, and Btnl6 may signal back to the cells that display them, consistent with our finding that Btnl1 attenuates epithelial cell sensitivity to IEL-derived cytokines (Bas et al., 2011). Yet, other BTNLSkint protein functions may not relate to γδ cell biology (Rhodes et al., 2016), consistent with which we show that Btnl4 is not critical for gut γδ T cell development. A similar diversity of immunological functions may describe avian B7-like B-G genes, a subset of which may shape and/or regulate large γδ cell compartments in birds (Kaufman et al., 1999).

A clear exception to the many parallels of Btnl1-mediated IEL selection and Skint1-mediated DETC selection is Skint1 expression by thymic epithelial cells (Boyden et al., 2008). Conversely, the restriction of Btnl1 to the intestine, both naturally and in villin-driven BiTg mice, clearly establishes that the signature gut IEL compartment is shaped extrathymically, even if progenitors are thymus derived, thus resolving a long-standing controversy (Le-françois, 1991; Poussier and Julius, 1994). This thymic independence may reflect a need to replenish gut IELs so as to maintain gut integrity post-thymic involution.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| CD3 APC Cy7 (17A2)  | BioLegend | Cat#:100222 |
| CD3 PerCPCy5.5 (145-2C11) | BioLegend | Cat#:100328 |
| TCRβ Brilliant Violet 421 (H57-597) | BioLegend | Cat#:109229 |
| TCRβ APC (H57-597) | BioLegend | Cat#:109212 |
| CD122 PE (TM)β1 | BioLegend | Cat#:123209 |
| CD122 Brilliant Violet 421 (TM)β1 | BioLegend | Cat#:123213 |
| CD122 APC (TM)β1 | BioLegend | Cat#:123213 |
| CD5 PE (53-7.3) | BD PharMingen | Cat#:553023 |
| CD24 FITC (M1/69) | eBiosciences | Cat#:11-0242-81 |
| CD24 PEcy7 (M1/69) | BD PharMingen | Cat#:560536 |
| CD8α PEcy7 (53-6.7) | BioLegend | Cat#:100722 |
| TCR Vδ4 FITC (GL-2) | BD | Cat#:552143 |
| TCR Vδ4 PE (GL-2) | BioLegend | Cat#:103030 |
| TCR Vγ7 (F2.67) | Institut Pasteur, Paris, France, Pablo Pereira | N/A |
| TCRVγ1 APC (2.11) | BioLegend | Cat#:141107 |
| TCRVγ4 APC (UC3-10A6) | BioLegend | Cat#:137708 |
| TCRβ BV421 (GL3) | BioLegend | Cat#:118119 |
| K67 FITC (B56/MOPC-21) | BD PharMingen | Cat#:556026 |
| CD45 Qdot 605 (30-F11) | eBiosciences | Cat#:93-0451-42 |
| CD8 Brilliant Violet 510 (53-7.3) | BD | Cat#:583069 |
| TCRβ PeCy7 (GL3) | BioLegend | Cat#:118124 |
| CD161/NK1.1 Brilliant Violet 650 (PK136) | BioLegend | Cat#:108735 |
| CD4 Brilliant Violet 786 (GK1.5) | BD | Cat#:563331 |
| CD8α AlexaFluor 700 (53-6.7) | BD | Cat#:557959 |
| CD25 APC (PC61) | BD | Cat#:557192 |
| GITR PE (DTA-1) | BD | Cat#:558119 |
| CD44 FITC (IM7) | BD | Cat#:553133 |
| CD45 RV Cy5.5 (MEL-14) | BD | Cat#:560513 |
| KLRG1 BV421 (2F1) | BD | Cat#:562897 |
| CD11c BV786 (HL3) | BD | Cat#:563735 |
| CD11b BV510 (M1/70) | BioLegend | Cat#:101245 |
| F4/80 PerCP-Cy5.5 (BM8) | BioLegend | Cat#:123128 |
| Ly6G APC (1A8) | BD | Cat#:560599 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Ly6C AlexaFluor 700 (AL-21) | BD | Cat#:561237 |
| CD103 PE (M290) | BD | Cat#:557495 |
| CD317 Brilliant Violet 650 (927) | BioLegend | Cat#:127019 |
| MHCII/IA/IE FITC (2G9) | BD | Cat#:553623 |
| CD86 Pe-Cy7 (GL1) | BD | Cat#:560582 |
| CD3 Brilliant Violet 421 (145-2C11) | BD | Cat#:562600 |
| CD19 Brilliant Violet 421 (1D3) | BD | Cat#:562701 |
| CD161/NK1.1 (lin) Brilliant Violet 421 (PK136) | BioLegend | Cat#:108735 |
| IgG1 PE (A85-1) | BD | Cat#:550083 |
| B220 (CD45R) AlexaFluor 700 (RA3-6B2) | BD | Cat#:557957 |
| IgM Brilliant Violet 786 (R6-60.2) | BD | Cat#:564028 |
| IgD PerCPCy5.5 (11-26c.2a) | BioLegend | Cat#:405710 |
| GL-7 AlexaFluor 647 (GL7) | BD | Cat#:561529 |
| CD95 PECy7 (Jo2) | BD | Cat#:557653 |
| CD138 Brilliant Violet 650 (281-2) | BioLegend | Cat#:142517 |
| CD21/35 FITC (7G6) | BD | Cat#:553818 |
| CD23 Brilliant Violet 421 (B-ly6) | BD | Cat#:563929 |
| DYKDDDDK-PE (Flag) | BioLegend | Cat#:627310 |
| DYKDDDDK-APC (Flag) | BioLegend | Cat#:627308 |
| HA-DyLight 650 | Thermo Fisher | Cat#:26186-D650 |
| 6x-Histidine-PE | Abcam | Cat#:Ab72467 |
| CD25 Brilliant Violet 421 (BC96) | Biolegend | Cat#:302630 |
| CD25 PE (BC96) | Biolegend | Cat#:302606 |
| CD3 Brilliant Violet 510 (OKT3) | Biolegend | Cat#:317332 |
| CD3 BUUV (UCHT 1) | BD Biosciences | Cat#:563546 |
| EpCAM eFlour® 660 (1B7) | eBioscience | Cat#:50-9326 |
| Streptavidin APC-Cy7 | Biolegend | Cat#:405208 |
| Streptavidin Brilliant Violet 421 | Biolegend | Cat#:405225 |
| TCRγδ PeCy7 (IMMU510) | Beckman Coulter | Cat#:41116015 |
| Vγ9 PC5 (IMMU360) | Beckman Coulter | Cat#:41116015 |
| Vγ9 PE (B3) | Biolegend | Cat#:331308 |
| Vδ1 APC (REA173) | Miltenyi | Cat#:130-100-519 |
| Vδ1 FITC (TS8.2) | Thermo Scientific | Cat#TCR2730 |
| Vδ2 PerCP (B6) | Biolegend | Cat#:331410 |
| Vγ2/3/4 biotin (23D12) | D Kabelitz and D Wesch, University of Kiel | N/A |
| Vγ3/5 biotin (56.3) | D Kabelitz and D Wesch, University of Kiel | N/A |
| Vγ8 biotin (R4.5.1) | D Kabelitz and D Wesch, University of Kiel | N/A |
| Hamster IgG Isotype Control | Biolegend | Cat#:400933 |
| LEAF-Purified anti-mouse CD3ε | Biolegend | Cat#:100331 |

