Butyrophilin-like proteins display combinatorial diversity in selecting and maintaining signature intraepithelial γδ T cell compartments

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Butyrophilin-like (Btnl) genes are emerging as major epithelial determinants of tissue-associated γδ T cell compartments. Thus, the development of signature, murine TCRγδ+ intraepithelial lymphocytes (IEL) in gut and skin depends on Btnl family members, Btnl1 and Skint1, respectively. In seeking mechanisms underlying these profound effects, we now show that normal gut and skin γδ IEL development additionally requires Btnl6 and Skint2, respectively, and furthermore that different Btnl heteromers can seemingly shape different intestinal γδ+ IEL repertoires. This formal genetic evidence for the importance of Btnl heteromers also applied to the steady-state, since sustained Btnl expression is required to maintain the signature TCR.Vγ7+ IEL phenotype, including specific responsiveness to Btnl proteins. In sum, Btnl proteins are required to select and to maintain the phenotypes of tissue-protective γδ IEL compartments, with combinatorially diverse heteromers having differential impacts on different IEL subsets.
from jawless vertebrates through to humans, many extra-
lymphoid tissues harbour distinct immune cell populations.
Those include tissue-resident memory (TRM) cells that
infiltrate tissues after antigen priming in lymphoid tissues, and
remain well-placed to respond to local antigen recurrence1. In
addition, various myeloid and lymphoid cells, including macro-
phages2, T-regulatory cells3 and γδ T cells become associated
with tissues developmentally, remaining in situ lifelong4,5. Such
cells are implicated in protecting tissue integrity, and γδ T-cell
function is causally linked to cancer, tissue inflammation and
defective wound healing6–12. In addition, the molecular phenotypes of local T cells com-
monly emphasise their relationships to specific anatomical
sites1. Thus, murine γδ T cells, which were revealed some 30
years ago to be prototypic tissue-associated T cells, display
tissue-restricted T-cell receptor (TCR) repertoires, including
Vγ5Vδ1 in the epidermis, Vγ6Vδ1 in the uterus, dermis and
lung, and Vγ7+ cells expressing a variety of Vδ chains in the
small intestine13. Nonetheless, how such TCRs contributed to
tissue protection remained enigmatic, particularly given that
Vγ5Vδ1+ dendritic epidermal T cells (DETC) were shown to
use innate receptors, specifically NKG2D, to respond rapidly to
epithelial cell dysregulation14,15.

Recently however, signature TCRs were shown to mediate the
tissue-specific selection of γδ T cells by members of the heretofore
enigmatic butyrophilin-like (Btnl) subfamily of B7 genes. Thus, Btnl1–/– mice mostly lack intestinal Vγ7− cells16, while mice
deficient in Skint1 (a Btnl-related gene) specifically lack Vγ5Vδ1+ DETC17,18. The conservation of this biology became evident when human colonic Vγ4+ cells were shown to be specifically regulated by BTNLI3,16,19, while Butyrophilin 3A1 (BTN3A1) and BTN2A120 were found to be critical for signature responses of human peripheral blood Vγ9Vδ2+ cells to low molecular mass phosphoantigens such as isopentenyl pyrophosphate21–23. Moreover, dysregulation of the BTN3–Vγ4+ axis has been implicated in celiac disease24. Thus, there is considerable interest in the mechanisms by which Skint/Btnl/BTN genes exert their effects.

Consistent with their regulation of γδ T-cell subsets defined by
their TCRs, Btnl/BTNl proteins have emerged as bona fide T-cell
selecting ligands akin to MHC or CD1. In addition, evidence from
cell culture and biochemical experiments argues that Btnl/BTNl/
BTN proteins may exert their impacts as heterodimers of Btnl1 + 6,
BTN11 + 8, and BTN3A1 + 2A1, respectively16,19,20,25. None-
theless, the functional significance of heterodimers has not been uni-
versally accepted26, with one concern being that the most compelling
evidence is based on cellular over-expression systems27,28.

This study has addressed this important issue by use of
genetics. By showing that Vγ5Vδ1+ DETC development depends
on Skint2 as well as on Skint1, and that Vγ7+ intestinal IEL
development depends on Btnl6 as well as on Btnl1, we now
provide formal genetic evidence that single Btnls are not sufficient
for IEL selection. Most unexpectedly, however, different Btnl
pairings had differential effects on IEL with different TCRs,
revealing a potential for combinatorial diversity that could finely
tune IEL repertoire composition. The major impacts of Skint1
and Btnl1 on IEL maturation occur during narrow time-windows
in early life. Beyond this, the sustained expression of Btnl genes
isherewith should be required to maintain signature intestinal
IEL phenotypes. In sum, epithelial Btnl proteins mediate a sus-
tained and complex regulation of local γδ T-cell compartments.

Results
DETC development requires Skint2. The normal, inthymic
development of Vγ5Vδ1+ DETC progenitors depends on Skint1,
as judged by severe DETC depletion in Skint1 hypomorphic
(FVB.Taconic), Skint1-deficient (Skint1Δ/Δ) [Δ denotes internal
deletion] or Skint locus deficient mice17,29,30. To ask whether
DETC development depends on at least one other Skint gene, we
used CRISPR to target Skint2, which seems evolutionarily con-
served across rodents possessing DETC31. To disrupt Skint2, we
introduced LoxP (fl) sites flanking the first and fifth protein-
coding exons (exons 2 and 6). However, as is common in CRISPR
strategies, a collateral outcome was an internal deletion spanning
those exons (Supplementary Fig. 1a). Those Skint2Δ/Δ mice
showed no Skint2 mRNA expression in ear skin (Fig. 1a) or
elsewhere, whereas wild-type (wt) Skint1 mRNA levels were
sustained.

Vγ5Vδ1+ DETC progenitors in the fetal thymus of ~E15.5 wt
mice show Skint1-dependent selective maturation, as indicated by
CD45RB and CD122 upregulation and CD24 and CD62L
downregulation30. Conspicuously, Vγ5Vδ1+ DETC progenitors
in Skint2Δ/Δ mice phenocopied those in Skint1 hypomorphs30
and Skint1Δ/Δ animals29, failing to mature to classic
wild-type (wt) controls, but showing compensatory increases in immature CD45RB+, CD122+, CD24hi and CD62L+ cells (Fig. 1b, c). Unsurprisingly, this maturation defect resulted in almost complete loss of mature DETC expressing the 17D1
epitope displayed by the Vγ5Vδ1 DETC TCR (Fig. 1d–f; Fig
Supplementary Fig. 1b). The so-called DETC-replacement
cells were TCRγδ+δ−, demonstrating that Skint2 deficiency did not cause pan-γδ deficiency (Fig. 1d, e). Moreover, although they
completely lacked 17D1+ DETC, some Skint2Δ/Δ mice harboured
Vγ5+ DETC-replacements (Fig. 1d–f) although their TCR expres-
sion was somewhat lower than wt Vγ5+ DETC, symptomatic of
defective selection18,30,32 (Fig. 1d, e). By contrast to the dramatic
change in the DETC compartment, Skint2Δ/Δ mice showed a largely
unchanged expression of MHC-class II+Langerhans cells with
dehich DETC share the epidermis (Fig.1f; Supplementary Fig. 1b).

The significance of these various phenotypic patterns notwithstanding,
DETC and LC counts showed some inter-individual variation (Fig
1f), indicative of the cells’ multifactorial regulation, although
there was no evident contribution of sexual dimorphism (Supple-
mentary Fig. 1c).

The cells with a TCR most closely related to Vγ5Vδ1+ DETC
are uterine and lung γδ T cells expressing Vγ6 paired with a Vδ1
chain identical to that in DETC. In the absence of a generally
available Vγ6-specific antibody, such cells were identified as
TCRγδ+Vγ4+Vδ2−Vδ5−, and in Skint2Δ/Δ mice such cells were
largely unaffected (Supplementary Fig. 1d,e), again phenocopying
Skint1 hypomorphs30. Collectively, these genetic data show that
Skint2 as well as Skint1 is critically required for the specific
maturation of Vγ5Vδ1+ DETC progenitors, supporting the
hypothesis that discrete γδ T-cell compartments are naturally
regulated by Btnl heteromers.

