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Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge

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Summary

Innate lymphoid cells (ILCs) have emerged as important players regulating the balance between protective immunity and immunopathology at mucosal surfaces. However, mechanisms that regulate ILCs effector functions during mucosal pathogenic challenge are poorly defined. Using mice infected with the natural mouse enteric pathogen *Citrobacter rodentium*, we demonstrate that lymphotoxin (LT) is essential for IL-22 production by intestinal ILCs. Blocking of LT β R signaling dramatically reduced intestinal IL-22 production after *C. rodentium* infection. Conversely, stimulating LT β R signaling induced IL-22 protection pathway in LT-deficient mice. Furthermore, exogenous IL-22 expression rescued LT β R deficient mice. IL-22 producing ILCs were predominantly located in lymphoid follicles in the colon, and interacted closely with dendritic cells. Finally, we find that an LT-driven positive feedback loop controls IL-22 production by ROR γ t⁺ ILCs via LT β R signaling in dendritic cells. Altogether, we show that LT β R signaling in gut lymphoid follicles regulates IL-22 production by ILCs in response to mucosal pathogen challenge.

Introduction

Innate lymphoid cells (ILCs) represent a heterogeneous population of innate lymphocytes in the mucosa, that participate in regulation of mucosal immune homeostasis (Colonna, 2009; Sawa et al., 2010; Spits and Di Santo, 2011). Although ILCs do not express antigen specific receptors like adaptive lymphocytes, they have the capacity to produce several cytokines upon stimulation that function to regulate the balance between protective immunity and destructive inflammation in the gut. However, the pathways that mediate crosstalk between ILCs and intestinal epithelial cells in response to mucosal bacterial infection remain poorly understood. *Citrobacter rodentium* (*C. rodentium*) is a natural mouse extracellular enteric pathogen that mimics human enteropathogenic *Escherichia coli* and enterohaemorrhagic *Escherichia coli* infections (Mundy et al., 2005). *C. rodentium* uses attaching and effacing

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lesion formation as a major mechanism to target and infect the intestinal epithelial layer, and therefore represents an excellent model to define the role of ILCs in intestinal immune homeostasis.

Lymphoid tissue inducer cells (LTi) belong to ILCs, and are critical for development of secondary lymphoid tissues during fetal development (Eberl et al., 2004; Randall et al., 2008; Roozendaal and Mebius, 2011; Spits and Di Santo, 2011). However, their role in regulation of mucosal immune responses in the gut is poorly defined. Recently, a CD4⁺ population of LTi cells within the intraepithelial lymphocyte compartment has been implicated in controlling mucosal bacterial infection (Sonnenberg et al., 2011b). Another ILC subset that expressed the NK cell marker NKp46 and is located in small and large intestine lamina propria has also been suggested to control *C. rodentium* infection (Cella et al., 2009; Satoh-Takayama et al., 2008). Both the intraepithelial LTi cells and the lamina propria NKp46⁺ cells, as well as other ILCs in the gut, express the transcriptional factor nuclear hormone receptor retinoic acid receptor-related orphan receptor gamma t (ROR γ t) for their development (Colonna, 2009; Eberl et al., 2004; Spits and Di Santo, 2011).

Recent studies have suggested that development of LTi cells and mucosal NKp46⁺ cells is distinct from classical NK cells (Colonna, 2009; Spits and Di Santo, 2011). The developmental relationship between NKp46⁺ and LTi cells remains an active area of research. Several studies suggested that ROR γ t⁺NKp46⁺ cells can originate from ROR γ t⁺ LTi cells (Cupedo et al., 2009; Vonarbourg et al., 2010). However, another report indicated distinct developmental programs of these populations, originally generated from a common fetal liver progenitor (Sawa et al., 2010). In contrast to LTi cells, NKp46⁺ cells require commensal microflora for their development (Sanos et al., 2009; Satoh-Takayama et al., 2008). However, this requirement has not been confirmed by other studies (Sawa et al., 2010; Sawa et al., 2011). Additionally, inactivation of the *Ncr1* gene (encoding NKp46) did not impact susceptibility to *C. rodentium* infection (Satoh-Takayama et al., 2009). Hence, the underlying function of mucosal NKp46⁺ cells in innate immune response is currently unclear.

The major functions of ILCs in mucosal immunity against *C. rodentium* infection as well as epithelial tissue repair are potentially mediated by the production of the cytokine, IL-22 (Cella et al., 2009; Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). IL-22 is a recently discovered cytokine of the extended IL-10 family that plays multiple roles in the regulation of mucosal immunity (Ouyang, 2010; Ouyang et al., 2011; Sonnenberg et al., 2011a). IL-22 signals through the IL-22R that is selectively expressed by intestinal epithelial cells. IL-22 can induce secretion of antimicrobial proteins from these epithelial cells, including RegIII γ and RegIII β , to kill *C. rodentium* (Zheng et al., 2008). Accordingly, IL-22-deficient mice show increased morbidity and mortality after *C. rodentium* infection (Zheng et al., 2008). IL-23 has been shown to promote IL-22 production (Sonnenberg et al., 2011b; Zheng et al., 2008). Furthermore, IL-23-deficient mice display reduced IL-22 levels and succumb to *C. rodentium* infection (Zheng et al., 2008). However, other potential regulators of IL-22 production by ILCs remain poorly understood.