**Chemicals, Peptides, and Recombinant Proteins**

| REAGENT | SOURCE | IDENTIFIER |
|----------|--------|------------|
| BrdU | Sigma-Aldrich | Cat#:B5002 |
| IL-2 | Immunotools | Cat#:12340024 |
| IL-15 | Immunotools | Cat#:12340155 |
| IL-3 | R&D Systems | Cat#:403-mL |
| IL-4 | R&D Systems | Cat#:404-mL |
| Amphotericin B | Thermo Scientific | Cat#:04195780D |
| Gentamicin | Sigma Aldrich | Cat#:G1272 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human recombinant IL-15 | Biolegend | Cat#: 570308 |
| Human recombinant IL-2 (Proleukin) | Novartis Pharmaceuticals, Supplied by Guy’s Hospital pharmacy | N/A |
| Metronidazole | Baxter healthcare | Cat#: FE3400G |

### Commercial Assays

| Assay Description | Source | URL | IDENTIFIER |
|------------------|--------|-----|------------|
| Deep sequencing: Amp2Seq (illumina MiSeq) | Irepertoire | http://www.irepertoire.com/ |  |
| Deep sequencing: immunoSEQ Platform | Adaptive biotechnologies | http://www.adaptivebiotech.com/immunoseq |  |
| Zombie NIR™ Fixable Viability Kit | Biolegend | Cat#: 423106 |  |
| Live/Dead Fixable Blue Dear Cell Stain Kit | Thermo Fisher | Cat#: L23105 |  |
| Click-IT EdU Alexa Fluor 647 Flow Cytometry Assay Kit | Invitrogen | Cat#: A10202 |  |
| Foxp3 Staining Buffer Set | eBioscience | Cat#: 00-5523-00 |  |
| RNAscope 2.0 HD Reagent Kit-Brown | ACD | Cat#: 320497 |  |
| Mouse TCS Purification System | abcam | Cat#: ab128749 |  |
| EZ-Link Sulfo-NHS-LC Biotinylation Kit | Thermo Fisher | Cat#: 21435 |  |
| Alexa Fluor 647 protein labeling kit | Thermo Fisher | Cat#: A20173 |  |
| KAPA Stranded RNA-seq Kit with RiboErase (HMR) | Roche | Cat#: 07962282001 |  |
| LS-Columns | Miltenyi Biotec | Cat#: 130-042-401 |  |

### Sequence-Based Reagents

| Reagents | Source | IDENTIFIER |
|----------|--------|------------|
| Primer sequences | See Table S1 | N/A |
| Mu:Btln1 | Advanced Cell Diagnostics | Cat#: 436641 |
| Mu:Btln4 | Advanced Cell Diagnostics | Cat#: 439811 |
| Mu:Btln6 | Advanced Cell Diagnostics | Cat#: 439821 |

### Deposited Data

| Data Type | Source | IDENTIFIER |
|-----------|--------|------------|
| RNA sequencing data | GEO: GSE85422 | N/A |

| Experimental Models: Cell Lines | Source | IDENTIFIER |
|-------------------------------|--------|------------|
| MODE-K cells | Dr. D. Kaiserlian, INSERM-U1111; Bas et al., 2011 | N/A |

### Experimental Models: Organisms/Strains

| Organism/Strain | Source | IDENTIFIER |
|-----------------|--------|------------|
| B6.Cg-Foxn1 < Nu > /J (nu/nu mice) | The Jackson Laboratory, Stock: 000819 | N/A |
| Nur77.gfp mice | University of Minnesota, USA, K. Hogquist | N/A |
| Btln1<sup>tm1(KOMP)Mbb</sup> mice | IMPC, Project ID: CSD67994 | N/A |
| Btln4<sup>tm1(KOMP)Mbb</sup> mice | IMPC, Project ID: CSD81524 | N/A |
| Villin-rtTA2-M2 mice | Erasmus University, Rotterdam, M. Smits | N/A |
| Btln1-Tg mice | This paper | N/A |
| Btln1<sup>tm1KOMP</sup>-<sup>tm1KOMP</sup> mice | This paper | N/A |
| R26-rtTA2-M2 mice | Hochedlinger et al., 2005 | N/A |