Indeed, the capacity of Skint1 and Skint2 to form physical
complexes in vitro and in vivo was validated when anti-
Skint1 immunoprecipitates from 293T cells transfected with
N-terminal Flag-tagged Skint1 and HA-tagged Skint2 were shown
to contain both Skint1 and Skint2, as detected by western blot (Supplementary Fig. 1f). Moreover, anti-Skint1 and anti-Skint2
antibodies could detect Skint1 and Skint2, respectively, in
western blots of anti-Flag immunoprecipitates from transgenic mice expressing an N-terminal Flag-tagged Skint1
construct (NF-Skint1Tg)32, but not from non-transgenic FVB
mice (Fig. 1g; long arrows). Note that the detection of anti-Flag
antibody chains in the FVB lysates (Fig. 1g; asterisks) validated
protein loading. Moreover, the specificity of Skint1 and Skint2
detection in the immunoprecipitates was verified by the failure
to detect actin in anti-Flag immunoprecipitates, despite its
detection in total input protein (Fig. 1g, lowest panel). The

ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-17557-y | www.nature.com/naturecommunications

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failure to detect Skint1 or Skint2 in total input protein is consistent with their very low levels of protein expression. The inefficiency of Skint1/Skint2 elution from the beads seemingly reflects a greater affinity of the anti-Flag antibody for Flag-tagged Skint proteins versus Flag peptide. This notwithstanding, the data show an evident capacity of Skint1 and Skint2 to associate in cell lines and in primary mouse tissue.

**Btnl genes exert hierarchical regulation of Vγ7+ IEL.** Small intestinal villus enterocytes express *Btnl1, Btnl4* and *Btnl6*...
genes\textsuperscript{13,34}. Whereas \textit{Bttnl}-deficient mice were substantially depleted of signature Vγ7\textsuperscript{+} intestinal IEL, \textit{Bttnl} deficiency had no obvious effect\textsuperscript{16}. Therefore, to test whether the heteromeric model also applied to the gut, we generated mice lacking \textit{Bttnl6}, which encodes a protein that can collaborate with \textit{Bttnl} to stimulate mature Vγ7\textsuperscript{+} intestinal IEL\textsuperscript{16}. To this end, we introgressed LoxP sites on either side of the 9-exon gene (Fig. 2a, right panel; \textit{Bttnl6}\textsuperscript{fl/−} mice). In parallel, to complement the \textit{Bttnl1KOMP} strain previously obtained from the International Knockout Mouse Consortium (\textit{Bttnl1KOMP})\textsuperscript{16}, we generated a floxed allelic \textit{Bttnl6} with LoxP sites flanking the first four coding exons (exons 2–5) (Fig. 2a, left panel). A constitutive, universal knockout of \textit{Bttnl6} (\textit{Bttnl6}\textsuperscript{ΔΔ} mice) was generated by crossing floxed \textit{Bttnl6} with Pgy-Cre mice\textsuperscript{35}, while intestinal epithelial cell (IEC)-specific knockouts of \textit{Bttnl6} (\textit{Bttnl6}\textsuperscript{Δgt} mice) and \textit{Bttnl1} (\textit{Bttnl1}\textsuperscript{Δgt} mice) were generated by crossing the floxed mice to Villin-Cre mice\textsuperscript{16} (Fig. 2a). The veracity of the different mutant mouse strains was evident from quantitative RT-PCR of \textit{Bttnl1}, \textit{Bttnl4} and \textit{Bttnl6} expression, and histologic RNAscope analysis of \textit{Bttnl1} and \textit{Bttnl6} (Supplementary Fig. 2a,b).

The intestinal IEL compartment of adult \textit{Bttnl1}\textsuperscript{Δgt} mice strikingly phenocopied the complete \textit{Bttnl1} knockout, displaying substantial and significant reductions of Vγ7\textsuperscript{+} cells, and of Vγ7Vδδ\textsuperscript{+} cells that we previously showed to be particularly affected by \textit{Bttnl1} deficiency\textsuperscript{16}; hence, the profound γδ-regulatory impact of \textit{Bttnl1} seems attributable exclusively to IEC (Fig. 2b, c). Most unexpectedly, however, \textit{Bttnl4}\textsuperscript{ΔΔ} and \textit{Bttnl6}\textsuperscript{Δgt} mice showed an overtly intermediate phenotype, with Vγ7\textsuperscript{+} IEL and Vγ7Vδδ\textsuperscript{+} IEL significantly reduced relative to either C57BL/6 or \textit{Bttnl6}\textsuperscript{fl/−} mice, as measured by a 33--50% decrease in their percentage representation among gut γδ cells or by a 2-fold drop in absolute numbers of Vγ7\textsuperscript{+} IEL, by contrast to the cells’ almost complete loss from \textit{Bttnl1}\textsuperscript{Δgt} mice (Fig. 2b–d).

The few residual IEL in \textit{Bttnl1}\textsuperscript{Δgt} mice showed major dysregulation of the signature Vγ7\textsuperscript{+} IEL phenotype, with many cells displaying low expression of CD122 (the IL-15R chain) and high Thy1 (CD90) expression (Fig. 2d, e; Supplementary Fig. 2c). Strikingly, such dysregulation was not true for \textit{Bttnl6}\textsuperscript{Δgt} or \textit{Bttnl6}\textsuperscript{ΔΔ} mice, in which residual IEL showed comparable phenotypes to controls (Fig. 2d, e; Supplementary Fig. 2c). Furthermore, whereas the Vγ7Vδ4 TCR mean fluorescence intensity (MFI) was somewhat lower in \textit{Bttnl1}-deficient mice, consistent with defective selection\textsuperscript{16}, it was unaltered in \textit{Bttnl1}\textsuperscript{Δgt} and \textit{Bttnl6}\textsuperscript{ΔΔ} mice (see Fig. 2c). Hence, \textit{Bttnl1} and \textit{Bttnl6} differentially affected Vγ7\textsuperscript{+} IEL development, with \textit{Bttnl6} required for the normal size of the IEL compartment, but not for the acquisition of the signature phenotype by residual Vγ7\textsuperscript{+} IEL. Moreover, the unique phenotype of \textit{Bttnl6}\textsuperscript{ΔΔ} mice was specific in that myriad immune subsets in the spleen were unaltered relative to controls (Supplementary Fig. 2d; Supplementary Table 1), as was reported for \textit{Bttnl1−/−} mice\textsuperscript{16}.

To test whether those Vγ7\textsuperscript{+} IEL that developed seemingly normally in \textit{Bttnl6}-deficient mice were \textit{Bttnl}-dependent, we generated mice lacking all three intestinal epithelial \textit{Bttnls}, by targeting sites upstream of the initiator ATG codons in \textit{Bttnl1} and \textit{Bttnl6}, respectively (note that those genes are transcribed in head-to-head orientation), thereby deleting over 130 kb in between, including the \textit{Bttnl4} gene (Fig. 2f). The resultant \textit{Bttnl4}\textsuperscript{ΔΔΔ} mice expressed no detectable \textit{Bttnl1}, \textit{Bttnl4} or \textit{Bttnl6} transcripts, and there was also reduced expression of the \textit{Bttnl2} gene that immediately flanks the deletion. Conversely, \textit{Psmb9}, which is more distal to the recombination point was unaffected (Supplementary Fig. 2e).

