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Lymphotoxin beta receptor signaling limits mucosal damage through driving IL-23 production by epithelial cells

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Abstract

The immune mechanisms regulating epithelial cell repair after injury remain poorly defined. We demonstrate here that lymphotoxin beta receptor (LT β R) signaling in intestinal epithelial cells promotes self-repair after mucosal damage. Using a conditional gene-targeted approach, we demonstrate that LT β R signaling in intestinal epithelial cells is essential for epithelial IL-23 production and protection against epithelial injury. We further show that epithelial-derived IL-23 promotes mucosal wound healing by inducing the IL-22-mediated proliferation and survival of epithelial cells and mucus production. Additionally, we identified CD4⁻CCR6⁺T-bet⁻ROR γ t⁺ lymphoid tissue inducer cells as the main producers of protective IL-22 after epithelial damage. Thus, our results reveal a novel role for LT β R signaling in epithelial cells in the regulation of intestinal epithelial cell homeostasis to limit mucosal damage.

INTRODUCTION

The intricate balance between immune defense and inflammation in the gut is a highly regulated process that requires interactions between the intestinal epithelium and the underlying immune system. A breakdown in this balance is believed to promote the induction and perpetuation of the chronic intestinal inflammation found in patients with intestinal inflammatory diseases^{1, 2}.

Lymphotoxin beta receptor (LT β R), a member of the TNFR superfamily of cytokines, has been shown to play a critical role in the regulation of mucosal immune responses^{3–6}. Like many other members of the TNFR superfamily, LT β R signaling can mediate both protective and pathogenic effects during intestinal inflammation. While inhibition of LT β R signaling has been shown to be beneficial in a T cell-mediated colitis model⁷, studies utilizing other chemically-induced and infectious colitis models suggest that LT β R signaling plays a

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Disclosure

The authors declare no conflict of interest.

protective role against intestinal injury^{4, 5, 8–11}. Additionally, LT β R-dependent production of IL-22 by innate lymphoid cells (ILCs) has recently been shown to be essential for protection against the bacterial pathogen *C. rodentium*^{4, 10}. However, the mechanisms whereby LT β R signaling contributes to protection during epithelial damage remain to be fully elucidated.

ILCs are a heterogeneous population of innate lineage negative lymphoid cells that rapidly produce several cytokines upon stimulation and participate in the regulation of immunity at mucosal surfaces^{12–14}. The current classification of ILC populations is based on the expression of specific transcription factors that regulate their development and function, and their cytokine profiles¹⁵. Group 3 ILCs (ILC3s) are dependent on the transcription factors ROR γ t and Gata3 for their development and include CD4⁺ and CD4⁻ lymphoid tissue inducer cell populations (LTi cells), NKp46⁺ and colitogenic NKp46⁻ ILC3s^{15–18}. A protective role for ILC3s during epithelial injury was suggested by a previous study showing that ROR γ t-deficient mice, which lack all ILC3 subsets, exhibited increased susceptibility to chemically-induced colitis¹⁹. In contrast, a pathogenic role for ILC3s has also been reported in experimental colitis models and in patients suffering from IBD^{16, 20}. However, the mechanisms that regulate the function of distinct ILC3s during mucosal damage are still poorly defined.

IL-22, IL-17, and IFN γ , the principal cytokines produced by ILC3s, have major effects on epithelial cells of many tissues and are critical in defense against mucosal bacterial pathogens^{15, 21, 22}. IL-22 has been identified as an important regulator of inflammation, particularly at the barrier surfaces of the skin, lung, and intestine^{14, 23}. However, dysregulation of IL-22 signaling may also be associated with disease progression, including tumorigenesis^{24–26}. Therefore, understanding the mechanisms controlling IL-22 production by distinct ILC3s populations during mucosal damage is critical for development of effective disease therapies.

In the present study, we investigate the role of LT β R-mediated signaling between intestinal epithelial cells and ILC3s in a model of mucosal wound healing. Our data suggest that, during epithelial injury, LT β R engagement in intestinal epithelial cells drives IL-23 production. The IL-23 then induces IL-22 production by CD4⁻ LTi cells thereby promoting epithelial cell repair and homeostasis. These results support a novel mechanism wherein LT β R-mediated cooperation between epithelial cells and LTi cells regulates intestinal homeostasis to limit mucosal damage.

RESULTS

LT β R signaling protects against intestinal epithelial injury by promoting IL-22-dependent epithelial cell repair and homeostasis

To define the role of LT β R signaling in mucosal wound healing, we utilized an acute epithelial injury-regeneration model, induced by transient administration of dextran sulfate sodium (DSS) in the drinking water. Since the genetic background of the mouse can significantly influence the outcome of DSS-treatments²⁷, we first generated neo-free LT β R-deficient and LT β R floxed mice by crossing tri-loxP conditional LT β R floxed mice⁴ with ubiquitous MeuC40 deleter mice²⁸, (all on C57BL/6 background) and selecting

recombinants with a deletion of the neo cassette (Supplementary Figure S1a,b). We then treated control neo-free LT β R floxed mice (*Ltbr^{fl/fl}*) and neo-free LT β R-deficient mice (*Ltbr^{-/-}*) with drinking water containing DSS for five days followed by normal water for three days. Compared to *Ltbr^{fl/fl}* mice, *Ltbr^{-/-}* mice lost body weight rapidly and displayed reduced survival (Figure 1a,b). Consistently, the disease score, assessed daily as an average of body weight loss and signs of rectal bleeding and diarrhea, was increased in *Ltbr^{-/-}* mice (Figure 1c). Importantly, these differences were not associated with altered food or water consumption (Supplementary Figure S1c). Macroscopic examination of the colons at day 8 revealed severe pathology and significant reduction of colon length in *Ltbr^{-/-}* mice compared to control animals (Figure 1d). Additionally, while the colon mucosa and submucosa of *Ltbr^{fl/fl}* mice exhibited low histological scores reflecting only slight infiltration of inflammatory cells, *Ltbr^{-/-}* mice exhibited increased histological scores reflecting severe destruction and necrosis of epithelial cells, loss of goblet cells, thickening of the submucosa, and increased infiltration of inflammatory cells (Figure 1e). Together, these data show that LT β R signaling is essential for mucosal wound healing following epithelial injury.

To define the mechanism whereby LT β R signaling conferred protection against DSS-induced colitis, we measured the expression of genes known to be involved in the induction or regulation of intestinal inflammation at day 5, when the response of the two groups was beginning to diverge. Surprisingly, *Ltbr^{fl/fl}* and *Ltbr^{-/-}* mice displayed similar colonic mRNA expression levels for several chemokines (*Cxcl1*, *Cxcl13*, *Ccl2*, and *Ccl20*), IL-17 family members (*Il17a*, *Il17c*, *Il17e*, *Il17f* and *Il17re*), key pro-inflammatory cytokines (*Il-1b*, *Il6*, *ifng*, and *tnf*) and regulatory molecules such as *Il10* and *Trim30a* (Supplementary Figure S1d). However, we found that *Il23a* and *Il22* mRNA expression were significantly reduced in the colons of DSS-treated *Ltbr^{-/-}* mice (Supplementary Figure S1d). To define the kinetics of IL-22 production, we next measured *Il22* mRNA expression during the injury and regeneration stages of colitis. While DSS-treated *Ltbr^{fl/fl}* mice displayed a marked up-regulation of *Il22* mRNA expression in the colon between day 5 and day 8 (Figure 1f), *Ltbr^{-/-}* mice failed to upregulate colonic IL-22 (Figure 1f,g), suggesting that LT β R signaling is required for IL-22 production during intestinal injury.