### Recombinant DNA

| DNA | Source | IDENTIFIER |
|-----|--------|------------|
| Doxycycline-inducible CMV promoter plasmid pTRE2 | Clontech | Cat#: 6241-1 |
| Packaging plasmid pCMVAr8.91 | Zufferey et al., 1997 | N/A |
| Packaging plasmid pHIT/G | Fouchier et al., 1997 | N/A |
| Lentiviral vectors pCSIGPW, pCSIGHW | This paper | N/A |
| cDNA BTNL3 (GenBank: NM_197975.2), BTNL8S (Short, GenBank: NM_024850), BTNL8 (Long, GenBank: NM_001040462) | This paper | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

For additional information about reagents and resources, contact the Lead Contact, Adrian Hayday at adrian.hayday@kcl.ac.uk.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Wild-type (WT) C57Bl/6 mice were obtained from Charles River and Harlan. 3 independently derived embryonic stem (e.s.) cells for $Btnl1^{-/-}$ (Btnl1<sup>tm1(KOMP)Mbp</sup>) and e.s. cells for $Bttl4^{-/-}$ (Btnl4<sup>tm1(KOMP)Mbp</sup>) were obtained from the international mouse phenotyping consortium (IMPC) (project IDs: CSD67994 and CSD81524). $Bttl1^{-del/del}$ mice were generated using CrisprCas Technology. Briefly: Two independent short guide RNAs, targeting the intronic region: between exon1 and 2 and between exon 5 and 6 were identified using the online tool: http://crispr.mit.edu/.

Intron1/2: CCAGCTCCAAGATCCCCCTTGGG Intron5/6: TCCATAGCACCTTATCCGGTTGG

The sg RNAs & PAM sequences were cloned into the g-RNA basic vector, translated in vitro, purified and co-injected with Cas9 into day 1 zygotes and transferred into pseudopregnant foster mice.

WT and $Bttl$-knockout lines were generated and maintained at The Francis Crick Institute’s Biological resource facilities. B6.Cg-Foxn1 < Nu > /J (NU/NU) mice were obtained from the Jackson Laboratory. Nur77.gfp mice were kindly provided by K. Hogquist (Moran et al., 2011). For timed pregnancies, mice were mated overnight and E0 was considered as the day a vaginal plug is observed. Both male and female mice aged between 1 and 35 weeks (as indicated) were used in this study. No gender-specific differences were observed.

Germ-free Mice and Food Antigen-free Nutrition

C57Bl/6 mice maintained on germ-free or on solid-food antigen-free diets were bred at the Institute for Medical Microbiology and Hospital Epidemiology, University of Marburg, Germany where all experiments were conducted according to the German animal protection law.

Germ-free (GF) mice were kept in plastic isolators (Metall and Plastik, Germany) with autoclaved food, bedding and water. Sterility of animals was checked bi-weekly by culturing faeces in thioglycollate medium under aerobic and anaerobic conditions for at least ten days. All handling procedures for GF mice were conducted in a laminar flow hood under sterile conditions. All experiments were conducted according to the German animal protection law.

Food antigen-free (FAF) mice were raised on an amino acid-containing diet for up to five generations. Pellets of FAF diet (ssniff, S7242-E014/-E714) contained all essential vitamins, minerals, trace elements, fat, dextrin, sucrose and free amino acids equimolar to the protein content of normal rodent chow (LASQCdietRod16, LASvendi).

Generation of Doxycycline Inducible $Bttl$-1 Transgenic Mice

Doxycycline (Dox)-inducible $Bttl$-1-Tg mice were generated by injection of $Bttl1^{-/-}$ blastocysts with a linearized cassette containing a TRE/CMV-promoter upstream of the $Bttl$-ORF. The TRE/CMV cassette has been previously described (Oppenheim et al., 2005). R26-rTA2-M2 (Hochedingler et al., 2003) or Villin-rTA2-M2 (Roth et al., 2003) mice were bred to homozygosity for $Bttl$-deficiency and backcrossed onto $Bttl$-Tg mice for 3 generations to facilitate global (R26) or local (Villin) induction of $Bttl$ transgene expression by doxycycline administered to drinking water (1mg/ml Dox, 2% sucrose).
Animal experiments were undertaken in full compliance with UK Home Office regulations and under a project license to A.H. (80/2480).

**Flow Cytometry**

Flow cytometry was performed using the following antibodies, coupled to the indicated fluorochromes (see key resources table).