When \textit{Bttnl4}\textsuperscript{ΔΔΔ} mice were contemporaneously compared with \textit{Bttnl1}\textsuperscript{ΔΔ}, \textit{Bttnl6}\textsuperscript{Δgt} and \textit{Bttnl4}-deleted mice (\textit{Bttnl4KOMP}) that we previously characterised\textsuperscript{16}, it was clear that \textit{Bttnl4}\textsuperscript{ΔΔΔ} mice (colour-coded green in Fig. 2g–i) largely phenocopied the near-ablation of Vγ7\textsuperscript{+} IEL in \textit{Bttnl1}-deficient mice (Fig. 2g, h). In both cases, residual Vγ7\textsuperscript{+} cells showed reduced Vγ7 and Vδ4 TCR MFI and failed to upregulate CD122 or downregulate Thy1, by comparison to IEL in control, \textit{Bttnl4}\textsuperscript{KOMP} or \textit{Bttnl6}-deficient strains (Fig. 2g, i). When we integrated data from large numbers of mice of the different strains described, it became clear that the strains’ respective Vγ7\textsuperscript{+} IEL compartments were consistent and stable over time for >120 days (Supplementary Fig. 2f).

In sum, further support for the heteromer hypothesis was provided by the developmental dependence of approximately one-third to one-half of intestinal Vγ7\textsuperscript{+} IEL on \textit{Bttnl6} as well as \textit{Bttnl1}. Nonetheless, an anticipated nuance was introduced in that there was a hierarchy of \textit{Bttnl} regulation, with Vγ7 IEL numbers depending almost completely on \textit{Bttnl1}, partially on \textit{Bttnl6}, and not on \textit{Bttnl4}, while the signature phenotypes of Vγ7\textsuperscript{+} IEL present in the different strains were largely dependent on \textit{Bttnl1}, but independent of either \textit{Bttnl4} or \textit{Bttnl6}.

\textit{Bttnl6} deficiency alters Vδ gene usage. Although Vγ7 usage denotes the signature intestinal γδ IEL compartment, Vδ usage is also limited to some degree, with Vδ4 (encoded by the \textit{Trdv2-2} gene) and Vδ7 predominating, whereas ≤10--15% of Vγ7\textsuperscript{+} cells express TCR Vδ6.3 (encoded by identical \textit{Trdv6D-I} and \textit{Trdv6N-1} genes) (Fig. 3a)\textsuperscript{19}. Conversely, slightly more Vγ7\textsuperscript{−} IEL in wt mice expressed Vδ6.3, although the TCR MFI was lower vis-à-vis Vγ7\textsuperscript{+}Vδ6.3\textsuperscript{+} cells, typical of unselected cells (Fig. 3a, top panels).

By contrast, Vδ6.3\textsuperscript{+} cells with high TCR MFI accounted for >30% of residual Vγ7\textsuperscript{+} IEL in both \textit{Bttnl6}-mutant strains, a highly
significant difference from controls (Fig. 3a–c, Supplementary Fig. 3). This unanticipated finding reflected the fact that Vγ7+Vδ6.3+ IEL numbers were essentially unaltered in Btnl6Δ/gut and Btnl6Δ/Δ mice versus wt mice, despite total Vγ7+ IEL being reduced by ~2-fold (above) (Fig. 3b). Although some Btnl1Δ/gut mice showed small increases in the percentage of Vδ6.3+ cells among Vγ7+ IEL (Fig. 3a, bottom plots; Supplementary Fig. 3), the absolute numbers of Vγ7+ IEL in this strain were so negligible as to make such comparisons somewhat unreliable (Fig. 3b). Indeed, the very few residual Vγ7+ IEL in Btnl146INDEL mice showed no significant increases in Vδ6.3 representation, although there was some reduction in Vδ6 usage (Fig. 3c, Supplementary Fig. 3a). In sum, Vγ7+Vδ6.3+ IEL showed essentially no requirement for Btnl6, by contrast to their dependence onBtnl1.
We therefore investigated whether Vy7+ Vδ6.3+ might be regulated by a Bn1n + Bn1n4 heteromer.

Vy7+ IEL respond to different Bn1n combinations. Biochemical and molecular evidence has shown that Bn1n + Bn1n6 function is mediated by Bn1n6 engaging Vy7, while Bn1n acts as a critical chaperone. Bn1n4 has near-identity to Bn1n6 in the region (CFG) that engages Vy7 and both are diverged from Bn1n (Fig. 4a, colour-coded orange, blue and red). To interrogate whether Bn1n4 might substitute for Bn1n6, we subjected primary IEL to co-culture with the MODE-K enterocyte cell line expressing either Bn1n + Bn1n6 (L1L6) or Bn1n + Bn1n4 (L1L4). The former pairing induced TCR downregulation and CD25 expression, as assessed by RNAscope at 8 days and 22 days (Fig. 3b,c), possibly consistent with their having been selectively expanded and matured by some combination of Bn1n4 and Bn1n.

To further investigate Vy7+ IEL regulation by Bn1n, Bn1n4 and Bn1n6, we expressed each separately and in combination in 293T cells. Surface display of Bn1n6 was highly inefficient, but was rescued by co-expression of Bn1n (Supplementary Fig. 4a). Conversely, Bn1n4 alone could travel to the cell surface (Supplementary Fig. 4a): hence, Bn1n6 and Bn1n4 are not strictly comparable. Vy7+ J76 transductants (see above) were strongly stimulated by cells co-expressing Bn1n1 + 6 but not by cells expressing Bn1n6 alone, nor by an admixture (Bn1n1/Bn1n6 sep) of cells expressing Bn1n6 with cells expressing Bn1n1 (Supplementary Fig. 4b, red). Conversely, 293T cells transduced with Bn1n4 alone showed some capacity to stimulate Vy7+ J76 cells, although this was clearly increased by co-expressing Bn1n1, but not by stimulating with an admixture of cells separately expressing Bn1n4 and Bn1n1 (Supplementary Fig. 4b, blue). These data evoke the activity of human BTNL3, a human Vy4+ -TCR ligand, which alone can provoke human Vy4+ -TCR downregulation, but whose effects are greatly amplified by BTNL8 co-expression.

In sum, Bn1n4 is evidently not required for Vy7+ IEL selection, but its capacity to stimulate Vy7+ IEL in vitro likely explains its capacity to select IEL, primarily Vy7+ Vδ6.3+, in Bn1n6-deficient mice. The starkly different phenotypes of Bn1n6-deficient and Bn1n1-deficient mice, argues that any Bn1n4-intrinsic capacity to select Vy7+ IEL relies in vivo on co-expression with Bn1n1. Added to this, our data show that the signature preferential responses of Vy7V84+ cells to Bn1n1 + 6 was seen only in cells from mice in which Bn1n6 was expressed. This conditioning might be enforced during developmental selection, and/or be maintained in the steady-state by Bn1n heteromers expressed in epithelial cells that juxtapose mature IEL. However, there has not hit hitherto been formal evidence of a maintenance function for Bn1n genes, beyond their roles in selection. We therefore investigated this by use of conditional knockout mice.

Phenotypic maintenance by Bn1n1 and Bn1n6. We crossed floxed Bn1n1 and Bn1n6 strains to tamoxifen-regulated Villin-Cre mice, in order to generate mice in which Bn1n1 and Bn1n6 were inducibly deleted in IEC. Indeed, there was sustained loss of Bn1n1 and Bn1n6 expression, as assessed by RNAscope at 8 days and 22 days
against each other in Vγ7+ chain usage in animals of indicated genotypes.

Over a 15-day period post tamoxifen-mediated Btnl1/Btnl6 (Fig. 5a), whereas there was no effect of tamoxifen treatment on Btnl1 deletion had occurred in enterocyte stem cells, as reported36. Thus, signature IEL could be deficient alters Vδ chain usage in animals of indicated genotypes. a FACS plots of TCRδ chain usage in animals of indicated genotypes. The Vδ4 and Vδ6.3 chains are plotted against each other in Vγ7+ IEL (left column) and Vγ7− cells (right column). b Quantification of Vγ7+ Vδ6.3+ (top) and Vγ7− Vδ6.3+ cell numbers (bottom), in animals of indicated genotypes. nCtrl: 12, nBtnl1Δ: 2, nBtnl6Δ:: 2, nBtnl6Δ::Δ: 3. Data are mean ± SD of a representative experiment. c Quantification of Vδ-chain usage in Vγ7+ IEL as depicted in quadrants (a) in animals of indicated genotypes nCtrl: 7, nBtnl1Δ: 2, nBtnl4KOMP: 3, nBtnl6Δ::: 5, nBtnl6Δ::Δ: 4. Statistical analysis: two-way ANOVA & Tukey’s multiple comparison post test. Data are mean ± SD of a representative experiment.