Lymphotoxin (LT) is a member of the TNF core family and plays a critical role in regulation of mucosal immune responses (Fu and Chaplin, 1999; Tumanov et al., 2007; Ware, 2005). The biologically active form of surface LT presents as a heterotrimeric complex (LT β ₂LT α ₁) that interacts specifically with LT β R. While LT is expressed mostly by lymphocytes, including T, B, NK, and LTi cells, LT β R is expressed on stromal, epithelial, DC, and myeloid cells, but not on lymphocytes (Fu and Chaplin, 1999; Tumanov et al., 2007; Ware, 2005). LT β R signaling in DCs regulates DC homeostasis (Kabashima et al., 2005; Summers Deluca and Gommerman, 2011). Evidence of cross-talk between LT-

expressing T cells and LT β R-bearing DC promotes T cell responses to a model antigen in mice (Summers-DeLuca et al., 2007; Summers Deluca and Gommerman, 2011). However, the role of LT in cross-talk between lymphocytes and DCs in host defense and autoimmune diseases remains to be fully elucidated. LT β R signaling plays a critical role in host defense against *C. rodentium* infection (Spahn et al., 2004), as all LT β R-deficient mice succumb early to *C. rodentium* infection (Spahn et al., 2004; Wang et al., 2010). In the present study, we explore the relationship between IL-22 and the LT β R pathway and define that LT β R signaling controls the IL-22 protective pathway in the gut via coordination of ROR γ ⁺ ILCs with DCs in lymphoid follicles.

Results

LT β R signaling controls innate IL-22 pathway in the gut

IL-22 is essential for control of early *C. rodentium* infection (Zheng et al., 2008). Since LT β R-deficient mice also succumb early to *C. rodentium* infection, we speculated that LT β R signaling might regulate IL-22 production. To test this hypothesis, we measured IL-22 levels in the colon of WT and LT β R-deficient mice early after *C. rodentium* infection. Compared to WT mice, expression of IL-22 was dramatically reduced in colon of *Ltbr*^{-/-} mice (Figure 1A). mRNA expression of IL-22 dependent antimicrobial proteins RegIII γ and RegIII β was also reduced in colon of *Ltbr*^{-/-} mice at day 5 post-infection (Figure 1A). In contrast to infected mice, basal IL-22 levels were comparable in the colon of naive WT and *Ltbr*^{-/-} mice (Figure 1A). These results suggest the critical role of LT β R signaling to control IL-22 production in the gut in response to mucosal bacterial infection.

LT β R-deficient mice display multiple immune abnormalities, including lack of lymph nodes and gut-associated lymphoid tissues, defects in DC and NK cell development and IgA production, and defects in central tolerance (Zhu et al., 2010). Therefore, in order to exclude developmental defects in *Ltbr*^{-/-} mice that might affect IL-22 production in the gut, we treated WT mice early after infection with soluble LT β R-Ig fusion protein that transiently blocks LT β R signaling. WT mice treated with LT β R-Ig at days 1 and 3 after infection showed severe pathology and around 50% of LT β R-Ig treated mice died after acute infection, consistent with previous reports (Wang et al., 2010). Remarkably, expression of IL-22 and IL-22-dependent antimicrobial proteins was severely reduced in the colon of LT β R-Ig treated mice at day 5 post-infection, compared to control mice (Figure 1B). These results suggest that LT β R signaling at the time of infection is required for IL-22 induction in the gut.

To further define whether LT β R signaling regulates innate mechanisms of IL-22 production, we treated Rag1-deficient mice with LT β R-Ig. This treatment reduced expression of both IL-22 and antimicrobial proteins in colon (Figure 1C), indicating the critical role of LT β R to control IL-22 production by innate lymphoid cells. LT β R is known to regulate expression of several chemokines and adhesion molecules responsible for migration of lymphoid cells to the gut (Fu and Chaplin, 1999; Ware, 2005). Therefore, impaired migration or development of IL-22 producing cells could result in reduced IL-22 levels in the gut. However, analysis of gut lymphoid cells revealed similar numbers of innate cell populations within the colon of WT and *Ltbr*^{-/-} mice (Figure S1). These results suggest that the defect in IL-22 production in LT β R-deficient mice is not due to the absence of innate cells capable to produce IL-22 in the gut. In fact, lamina propria lymphocytes from *Ltbr*^{-/-} mice were capable to produce IL-22 after IL-23 stimulation *in vitro* (Figure S1, C and D), suggesting no intrinsic defect in production of IL-22 by these cells. Thus, our data demonstrate that direct LT β R signaling is essential for control of innate IL-22 pathway in the gut after mucosal bacterial infection.