**Antibodies for mouse:** CD3 APC Cy7 (17A2); CD3 PerCP-Cy5.5 (145-2C11); TCRγ/δ Brilliant Violet 421 (H57-597); TCRγ/δ APC (H57-597); CD112 PE (TM1); CD112 Brilliant Violet 421 (TM1); CD122 APC (TM1); TIGIT PE (GIGD7); CD45RB APC Cy7 (C363-16A); Thy1.2 Brilliant Violet 510 (53-2.1); Lag3 PerCP-efluor 710 (C9B7W); CD5 PE (53-7.3); CD24 FITC (M1/69); CD24 PE-Cy7 (M1/69); CD8α PE-Cy7 (53-6.7); CD8α PE-Cy7 (53-6.7); TCR Vα4 FITC (GL-2); TCR Vδ4 PE (GL-2); CD8δ PerCP-Cy5.5 (YTS156.7.7); CD25 PerCP-Cy5.5 (PC61); CD69 PE-Cy7 (H1.2F3); CCR9 PE-Cy7 (CW-1.2); CD44 PE-Cy7 (IM7); TCRγ/δ (F2.67) was provided by Pablo Pereira (Institut Pasteur, Paris, France); TCRVγ1 APC (2.11); TCRVγ4 APC (UC3-10A6); TCRα BV421 (GL3); Ki67 FITC (B56/MOPC-21); CD45 Qdot 605 (30-F11); CD5 Brilliant Violet 510 (53-7.3); TCRδ PeCy7 (GL3); CD161/NK1.1 Brilliant Violet 650 (PK136); CD4 Brilliant Violet 786 (GK1.5); CD8α AlexaFluor 700 (53-6.7); CD25 APC (PC61); GITR PE (DTA-1); CD44 FITC (IM7); CD62L PerCP-Cy5.5 (MEL-14); KLRG1 BV421 (2F1); CD11c BV786 (HL3); CD11b BV510 (M1/70); F4/80 PerCP-Cy5.5 (BM8); Ly6G APC (1A8); Ly6C AlexaFluor 700 (AL-21); CD103 PE (M290); CD317 Brilliant Violet 650 (927); MHCII/IA/IE FITC (2G9); CD86 Pe-Cy7 (GL1); CD3 Brilliant Violet 421 (145-2C11); CD19 Brilliant Violet 421 (1D3); CD161/NK1.1 (lin) Brilliant Violet 421 (PK136); IgG1 PE (A85-1); B220 (CD45R) AlexaFluor 786 (RA3-6B2); IgM Brilliant Violet 786 (R6-60.2); IgD PerCP-Cy5.5 (11-26c.2a); GL7 AlexaFluor 467 (GL7); CD95 PE-Cy7 (Jo2); CD138 Brilliant Violet 650 (281-2); CD21/35 FITC (7G6); CD23 Brilliant Violet 421 (B-ly6). **Antibodies for human:** CD25 Brilliant Violet 421 (BC96); CD25 PE (BC96); CD3 Brilliant Violet 510 (OKT3); CD3 BUV (UCHT 1); EpCAM eFlour 660 (1B7); Streptavidin APC-Cy7; Streptavidin APC-Cy7; TCRγ/δ PeCy7 (IMMU360); Vγ9 PC5 (IMMU360); Vγ9 PE (B3); Vδ1 APC (REA173); Vδ2 PerCP (B6); Vγ2/3/4 biotin (23D12), Vγ3/5/6 biotin (56.3) and Vγ8 biotin (R4.5.1) were provided by D. Kabelitz and D. Wesch (University of Kiel). **Other antibodies:** DYKDDDDK-PE (Flag); DYKDDDDK-APC (Flag); HA-DyLight 650; 6x-Histidine-PE.

Commercial antibodies were purchased from Biolegend, eBioscience, BD-Bioscience, Thermo Fisher Scientific or Miltenyi (see key resource Information). Viability dyes (near IR or Blue) were from Invitrogen. Anti TCRVγ/δ (F2.67) was purified from hybridoma supernatant using the mouse TCS purification system (Abcam) and conjugated to biotin or AF647 (see key resources table).

**Flow Cytometry data analysis** was performed on FlowJo (Version 9.9).

**Plasmids, Cloning, RT-PCR, Transfection and Lentiviral Transduction**

The self-inactivating lentiviral vector pCSIGPW (SFFV promoter – Multiple Cloning Site [MCS] – IRES-GFP – CMV promoter – Puromycin) was constructed by replacing the Puromycin59/mir cassette from the pAAM vector (Pertel et al., 2011) by a custom EcoRI-Xhol-Pmel-NotI-BamHI-Xbal-MulI MCS. The IRES-GFP cassette was cloned by PCR from the pIRE2-eGFP vector (Clonetech) using the BamHI/XbaI sites. The CMV promoter was cloned by PCR from the pCDNA3.1+ vector (Thermo Fischer Scientific) using the XhoI-PmeI-NotI-BamHI-XbaI-MluI MCS. The IRES-GFP cassette was cloned by PCR from the pIRES2-eGFP vector (Clonetech) using the EcoRI-XhoI sites. The Puromycin resistance gene was cloned by PCR from the pGIPZ vector (Dharmacon) using the ClaI/AgeI sites. The puromycin resistance gene was cloned by PCR from the pLHCX vector (Clontech).

cDNAs were (sub-)cloned into pCSIGPW or variant vectors (see Supplemental Information). Btn1, Btn4 and Btn6 were previously described (Bas et al., 2011). BTNL3 (GenBank:NM_197975.2), BTNL8S (GenBank:NM_024850), and BTNL8 (GenBank:NM_001040462) were cloned from Caco-2 cells by conventional RT-PCR, using the following primers (See Table S2):

| BTNL3 For 5'-GAATATCCATGGCCTTTGTCG-3' | BTNL3 Rev 5'-GTCCTCTGCTGCATCCCC-3' |
| BTNL8 For 5'-CCATTCGCAAGAAACACATCCATG-3' | BTNL8S Rev 5'-TATGGGTATACGTTTTCGATCG-3' |
| BTNL8 Rev 5'-GGGGATGTTGATATCATCTAC-3' | |

FLAG, HA and HIS tags were added downstream of the putative leader peptides by overlapping PCR. Human full-length TCR γ and δ chains were cloned (Xhol / NotI, pCSIGPW) using the following primers (See Table S2):

| Vγ2/3/4 For 5'-ATGCAGTGGGCCCTAGGCG-3' | Vγ8 For 5'-ATGCTGGTGCTCTAGTCCTGC-3' |
Expression of BTNL3 and BTNL8 was checked by conventional RT-PCR using the primers indicated above. BTN3A1, BTN3A2, EPCAM and GAPDH were used as control genes (See Table S2):

- BTN3A1 For: 5'-AGTATCCTCTGATATGCAGCATG-3'
- BTN3A1 Rev: 5'-GGAGGAACTCTCTTCTTCTTTAC-3'
- BTN3A2 For: 5'-TGGTATCTCTTGATATGCAGCATAG-3'
- BTN3A2 Rev: 5'-AGAGCATCAGGCTGACTTATTGG-3'
- EPCAM For: 5'-GCCGCCACCATGGCGCCCCCGCAG-3'
- EPCAM Rev: 5'-TTATGCATTGAGTTCCCTATGCA-3'
- GAPDH For: 5'-GAAGGTGAAGGTCGGAGTC-3'
- GAPDH Rev: 5'-GAAGATGGTGATGGGATTTC-3'

Transfections were carried out in HEK293T cells using PEI (3:1 PEI:DNA ratio, Polysciences). Btnl/BTNL expression was checked 48h post-transfection. Lentiviral particles were produced in HEK293T cells by co-transfection of pCSIGPW or pCSIGHW either empty or containing Btnl/BTNL cDNAs, pCMV/D8.91 (HIV-1 tat/rev/gag/pol) (Zufferey et al., 1997), and pHIT/G (MLV env) (Fouchier et al., 1997). Transduced cells were treated with puromycin and hygromycin 48h post-transduction for 7 days, sorted on the basis of GFP expression and used for functional assays.