IL-22 has been shown to exert its anti-inflammatory effects via the induction of a wide range of anti-microbial peptides that help to prevent the lethal dissemination of intestinal microbiota, and via reinforcing the mucus barrier and enhancing epithelial cell regeneration²⁹⁻³². Unexpectedly, we found comparable mRNA levels of *Reg3b*, *Reg3g* and *Defb4* in the colons of DSS-treated *Ltbr^{fl/fl}* and *Ltbr^{-/-}* mice (Figure 1h). Additionally, similar levels of bacterial dissemination were found in the liver and spleen of DSS-treated *Ltbr^{fl/fl}* and *Ltbr^{-/-}* mice at day 8 (Figure 1i), suggesting that the sensitivity of *Ltbr^{-/-}* mice to DSS-induced colitis is not mediated by the pathogenic dissemination of commensal bacteria. We therefore hypothesized that the sensitivity of *Ltbr^{-/-}* mice is caused by a defect in the promotion of IL-22-dependent tissue protective responses. To test this, we compared the induction of IL-22-associated mucins, which are thought to protect against intestinal inflammation^{29, 33}, between DSS-treated *Ltbr^{fl/fl}* and *Ltbr^{-/-}* mice. *Muc2*, *Muc3*, *Muc4* and *Muc13* mRNA levels were up-regulated in the colons of control mice during DSS-induced

colitis whereas the induction of these mucins was impaired in *Ltbr*^{-/-} mice (Figure 1j). Moreover, Muc2 protein, the most abundant mucin in the large intestine^{33, 34}, was expressed at a lower level in the colons of *Ltbr*^{-/-} mice during DSS-induced injury (Figure 1k). Since mucins can protect epithelial cells from damage during intestinal inflammation^{29, 33}, we next addressed whether there was increased epithelial cell death in the colon of *Ltbr*^{-/-} mice. We found that, with respect to *Ltbr*^{fl/fl} mice, *Ltbr*^{-/-} mice exhibited an increase in the number of apoptotic epithelial cells at day 8 (Figure 1l). Consistent with this observation, expression of the anti-apoptotic factors *Bcl2* and *Bclxl* was significantly reduced in the colon of *Ltbr*^{-/-} mice at day 5 (Figure 1m). Moreover, analysis of epithelial cell proliferation following BrdU pulse injection revealed that intestinal epithelial cell proliferation was markedly reduced in *Ltbr*^{-/-} mice (Figure 1n). Collectively, these data suggest that LTβR-mediated IL-22 production promotes intestinal wound healing by inducing the regeneration of the epithelial layer.

Exogenous IL-22 expression rescues LTβR-deficient mice from epithelial injury

To determine whether IL-22 production is sufficient to promote tissue repair upon DSS-induced epithelial injury, we intravenously injected control and *Ltbr*^{-/-} mice with an IL-22-expressing or control plasmid 24h after initiation of DSS treatment. IL-22-expressing plasmid significantly reduced the severity of DSS-induced colitis in *Ltbr*^{-/-} mice as indicated by reduced body weight loss, increased survival, decreased disease score, and reduced colon shortening and pathology (Figure 2a–d). These macroscopic observations were corroborated with histological analysis of colon sections which revealed reduced colon inflammation and reduced histological scores in IL-22-expressing plasmid-treated *Ltbr*^{-/-} mice (Figure 2e). Furthermore, exogenous IL-22 expression also increased *Muc2*, *Muc3*, *Muc4* and *Muc13* mRNA expression in the colons of DSS-treated *Ltbr*^{-/-} mice (Figure 2f). Taken together, these data suggest that IL-22-mediated induction of mucins is the critical mechanism downstream of LTβR signaling that promotes wound healing after epithelial injury.

LTβR signaling promotes epithelial wound healing through IL-22 production by CD4⁻ LTi cells

To identify which cells require LTβR signaling for IL-22 production during intestinal injury, we compared *Il22* mRNA expression in distinct innate and adaptive cell populations isolated from the colonic *lamina propria* (LP) of RORγt-GFP^{+/-}*Ltbr*^{+/-} and RORγt-GFP^{+/-}*Ltbr*^{-/-} reporter mice treated with DSS for five days. We found that *Il22* expression was markedly reduced in CD4⁻ and CD4⁺ LTi cells isolated from RORγt-GFP^{+/-}*Ltbr*^{-/-} mice (Figure 3a), indicating that IL-22 production by these cells is LTβR-dependent. Interestingly, *Il22* expression by NKp46⁺ ILC3 cells was not significantly impaired in the absence of LTβR signaling (Figure 3a). Importantly, we found comparable numbers and frequencies of CD4⁻ LTi, CD4⁺ LTi, NKp46⁺ ILC3, RORγt⁺ T cells, CD4⁺ T cells, NK cells, and B cells in the LP of *Ltbr*^{+/-} and *Ltbr*^{-/-} mice (Figure 3b and Supplementary Figure S2) indicating no defect in the recruitment of IL-22 producing cells to the LP in response to epithelial injury in the absence of LTβR signaling.

Production of IL-22 by ILCs has been shown to be triggered by IL-23R stimulation^{21, 22}. We found that ILCs and ROR γ ⁺ T cells isolated from the colons of *Ltbr*^{+/+} and *Ltbr*^{-/-} mice displayed similar levels of IL-23R expression (Figure 3c). Furthermore, LP ILCs isolated from *Ltbr*^{-/-} mice were able to produce IL-22 after *in vitro* stimulation with IL-23 (Figure 3d), indicating that ROR γ ⁺ ILCs from *Ltbr*^{-/-} mice do not have an intrinsic defect in IL-22 production.

To characterize the IL-22-producing ILC subsets, we purified colonic LP cells from DSS-treated *Ltbr*^{fl/fl} mice and stimulated them with IL-23. The majority of CD45⁺Lin⁻ROR γ ⁺ IL-22-producing cells expressed IL-7R α , c-Kit, Thy1.2, and Sca-1, but were poor producers of IL-17A or IFN γ (Figure 3e). Furthermore, the majority of these cells did not express the surface marker NKp46 (4.2% NKp46⁺ vs 95.8% NKp46⁻) confirming them as LTi cells. As T-bet participates in the differentiation of CCR6⁻ LTi cells to IL-22 producing NKp46⁺ ILCs during mucosal immune responses^{15, 35}, we sought to determine whether LTi cells and NKp46⁺ ILCs expressed T-bet during DSS-induced injury. We found that both CD4⁻ and CD4⁺ LTi cells were CCR6⁺T-bet⁻ whereas NKp46⁺ ILC3 cells were CCR6⁻ T-bet⁺ (Figure 3e). Remarkably, we found that the frequency of IL-22-producing CD4⁻ LTi cells was higher than the frequency of IL-22-producing CD4⁺ LTi cells (77.2% vs 22.8%, respectively) (Figure 3e), suggesting that CD4⁻ LTi cells represent a predominant source of IL-22 during epithelial injury.

To further delineate the contribution of CD4⁻ and CD4⁺ LTi cells to protection against intestinal injury, *Rag1*^{-/-} mice were treated with anti-CD4 or anti-Thy1 antibodies to deplete CD4⁺ LTi cells or both CD4⁻ and CD4⁺ ILCs, respectively. Depletion of Thy1⁺ ILCs in DSS-treated *Rag1*^{-/-} mice induced severe pathology and dramatically reduced colonic IL-22 expression (Figure 3f-i). In contrast, anti-CD4 treatment only partially reduced colonic IL-22 expression, yet it had no effect on mortality, body weight loss, or colon shortening in DSS-treated *Rag1*^{-/-} mice (Figure 3f-i), suggesting that although CD4⁺ LTi cells may contribute to LT β R-mediated IL-22 production during intestinal injury, they are dispensable for protection. Collectively, these results suggest that LT β R signaling promotes epithelial wound healing through the induction of IL-22 production by CD4⁻ LTi cells.

LT β R signaling in epithelial cells protects against intestinal epithelial injury by promoting IL-23-driven IL-22-dependent tissue protective responses

To identify which LT β R-expressing cells are essential for controlling IL-22 production during epithelial injury, we generated reciprocal bone marrow chimeric mice. Following DSS treatment, we observed reduced survival, increased body weight loss, and reduced colonic IL-22 expression in WT \rightarrow *Ltbr*^{-/-} and to a lesser extent in *Ltbr*^{-/-} \rightarrow WT chimeras with respect to WT \rightarrow WT chimeras (Figure 4), indicating that LT β R expression on both radioresistant cells and bone marrow-derived cells contributes to protection against epithelial injury. Additionally, mice lacking LT β R in CD11c⁺ cells (CD11c-*Ltbr*^{-/-}) displayed increased body weight loss, reduced survival, and reduced colonic IL-22 levels (Supplementary Figure 3), indicating a role for LT β R signaling in DCs in protection against epithelial injury, in line with previous studies in an infectious colitis model¹⁰.