Fig. 3 Btnl6 deficiency alters Vδ gene usage. a FACS plots of TCRδ chain usage in animals of indicated genotypes. The Vδ4 and Vδ6.3 chains are plotted against each other in Vγ7+ IEL (left column) and Vγ7− cells (right column). b Quantification of Vγ7+ Vδ6.3+ (top) and Vγ7− Vδ6.3+ cell numbers (bottom), in animals of indicated genotypes. nCtrl: 12, nBtnl1Δ: 2, nBtnl6Δ:: 2, nBtnl6Δ::Δ: 3. Data are mean ± SD of a representative experiment. c Quantification of Vδ-chain usage in Vγ7+ IEL as depicted in quadrants (a) in animals of indicated genotypes nCtrl: 7, nBtnl1Δ: 2, nBtnl4KOMP: 3, nBtnl6Δ::: 5, nBtnl6Δ::Δ: 4. Statistical analysis: two-way ANOVA & Tukey’s multiple comparison post test. Data are mean ± SD of a representative experiment.

following the start of 5 days’ of tamoxifen administration (Fig. 5a), whereas there was no effect of tamoxifen treatment on mice lacking the relevant Cre allele (Fig. 5a; middle column). Durable loss of Btnl1 and Btnl6 expression suggested that gene deletion had occurred in enterocyte stem cells, as reported36. Over a 15-day period post tamoxifen-mediated Btnl1/Btnl6 deletion, no significant reduction was apparent in the representation of Vγ7+ intestinal IEL, particularly by comparison to the reduced numbers seen in constitutively deleted Btnl1Δ/Δ mice and Btnl1Δ/Δ mice (Fig. 5b). Thus, signature IEL could be maintained at steady-state for at least two weeks in the absence of either Btnl1 or Btnl6.

Nonetheless, to investigate whether there might be more immediate effects of Btnl1/Btnl6 deletion, we applied tamoxifen daily for 3 days, and examined IEL 3 days later (Fig. 5c). Within this short time-frame, CD122 expression was markedly reduced on a large percentage of Vγ7+ IEL in both Btnl1Δ/ΔVcreERT2+ and Btnl6Δ/ΔVcreERT2+ mice (Fig. 5d). While this echoed the limited expression of CD122 by residual Vγ7+ IEL in constitutive Btnl1−/− mice, it seemed a priori to conflict with sustained CD122 expression in constitutive Btnl6−/− mice (above). This conflict, however, was resolved by the finding that in mice acutely depleted of Btnl6, rapid CD122 downregulation was mostly limited to approximately half of Vγ7Vδ6.3+ IEL (Fig. 5d). Indeed, Vγ7Vδ6.3+ IEL (that are
disproportionately enriched in constitutive Btnl6−/− mice) were much less affected by acute depletion of Btnl6 versus Btnl1 (Fig. 5d), providing another example of the differential effects of Btnl proteins on different Vγ7+ IEL subsets.

In this regard, we hypothesise that Vγ7Vδ6.3+ IEL may have been selected on Btnl1+4 even in wt mice, with their CD122 expression likewise maintained by Btnl1+4; hence, they were essentially insensitive to acute Btnl6 depletion, phenocopying Vγ7Vδ6.3+ IEL and some Vγ7Vδ4+ IEL in constitutive Btnl6−/− mice. Evidence in support of this hypothesis was provided by further analysis of Vδ usage by Vγ7+ IEL, which was essentially unaffected at 3 days following Btnl6 depletion, but which was significantly skewed toward Vδ6.3+ cells by day 56 (Fig. 5e, f). This would be consistent with natural IEL turnover favouring newly-maturing Vγ7Vδ6.3+ IEL versus Vγ7Vδ4+ IEL, since followingBtnl6 deletion, the former could more efficiently engage Btnl1+4.

The differential impacts of Btnl1, 4 and 6 on different IEL subsets might reflect their different spatio-temporal regulation. We therefore analysed single-cell RNA data available from studies in which distinct small intestinal populations were investigated. Consistent with our and others’ studies34,38, all three Btnls were restricted to enterocytes and enterocyte progenitors (Supplementary Fig. 5a), and spatially each peaked...
Fig. 4 Vγ7+ IEL respond to different Btnl pairings. a Left: Alignment of the IgV-domain sequences of Btnl1, Btnl4 and Btnl6. Canonical Ig-fold β-strands [A, B, C, C', D, E, F, G] are indicated with arrows. CFG face motifs previously shown in Btnl6 to be critical for the response of Vγ7+ cells are highlighted in orange (AQTPT/SRFSE/SRFSA), blue (QF/HF/HH) and red (SVEVS/YDEA/VEEAI). Right: Cartoon representation of the IgV-domain of Btnl6, derived with 3D-JIGSAW from the crystal structure of BTN3A1 (PDB accession code 4F80), with the same annotation as in (a). Side chains are displayed for the two residues that differ in the CFG face motifs of Btnl6 versus Btnl4 (Ala versus Glu, Glu versus Asp). b TCR downregulation (left) and CD69 upregulation (right) by J76 cells expressing a Vγ7Vδ4 TCR and co-cultured with 293T transiently transfected with Btnl1 in combination with Btnl4 wild-type (L4WT) or mutated in the CFG region as indicated on the X-axis. Results are normalised to 293T transfected with empty vector (EV). Data are represented as mean ± SD of duplicate co-cultures, representative of n = 2 independent experiments. FC, fold change. c Experimental setup to analyse IEL from various KO strains in co-cultures with MODE-K cells overexpressing either Btnl1 and Btnl4 (L1L4) or Btnl1 and Btnl6 (L1L6). IELs are isolated from indicated mouse strains which can display distinct combinations of Btnl molecules on the epithelial surface during development. Following isolation, IEL were co-cultured o.n. with MODE-K cells displaying either Btnl1+ or Btnl1+ knockout on their surface. MODE-K cells transduced with empty vector (EV) were used as control. d IEL response to MODE-K cells expressing different Btnl dimers (L1L4 or L1L6) was measured by analysing CD25+ cells gated on Vγ7+ cells in animals of indicated genotypes. Data are mean ± SEM of five independent experiments, n Btnl1KO: 8, nBtnl4KO: 9, nBtnl14Indel: 10, nBtnl14Indel: 15. Statistical analysis: two-way ANOVA & Tukey’s multiple comparison post test. *IEL response to MODE-K cells expressing different Btnl dimers (L1L4 or L1L6) was measured as %CD25+ cells and further gated on Vγ7+/Vδ4+ (left) or Vγ7+/Vδ6+ (right) cells in animals of indicated genotypes. Data are mean ± SEM of five independent experiments, nBtnl1KO: 13, nBtnl4KO: 9, nBtnl14Indel: 10, nBtnl14Indel: 15. Statistical analysis: two-way ANOVA & Tukey’s multiple comparison post test.

in the middle of the basal-apical villus axis (regions V2–V4) (Supplementary Fig. 5b), aligning with the distribution of Vγ6+ IEL. In sum, there was no obvious difference in spatio-temporal expression that might explain the proteins’ differential effects, although there was an apparent hierarchy of RNA expression levels—Btnl1 >> Btnl6 > Btnl4— that evoked the hierarchy of the genes’ effects on Vγ7+ IEL.