IL-22 is an essential protection pathway downstream of LT β R signaling

Surface LT and LIGHT (TNFSF14) are two known ligands for LT β R (Fu and Chaplin, 1999; Ware, 2005). To define whether LIGHT or LT controls IL-22 production in the gut after infection, we orally infected WT, LT β - and LIGHT- deficient mice with *C. rodentium*. Clearly, IL-22 expression was reduced in the colon of *Ltb^{-/-}* (Figure S2A), but not in *Tnfsf14^{-/-}* mice (Figure S2D), suggesting the essential role of surface LT to control IL-22 production after infection. Expression of LT on adaptive immune cells was dispensable for IL-22 production, because mice that lack surface LT specifically on T and B cells (T,B-*Ltb^{-/-}*) were able to express similar mRNA levels of IL-22 and antimicrobial proteins RegIII γ and RegIII β compared to WT mice after infection (Figure S2A-C). Consistently, *Tnfsf14^{-/-}* mice and T,B-*Ltb^{-/-}* mice survived *C. rodentium* infection (Wang et al., 2010). To define whether LT β R signaling is sufficient to induce IL-22 production in the gut, we treated LT-deficient mice with agonistic anti-LT β R antibody. We found that anti-LT β R treatment greatly induced expression of IL-22 in the colon early after infection (Figure 2A), increased production of antimicrobial proteins (Figure 2B) as well as reduced bacterial dissemination into peripheral organs (Figure 2C). These results suggest that LT β R signaling is sufficient to promote IL-22 pathway following mucosal bacterial infection *in vivo*. However, it was not clear whether IL-22 is the essential protection pathway downstream of LT β R signaling because LT β R could regulate other protection mechanisms. To address this question, we tested whether *Ltbr^{-/-}* mice could be rescued from lethal *C. rodentium* infection by using hydrodynamic IL-22 injection. Remarkably, a single IL-22 injection 6 hours after infection was able to rescue *Ltbr^{-/-}* mice from an otherwise lethal *C. rodentium* infection. More than 60% of *Ltbr^{-/-}* mice treated with IL-22 survived the infection (Figure 2D, and E). *Ltbr^{-/-}* mice treated with control vector showed severe colon inflammation, loss of goblet cells, ulcerations of epithelial layer, developed severe diarrhea and all died by day 12 post infection (Figure 2D-G). In contrast, histological analysis of colon sections at day 9 post-infection of IL-22 treated *Ltbr^{-/-}* mice revealed reduced colon inflammation and reduced histopathology score (Figure 2F, and G). These data suggest that IL-22 is an essential pathway downstream of LT β R signaling to protect mice against mucosal bacterial pathogen.

IL-22 is produced in lymphoid follicles by innate ROR γ t⁺ cells

Our data suggested that LT β R signaling controls the innate IL-22 production in response to mucosal bacterial infection. However, the innate cell populations that are regulated by LT β R signaling remain unclear. Analysis of IL-22 expression in total cell populations from colon revealed predominant expression of IL-22 in lamina propria lymphocytes (LPL), compared to intraepithelial lymphocytes (IEL) (Figure 3A). To further define the innate cell source of IL-22, we next measured expression of IL-22 in purified innate cell populations from lamina propria after infection. Because all ILCs require the transcriptional factor ROR γ t for their development (Eberl et al., 2004; Spits and Di Santo, 2011), we utilized ROR γ t-GFP^{+/+} reporter mice that are heterozygous for insertion of a green fluorescent protein (GFP) reporter into the *Rorc* gene (Eberl and Littman, 2004) to determine IL-22 expression from the sorted cells. We found that LT_i cells were the predominant IL-22 producing population in the lamina propria (Figure 3B, and Figure S3). Furthermore, we crossed *Ltbr^{-/-}* mice to ROR γ t-GFP^{+/+} reporter mice to allow isolation of ROR γ t⁺ ILCs from LT β R-deficient mice by flow cytometry. Expression of IL-22 was greatly reduced in LT_i cells from ROR γ t-GFP^{+/+}/*Ltbr^{-/-}* mice, compared to ROR γ t-GFP^{+/+}/*Ltbr^{+/+}* control mice (Figure 3B), suggesting that, in the absence of LT β R signaling the LT_i cells are unable to produce IL-22 after infection.

To better define the location of IL-22 producing cells in the gut, we stained colon sections with anti-IL-22 antibody at day 5 post infection. Surprisingly, most of IL-22 expressing cells were not randomly scattered in lamina propria, but were predominantly located in isolated

lymphoid follicles (ILF) and colonic patches (Figure 3C-D). Consistently, we found predominant IL-22 expression in ILF-containing areas microdissected from colon tissue, compared to ILF-negative areas (Figure 3E). Although the DCs concentrated in B cell areas, they did not produce IL-22. However, we observed that they were in close contact with ROR γ ⁺ IL-22 producing cells (Figure 3C, and D). Furthermore, colon lamina propria DCs purified from infected mice were able to activate naïve LTi cells to produce IL-22 when co-cultured *in vitro* (Figure 3F), suggesting that cooperation of DC and LTi cells promotes efficient IL-22 production after infection. Thus, our results suggest that IL-22 is produced predominantly by innate ROR γ ⁺ cells that are located within gut-associated lymphoid tissues.

LT expression on ROR γ ⁺ cells is essential for control of IL-22 production and protection of mice against *C. rodentium* infection

Since our data suggested the critical role of LT β R signaling in controlling innate IL-22 pathway, we next tested whether LT expression on innate ROR γ ⁺ cells is essential for IL-22 production. In order to test this, we utilized mice with specific inactivation of surface LT β ₂LT α ₁ complex on ROR γ ⁺ expressing cells (ROR γ -*Ltb*^{-/-} mice), by crossing LT β floxed mice (Tumanov et al., 2002) with ROR γ -Cre transgenic mice (Eberl et al., 2004). We found that IL-22 expression in colon tissue or in colon lamina propria cells was dramatically reduced in ROR γ -*Ltb*^{-/-} mice, compared to WT mice (Figure 4A). Reduced expression of IL-22 in colon correlated with reduced expression of IL-22 dependent antimicrobial proteins RegIII γ and RegIII β (Figure 4B). Thus, our data suggest that surface LT expression on ROR γ ⁺ cells is essential to control the IL-22 production in the gut.