Since WT→*Ltbr*^{-/-} chimeras displayed increased morbidity and colon pathology compared to *Ltbr*^{-/-}→WT chimeras (Figure 4), we hypothesized that LTβR signaling in intestinal epithelial cells may be important for IL-22-mediated wound healing after epithelial injury. To test this, we generated mice with specific inactivation of LTβR in epithelial cells (*Vil-Ltbr*^{-/-} mice). Compared to *Ltbr*^{fl/fl} mice, DSS-treated *Vil-Ltbr*^{-/-} mice displayed increased body weight loss, reduced survival, reduced colon length, and severe colon inflammation (Figure 5a–d) indicating that LTβR signaling in epithelial cells is essential for protection against epithelial injury. Comparable numbers of CD4⁺ and CD8⁺ T cells, CD11c⁺ cells, neutrophils and RORγt⁺ ILCs were found in the colons of *Ltbr*^{fl/fl} and *Vil-Ltbr*^{-/-} mice (Supplementary Figure S4), suggesting that the migration or expansion of these cells to the LP during DSS-induced injury is not impaired in the absence of LTβR signaling in epithelial cells. However, epithelial cell proliferation and the expression of the anti-apoptotic factors *Bcl2* and *Bclxl* were significantly reduced in the colons of *Vil-Ltbr*^{-/-} mice (Figure 5e,f). Accordingly, *Il22*, *Muc2*, *Muc4* and *Muc13* expression was reduced in the colons of *Vil-Ltbr*^{-/-} mice (Figure 5g,h). These results indicate that LTβR signaling in epithelial cells is critical for the induction of IL-22-dependent tissue protective responses.

IL-23 is the principal cytokine required for induction of IL-22 in the gut^{21, 22}. Based on our findings that LTβR signaling in epithelial cells is essential for colonic IL-22 production and that *Il23a* mRNA expression was significantly reduced in the colons of *Ltbr*^{-/-} mice (Figure 1f and Supplementary Figure S1d), we hypothesized that LTβR expression in epithelial cells may be necessary for IL-23 production. We found that with respect to *Ltbr*^{fl/fl} mice, *Il23a* mRNA expression and IL-23 protein production were significantly reduced in DSS-treated *Vil-Ltbr*^{-/-} mice (Figure 5i,j), suggesting that LTβR signaling in epithelial cells is indeed required for IL-23 production during DSS-induced injury. Furthermore, hydrodynamic injection of a construct encoding both p19 and p40 IL-23 subunits, restored *Il22* mRNA expression in *Vil-Ltbr*^{-/-} mice (Figure 5k), providing additional evidence that LTβR expression by epithelial cells regulates the IL-23-IL-22 axis.

Epithelial cells can produce IL-23 in response to mucosal damage in a LTβR-dependent manner

To determine which cells produce IL-23 during DSS-induced epithelial injury, we generated bone marrow chimeric mice using WT and *Il23a*^{-/-} mice³⁶ and treated them for 5 days with DSS. Unexpectedly, we found that colonic *Il23a* mRNA expression was comparable in WT→WT and *Il23a*→WT mice whereas it was greatly reduced in WT→*Il23a*^{-/-} mice (Figure 6a). These results suggest that *Il23a* is mainly produced by radioresistant cells in WT mice during DSS-induced colitis. We also observed that WT→*Il23a*^{-/-} mice displayed increased pathology with significant reduction of colon length and increased disease score after DSS treatment, compared to WT→WT and *Il23a*→WT chimeras (Figure 6b), suggesting that *Il23a* expression by radioresistant cells contributes to protection against intestinal inflammation.

To further define the cell types responsible for IL-23 production following epithelial injury, we induced colitis in IL-23-GFP^{+/-} reporter mice³⁶. Surprisingly, we found that IL-23-driven GFP expression was up-regulated in epithelial cells, as well as DCs and macrophages

(Figure 6c and Supplementary Figure 5) at day 5 post DSS treatment, indicating that epithelial cells are capable of producing IL-23 during epithelial injury. Furthermore, inhibition of LT β R signaling using an LT β R-Ig fusion protein reduced both IL-23-driven GFP and colonic *Ii23a* and *Ii22* mRNA expression (Figure 6c,d), suggesting that LT β R signaling promotes IL-23 production by epithelial cells during mucosal damage. In contrast to LT β R-Ig treatment, activation of the LT β R pathway using agonistic α LT β R antibody was able to upregulate colonic *Ii23a* and *Ii22* expression during epithelial injury (Figure 6e).

To confirm the ability of LT β R signaling to directly induce IL-23 production by epithelial cells, we stimulated both transformed colonic epithelial cells (CMT-93 cells) as well as primary intestinal epithelial cells with agonistic α LT β R antibody. Agonistic α LT β R antibody treatment significantly induced *Ii23a* mRNA expression and IL-23 protein production (Figure 6f,g), suggesting that direct activation of LT β R on epithelial cells can induce IL-23. Furthermore, IL-23 production was also induced after stimulation with LPS (Figure 6f,g), indicating that epithelial cells can produce IL-23 in response to TLR4 agonists. Consistently, *Ii23a* expression was reduced in colon epithelial cells of *Vil-Ltbr*^{-/-} mice during DSS induced injury (Figure 6h). Together, these data indicate that LT β R signaling in intestinal epithelial cells promotes IL-23-production during mucosal damage.

Given that IL-23 is composed of p19 (Ii23a) and p40 (Ii12b) subunits, we then investigated the ability of epithelial cells to induce *Ii12b* expression during DSS-induced injury. *Ii12b* expression was upregulated in both purified colonic epithelial cell fraction and sorted colonic epithelial cells (Supplementary Figure 6a,b). To further define the role of *Ii12b* production by radioresistant cells in mucosal protection, we generated reciprocal bone marrow chimeric mice. Although both radioresistant and bone marrow derived cells contributed to *Ii12b* expression during DSS-induced injury, mice lacking *Ii12b* in radioresistant cells displayed increased disease score, reduced colon length, and reduced colonic IL-22 production during DSS-induced injury (Supplementary Figure 6c-f). These results indicate that *Ii12b* can be expressed by epithelial cells and participate in epithelial repair after injury. Taken together, these data suggest that LT β R signaling in intestinal epithelial cells is critical for IL-23 production for intestinal epithelial cell repair and intestinal homeostasis after injury.

DISCUSSION

Interactions between epithelial cells and ILCs during epithelial damage and how these cells contribute to disease pathogenesis remains unclear. Our results suggest that during epithelial injury, activation of LT β R triggers IL-23 production by intestinal epithelial cells. IL-23 in turn promotes IL-22 production by CD4⁻CCR6⁺T-bet⁻ LTi cells. The IL-22 then induces intestinal epithelial cell proliferation and survival, and the production of mucus thereby promoting mucosal wound healing. These results provide evidence that support a novel mechanism of protection against intestinal injury in which the LT β R-dependent production of IL-23 by epithelial cells regulates mucosal wound healing.

IL-23 is critical for IL-23R-mediated production of IL-22 by ILCs^{21, 22}. A dichotomy between beneficial and pathogenic roles for IL-23R-responsive innate and adaptive cell populations has been described in the DSS-induced colitis model^{37, 38}. DCs and

macrophages are thought to be the major IL-23 producers during infection, inflammation and cancer progression in the colon, although epithelial cells can also contribute to IL-23 production³⁹⁻⁴². Accordingly, we have previously demonstrated that LT β R-mediated production of IL-23 by DCs promotes IL-22 production by ROR γ t⁺ ILCs during mucosal bacterial infection¹⁰. Additionally, our results using CD11c-*Ltbr*^{-/-} mice suggest that LT β R signaling in DCs contributes to protection against DSS-induced injury. Unexpectedly, in the current study we find that IL-23 and IL-22 production during epithelial injury are impaired in the absence of LT β R signaling in intestinal epithelial cells. We demonstrate that activation of LT β R signaling can promote IL-23 production by epithelial cells, identifying a previously unrecognized mechanism of LT β R-dependent regulation of intestinal wound healing. Our results indicate that both p19 and p40 subunits of IL-23 can be expressed by epithelial cells during DSS-induced injury. These results suggest that following epithelial damage, epithelial cells represent an important source of IL-23 for mucosal repair.

LT β R signaling is known as a key regulator of lymphoid organ development and maintenance, however its function outside of lymphoid organs is still elusive. In the present study we reveal that LT β R signaling in intestinal epithelial cells is essential for IL-22 production by ROR γ t⁺ ILC3s. Although mice with specific inactivation of LT β R in epithelial cells (*Vil-Ltbr*^{-/-} mice) were highly sensitive to DSS-induced injury, LT β R signaling in other cells could also contribute to protection. Indeed, a protective role for LT β R activation in DCs and macrophages was recently described in infectious and chemically-induced colitis models^{4, 9, 10}.