Response maintenance by Btnl1 and Btnl6. To further investigate the requirement for sustained expression of Btnl genes, we examined IEL at 54 days after gut epithelium-specific deletion of the whole Btnl1,4,6 locus, making comparisons with wt mice and constitutive Btnl14Indel mice (Fig. 6a). (Note, acute loss of Btnl1,4,6 could not be examined because of variable penetrance of locus deletion until 1-month post tamoxifen treatment.) Locus loss for ∼8 weeks again failed to diminish Vγ7+ IEL numbers (Fig. 6b, middle panel, light green bars), supporting the conclusion that steady-state maintenance of Btnl-selected Vγ7+ IEL numbers does not require sustained Btnl expression. Moreover, there was no significant increase in Vδ6.3+ cells consistent with there being no Btnl1 + 4 heteromers to promote their selective advantage (Fig. 6c). Interestingly, however, induced Btnl1,4,6 locus deletion also phenocopied constitutive Btnl14Indel mice in that the capacity of co-cultured Vγ7+ IEL to respond preferentially to Btnl1 + 6 versus Btnl1 + 4 was lost over time (Fig. 6d). Diminished responses to Btnl1 + 6 were seen for Vγ7Vδ4+ IEL and particularly for Vγ7Vδ6.3+ cells (Fig. 6e). This provides further support for the hypothesis that Btnl6 needs to be sustained to establish and to maintain the phenotype of cells that preferentially respond to Btnl1 + 6.

Discussion y6 T cells, particularly those residing within extralymphoid tissues, have been increasingly implicated in the regulation of tissue maintenance and protection against cancer. Nonetheless, the cells’ biologies remain poorly elucidated. Germane to this, a substantive advance was made by the discovery that different compartments of mouse and human y6 T cells are critically and specifically regulated by butyrophilin and butyrophilin-like (Btnl) proteins. Moreover, recent cell biological, molecular and biochemical data fuelled the hypothesis that the active forms of Btnl proteins may be heterodimers, although there was heretofore no formal evidence supporting this in vivo. This study now provides genetic evidence for the importance of Skint/Btnl proteins functioning collaboratively, as would be the case for heteromers. In addition, our approach has revealed some surprising findings that emphasise the importance of genetics in understanding cell regulatory mechanisms.

Thus, signature murine skin y6 IEL are shown to depend upon Skint1 + Skint2 and the normal intestinal y6 IEL compartment shown to depend upon Btnl1 + Btnl6. However, whereas Btnl2+/−/− mice lacked the great majority of Vγ7+ IEL, ~50% were retained in different strains of Btnl6-deficient mice. In seeking to understand this unanticipated hierarchy of Btnl proteins, we identified a potential of Btnl4 to substitute for Btnl6. However, whereas Vγ7+ IEL from wt mice ordinarily responded better in vitro to Btnl1 + Btnl6 versus Btnl1 + Btnl4, this was not so in Btnl6-deficient mice wherein the compartment of mature Vγ7Vδ6.3+ and Vγ7Vδ4+ IEL, that was presumably selected by Btnl1 + Btnl4, responded comparably well to Btnl1 + Btnl4.

The CD122hi phenotype of most Vγ7+ IEL was reduced when Btnl1 was acutely depleted, providing formal evidence that sustained expression of a Btnl gene-product is required to maintain the signature status of the wt y6 IEL compartment. By contrast, only a fraction of Vγ7+ IEL showed CD122 downregulation when Btnl6 was acutely depleted. Moreover, the unaffected cells were enriched in Vδ6.3+ cells, phenocopying the repertoire composition in constitutive Btnl6-deficient mice. These data are consistent with the hypothesis that whereas Btnl4 is not required for the selection and/or maintenance of any Vγ7+ IEL, some IEL in wt mice have naturally selected on Btnl1 + Btnl4 while others selected on Btnl1 + Btnl6. Indeed, we propose that discrete Btnl heteromers ordinarily select those cells that respond most strongly to them and/or that they condition the responses of the cells they select. Thereafter, the Btnl heteromer on which the cells are selected is required to maintain the cells’ signature phenotype. In sum, Btnl proteins operate in different combinations (i.e. show combinatorial diversity) in refining and regulating the composition of IEL compartments.

The biophysical basis for the preference of some cells for Btnl1 + Btnl6 versus Btnl1 + Btnl4 is unresolved. Btnl6 and Btnl1 physically associate, either directly or via an intermediate, and in this complex Btnl6 seemingly interacts directly with Vγ7+. Although there is currently less evidence available for the direct interaction of Btnl4 with Btnl1, it is likely that the two associate given the Btnl1-dependence of essentially all Vγ7+ IEL, and the capacity of Btnl1 co-expression to greatly increase the impact of Btnl4 on Vγ7+ IEL. In this regard, the association of BTN2A1 and BTN3A1, which are jointly required to regulate human Vγ9Vδ2 cells, only became evident after chemical cross-linking. Nonetheless, some capacity of some mouse or human Btnl
proteins (e.g. Btnl4; BTNL3), when over-expressed, to traffic to the cell surface and to regulate γδ IEL, albeit suboptimally, leaves open the possibility that non-heteromeric complexes might be active, e.g., in disease settings in which BTN1 proteins might be dysregulated.

Btnl1-dependence has to date been attributed solely to Vγ7, and so an influence of Vδ chains on the Btnl response was a priori surprising. Possibly pairings with particular Vδ chains might affect the response by altering the quaternary structures of TCRs. Alternatively, Btnl1 + 4-responsive IEL may comprise qualitatively distinct cells whose responsiveness might reflect their development along a distinct pathway: indeed, Vδ6.3 expression has been associated with PLZF-expressing innate-like lymphocytes. This issue will be addressed by single-cell transcriptomics.

**Figure Legends:**

a) Tamoxifen 5x
Analysis day 8/15/22
Btnl6f/fVcreERT2
Btnl6f/fVcreERT2
Btnl1f/fVcreERT2

b) % Vγ7+ CD3+ cells

| Group | Tamoxifen 8/15/22 |
|-------|------------------|
| control | 45.7 ± 0.4 |
| Btnl6f/fVcreERT2- | 50.4 ± 0.5 |
| Btnl6f/fVcreERT2 | 52.9 ± 0.5 |
| Btnl1f/fVcreERT2- | 47.9 ± 0.5 |
| Btnl1f/fVcreERT2 | 45.7 ± 0.5 |