To define whether the IL-22 pathway is an essential protection mechanism downstream of LT signaling we delivered exogenous IL-22 by hydrodynamic injection of IL-22 expressing plasmid to ROR γ -*Ltb*^{-/-} mice. Exogenous IL-22 rescued ROR γ -*Ltb*^{-/-} mice, as 90% of mice treated with IL-22 survived the infection and did not lose weight compared to mice treated with control vector (Figure 4C). ROR γ -*Ltb*^{-/-} mice rescued by IL-22 delivery showed reduced colonic inflammation and bacterial dissemination to peripheral organs, compared to ROR γ -*Ltb*^{-/-} mice treated with control vector (Figure 4D, and E). Thus, LT expression on innate ROR γ ⁺ cells is essential to control the IL-22 protective pathway.

LT β R signaling on DCs is required for IL-22 production

Our results suggested that LT expression directly regulates IL-22 production from ROR γ ⁺ cells. Therefore, we considered two potential mechanisms to explain how LT regulates IL-22 production. We first considered that direct interactions among innate ROR γ ⁺ cells expressing both surface LT and LT β R leads to IL-22 production. We also considered an indirect mechanism, where ROR γ ⁺ cells produce IL-22 via interaction with accessory LT β R expressing cells. To test these possibilities, we used conditional gene targeting approach by inactivating *ltbr* in different cell types. We first ruled out the direct mechanism, because mice with specific inactivation of *ltbr* in ROR γ ⁺ cells produced similar levels of IL-22 and antimicrobial proteins after infection, compared to WT mice (Figure S4). Since our data showed a close interaction of ROR γ ⁺ cells with DCs in lymphoid follicles, we proposed that LT signaling by ROR γ ⁺ cells could trigger LT β R on DCs to provide additional signals for IL-22 production by ROR γ ⁺ cells as a positive feedback loop mechanism. In order to resolve this hypothesis, we inactivated the *ltbr* gene specifically in DCs by crossing LT β R floxed mice (Wang et al., 2010) with CD11c-Cre transgenic mice (Stranges et al., 2007), generating CD11c-*Ltbr*^{-/-} mice. We found an efficient deletion of the *ltbr* gene and mRNA in bone marrow derived DCs from CD11c-*Ltbr*^{-/-} mice (Figure S5A-B). Since CD11c is also expressed at lower levels in NK cells (Laouar et al., 2005; Stranges et al., 2007), we cannot exclude the possibility of *ltbr* gene deletion in a fraction of NK cells.

However, CD11c-Cre expression unlikely affects the development or function of NK cells, since the number of NKp46⁺ and LTI cells was not affected in CD11c-*Ltbr*^{-/-} mice (Figure S5A-B, and data not shown). CD11c-*Ltbr*^{-/-} mice infected with *C. rodentium* showed significant reduction of body weight compared to WT mice, but were able to survive infection (Figure 5A). CD11c-*Ltbr*^{-/-} mice showed focal colon bacterial lesions in colon (Figure 5B), and were not able to control bacterial dissemination to peripheral organs (Figure 5C-E). These data suggest that LTβR signaling in DCs is required for efficient immune response to mucosal pathogen. To define whether LTβR signaling on DCs is required for IL-22 production, we measured IL-22 production in colon of CD11c-*Ltbr*^{-/-} mice early after infection. We found that both IL-22 mRNA and protein expression were greatly reduced in the colon of CD11c-*Ltbr*^{-/-} mice compared to WT mice (Figure 5F, and G). Consistently, expression of IL-22-dependent antimicrobial protein RegIIIγ was reduced in colon of CD11c-*Ltbr*^{-/-} mice (Figure 5H). These results suggest that LTβR signaling in DCs is required for effective IL-22 production in the gut post-infection.

To further define the mechanism of IL-22 production by LTI cells, we measured expression of cytokines in colon lamina propria DCs isolated from WT and *Ltbr*^{-/-} mice at day 5 post infection. We found that expression of IL-23, a potent inducer of IL-22 (Sonnenberg et al., 2011b; Zheng et al., 2008), was reduced in DCs purified from colon of *Ltbr*^{-/-} mice, compared to WT mice (Figure 5I). In contrast to IL-23 expression, DCs from WT and *Ltbr*^{-/-} mice expressed similar levels of IL-1β and IL-6 (Figure 5I). These results suggest that LTβR signaling activates IL-22 pathway indirectly via IL-23 production by DCs.

Discussion

Innate lymphoid cells (ILCs) represent a mechanism to regulate the immune response to pathogens at mucosal barriers. However, the mechanism that controls their effector functions is not well defined. In this study, we identified LTβR signaling as an essential regulator of IL-22 protection pathway in the gut. We demonstrate that LT controls IL-22 production by ILCs in response to enteric bacterial pathogen *C. rodentium*. LT from innate RORγt⁺ cells but not from adaptive T or B cells was essential for IL-22 production. IL-22-producing RORγt⁺ cells localized predominantly in gut B cell follicles and interacted closely with DCs. We demonstrate that LT controls IL-22 production by RORγt⁺ ILCs indirectly via LTβR signaling in DCs. Therefore, LT represents a regulator of IL-22 protection pathway by ILCs to control mucosal bacterial infection.