Recent studies have implicated the role of the gut microbiota in the pathogenesis of intestinal inflammation^{2, 43}. LT β R signaling, the IL-22 pathway and ROR γ t⁺ ILC3s have been identified as regulators of commensal microbiota composition^{31, 44, 45}. The comparable colonic expression of antimicrobial peptides and bacterial dissemination observed between *Ltbr*^{fl/fl} and *Ltbr*^{-/-} mice suggested that the sensitivity of *Ltbr*^{-/-} mice to DSS-induced colitis was not primarily mediated by the dissemination of commensal bacteria. Instead, we found that, during epithelial injury, LT β R signaling regulates epithelial cell homeostasis via the IL-22-dependent promotion of mucins expression and proliferation/survival of intestinal epithelial cells.

While ROR γ t⁺ ILC3s have been shown to be essential for protection against intestinal inflammation^{4, 19, 43}, accumulating evidence suggests that, in response to IL-23, the production of IL-22 by distinct ROR γ t⁺ ILC3 subsets may differentially impact the outcome of intestinal inflammation. Indeed, exuberant IL-22 responses together with IL-17 and IFN γ production, by colitogenic NKp46⁻ ILC3s has been shown to promote intestinal immunopathology^{16, 26}. These observations were corroborated by the presence of IL-23-responsive ILCs in the inflamed tissue of IBD patients²⁰. In contrast, IL-22 production by NKp46⁺ ILC3s and LTi cells has been shown to inhibit intestinal inflammation in the *C. rodentium*-induced colitis model^{10, 21}. Our results show that CCR6⁺Tbet⁻ CD4⁻ and CD4⁺ LTi cells but not NKp46⁺ ILC3s, colitogenic NKp46⁻ ILC3s or T cells were the primary source of IL-22 during DSS-induced injury. NKp46⁺ ILC3s represented less than 5% of IL-22-producing ILC3s and IL-22 production by those cells was LT β R-independent, in agreement with previous results⁴⁶. A recent study suggested a critical role for CD4⁺ LTi

cells in IL-22-mediated protection against infectious colitis⁴⁷, whereas the function of CD4⁻ LTi cells in mucosal homeostasis remained obscure. Unexpectedly, we found that depletion of CD4⁺ LTi cells in *Rag1*^{-/-} mice only partially reduced colonic IL-22 levels but had no effect on colon immunopathology and mice survival, indicating that CD4⁺ LTi cells are not critical for protection against DSS-induced intestinal injury. This observation might be explained by the fact that the vast majority (approximately 75%) of IL-22-producing LTi cells did not express CD4. Therefore, our results suggest that CD4⁻ LTi cells are the main producers of IL-22 during epithelial injury. Since the developmental plasticity between ROR γ ⁺ ILC subsets remains controversial^{15, 17}, it is important to determine the role of LT in the regulation, and function of CD4⁻ and CD4⁺ LTi subsets in future studies.

Increased LT expression has previously been found in the mucosa of IBD patients⁴⁸. Additionally, chronic overexpression of LT, as a transgene, has been connected with the formation of tertiary lymphoid tissue, chronic inflammation, immunopathology, and tumor progression⁴⁹. Therefore, future studies will be required to better define whether LT expression by distinct ILC populations correlates with disease severity in human IBD patients. In summary, our findings suggest that the LT β R-dependent IL-23 production by epithelial cells limits mucosal damage via induction of IL-22 by CD4⁻ LTi cells and that the manipulation of LT β R signaling may represent a novel therapeutic avenue in inflammatory diseases of the intestine.

METHODS

Mice

C57BL/6 and *Rag1*^{-/-} were originally obtained from The Jackson Laboratory (Bar Harbor) and bred at Trudeau Institute. Neo-free *Ltbr*^{+/+} and *Ltbr*^{fl/fl} mice were generated from LT β R tri-loxP floxed mice⁴ by crossing with MeuCre40 mice²⁸, all on C57BL/6 background. Vil-*Ltbr*^{-/-} mice were generated by crossing neo-free *Ltbr*^{fl/fl} mice with Vil-Cre mice. *Ltb*^{-/-}, ROR γ ^t-GFP^{+/-}, CD4-*Ltb*^{-/-}, IL-23-GFP^{+/-}, and *Il12b*^{-/-} mice were described previously^{4, 18, 36, 50}. All mice used in this study were on C57BL/6 background and maintained under specific pathogen free conditions. When possible, age/sex matched controls or littermate controls were co-housed with experimental mice. When this was not possible, bedding exchange was performed for 2 weeks prior to initiation of the experiment. All animal studies were performed in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the Trudeau Institute.

Generation of bone marrow chimeras

Recipient mice were irradiated twice for a combined dose of 950 rad and reconstituted with 4×10^6 total bone marrow cells. Recipient mice were kept on antibiotic-containing food for 4 weeks after the bone marrow transfer and used for experiments 6–8 weeks after the transfer.

DSS-induced colitis and study design

Mice were given 3.5–5% (w/v) DSS (M.W.=36000–50000 Da; MP Biomedicals) in their drinking water for 5 days replaced then by regular water for an additional 3–6 days. For Thy1.2- and CD4-depletion experiments, mice were i.p. injected with 150 µg of rat αCD4 (GK1.5), αThy1.2 (30H12), or IgG2b isotype control at days 0 and 3. To inhibit LTβR signaling in WT mice and IL-23-GFP^{+/-} mice, 150 µg of mouse LTβR-Ig fusion protein or control IgG antibody (Biogen Idec) was i.p. injected at days 0 and 3. To activate LTβR signaling, 100 µg of agonistic αLTβR antibody (ACH6, Biogen Idec) or control antibody (MOPC) was i.p. injected at days 3 and 5.

Assessment of DSS-induced colitis

Mice were weighed daily. The disease score was determined as an average of body weight loss (0 points, no weight loss; 1 point, weight loss of 1 to 5%; 2 points, weight loss of 5 to 10%; 3 points, weight loss of 10 to 20%; 4 points, weight loss >20%), signs of rectal bleeding (0 points, no blood in feces; 2 points, presence of blood in feces; 4 points, gross bleeding) and stool consistency (0 points, well-formed pellet; 1 point, soft pellet; 2 points, loose stool; 4 points, diarrhea). The scores were added to obtain a disease score ranging from 0 (healthy) to 12 (maximal activity of the disease). Postmortem, colons were removed and their length was measured from rectum to cecum. Swiss rolls of the colon were fixed in 10 % buffered formalin, and 4–5 µm paraffin-embedded sections were stained with hematoxylin and eosin. The severity of colitis was blindly determined as an average of colonic epithelial damage [0 points, normal epithelium; 1 point, hyperproliferation, irregular crypts, and goblet cell loss; 2 points, mild to moderate crypt loss (10–50%); 3 points, severe crypt loss (50–90%); 4 points complete crypt loss with the surface of the epithelium intact; 5 points, small to medium size ulcer (<10 crypts width); 6 points large ulcers (>10 crypts width)], and the degree of inflammatory cell infiltration in the mucosa (0 points, normal infiltration; 2 points, modest infiltration; 3 points, severe infiltration), the submucosa (0 points, normal infiltration; 1 point, modest infiltration; 2 points, severe infiltration) and the muscularis mucosae (0 points, normal infiltration; 1 point, moderate to severe infiltration). Scores for epithelial damage and inflammatory cell infiltration were added, resulting in a total scoring range of 0 to 12.

Hydrodynamic IL-22 and IL-23 plasmid injection

10 µg of IL-22-expressing plasmid (pRK-mIL-22, Genentech), IL-23 p19-p40 expressing plasmid (IL-23Fc)⁴⁴ or control plasmid (p-RK) in 1.7 mL of TransIT-EE Hydrodynamic Delivery Solution (Mirus Bio) were i.v. injected in the tail vein one day after start of DSS treatment.

RNA isolation, reverse transcription and real-time PCR

Total RNA from cells or frozen tissues was isolated using the RNeasy Mini Kits (Qiagen). cDNA was synthesized using MLV Reverse Transcriptase (Promega) and random primers (Invitrogen). Quantitative real-time PCR reactions were performed using Power Sybr Green PCR master mix and the 7500 cycler (Applied Biosystems). Relative mRNA expression of the target gene was determined using the comparative 2^{-C_t} method.