Quantitative RT–PCR
Samples were stored in RNAlater (Ambion) or directly frozen in RLT buffer prior to RNA purification (QIAGEN RNeasy kit). cDNA was generated using Superscript-II (Invitrogen) and analyzed using Sybr-green assay (Invitrogen) using a ViiA7 Real-time PCR machine (Applied Biosystems) (See Table S2).

Primers for Murine qPCR
- Btnl For: 5'-TGACCAGGAGAAATCGAAGG-3'
- Btnl Rev: 5'-CACCGAGCAGGACCAATAGT-3'
- Btnl4 For: 5'-CATTCTCCTCAGAGACCCACACTA-3'
- Btnl4 Rev: 5'-GAGAGGCCTGAGGGAAGAA-3'
- Btnl6 For: 5'-GCACCTCTCTGGTGAAGGAG-3'
- Btnl6 Rev: 5'-ACCGTCTTCTGGACCTTTGA-3'
- b-Actin For: 5'-CAGCTTCTTTGCAGCTCCTT-3'
- b-Actin Rev: 5'-CACGATGGAGGGGAATACAG-3'
- Sox-13 For: 5'-CTCCAGGCCTTCCCCAGAC-3'
- Sox-13 Rev: 5'-CATGGACTTCCAGCGAGAAC-3'
- Rorc For: 5'-GGTGACCAGCTACCAGAGGA-3'
- Rorc Rev: 5'-CCACATCAGTGAATGGCCTCA-3'
- Tbp For: 5'-GGGGAGCTGTGATGTGAAGT-3'
- Tbp Rev: 5'-CCAGGGAAATAATTCTGGCTCA-3'
- CycloFor: 5'-CAAATGCTGGACCAAACACAA-3'
- Cyclo Rev: 5'-CCATCCAGGCCATCAGTCTTG-3'

Southern Blotting
Southern blots were performed with probes generated using a Dig-Probe labeling kit; blots were hybridized in DIG-Easy-hyb buffer overnight, and developed using the DIG–Luminescence Detection Kit (Sigma-Aldrich). For probe sequences see Supplementary Information. DIG labeled probes for Southern blotting were generated using the following primers (See Table S2):

- Btnl1 For: 5'-ACTGGCTTCCTCAGAGACCAAT-3'
- Btnl1 Rev: 5'-CAGCTTCTCAGAGCCAGCACACTA-3'
- Btnl4 For: 5'-CATGGACTTCCAGGAGAATA-3'
- Btnl4 Rev: 5'-AGGAGGGAGGAGGGAAGAA-3'
- Btnl1-Tg-Ex3 For: 5'-GGTTTTCTGTGAAGGGACCA-3'
- Btnl1-Tg-Ex4 Rev: 5'-GGTCTGCAACTCAGTCTTG-3'
RNAscope
RNAscope was performed on paraffin embedded sections using probes and kits obtained from Advanced Cell Diagnostics using the RNAscope 2.0 HD Reagent Kit-BROWN. Reference sequences are as follows: Btrn1, GenBank:NM_001111094.1 (576-1723); Btrn4, GenBank:NM030748.1 (560-968); Btrn6, GenBank:NM_030747.1 (245-1552) (See Table S2).

Isolation of Murine Intestinal Intra-epithelial Lymphocytes (IEL)
IEL were isolated from mouse small intestine as previously described (Wencker et al., 2014). Small intestine was opened and washed in PBS, cut into 1cm pieces and incubated for 20min in RPMI 1640 supplemented with 1% penicillin/streptomycin (pen/strep), 10% fetal calf serum (FCS) and 1mM dithiothreitol on a turning wheel. Tissues were washed and vortexed in RPMI, then passed through a 70 μm nylon cell strainer twice, and centrifuged on a 20/40/80% Percoll density gradient at 700 g for 30min. IEL were harvested from the 40 to 80% Percoll interface.

Spleen and Mesenteric Lymph Node Immunophenotyping
Comprehensive immunophenotyping of Btn1-/- mice was performed using a platform developed by the Wellcome Trust Infection and Immunity Immunophenotyping (3i) consortium (www.immunophenotyping.org). In brief, Spleen and MLN were digested with collagenase (1mg/ml)/DNAse (0.1 mg/ml) in 2% FCS PBS (+ Ca/Mg) for 20 minutes at 37˚C and filtered through 30μm cell strainers. Cells were plated on 96 well V-bottom plates, washed in PBS and stained with Zombie Near-IR (Biolegend) for live/dead discrimination. Antibody stains were performed at 4˚C for 20mins. Full details regarding phenotyping panels are included in Table S3. Samples were acquired on a BD LSR Fortessa X-20 equipped with 405nm (40mW), 488nm (50mW), 561nm (50mW), and 640nm (100mW) lasers.