Previous studies identified IL-22 as a critical cytokine to control *C. rodentium* infection (Zheng et al., 2008). This IL-22 protective effect is mediated by production of antimicrobial proteins by epithelial cells that specifically express IL-22R. Additionally, IL-22R signaling is required for survival and regeneration of intestinal epithelial cells after injury as well as for mucus production (Sugimoto et al., 2008; Zenewicz et al., 2008). Innate immune cells are critical for production of this IL-22 that provides host protection after enteric *C. rodentium* infection (Zheng et al., 2008). IL-23 was shown as a critical regulator to promote IL-22 production by ILC *in vitro* and *in vivo* (Sonnenberg et al., 2011b; Zheng et al., 2008). However, other pathways that influence IL-22 production remained largely unknown. Our data show that the LTβR pathway is an essential regulator of IL-22 production by innate immune cells in response to enteric bacterial infection. Since LTβR deficient mice display multiple defects in components of both innate and adaptive immune system, it is important to define whether IL-22 pathway is an essential LTβR-dependent mechanism that might impart protection against *C. rodentium*. By using both agonistic anti-LTβR stimulation in LT deficient mice and reconstitution of IL-22 expression in LTβR-deficient mice we demonstrate that IL-22 is a critical protection pathway downstream of LTβR signaling.

Our next experiments aimed to define which LT-expressing cell populations are essential for IL-22 production after infection. Our results suggest that LT expression by innate but not adaptive immune cells is critical for IL-22 production and host protection, because mice with specific inactivation of surface LT on T and B cells did not show defects in IL-22 production. Recent studies identified a heterogeneous population of ILCs in the gut capable of producing IL-22 upon stimulation (Colonna, 2009; Sonnenberg et al., 2011a; Spits and Di Santo, 2011). In particular, mucosal $ROR\gamma^t^+NKp46^+$ cells were suggested as a major source of IL-22 production in the small intestine lamina propria (Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008). In contrast to $ROR\gamma^t^+NKp46^+$ cells, LT_i cells did not produce IL-22 in the small intestine lamina propria from naïve mice (Luci et al., 2009; Sanos et al., 2009), although other studies reported IL-22 expression also by LT_i cells (Marchesi et al., 2009; Sawa et al., 2010). It remains possible that cytokine production by LT_i cells might change under ongoing bacterial infection conditions. A recent study reported that LT_i cells in the intraepithelial lymphocyte compartment were, in fact, the major IL-22 producing cells after *C. rodentium* infection (Sonnenberg et al., 2011b). Yet another study suggested that DCs could also contribute significantly to IL-22 levels soon after *C. rodentium* infection (Zheng et al., 2008). In contrast to this earlier study, we found that DCs were not major producers of IL-22 after infection. However, we observed that DCs were in close contact with IL-22 producing $ROR\gamma^t^+$ cells in B cell follicles. Our results using $ROR\gamma^t$ -GFP^{+/+} reporter mice showed that LT_i cells were the predominant producers of IL-22 in the lamina propria early after infection. Furthermore, expression of IL-22 was greatly reduced in LT_i cells from *Ltbr*^{-/-} mice, suggesting that in the absence of LTβR signaling the LT_i cells are unable to produce IL-22 after infection. However, we found no intrinsic defect in IL-22 production by lamina propria ILCs from *Ltbr*^{-/-} mice since these cells were able to produce IL-22 after *in vitro* stimulation with IL-23, consistent with a recent report (Satoh-Takayama et al., 2011). Our results are also consistent with the recent study (Sonnenberg et al., 2011b) and extend our current understanding to suggest that LT_i subset of ILCs actively participate in host defense by regulating IL-22 protection pathway in the gut.

ILCs produce various TNF family cytokines and chemokine receptors, including TNF, LTα, LTβ, LIGHT, TRANCE, CCR7, CCR6, and CXCR5 (Luci et al., 2009; Marchesi et al., 2009; Vonarbourg et al., 2010; Wang et al., 2010). Our study revealed that a genetic inactivation of a single cytokine, surface LT on $ROR\gamma^t^+$ ILCs, resulted in a dramatic defect in the production of IL-22 by these cells, leading to a 100% mortality rate to *C. rodentium* infection in mice. These results demonstrate that LT expression on $ROR\gamma^t^+$ ILCs is critical for control of IL-22 protection pathway in the gut.

In contrast to LT, expression of LTβR on ILCs was dispensable for IL-22-mediated protection, suggesting an indirect LT-dependent mechanism of IL-22 production by $ROR\gamma^t^+$ ILCs. Remarkably, IL-22 producing cells were not randomly scattered in the gut, but predominantly located in organized secondary lymphoid tissues in the colon, and closely interacted with DCs. By using a conditional gene targeting approach, we demonstrate that LTβR signaling in DC is important for IL-22 production and bacterial clearance. We also cannot exclude the potential contribution of LTβR signaling by other cells, in addition to DC, because IL-22 levels were only partially reduced in colon of *CD11c-Ltbr*^{-/-} mice compared to *Ltbr*^{-/-} mice. *Ltbr*^{-/-} mice have multiple defects in development of gut-associated lymphoid tissues which may additionally affect IL-22 production independently of LTβR signaling in DCs.