Flow cytometry

Fluorochrome-conjugated antibodies against mouse CD45 (30-F11), CD3 (145-2C11), CD8 (53-6.7), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), Gr1 (RB6-8C5), TER119 (TER119), NKp46 (29A1.4), CD4 (RM4-5), Thy1.2 (53-2.1), SCA1 (D7), CCR6 (29-2L17), c-Kit (2B8), IL-7R α (A7R34), IL-22 (1H8PWSR), IL-17A (TC11-18H10.1), IFN γ (XMG1.2), T-bet (4B10), ROR γ t (2B2), EpCAM (G8.8) were purchased from eBioscience, BD Pharmingen, or Biolegend. For intracellular cytokine staining, cells were stimulated with IL-23 (50 ng/ml) in the presence of brefeldin A (10 μ g/ml) for 4 hours at 37°C. Cell sorting and flow cytometry were performed using an Influx cell sorter and a LSRII flow cytometer (BD Biosciences), respectively. Data were analyzed using FlowJo vX software (TreeStar).

Determination of bacterial dissemination

Spleens and livers were aseptically removed and homogenized in sterile PBS. Viable counts were determined by plating serial dilutions on sheep blood agar plates.

ELISA analysis

IL-22 and IL-23 concentrations were measured in culture supernatants or colon extracts using specific R&D Duoset and eBioscience ELISA kits, respectively, according to the manufacturer's instructions.

Epithelial cell line CMT-93

Mouse colonic CMT-93 epithelial cells were obtained from American Type Culture Collection. CMT-93 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, penicillin and streptomycin. Cells were serum starved for 24h before *in vitro* stimulation with LPS from *E. coli* O111:B4 (Invitrogen) or agonistic α LT β R antibody (ACH6, Biogen Idec) for 6h (gene expression) or 24h (ELISA).

Statistical analysis

Statistical analysis was performed using the two-tailed Student's t-test or the Mann & Whitney test when appropriate in Prism V5 (GraphPad Software). P values <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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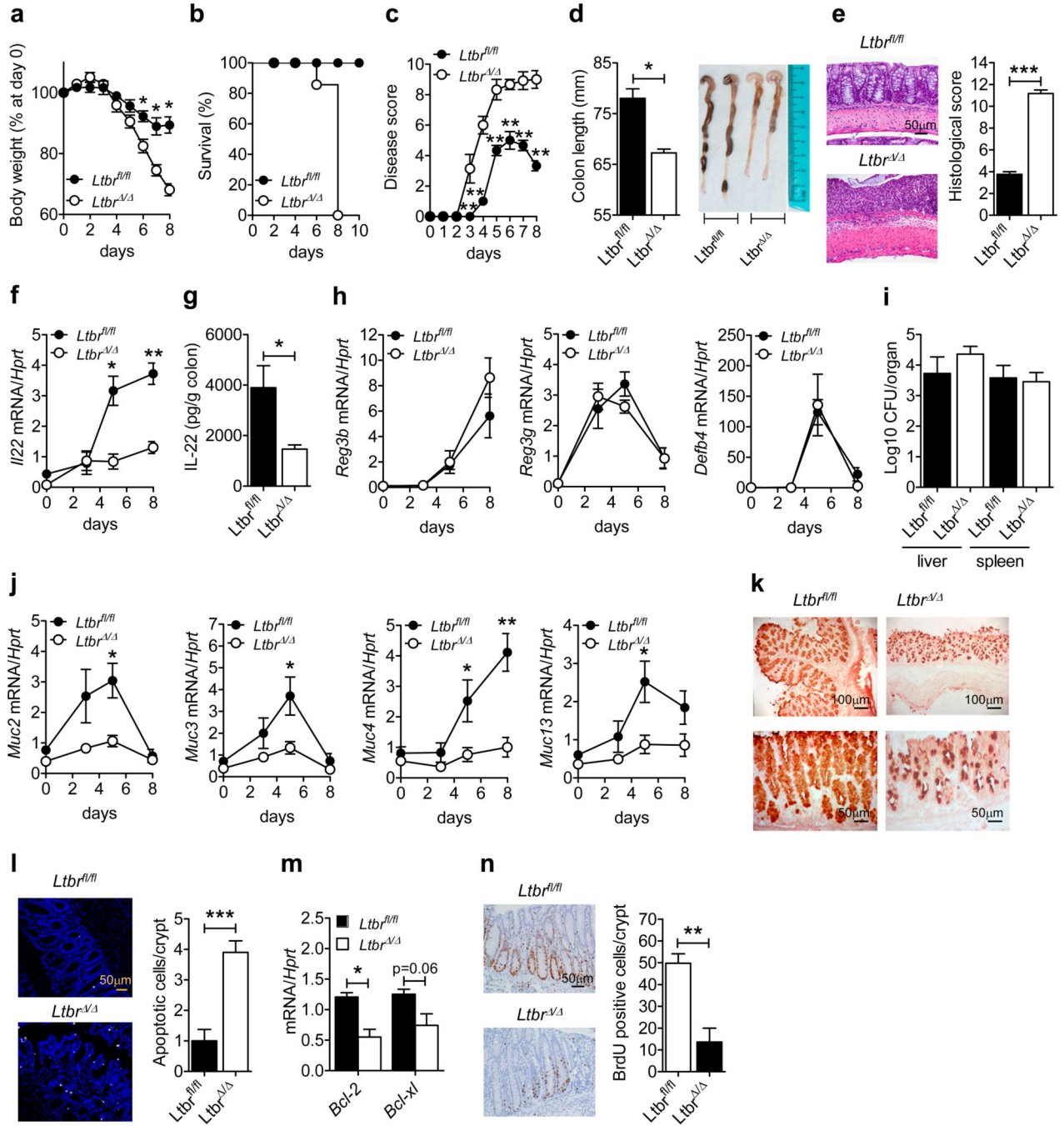


Figure 1. LT β R signaling protects against intestinal epithelial injury by promoting IL-22-dependent epithelial cell repair and homeostasis. *Ltbr^{fl/fl}* and *Ltbr^{-/-}* mice (n=5) were treated for 5 days with 3.5% DSS. **(a)** Weight loss, **(b)** survival, **(c)** disease score and **(d)** length and photographs of colons at day 8. **(e)** Hematoxylin and eosin staining and histological score of colon sections at day 8. **(f)** Kinetics of colonic *Il22* mRNA expression. **(g)** IL-22 production in the supernatants of colons collected at day 5 and cultured for 24h. **(h)** Kinetics of colonic mRNA expression of indicated anti-microbial proteins. **(i)** Bacterial titers in liver and spleen

at day 8. **(j)** Kinetics of colonic expression of indicated mucin genes. **(k)** Immunohistochemical staining of Muc2 in sections of colons collected at day 5. **(l)** Colon sections were stained with TUNEL (white) to detect apoptotic cells at day 8. Nuclei were stained with Hoechst (blue). The number of apoptotic cells was determined by analyzing ten crypts per colon section. **(m)** Colonic expression of anti-apoptotic factors at day 5. **(n)** Intestinal epithelial cell proliferation was analyzed with BrdU pulse injection on day 8. Ten crypts from the colons of 5 mice per genotype were analyzed. For mRNA expression analysis, Ct values were normalized to *Hprt* expression. Data are representative of two (**g, i, k–n**) or three independent (**a–f, h, j**) experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test). Error bars represent SEM.