MODE-K Co-culture Assays
Cells were co-cultured in RPMI 1640 supplemented with 10% FCS, Pen/Strep, 2.5% HEPES, 1% Glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 0.2% β-mercapto-ethanol (GIBCO) and cytokines including IL-2 (10U/ml), IL-15 (10 ng/ml) (Immunotools), IL-3 (100U/ml), IL-4 (200U/ml) (R&D). 10⁵ MODE-K were seeded in 48-well plates 24h prior to the addition of 10⁵ unsorted or (where indicated) positively FACS-sorted (CD45+Vγ7+) IEL and incubated for 16-18h in 10% CO₂ unless indicated otherwise. For transwell assays, 2x10⁵ MODE-K cells were seeded onto 24-well transwell plates (3 μm pore size - Corning) 24h prior to the addition of 3x10⁵ IEL, either in direct contact (below), sequestered from (above), or split 50:50 with MODE-K cells (above and below the transwell).

IEL Stimulation
96-well U bottom plates were coated overnight with 10 μg/ml LEAF-Purified anti-mouse CD3-ε or Hamster IgG Isotype control (Biolegend) at 4˚C and washed once with PBS 1x before seeding IEL. 100,000 IEL were seeded per well. Cells were incubated at 37˚C for 16-18h in 10% CO₂ prior to analysis.

Confocal Imaging
Proximal small intestine (SI) samples were fixed in Zamboni’s fixative, blocked with normal goat serum and stained with antibodies against TCRβ, TCRδ, TCRVδ4 (encoded by TRDV2-2) (GL2), CD3 and Vγ7. Z-Sections were acquired on a confocal-LSM-710 microscope (Zeiss) and processed and analyzed using Imaris Software (Bitplane Scientific Solutions).

Bone Marrow Chimeras and Adoptive IEL Transfers
10-12 week old recipient mice were irradiated with 950Rads 24h, injected (IV) with 5-10x10⁶ donor bone marrow cells and analyzed 4-12 weeks later.

RNA Sequencing
Vγ7⁺CD122⁺ and Vγ7⁺CD122⁻ IEL were sorted from from pooled D14-17 pups directly into RLT buffer. RNA was prepared using the RNA-Micro-plus kit (QIAGEN). RNA libraries were generated using the KAPA Stranded RNA-seq Kit with RiboErase (HMR) (KAPA BIOSYSTEMS). Paired-end sequencing on HiSeq 2500 (illumina) using rapid run chemistry (read length: 100bp).

Human Samples and Primary Lymphocyte Isolation
Endoscopic biopsies were obtained from the ascending colon of adult donors undergoing routine diagnostic colonoscopy after informed consent and in compliance with local ethical approval (REC number 07/H0803/237). Excess resected skin discarded at the time of cutaneous or reconstructive surgery was obtained from adult donors after informed consent and in compliance with local ethical approval (REC number 06/Q0704/18). This study was conducted adhering to the principles of the Declaration of Helsinki.

Primary gut lymphocytes were obtained using an adaptation of the method of Kupper and Clarke (Clark et al., 2006) (Figure S7F). Skin lymphocytes were isolated using the method as originally described (Clark et al., 2006). 9mm x 9mm x 1.5mm Cellfoam matrices
(Cytomatrix PTY Ltd), were autoclaved and incubated in 100mg/mL rat tail collagen I (BD Biosciences) in PBS for 30min at 37°C, and washed twice in PBS. In compliance with local ethical approval, 12 endoscopic biopsies were taken from the ascending colon of donors. Biopsies were washed for 20min in 5mL wash medium (RPMI 1640 10% FCS, β-mercaptoethanol, penicillin [500U/ml], streptomycin [500 μg/ml], metronidazole [5 μg/ml, Pharmacy department, Guy’s Hospital], gentamicin [100 μg/ml, Sigma-Aldrich] and amphotericin 12.5 μg/ml [Thermo Fisher Scientific]). One endoscopic biopsy was placed on top of each matrix, which was inverted, and pressure applied, to crush the biopsy into the matrix. The matrices were placed into a 24-well plate (1 per well) and covered with 2mL RPMI 1640 (supplemented with 10% FCS, β-mercaptoethanol, penicillin [100U/ml], streptomycin [100 μg/ml], metronidazole [1 μg/ml], gentamicin [20 μg/ml], amphotericin [2.5 μg/ml]), IL-2 (100U/mL, Novartis Pharmaceutical UK) and IL-15 (10ng/mL, Biolegend). 1 ml of medium was aspirated every second day and replaced with complete medium containing 2x concentrated cytokines. Cells were harvested and residual biopsy and empty wells were washed with PBS 0.02mM HEPES. The cell suspension was passed through a 70 μm nylon cell strainer, centrifuged at 400 g for 5min and resuspended in complete medium without additional cytokine and placed into co-culture immediately. Lymphocytes were used after 5-7 days of culture.

PBMC were isolated by Ficoll gradient from blood obtained from the blood donation service.

**Human Epithelial Cell Isolation**
Colonic samples were incubated with 5 mM 1,4-dithiothreitol (Sigma), followed by enzymatic digestion with 1.5mg/ml collagenase VIII (Sigma) and 0.05 mg/mL DNase I (Sigma). EpCAM+ cells were sorted by flow cytometry directly into RLT lysis buffer. RNA and cDNA were prepared as described above.

**HEK293T Co-culture Assay**
5x10^5 HEK293T cells, transduced with either empty vector (EV), BTNL3, BTNL8 or BTNL3+8 and 2x10^5 freshly harvested primary human lymphocytes were co-cultured in 96-well plates with complete medium (see Supplementary Information) without supplementary cytokine and incubated at 37°C at 5% CO2 for 16hrs (Figure S7F).

**Deep Sequencing**
Mouse TRDV gene: Amplification and sequencing of TCRδ CDR3 from RNA purified from sorted Vγ7+ IEL was performed using the Amp2Seq Platform (iRepertoire).Human TCRG Vγ gene: Amplification and sequencing of TCRγ CDR3 was performed using the immunoSEQ Platform (Adaptive Biotechnologies).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistics**
Unless stated otherwise, bar/spider charts display mean ± SD and p values were derived from unpaired two tailed t tests, assuming equivalent SD (ns > 0.05).