Based on our results, we propose a model of regulation of IL-22 production in the gut to control mucosal bacterial infection as shown in Figure 6. LT expression by $ROR\gamma^t^+$ ILCs is necessary for IL-22 production following invasion of mucosal bacterial pathogen. LT

signaling by ROR γ ⁺ ILC promotes the development of lymphoid follicles in the gut. Lymphoid follicles provide the necessary microenvironment for close interaction between ROR γ ⁺ ILCs and DCs. Interplay between ROR γ ⁺ ILCs and DCs then promotes IL-23 secretion by DCs that, in turn, activates IL-22 production by ROR γ ⁺ cells as a positive feedback loop. Since both LT (LT β ₂LT α ₁) and LT β R exist only as membrane bound molecules, DCs and ROR γ ⁺ ILCs interaction likely require direct cell contact. IL-22 activates IL-22R on epithelial cells which leads to production of antimicrobial proteins RegIII γ and RegIII β to eliminate the mucosal bacterial pathogen. Thus, our study opens avenues to explore the role of ILCs and DC cooperation in host defense and in pathogenesis of autoimmune diseases.

It was recently proposed by several studies that host-protective functions of ROR γ ⁺ ILCs evolutionary preceded the acquisition of lymphoid tissue-inducing functions (Lane et al., 2009; Sonnenberg et al., 2011b). However, our study suggests that gut-associated lymphoid tissues provide an environment where ROR γ ⁺ ILCs coordinate closely with cells of adaptive immune system. It is also possible that LT signaling by ROR γ ⁺ ILCs also participates in the maintenance of gut-associated lymphoid tissues. Therefore, the mechanism of interplay and coordination of ILCs with components of adaptive immune system will be an exciting area of future research.

In animal models of colitis, expression of IL-22 by both innate and adaptive immune cells regulates colon inflammation in both DSS-induced and T-cell induced colitis models (Sugimoto et al., 2008; Zenewicz et al., 2008). Other studies show that LT production by B cells promotes colon inflammation in a DSS-induced inflammation model (Jungbeck et al., 2008; Lochner et al., 2011), while ROR γ ⁺ ILCs reduce colon inflammation in this model (Sawa et al., 2011). In light of our results, it would be important to determine whether LT by innate ROR γ ⁺ cells can also participate in prevention of colon inflammation in DSS-induced colitis and other models of colon inflammation.

Elevated IL-22 levels have been reported in IBD patients (Ouyang, 2010). Although the role of IL-22 in IBD is complex, most studies suggest a protective role of IL-22 in IBD, by regulating epithelial barrier function, regeneration of epithelial cells, and production of antibacterial proteins (Sonnenberg et al., 2011a; Sugimoto et al., 2008; Zenewicz et al., 2008). However, a pathogenic role of IL-22 has been shown in other organs, for example, IL-22 promotes psoriasis (Ouyang, 2010). Therefore, careful design of IL-22 modulating agents is required for the development of potential therapeutic approaches. Our data suggest that LT β R signaling regulates IL-22 production by ILCs in the gut and that pharmacological manipulation of LT β R signaling could represent a therapeutic avenue in autoimmune diseases.

Experimental Procedures

Mice

C57BL/6 and *Rag1*^{-/-} mice were purchased from Harland Teklad. *Ltb*^{-/-}, *Tnfsf14*^{-/-}, and *Ltbr*^{-/-} mice were backcrossed onto C57BL/6 background 13, 11 or 10 generations, respectively, and maintained under specific pathogen-free conditions as described (Wang et al., 2010). *Rorc*^{-/-} (Eberl and Littman, 2004) mice were purchased from The Jackson Lab. CD11c-*Ltbr*^{-/-} and ROR γ -*Ltbr*^{-/-} mice were generated by crossing LT β R floxed mice (Wang et al., 2010) with CD11c-Cre (Stranges et al., 2007) and ROR γ -Cre (Eberl et al., 2004), respectively. *T,B-Ltbr*^{-/-} mice were described previously (Tumanov et al., 2002; Tumanov et al., 2003). Animal care and use were in accordance with institutional and National Institutes of Health guidelines and all studies were approved by the Animal Care and Use Committee of the University of Chicago.

***Citrobacter rodentium* infection**

To induce bacterial colitis in mice, mice were orally gavaged with 2×10^9 cfu *C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection), as previously described (Zheng et al., 2008). Briefly, mice were fasted for 8 h before oral inoculation of *C. rodentium* culture in a total volume of 0.2ml per mouse. Bacteria were prepared by shaking at 37°C overnight in LB broth. Concentration was assessed by measuring absorbance at OD600. Bacterial culture was serially diluted and plated after each inoculation to confirm the colony-forming units (CFUs) administered. Body weight was measured a day before and then frequently during the course of disease.

LT β R-Ig and anti-LT β R antibody treatment

The LT β R-Ig used in this study has been previously described (Wang et al., 2010). Briefly, cDNA encoding the extracellular domain of murine LT β R was fused with the Fc portion of human IgG, transfected into BHK/VP16 cell, and the supernatant collected. The anti-LT β R agonistic antibody (3C8) was kindly provided by C. Ware (La Jolla Institute for Allergy and Immunology, La Jolla, CA).

Isolation of intraepithelial lymphocytes (IEL), lamina propria mononuclear (LPL) cells and epithelial cells from mouse colon

IELs, LPLs and colonic epithelial cells were isolated as described (Wang et al., 2010).