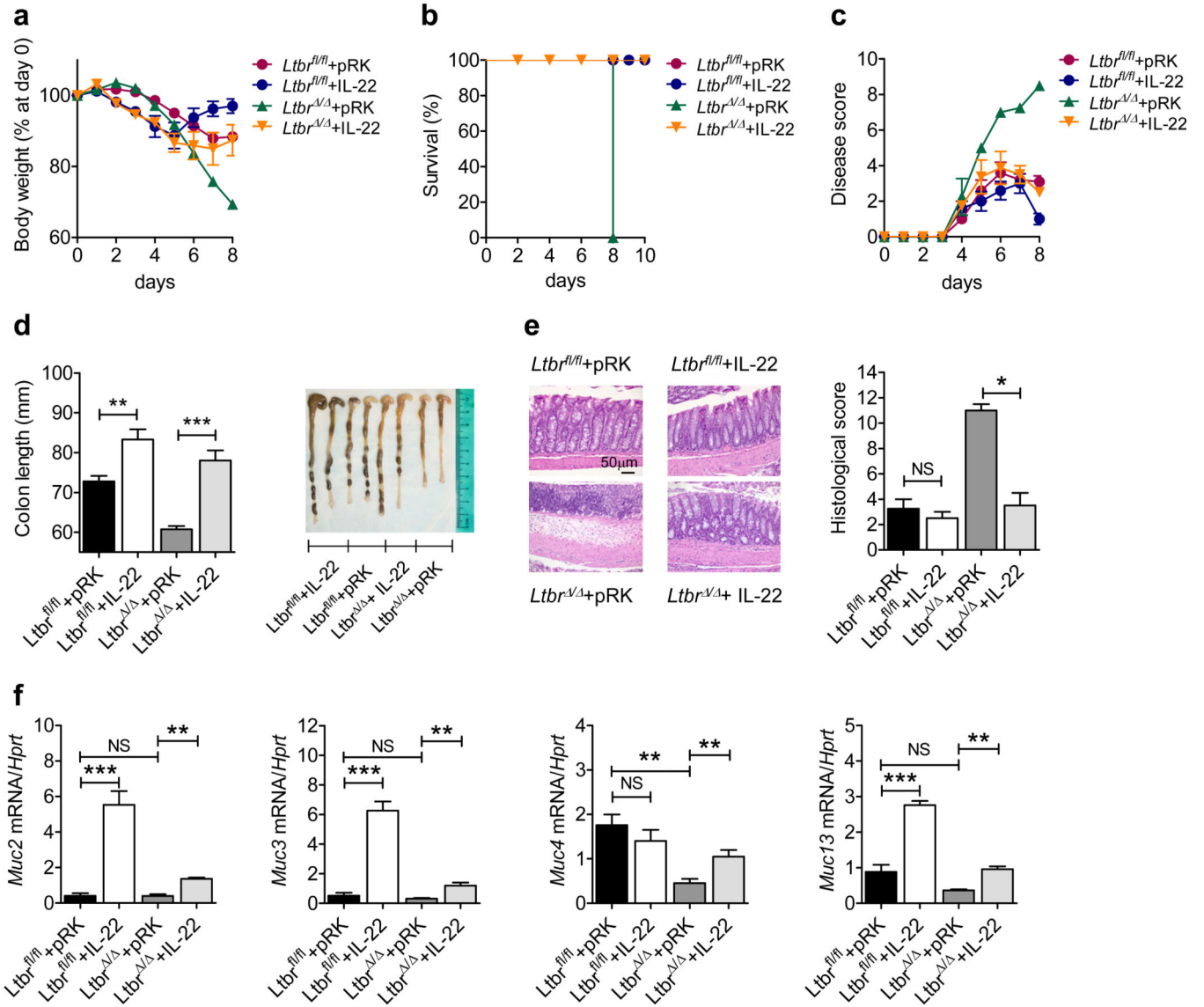


Figure 2. Exogenous IL-22 expression rescues LTβR-deficient mice from epithelial injury. *Ltbr^{fl/fl}* and *Ltbr^{Δ/Δ}* mice (n=5) were treated for 5 days with 3.5% DSS. 10 μg of plasmid encoding empty vector (pRK) or IL-22-expressing plasmid were injected intravenously at day 1. (a–c) Weight loss (a), survival (b), and clinical disease score (c). (d) Length and photographs of colons at day 8. (e) Hematoxylin and eosin staining and histological score of colon sections at day 8. (f) Colonic expression of mucin genes at day 8. Data are normalized to *Hprt*. **P*<0.05; ***P*<0.01; ****P*<0.001; NS, not significant (Student’s *t*-test). Data are representative of two independent experiments. Error bars represent SEM.

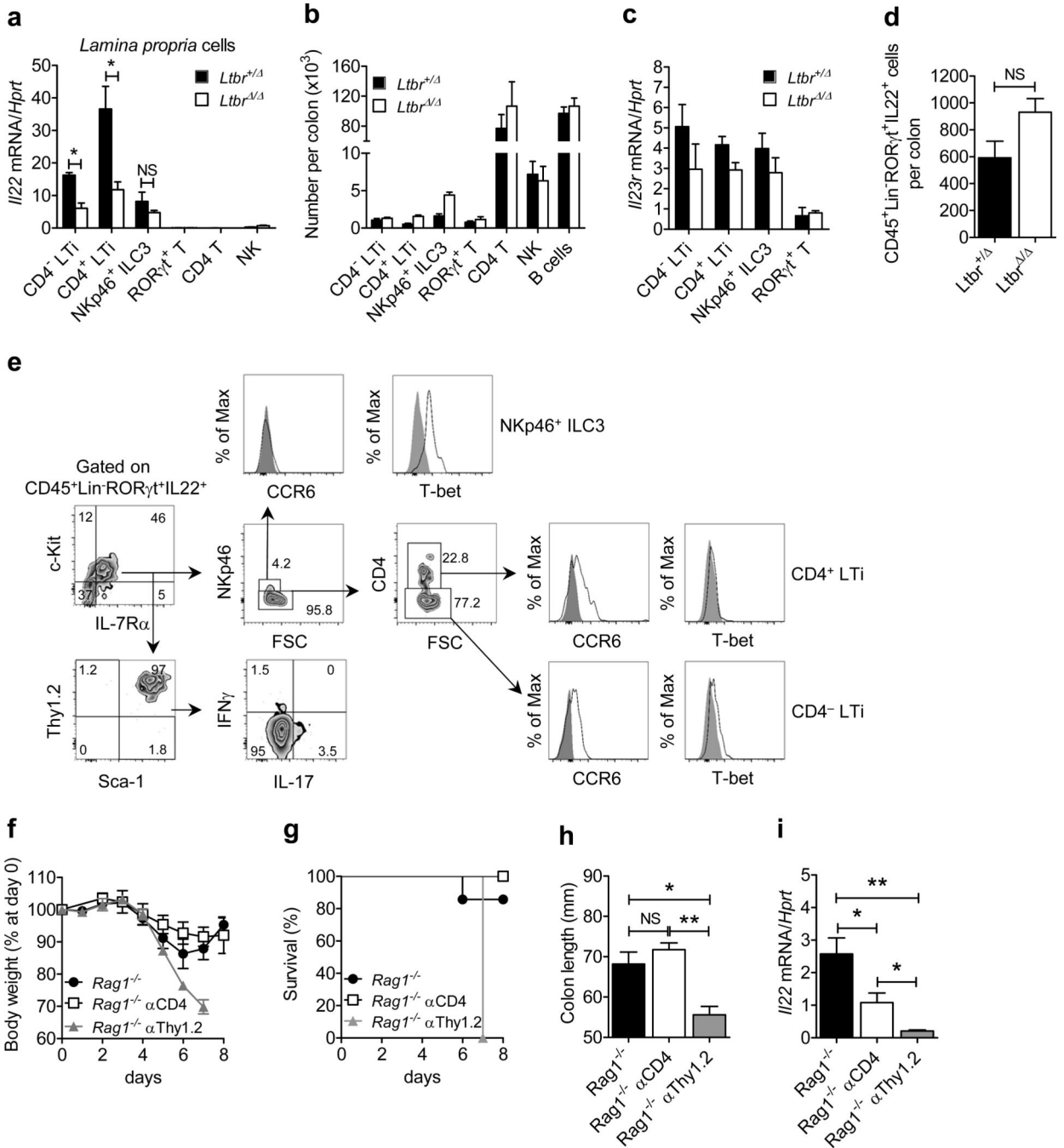
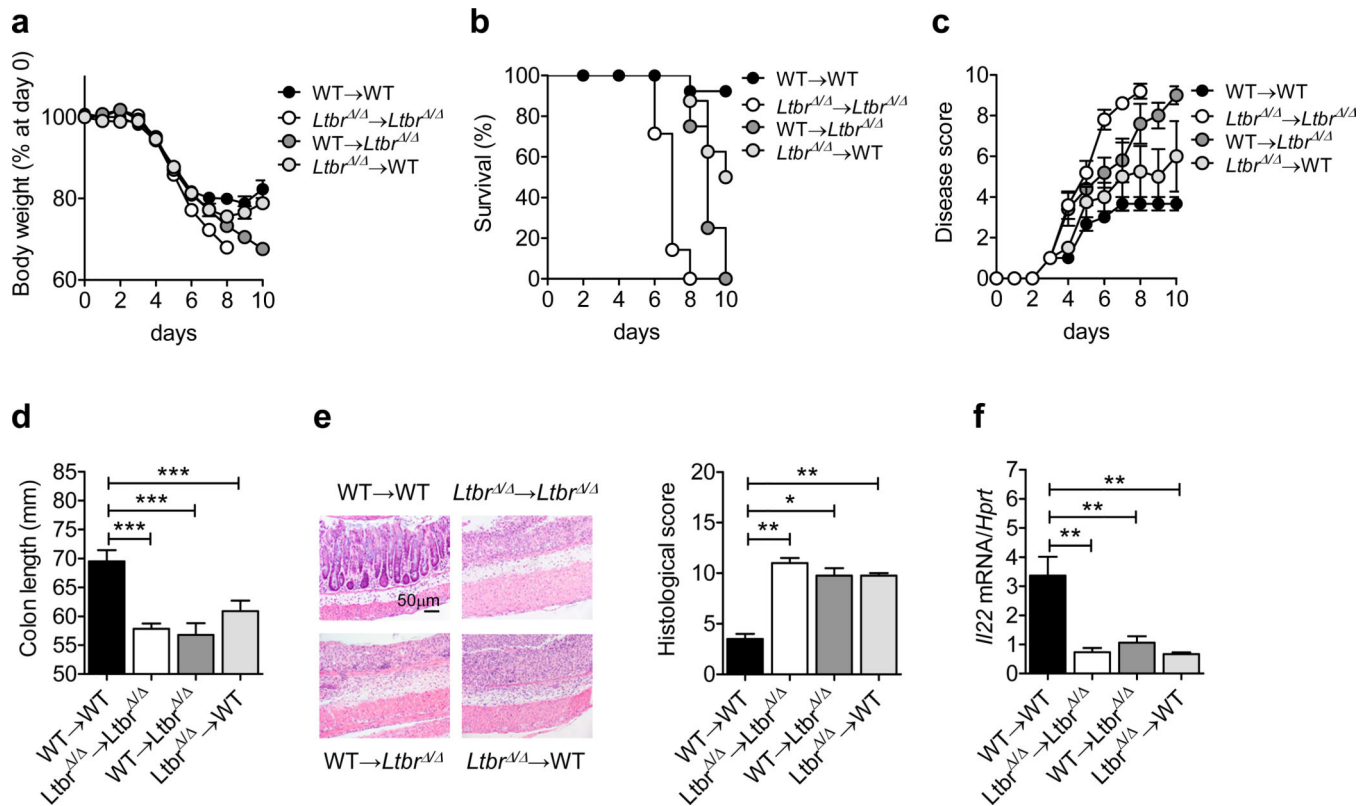