**Imaris Image Analysis**
Confocal microscopy was performed using a LSM710 laser scanning confocal microscope (Zeiss) with a 40x oil objective (numerical aperture 1.3). 3D image analysis on z-stacks was carried out using Imaris (Bitplane). The surfaces tool was used to identify CD3+ cells. Volumes outside of these structures were set to zero in each of the channels to create masks.

**Bioinformatics Analysis of RNA Sequencing**
101 base-pair paired-end reads were aligned and quantified using RSEM (v1.2.11) (Li and Dewey, 2011) with Bowtie2. Reads were aligned to a transcriptome constructed from the mm10 mouse genome and a UCSC knownGene gtf file. A mean alignment rate of 57.4 million fragments per sample was observed. Using the gene level quantification, only detected genes (mean TPM value across all samples > 1; 13,313 genes) were selected. Differential expression between the CD122hi and CD122lo Vγ7+ IEL groups using DESeq2 (Love et al., 2014) was identified by taking into account the paired structure within the replicate groups. Using an FDR of 0.01 2664 phenotype dependent gene expression effects were identified.

**DATA AND SOFTWARE AVAILABILITY**

**Data Resources**
The accession number for the RNA sequencing data reported in this paper is GEO: GSE85422.
Figure S1. Phenotypic Differences between CD122^{hi} V_{γ7}^{+} IELs and Other IEL Subsets, Related to Figure 1

(A) Cell surface phenotype of V_{γ7}^{+}, V_{γ7}^{-} (CD3^{+}TCR_{β}^{-}V_{γ7}^{-}) and ab (TCR_{β}^{+}) IEL from 3-5 week old (W3-5) C57Bl/6 (WT) mice (n ≥ 7). (B) Cell surface phenotype of WT V_{γ7}^{+}CD122^{hi} versus V_{γ7}^{+}CD122^{lo} IEL (n ≥ 7). (C) Heat map of genes differentially expressed (log-2-FoldChange) between V_{γ7}^{+}CD122^{hi} and V_{γ7}^{+}CD122^{lo} IEL sorted from D14-D17 WT mice. Data generated by RNA sequencing (‘cell cycle’ & ‘cell surface’ GO terms annotated). Values scaled to their median value across the samples. (D) 3hr EdU incorporation in vivo in V_{γ7}^{+} versus V_{γ7}^{-} IEL from D28 WT mice assessed by flow cytometry in indicated IEL subsets (V_{γ7}^{-} are CD3^{+}TCR_{β}^{-}V_{γ7}^{-}). Data are representative of 1 (C) or ≥ 3 (A,B) independent experiments. Panel (D) presents data pooled from 3 independent experiments. All error bars represent mean ± SD. Related to Figure 1.
(legend on next page)
Figure S2. Local Intestinal Development of CD122<sup>HI</sup> V<sub>γ7</sub>+ IELs, Related to Figure 2

(A) Deep sequencing of TCR V<sub>d</sub> chain usage in WT V<sub>γ7</sub>+ IEL sorted from W7-10 CS7B/L6 (WT) mice (n = 3). (B) Absolute numbers of WT V<sub>γ7</sub>+ and V<sub>γ7</sub>+GL2+ thymocytes from WT mice assessed by flow cytometry. C-D) Cell surface phenotype of WT V<sub>γ7</sub>+ IEL and thymocytes at indicated time points. (E) Cell surface phenotype of V<sub>γ7</sub>+ thymocytes and IEL isolated from W3-5 WT mice (n = 5). (F) γ<sub>H</sub> IEL composition (left), cell count (middle) and cell surface CD122 expression (right) in WT versus alymphoplasia (aly/aly) mice. (G) Longitudinal RNAseq analysis of Btn1 expression during gut development. (H) Gene expression by qRT-PCR along the length of the gut in WT mice (n ≥ 3). (I) Gene expression by qRT-PCR in the thymus of WT and Btn1<sup>−/−</sup> animals compared to the proximal small intestine. (J) Organization of WT and targeted loci for Btn1<sup>−/−</sup>, Btn1<sup>indel/indel</sup> and Btn4<sup>−/−</sup> mice. Grey: untranslated region; green: translated region; orange: inserted targeting cassette. Knockout ES cell clones were obtained from the international mouse consortium IKMC-ID 67994 (Btnl1) and 81524 (Btnl4). (K) Southern blot for targeting of alleles in Btn1<sup>−/−</sup> and Btn4<sup>−/−</sup> mice. Genomic DNA was digested using the indicated enzymes (arrowheads). Probes targeting the indicated regions were generated to detect the WT and targeted alleles. Data are representative of ≥ 1 (A,K) or ≥ 2 (C,E,G,H,I) independent experiments. Some panels include data pooled from 2 (F), > 3 (D) or > 6 (B) independent experiments. All error bars represent mean ± SD. Related to Figure 2
Figure S3. *Btnl1* Has No Detectable Effect on the Systemic T, B, and Myeloid Cell Compartments, Related to Figure 3