Tissue collection, histology, histological score and CFU counts

Colons were dissected from the mice and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue sections were stained with H&E to evaluate tissue pathology. Histological scoring was performed using a modified scoring system described previously (Zheng et al., 2008). In brief, the presence of rare inflammatory cells in the lamina propria were counted as: 0; 1- confluence of inflammatory cells; 2- extending into the submucosa; 3- transmural extension of the inflammatory cell infiltrate. For epithelial damage, absence of mucosal damage was counted as 0, discrete focal lymphoepithelial lesions were counted as 1, mucosal erosion/ulceration was counted as 2, and a score of 3 was given for extensive mucosal damage and extension through deeper structures of the bowel wall. The two subscores were added and the combined histological score ranged from 0 (no changes) to 6 (extensive). For CFU count fecal samples were collected and weighted, then homogenized in sterile phosphate-buffered saline. Serially diluted homogenates were plated on MacConkey agar plates (Sigma). *C. rodentium* colonies were identified as pink colonies after overnight incubation at 37°C. Spleens and livers were aseptically removed and homogenized. Organs colonization was assessed as described for fecal specimens.

Hydrodynamic IL-22 delivery

Mice were restrained in conical restrainer with a heating element to dilate blood vessels. 10 μ g of IL-22 expressing plasmid (pRK-mIL-22, Genentech) or control vector (pRK) were injected in tail vein 6h after *C. rodentium* infection in 1.6 ml of TransIt-EE Hydrodynamic Delivery Solution (MIR 5340, Mirus Inc) over a period of 3-5 sec.

ELISA

Terminal colon pieces 1 centimeter length were cut in small pieces and incubated in 0.3ml of RPMI 1640 medium containing 10% FCS, amphotericin, gentamicin, penicillin and streptomycin for 48h in tissue plates, as previously described (Zheng et al., 2008). IL-22 in supernatants was measured by ELISA (R&D Systems) according to manufactures recommendations.

Flow cytometry and antibodies

Flow cytometry analysis was performed on FACSCanto, and FACSAria II (BD Biosciences) instruments and analyzed using FlowJo software (Tree Star Inc.). All antibodies were purchased from BD Biosciences or eBioscience. Intracellular for IL-22 and ROR γ t was performed as described previously (Sawa et al., 2010).

RNA isolation and real-time reverse transcriptase PCR

RNA from cells or tissues was isolated using RNeasy Micro or Mini Kit (Qiagen). cDNA synthesis and real-time RT-PCR was performed as described (Wang et al., 2010).

Immunohistochemistry

Colons was isolated from mice and extensively washed in PBS, then fixed for 1h in 4% (wt/vol) paraformaldehyde/PBS. After three washes in PBS, paraformaldehyde-fixed organs were incubated in a 30% (wt/vol) sucrose/PBS solution overnight. Fixed tissues were embedded in optimum cutting temperature compound (Sakura Finetek) and frozen at -70C. 6-8 mm cryostat sections were stained with IL-22 (Alexa 555-conjugated, clone 8E11, Genentech) and ROR γ t (hamster polyclonal antibody), as previously described (Eberl et al., 2004; Zheng et al., 2008). After being stained, slides were dried, mounted with ProLong Gold (Invitrogen) and examined with Nikon A1 confocal microscope (Nikon, Inc.), or Zeiss Axiovert 200M microscope (Carl Zeiss, Inc.). Images were processed with NIH ImageJ v. 1.42 software.

Statistical analysis

Comparisons of data were analyzed by two-tailed Student's t test using GraphPad Prism 5.0 program. Data from such experiments are presented as mean values \pm S.E.M. $P < 0.05$ was considered significant. For survival curves statistics were done using the log rank (Mantel-Cox) test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The Lymphotoxin pathway regulates IL-22 production by ROR γ ⁺ innate lymphoid cells
- LT β R signaling in gut DCs triggers the innate immune response to mucosal pathogens
- ROR γ ⁺ ILCs communicate with DCs in gut lymphoid follicles for IL-22 production
- IL-22 represents an essential protection pathway downstream of LT β R signaling