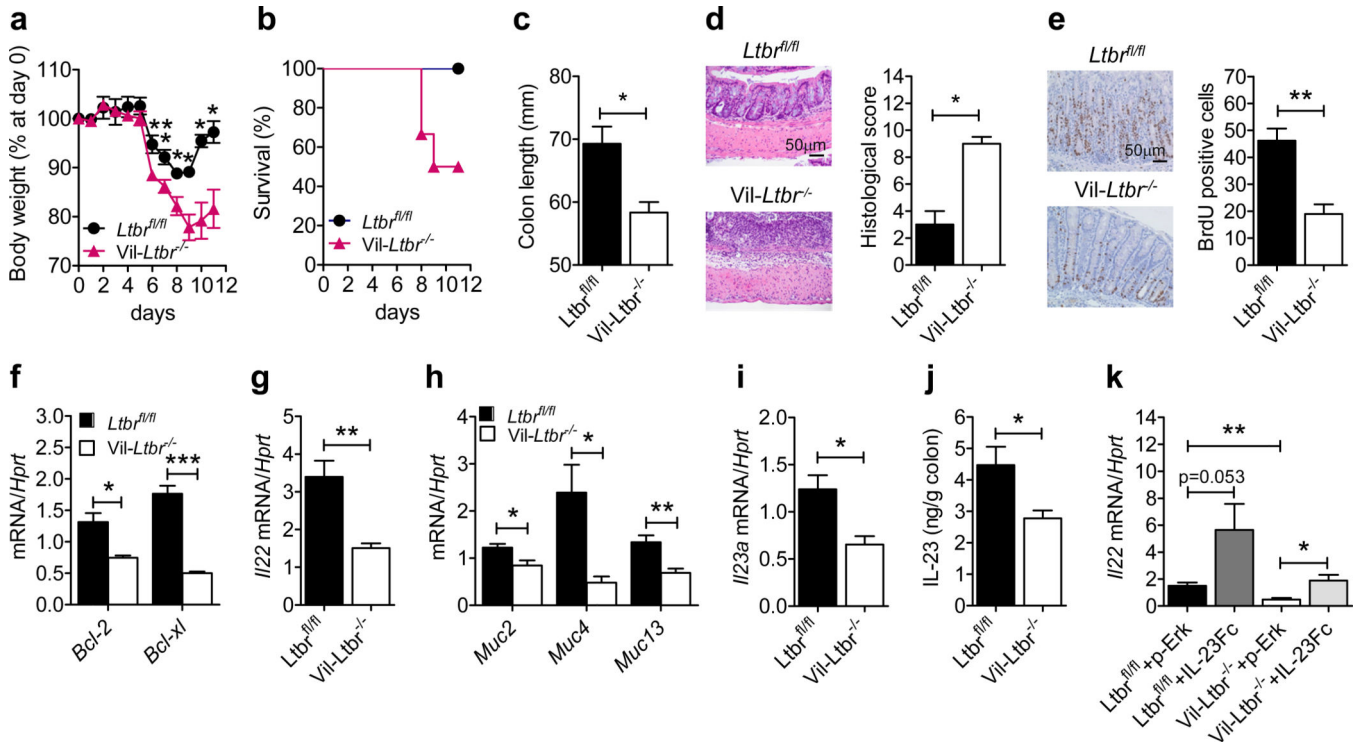
Figure 3.

LTβR-dependent IL-22 production by CD4⁻CCR6⁺T-bet⁻ LTi cells is the primary source of protective IL-22 during epithelial injury. (a-c) Lamina propria (LP) cells were purified from the colon of RORγt-GFP^{+/-} *Ltbr*^{+/-} and RORγt-GFP^{+/-} *Ltbr*^{-/-} reporter mice treated with 3.5% DSS for 5 days (n=3). (a) Expression of *Il22* mRNA in sorted CD4⁻ (CD45⁺RORγt⁺CD3⁻CD4⁻NKp46⁻) and CD4⁺ LTi cells (CD45⁺RORγt⁺CD3⁻CD4⁺NKp46⁻), NKp46⁺ ILC3s (CD45⁺RORγt⁺CD3⁻CD4⁻NKp46⁺), RORγt⁺ T cells (CD45⁺RORγt⁺CD3⁺CD4⁺NKp46⁻), CD4⁺ T cells (CD45⁺RORγt⁻