**Figure e:**

- gate: TCRγCD3^+ |
- CD122-Pe |

**Figure f:**

- Vδ6.3-BV711 |
- CD3-APC-Cy7 |

By single-cell transcriptomics.
Fig. 5 Depletion of individual Btnl genes does not impact Vγ7 IEL numbers but differentially affects CD122 expression. a Top: Experimental scheme to analyse the effect of Btnl1 and Btnl6 tamoxifen-mediated depletion at different timepoints. Bottom: RNAscope analysis for Btnl1 and Btnl6 in animals of indicated genotypes at 3 or 17 days post tamoxifen administration. Data are representative micrographs from one time course experiment with numbers of gut sections stained per genotype as: day 8: nCtrl: 4, Btnl1f/f-VcreERT2: 2, Btnl6f/f-VcreERT2: 4, day 22: nCtrl: 4, Btnl1f/f-VcreERT2: 1, Btnl6f/f-VcreERT2: 2; scale bar: 200 μm. b Quantification of Vγ7+ IEL cells at indicated timepoints post tamoxifen (red and blue side arrows denote for comparison the average percentage of Vγ7+ IEL in full knockout animals (see also Figs. 1–3). Data are mean ± SD, day 8: nCtrl: 6, Btnl1f/f-VcreERT2: 2, Btnl6f/f-VcreERT2: 3, day 22: nCtrl: 2, Btnl1f/f-VcreERT2: 1, Btnl6f/f-VcreERT2: 4; day 15: nCtrl: 2, Btnl1f/f-VcreERT2: 3, Btnl6f/f-VcreERT2: 3; day 30: nCtrl: 2, Btnl1f/f-VcreERT2: 1, Btnl6f/f-VcreERT2: 4; c Top: Experimental scheme to analyse the effect of Btnl1 and Btnl6 tamoxifen-mediated depletion after 3 days. Bottom: V6-chain usage in Vγ7+ IEL in control (black), Btnl1f/f/VillinCreERT2+ (orange), and Btnl6f/f+ (purple) and Btnl6f/f/VillinCreERT2+ (light blue) animals. The V64 and V66.3 chain gated on Vγ7+ IEL are plotted against each other. d Histogram of surface CD122 expression in indicated subpopulations of Vγ7+ IEL in animals of indicated genotypes. e Percentage of Vγ7 cells (left) and usage of the V64 and V66.3 chain (right) in Vγ7+ IEL. 56 days after tamoxifen in Btnl1f/f/VillinCreERT2+ (black) and Btnl6f/f/VillinCreERT2+ (blue) animals. f Quantification of V6-chain usage in Vγ7+ IEL in control, Btnl1f/f/VillinCreERT2+ and Btnl6f/f/VillinCreERT2+ knockout animals, 3 (left graph) and 56 days (right) after tamoxifen administration. Mean ± SEM from two experiments per timepoint, left graph: nCtrl: 6, Btnl1f/f-VcreERT2: 3, Btnl6f/f-VcreERT2: 6; nBtnl6f/f+; 2, right graph: nCtrl: 5, Btnl6f/f-VcreERT2: 15.

Because the murine gut epithelium expresses Btnl1, Btnl4 and Btnl6, it is also not obvious why Btnl1 + Btnl6 is the dominant selecting combination, although this might reflect expression levels (considered above), a prospect which cannot be investigated at the protein level until appropriate reagents are available.

Intriguingly, Vγ7+ IEL numbers did not decline over many weeks following acute Btnl gene locus ablation. This was surprising given that the IEL showed reduced expression of CD122, the receptor for IL-15 which is an important IEL growth factor. Possibly, Vγ7+ IEL were still able to compete for IL-15 because of the reduction in receptor expression by most such cells. Alternatively, the impact of reduced IL-15R expression on IEL might become evident by assessing the cells’ replenishment in mice following infection or injury. The Btnl-dependence of sustained CD122 expression is also interesting in the light of reports that IL-15 regulates mucosal T-cell mobility within the gut, as part of immune surveillance.

This scenario may model human disease settings where BTN1 protein becomes altered, e.g. by inflammation or other gut pathophysiology. However, the consequences may be greater than in mouse, because to date the potential to make only one type of heteromer (BTN1L3 + 8) has been identified in the human colon. Hence, the reduced expression of either BTN1 protein, as has been reported in colon cancer (www.oncomine.org), might undermine the capacity to sustain the normal IEL repertoire and its functions in tissue maintenance, that have seemingly been conserved from agnathans to Homo sapiens. Finally, we note that future studies should investigate whether Skint/Btnl/BTN1 heteromers exert cell-autonomous effects on the epithelial cells that express them, outside of the impacts on their local lymphocyte compartments.

Methods

RNAscope. RNAscope was performed using probes for Btnl1 and Btnl6 according to the manufacturer’s instructions. RNAscope was performed on paraffin-embedded sections using probes and kits obtained from Advanced Cell Diagnostics/biotechene using the RNAscope 2.0 HD Reagent Kit-BROWN. Reference sequences are as follows: Btnl1, GenBank:NM_01111094.1 (576–1723); Btnl4, GenBank:NM_030746.1 (560–968); Btnl6, GenBank:NM_030747.1 (245–1552) and images were acquired using a Zeiss Axio/scan Z1 slide scanner and Zen Image acquisition software (Zen Blue, v2.6 Carl Zeiss Microscopy).

Tissue-specific deletion of genes. Tissue-specific deletion of genes was achieved by crossing floxed Cre-transgenic lines: Pkg-Cre (MGI: 2178050), Villin-Cre (MGI: 3053819) and VillinCre/ERT2 (MGI: 3053826). Tamoxifen (Sigma, T5648) dissolved in corn oil (Sigma, C8667) was administered on consecutive days as indicated via i.p. injection and animals were sacrificed on indicated timepoints. Successful deletion was confirmed by qPCR.

Spleen immunophenotyping. Comprehensive immunophenotyping of Btnl6+/- mice was performed using a platform developed by the Wellcome Trust Infection and ImmunityImmunophenotyping (3i) consortium (www.immunophenotyping.org)39. In brief, Spleen and MLN were digested with collagenase (1 mg/ml)/DNAse (0.1 mg/ml) in 2% FCS PBS (+/−Ca/Mg) for 20 min at 37 °C and filtered through 30 μm 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 20 min. Full details regarding phenotyping panels are included in Table S1. Samples were acquired on a BD LSR Fortessa X-20 equipped with 405 nm (40 mW), 488 nm (50 mW), 561 nm (50 mW) and 640 nm (100 mW) lasers.

Mice. Wild-type (WT) C57BL/6J and FVB mice were obtained from Jackson Laboratories. NF-Skint1 +/-, Btnl1-KOMP, Btnl4-KOMP and Skint1-/-+ mice have been previously described16,29,32. Genetically engineered mice were generated at the Francis Crick Institute’s Transgenic Facility. The sg RNAs & PAM sequences (see Table 1) 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 by the Francis Crick Institute’s Transgenic Facility. Targeted animals were identified and validated by PCR and later genotyped using the Transnetyx platform. All animals were maintained at The Francis Crick Institute’s Biological resource facilities with a 12 h light/dark cycle and access to food and water ad libitum, temperature 19–23 °C, 55 ± 10% humidity. Animal experiments were undertaken in full compliance with UK Home Office regulations and under a project license to A.C.H. (7009056).

Generation of Skint1, Skint2 rat monoclonal antibodies. Rat monoclonal antibodies against Skint1 and Skint2 were generated by immunization of Lou/c rats with purified GST-tagged human Skint1 or Skint2 extracellular domain, respectively. Hybridoma cells were generated and binding to Skint1 or Skint2 protein was analysed by enzyme-linked immunosorbent assay (ELISA). Positive hybridoma supernatants were further assayed for their potential in immunoblotting. Hybridoma clones Skint1 2G2 and Skint2 3G8 (both IgG2a/k) were recloned by limiting dilution to obtain stable monoclonal cell lines.

Quantitative RT-PCR. Samples were stored in RNAlater (Ambion) or directly frozen in RLT buffer prior to RNA purification with DNAse digest (QIAGEN RNaseasy kit). cDNA was generated using Superscript-II (Invitrogen) and analysed using Sybr-green assay (Invitrogen) using a Quant-studio 5 or Viaa7 Real-time PCR machine (Applied Biosystems) and qPCR primers indicated in Table 2.