(A) γδ IEL composition in adult WT versus *Btnl1*+/-/+indel/indel mice (n = 3) (B) Mesenteric Lymph Node (MLN) and (C) Splenic immune compartments of WT, *Btnl1*+/- and *Btnl1*+/-/ mice analyzed by flow cytometry (n = 7–9). (D) TCRVγ chain usage in MLN and splenic lymphocytes harvested from WT, *Btnl1*+/- and *Btnl1*+/-/ mice assessed by flow cytometry (n = 8). (E) Vγ7+ and Vγ7+GL2+ thymocytes from WT or *Btnl1*+/- and *Btnl1*+/-/ mice assayed by flow cytometry to enumerate total cell counts (left) and cell surface phenotype (right) at three time points. Panels (A-D) are representative of 1 experiment. Panel E-left presents data pooled from ≥ 2 independent experiments. All error bars represent mean ± SD. See Table S3.
Figure S4. Impact of Btnl1 on Intestinal Engraftment, Expansion, and Retention of CD122HI Vγ7+ IELs, Related to Figure 4
(A) K67+ expression in Vγ7+ IEL isolated from WT versus Btnl/C0/C0 mice (n = 4-27). (B) Cell surface phenotype of Btnl/C0/C0 Vγ7+CD122HI versus Vγ7+CD122LO and Vγ7+GL2+CD122HI versus Vγ7+GL2+CD122LO IEL displayed in Figure 4B (n ≥ 8). (C) Irradiated TCRδ KO mice reconstituted with WT bone marrow (BM) were analyzed for γδ IEL composition at the indicated time-points after BM transfer (n ≥ 3). (D) IEL isolated from WT W4-5 mice were column-purified using CD45 microbeads and adoptively transferred intravenously into W6 TCRδ/C0/C0 or TCRδ/C0/C0 Btnl/C0/C0 hosts. γδ T cell composition was assayed 2-3 weeks later by flow cytometry (n ≥ 5). Data are representative of 1 (C), 2 (D) or ≥ 3 (B) independent experiments. Bar graph displays mean ± SD. All error bars represent mean ± SD.
Figure S5. Inducible Btnl1 Transgene Expression and Its Impact in Adult Mice, Related to Figure 5
(A) Schematic representation of the WT Btnl1 locus (top) and TRE-Btnl1 transgene construct (bottom). Grey: untranslated region; green: translated region; orange: upstream-tetracycline response element/CMV promoter and downstream-β-globulin/polyA. (B) Southern blot to detect transgene insertion. Genomic DNA was digested with EcoRI as indicated (arrowheads) and a probe (blue bar) targeting the indicated region (Exon3/4 boundary in ORF) was generated to detect the WT and targeted allele (n = 2). (C-F) W7-13 (ADULT) mice of indicated genotypes on a Btnl1+/C0/C0 background were administered doxycycline water (1mg/ml Dox, 2% sucrose) or ctrl water (2% sucrose) for 1-2 weeks. (C) Gene expression by qRT-PCR in proximal small intestine of adult mice following the indicated treatment. (D) γδT cell composition (left) and absolute cell counts (right) assessed by flow cytometry in adult mice following the indicated treatment (sugar, n = 3-5; rest, n = 4-10). (E) Ki67 and cell surface CD122 expression in Vγ7+ IEL from adult mice following the indicated treatment. (F) Ki67 expression in Vγ7+ versus Vγ7− and TCRβ+ IEL from adult mice following the indicated treatment (n ≥ 9). Data are representative of 2 (B), or 3 independent experiments. Some panels include results pooled from 2 (C) or ≥ 3 (D-F) independent experiments. All error bars represent mean ± SD.
Figure S6. Co-expression of Btnl1 and Btnl6 and Their Impact on Vγ7+ IELs, Related to Figure 6
(A) Cell surface expression of FLAG-Btnl1, HIS-Btnl4 or HA-Btnl6 co-transfected in MODE-K cells. Histogram overlays show the expression of each BTN L after gating on GFP+ cells (numbers in brackets indicate geometric mean fluorescence intensity, gMFI). (B) Primary small intestinal IEL cultured for the indicated times with MODE-K cells transduced with constructs expressing an empty vector (EV) versus Btnl1+Btnl6 (L1+6) (n = 7). (C) Representative plots of cell surface CD122 and CD25 expression on Vγ7+ cells after the indicated overnight culture conditions (n = 21). (D) Cell surface CD25 expression in positively FACS-sorted Vγ7+ IEL after overnight co-culture with MODE-K cells expressing EV versus L1+6 (n = 4). (E) Cell surface CD25 expression in primary Vγ7+ IEL after overnight co-culture with the indicated MODE-K transductants in the presence PP2, PP3 or vehicle. Data are representative of representative of 2 (A,D), or > 5 (C) independent experiments. Some panels (B,E) present data pooled from 2 independent experiments. All error bars represent mean ± SD.
Figure S7. Human Intestinal γδ Cells and the Selective Impact on Them of BTNL3 and BTNL8 Co-expression, Related to Figure 7

(A) FACS-sorted γδ T cells harvested from human intestinal tissue were analyzed by deep sequencing for TCR Vδ chain usage. (B) Schematic illustrating the murine and human Btn2/BTN2 and Btn9/BTN9 loci, adapted from the NCBI gene viewer. (C) Conventional RT-PCR analysis of BTN3A2, BTN3L and BTN8 expression in the indicated tissues. (D) Conventional RT-PCR analysis of BTN3A1, BTN3L, BTN8, EPCAM and TCR Vγ2/3/4 expression in the indicated samples. (E) Cell surface expression of FLAG-BTN3L, FLAG-BTN8S or FLAG-BTN8 co-transfected in HEK293 cells with the indicated constructs. Histogram overlays show the expression of each BTN after gating on GFP+ cells (numbers in brackets indicate geometric mean fluorescence intensity, gMFI). (F) Schematic illustrating the method of human intestinal tissue-resident lymphocytes isolation and co-culture with HEK293 transductants. (1) Endoscopic biopsies recovered from ascending colon of healthy donors. (2) Washed in complete media supplemented with antibiotic. (3) 1 biopsy applied to each matrix. (4) Culture for 5-7 days in complete medium supplemented with antibiotics, IL-2 and IL-15. (5) Co-culture with HEK293 cell lines transduced with EV, L3, L8 or L3+8. (G) Cell surface CD25 expression on indicated subsets of human gut-derived lymphocytes after co-culture with EV versus L3+8-transduced HEK293 cells. (H) Gating parameters for sorting of Btnl3+8-responsive human gut-derived lymphocytes. (I) TCRVγ chain usage (left) and cell surface TCRγδ expression (right) in gut-derived γδ T cells (isolated from a donor unresponsive to BTN3L+8) after co-culture with EV versus L3+8-transduced HEK293 cells.