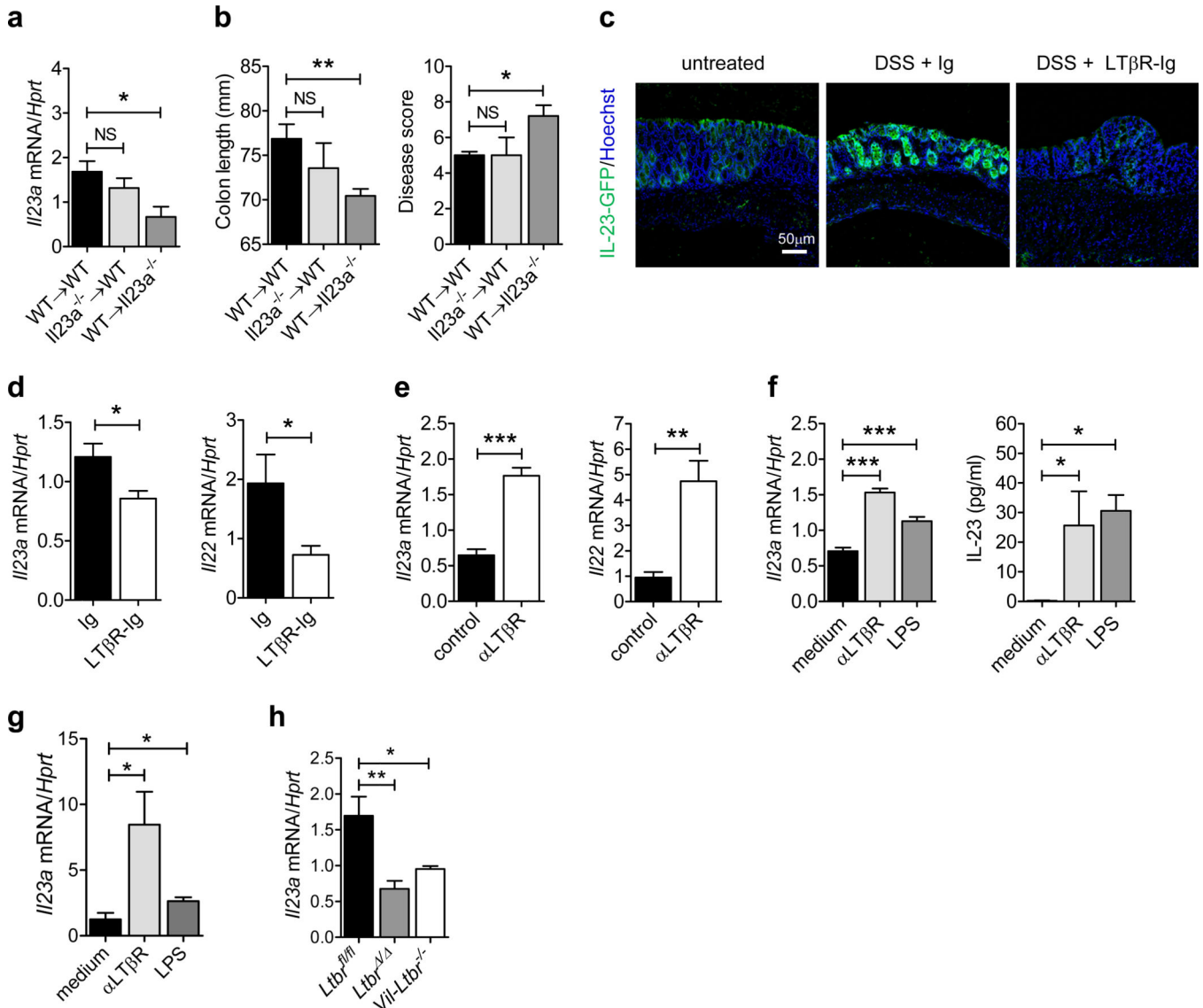
CD3⁺CD4⁺NKp46⁻) and NK cells (CD45⁺RORγt⁻CD3⁻CD4⁻NKp46⁺). **(b)** Number of indicated LP cell populations per colon. **(c)** Expression of *Il23r* mRNA in sorted RORγt⁺ ILCs and RORγt⁺ T cells. **(d and e)** Colonic LP cells from *Ltbr^{fl/fl}* and *Ltbr^{-/-}* mice treated with 3.5% DSS for 5 days (n=3 per group) were stimulated *ex vivo* for 4 hours with IL-23 (50 ng/ml). The number **(d)** and surface and intracellular marker analysis **(e)** of colonic LP CD45⁺Lin⁻RORγt⁺ IL-22-producing cells was evaluated by flow cytometry. **(f–i)** Rag1^{-/-} mice (n=4) were treated for 5 days with 3.5% DSS. Isotype control, αCD4 (150 μg/mouse) and αThy1.2 (150 μg/mouse) were injected i.p. at days 0 and 3. **(f)** Weight loss, **(g)** survival, and **(h)** colon length evaluated at day 8. **(i)** Expression of colonic *Il22* mRNA at day 8. For mRNA expression, data are normalized to *Hprt*. **P*<0.05; ***P*<0.01; ****P*<0.001; NS, not significant (Student's *t*-test). Data are representative of two independent experiments. Error bars represent SEM.

**Figure 4.**

Expression of LTβR on both radioresistant and hematopoietic cells is required for control of intestinal inflammation. Bone marrow cells from C57BL/6 (WT) or *Ltbr*^{-/-} mice were transferred into lethally-irradiated WT or *Ltbr*^{-/-} mice. Six weeks later, WT→WT (n=16), *Ltbr*^{-/-}→*Ltbr*^{-/-} (n=9), WT→*Ltbr*^{-/-} (n=12) and *Ltbr*^{-/-}→WT (n=9) chimeric mice were treated for 5 days with 5% DSS. (a–d) Weight loss (a), survival (b), clinical disease score (c), and length and photographs of colons at day 10 (d). (e) Hematoxylin and eosin staining and histological score of colon sections from chimeric mice at day 10. (f) *I122* mRNA expression in the colon of chimeric mice (n=5) at day 10. Data are normalized to *Hprt*. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test). Data are representative (e, f) or a combination (a–d) of two independent experiments. Error bars represent SEM.

**Figure 5.**

LT β R signaling in epithelial cells inhibits intestinal epithelial injury by promoting IL-23-driven IL-22-dependent tissue protective responses. *Ltbr^{fl/fl}* and *Vil-Ltbr^{-/-}* mice (n=5) were treated for 5 days with 3.5% DSS. (a) Weight loss and (b) survival. (c) Colon length, and (d) hematoxylin and eosin staining and histological score of colon sections at day 11. (e) Intestinal epithelial cell proliferation was analyzed after BrdU pulse injection on day 8. Ten crypts from the colons of 5 mice per genotype were analyzed. (f–j) Expression of the indicated genes (f–i) and IL-23 production in culture supernatants of colons (j) was evaluated at day 5. (k) *Ltbr^{fl/fl}* and *Vil-Ltbr^{-/-}* mice (n=3–4) were treated for 5 days with 5% DSS. 10 μ g of plasmid encoding empty vector (pRK) or IL-23-expressing plasmid (IL-23Fc) was injected intravenously at day 1. Colonic expression of *Il22* mRNA was evaluated at day 5. For mRNA expression, data are normalized to *Hprt*. * P <0.05; ** P <0.01; *** P <0.001 (Student's *t*-test). Data are representative of two (e, j, k) or three (a–d, f–i) independent experiments. Error bars represent SEM.

**Figure 6.**

Epithelial cells produce IL-23 in response to mucosal damage in a LTβR-dependent manner.

(a and b) Bone marrow cells from C57BL/6 (WT) or *Il23a*^{-/-} mice were transferred into lethally-irradiated WT or *Il23a*^{-/-} mice. Six weeks later, WT→WT, *Il23a*^{-/-}→WT and WT→*Il23a*^{-/-} chimeric mice were treated for 5 days with 5% DSS. (a) *Il23a* mRNA expression in the colon of chimeric mice at day 5. (b) Colon length and clinical disease score at day 5. (c and d) Mice were treated for 5 days with 3.5% DSS or left untreated. LTβR-Ig fusion protein or control IgG was administered i.p. at days 0 and 3 (150 μg/mouse). (c) IL-23-driven GFP expression was evaluated at day 5 in the colon of IL-23-GFP^{+/-} mice by GFP staining (green). Nuclei were stained with Hoechst (blue). (d) *Il23a* and *Il22* expression was evaluated in the colon of C57BL/6 mice at day 5. (e) C57BL/6 mice were treated for 5 days with 3.5% DSS. Agonistic αLTβR or control antibody was injected i.p. at days 0 and 3 (100 μg/mouse). Colonic *Il23a* and *Il22* mRNA expression was evaluated at day 5. (f) CMT-93 epithelial cells were stimulated with agonistic αLTβR antibody (5 μg/ml)

or LPS (1 $\mu\text{g/ml}$). *Il23a* mRNA expression and IL-23 protein production in culture supernatant was measured. **(g)** *Il23a* mRNA expression in sorted EpCAM⁺ CD45⁻ colon epithelial cells from WT mice, stimulated *in vitro* with agonistic $\alpha\text{LT}\beta\text{R}$ antibody (5 $\mu\text{g/ml}$) or LPS (1 $\mu\text{g/ml}$) for 12h. **(h)** *Il23a* mRNA expression in sorted EpCAM⁺ CD45⁻ colon epithelial cells at day 5 after DSS treatment. For mRNA expression, data are normalized to *Hprt*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test (**a, b, d, e, f** left panel, **g, h**) or two tailed Mann-Whitney test (**f** right panel)). Data are representative of two independent experiments with 3–7 mice per group. Error bars represent SEM.