Isolation of mouse intestinal IEL. Mouse IEL were isolated from small intestine18. Briefly, small intestine was opened, washed in PBS, cut into 0.5-cm-long pieces and...
Fig. 6 Response and maintenance by Btnl1 and Btnl6. a Left: Targeting strategy to generate animals harbouring a floxed Btnl146 locus, which can be excised after tamoxifen administration. Administration of tamoxifen results in Btnl1 and Btnl6 expression loss in the IEC. Right: Experimental design for IEL analysis (c, d) and co-culture experiment (e) following Btnl146 locus depletion. During development Btnl molecules are expressed on the IEC and only after tamoxifen depletion Btnl expression is lost. Following loss of Btnl expression, IELs are harvested and subjected to co-cultures with MODE-K cells expressing specific Btnl combination.

b Quantification of αβ (left), Vγ7+ (middle) and Vγ1+ (right) T cells following Btnl146 locus depletion. nctrl: 9, nBtnl146VcreERT2+: 14. Data are mean ± SD.

c Quantification Vδ chain usage in Vγ7+ cells in animals of indicated genotypes under indicated conditions. nctrl: 6, nBtnl146VcreERT2+: 8. Data are mean ± SD.

d Co-culture of MODE-K cells transduced with EV, L1L4 or L1L6 with IEL from control, or Btnl146f/f;VillinCreERT2+ animals. Controls are pooled: Btnl146f/f,VillinCreERT2+ that did not receive tamoxifen and Btnl146f/f,VillinCreERT2– animals that did receive Tamoxifen. Data are mean ± SD (nctrl: 6, nBtnl146VcreERT2+: 8). Statistical analysis two-way ANOVA & Tukey’s multiple comparison post test.

e IEL response in co-cultures of MODE-K cells transduced with EV, L1L4 or L1L6 with IEL from control or Btnl146f/f;VillinCreERT2+ animals that did receive tamoxifen in Vγ7Vδ4+ (left graph) and Vγ7Vδ6.3+ (right graph) cells. nctrl: 6, nBtnl146VcreERT2+: 8.
IEL cultures

IEL cultures were performed19. Briefly, the medium was reduced and 10% unsorted IEL suspended in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% pen/strep, 10 g/ml d-glucose, l-glutamine, 1% penicillin streptomycin and 2×5×10^5 transiently transfected 293T cells, followed by co-culture for 5 h. The ear epidermis was separated from dermis, minced with razor blades. Samples were digested with Milleniyi Multi Tissue Dissociator kit 1, according to the manufacturer’s instructions. Brieﬂy, samples were transferred to GentleMACS C tubes containing 2.5 mL digestion mix (100 μL Enzyme D, 50 μL Enzyme R and 12.5 μL Enzyme A) directly boiled for analysis. Beads, Eluate or samples were mixed with NuPAGE LDS Sample Buffer supplemented with 1xNuPAGE reducing agent and separated by electrophoresis on NuPage 4–12% Bis-Tris protein gels (Thermo Fisher) and then transferred onto PVDF membranes. Membranes were blocked in PBS-0.1% Tween 20 and incubated with primary antibodies overnight. Membranes were washed three times with PBS-0.1% Tween 20 and incubated with secondary antibodies, washed again and developed using ECL detection reagents (Merck).

Biochemistry

Cells were lysed for 30 min in ice cold RIPA buffer with protease inhibitors (Roche) and phosphatase inhibitors (Phosphatase inhibitor cocktails 2 & 3, Sigma) and spun at 20,000 × g for 15 min at 4 °C. Protein concentrations in supernatants was determined using a BCA kit (Pierce).

Immunoprecipitation from cell lysates

Lysates were pre pared on Protein G Sepharose (Millipore-Sigma) for 1 h, incubated with antibodies for 1 h followed by incubation with 1% BSA blocked Protein G beads for a further hour. Following three washes in RIPA buffer immunoprecipitates or samples were mixed with 1xNuPAGE lysis buffer and incubated at 4 °C for 16–18 h at 4 °C with 10% CO2. Immunoprecipitated samples were eluted with 3xFlag peptide to obtain the eluate or beads were coated beads overnight at 4 °C and filtering through 70 μm cell strainers. Single-cell suspensions were stained with Live/Dead Aqua for dead cell exclusion, followed by Fc-block and surface stain with specific antibodies.

Preparation of lung and uterus y0 cells

Lungs and uteri from experimental mice were collected in medium and minced with razor blades. Samples were digested with Milleniyi Multi Tissue Dissociator kit 1, according to the manufacturer’s instructions. Brieﬂy, samples were transferred to GentleMACS C tubes containing 2.5 mL digestion mix (100 μL Enzyme D, 50 μL Enzyme R and 12.5 μL Enzyme A) directly boiled for analysis. Beads, Eluate or samples were mixed with NuPAGE LDS Sample Buffer supplemented with 1xNuPAGE reducing agent and separated by electrophoresis on NuPage 4–12% Bis-Tris protein gels (Thermo Fisher) and then transferred onto PVDF membranes. Membranes were blocked in PBS-0.1% Tween 20 and incubated with primary antibodies overnight. Membranes were washed three times with PBS-0.1% Tween 20 and incubated with secondary antibodies, washed again and developed using ECL detection reagents (Merck).

Bioinformatics analysis

Raw gene counts were obtained from GSE109413 (Moor et al.) and GSE92332 (Haber et al.) and each cell-set was preprocessed using Seurat56 (version 3.1.1.9023). In the case of the data from Moor et al. data, cells with <200 and >3000 detectable genes and cells with percentage mitochondrial expression greater 5% were removed. For all cell-sets, the total counts were scaled to 1e4 counts, a log transformation applied and genes were z-score across all cells.

Preparation of epidermal sheets

Ear epidermis was separated from dermis following incubation in 0.5 M ammonium thiocyanate for 35 min at 37 °C. Isolated epidermal sheets were fixed with ice cold acetone at −20 °C. The samples were blocked in 5% FCS for 1 h at room temperature and stained for 1 h at 37 °C using Vy 3 TCR-TCIT (clone 536, BD), MHIC 1/A-I-E-F/647 (clone M5/114.52.2, Bio-Legend) and CD45-eFluor450 (clone 30-F11, ebioscience) antibodies. Tissue samples were mounted on microscope glass in Prolong Gold mounting medium under a stereo microscope to ensure flat epidermal mounting. Confocal images were recorded using Leica SP5 confocal microscope with 40x 1.25 NA HCX PL APO CS lens. Three confocal renders 387.5 x 387.5 μm size were acquired from each epidermal sheet. Image quantification was performed using 2D Bioservices Developer software (version XD2.7). Each channel in a process was processed with Gaussian filter followed by application of automated multi-threshold segmentation. Individual cells (CD45+, Langerhans cells, and T cells) were detected based on their relative intensity in CD45, MHIC II and TCR channels, respectively. Cell number and morphology were measured for each cell type.

Cell lines

HEK293T cells (FCI) were maintained in DMEM supplemented with 12% Bis-Tris protein gels (Thermo Fisher) and 500 μg/ml hygromycin (Thermo Fisher). Transgenic J76 cells18 were maintained in RPMI 1640 l-glutamine, 10% FCS and 1% penicillin-streptomycin. All cell culture reagents were from Thermo Fisher.

Cell line co-culture.

In all, 5 × 10^6 transfected J76 was mixed in 96-well plates with 2 × 10^5 transiently transfected 293T cells, followed by co-culture for 5 h.

Plasmids and transfection

Overlap-extension PCR (OE-PCR) was used to replace the GFG regions of Btn14 with those of Btn1 on plasmids encoding Btn1/4/6/19. HEK293T cells were transfected with the indicated combinations of FLAG-Btn1, HA-Btn4, His-Btn4 and empty vector (EV) encoding plasmids. The medium was replaced 18 h after transfection and cells were harvested at 48 h and used for the co-culture assay. For antibodies see Table 3.

Preparation of epidermal sheets

Ear epidermis was separated from dermis following incubation in 0.5 M ammonium thiocyanate for 35 min at 37 °C. Isolated

| Table 1 Oligos and repair templates used for generation of floxed mice. |
|-------------------------|-----------------|
| Short guide oligo (sg) and homology repair (HDR) | Sequence 5’-3’ |
| Btnl6-5-sg-2 | TACCTGGGGGAGGATTAGG |
| Btnl6-3-sg-2 | AGGATTCACGACCATGAG |
| Btnl6-5-HDR template | CAGCCCAGATCTGGCAGGGGAAAT |
| Btnl6-3-HDR template | GTCTCCAGGTCTGCTGCTGCTG |
| Btln1 Int12 sg-3 | CAGGGCCTTCTGTCGTCG |
| Btln1 Int56 sg-2 | CAGAGGAGGCAGAGAGGAGG |
| Btln1-HDR template_1 | TCTTTATCATGGGGCCTCTACGGGAACGC |
| Flow Cytometry acquisition was performed using BD-FACS/Divia Software. | |
| Data analysis was performed using FlowLo v10.6.1 (FlowJo, LLC, Ashland OR). |

Table 2 qPCR primers.

| Target | Forward | Reverse |
|--------|---------|---------|
| Mu-Btnl1 | TGACCCAGGAAAAGCAGAGAA | CACCCGAGCAGGACCAATAGT |
| Mu-Btnl4 | CATCTCTAGCAGGACTACAT | GAGGGGCTGTGAGGAGGAGAG |
| Mu-Btnl6 | GACCTGCTCAGTTGCTGAGGAGG | AGGCACTGCCGACCTGAGATT |
| Mu-Psmb9 | TGGCTGTTGGGCTGCTGCTGCTG |
| Mu-Btnl2 | TTTGCTATGATGACCTTCC |

Bold letters indicate the PAM, underlined letters indicate the restriction site and italic letters indicate loxP.
Table 3 Antibodies used for flow cytometry, western blotting and microscopy.

| Antibodies          | Clone      | Source                | Identifier/Cat no. | Dilution |
|---------------------|------------|-----------------------|--------------------|----------|
| CD3 APC Cy7         | 17A2       | BioLegend             | 100222             | 1:400    |
| TCRβ BV421          | H57-597    | BioLegend             | 109229             | 1:300    |
| CD122 PE            | TM-β1      | BioLegend             | 123209             | 1:200    |
| Thy-1.2 BV 510      | S3-2.1     | BioLegend             | 140319             | 1:100    |
| Lag3PerC Pefluor 710| C9B7W      | ebioscience           | 46-2231-80         | 1:300    |
| CD24 BV650          | M1/69      | BD                    | 563545             | 1:400    |
| CD8α PE Cy7         | S3-6.7     | BioLegend             | 100722             | 1:200    |
| TCR V64 FITC        | GL-2       | BD                    | 552143             | 1:100    |
| TCR V64 PE          | GL-2       | BioLegend             | 134905             | 1:100    |
| CD8β PerC P Cy5.5   | YT5156.7.7 | BioLegend             | 126610             | 1:200    |
| TCR Vγ 14 FITC      | 2.1T       | BioLegend             | 141103             | 1:100    |
| TCR Vγ 4 APC        | UC3-10A6   | BioLegend             | 137708             | 1:100    |
| TCR6 BV421          | GL3        | BioLegend             | 118119             | 1:200    |
| TCR8 Pe             | GL3        | BioLegend             | 118108             | 1:800    |
| TCR6 Pe Cy7         | GL3        | BioLegend             | 118124             | 1:200    |
| CD4 BV 510          | RM4-5      | BioLegend             | 100559             | 1:100    |
| TCR V66.3/2 BV711   | 8F4H7B7    | BioLegend             | 744476             | 1:100    |
| CD25 PerC P Cy5.5   | PC61       | BioLegend             | 102030             | 1:200    |
| TCR V66.3/2-PE      | 8F4H7B7    | Pharrmingen           | 555321             | 1:300    |
| TCR Vγ 7            | F2.67      | Institut Pasteur, Paris, P. Pereira | N/A | 1:400    |
| CD45 Rδ FitC        | C363.16 A  | ebioscience           | 11-0455-82         | 1:100    |
| Vγ 5 APC            | 7-17       | BioLegend             | 137506             | 1:200    |
| TCR6 PerC Pefluor 710| GL3       | BioLegend             | 46-5711-82         | 1:200    |
| TCR6 AF647          | GL3        | BioLegend             | 118134             | 1:200    |
| CD45 PB             | HI30       | BioLegend             | 304022             | 1:100    |
| CD69 PE             | HI.2F3     | ebioscience           | 12-0691-93         | 1:200    |
| CD3 PerC P Cy5.5    | SK7        | BioLegend             | 344808             | 1:200    |
| DYDDDDD Pe Cy7      | L5         | BioLegend             | 637324             | 1:300    |
| HA AF647            | 16B12      | BioLegend             | 682404             | 1:200    |
| HIS PE              | J095G46    | BioLegend             | 362603             | 1:100    |
| CD24-2BV650         | M1/69      | BD                    | 563545             | 1:400    |
| CD62L BV421         | MEL-14     | BioLegend             | 104436             | 1:100    |
| CD44 Pe Cy7         | IM-7       | BioLegend             | 103030             | 1:100    |
| CD45 eVolve 605     | 30-F11     | ebioscience           | 83-0451-42         | 1:100    |
| TCR Vγ 5-PE         | S36        | BioLegend             | 137504             | 1:100    |
| Vγ 5V61             | 17D1, Supernatant | Yale, US, R. Tigelaar, J. Lewis | N/A | 1:2    |
| TCR-Vγ 5F5 FitC     | S36        | BD                    | 553229             | 1:300    |
| MHC I- A/1-E Af647  | M5/1415.2  | BioLegend             | 107618             | 1:500    |
| CD45 efluor 450     | 30-F11     | ebioscience           | 48-0451-82         | 1:200    |
| Skint1              | 2G2        | Monoclonal Antibody core facility (Helmholtz Zentrum Munich) | 100ul SN/IP |
| Skint2              | 3G8        | Monoclonal Antibody core facility (Helmholtz Zentrum Munich) | 110000 |
| Flag                | M2         | Merck                 | F1804              | 1:5000   |
| Goat anti rat HRP   | Thermo Fisher | Thermo Fisher | 31470              | 1:5000   |
| Goat anti mouse HRP | Thermo Fisher | Thermo Fisher | 31446              | 1:5000   |
| Flag magnetic beads | M2         | Sigma                 | M8823              | 5µl/IP   |

**Statistical analysis.** Summary data are represented as mean ± SD if representative experiments are shown or mean ± SEM if summarized data are shown as indicated in individual figures. Numbers of animals per group are indicated in individual figures. Control groups includes animals that are wt, heterozygous or homozygous without the respective Cre transgene. Heterozygous animals are comparable to WT animals.

**Modelling software.** Figures for all modelling data were generated in PyMOL v2.0.7 (Schrodinger LLC). 3D-JIGSAW was used to generate 3D models of proteins and perform docking simulations, respectively.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

This work did not include any data which mandated deposition in public databases. Associated raw data are provided in the main and/or supplementary figures. Relations to summary data charts are indicated and a full list of figures with associated raw data is provided in the reporting summary linked to this article. Raw gene counts were obtained from GSE109413 (Moor et al.) and GSE92332 (Haber et al.). For bioinformatics single-cell analysis scripts are available on github: https://github.com/ajandke/Jandke_etal_naturecomms. Immunophenotyping data for pipeline procedure can be found under https://www.mousephenotype.org/data/secondaryproject/3i.

**Code availability**

The full source codes have not been released. Publicly available servers can be accessed online for 3D-JIGSAW (https://bmm.crick.ac.uk/~svc-bmm-3djigsaw/). For bioinformatics single-cell analysis, scripts are available on github: https://github.com/ajandke/Jandke_etal_naturecomms.

Received: 5 November 2019; Accepted: 26 June 2020; Published online: 28 July 2020

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and to scientific facilities support at King’s College London. This work was supported by a Wellcome Trust Investigator Award, 106292/Z/14/Z and the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001093), the UK Medical Research Council (FC001093), and the Wellcome Trust (FC001093). L.M. was supported by the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 792383. D.M. received studentships from the King’s Bioscience Institute and the Guy’s and St. Thomas’ Charity Prize PhD program in Biomedical and Translational Science.

**Author contributions**
A.J., D.M., L.M., D.U., A.L. and P.V. designed and undertook experiments; P.E. performed bioinformatics analysis on public datasets; T.Ni, T.Na. and H.T. provided Skint1-ko mice; R.F. generated antibodies; A.J. and A.H. designed the study and wrote the paper.

**Competing interests**
A.C.H. is equity holder in GammaDelta Therapeutics and in Adaptate Biotherapeutics. The remaining authors declare no competing interests.

**Additional information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17557-y.

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