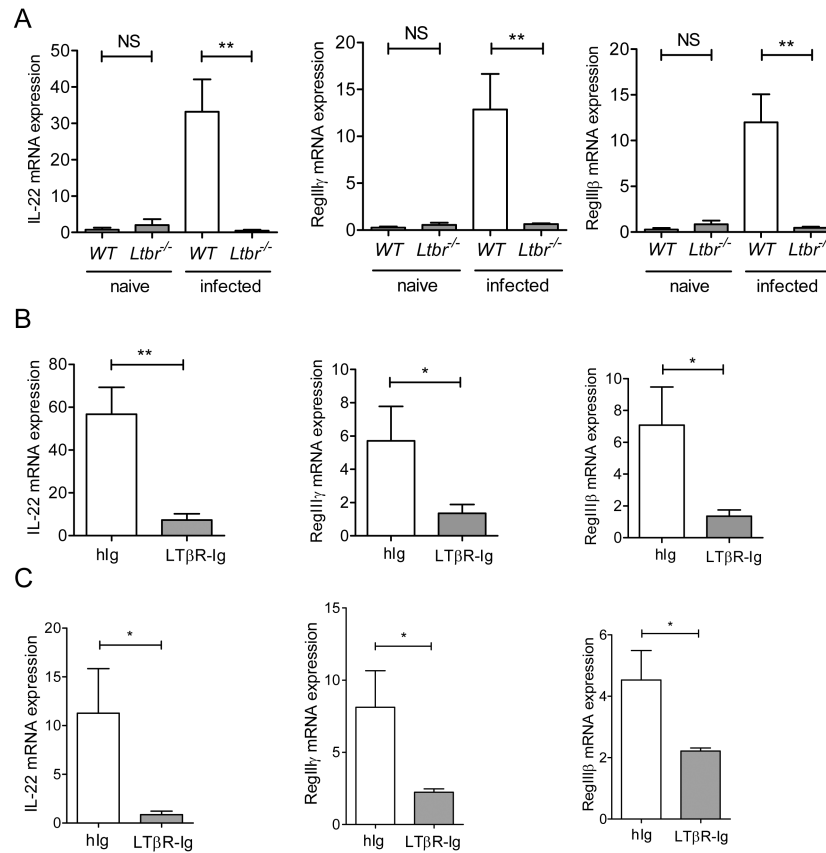


Figure 1. LT β R signaling controls innate IL-22 pathway in the gut

A. IL-22 levels in the colon of naïve and infected WT and *Ltbr*^{-/-} mice. *Ltbr*^{-/-} and WT mice (n=5/group/experiment) were orally inoculated with *C. rodentium*. mRNA expression of IL-22 and IL-22- dependent RegIII γ and RegIII β antimicrobial proteins in the colon were measured by real-time PCR on day 5 post infection. Naïve WT mice were used as control. **B.** WT mice were treated with 150 μ g of LT β R-Ig, or control human Ig (hlg) at day 0 and 3 post infection. Expression of IL-22 and RegIII γ and RegIII β antimicrobial proteins were measured in colon by real-time PCR at day 5 post infection. **C.** *Rag1*^{-/-} mice were treated intraperitoneally with 150 μ g of LT β R-Ig at day 0 and 3 post infection, and expression of IL-22, and RegIII γ and RegIII β antimicrobial proteins was measured by real-time PCR at day 5 post infection. **A-C.** Data represent means \pm s.e.m. n=5, *p<0.05, **p<0.01. Data represent one of three independent experiments with similar results. Real-time PCR data were normalized to *hprt* expression.

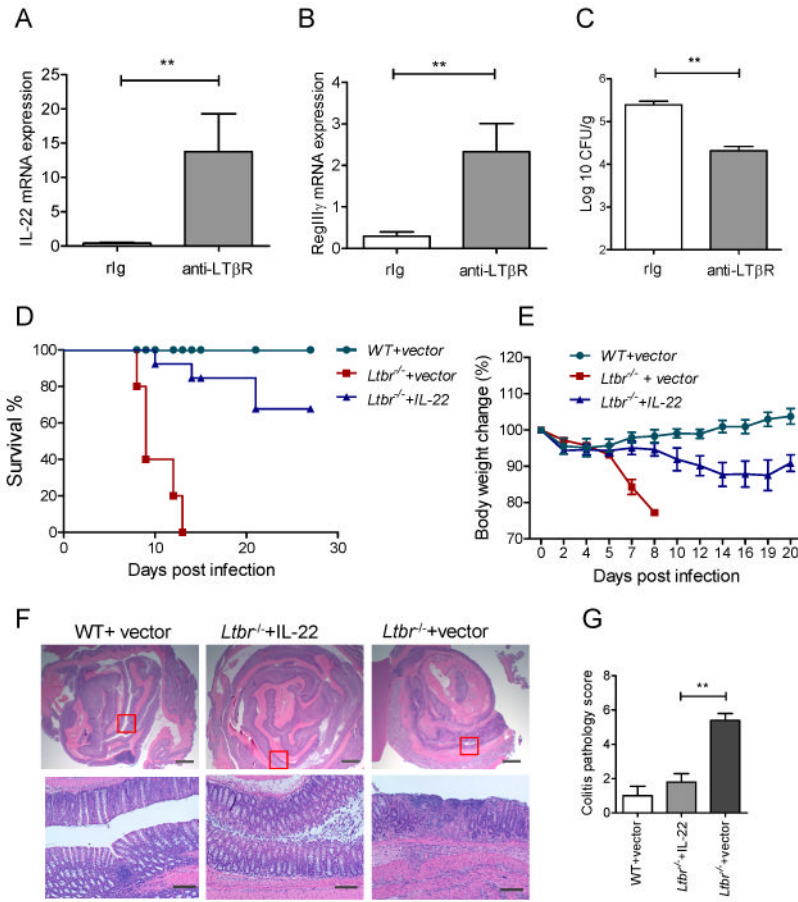


Figure 2. IL-22 is an essential protection pathway downstream of LTβR signaling
A-C. Stimulation of LTβR signaling promotes IL-22 protection pathway. *Ltbr*^{-/-} mice were injected intraperitoneally with 75μg of agonistic anti-LTβR antibody or control rat Ig (rIg) at day 0 and 3 post infection with *C. rodentium*. mRNA expression of IL-22 (**A**) and RegIIIγ (**B**) were measured in colon by real-time PCR at day 4 post infection. **C.** Bacterial titers in liver at day 4 post infection. n=4, **p<0.01, Data represent means ± s.e.m. **D-G.** Hydrodynamic injection of IL-22 expressing plasmid rescues *Ltbr*^{-/-} mice from lethal *C. rodentium* infection. IL-22 expressing plasmid or control vector were intravenously injected to WT or *Ltbr*^{-/-} mice 6h after *C. rodentium* infection. n=10-13/group. Survival (**D**) and body weight change (**E**) are shown. ***p<0.001 between IL-22 treated *Ltbr*^{-/-} and control untreated *Ltbr*^{-/-} mice by Mantel-Cox log-rank test. Data combined from two experiments with similar results. **F.** Representative hematoxylin and eosin staining of colons at day 9 post infection. Red boxes in top panel are shown at higher magnification at lower panel. Bars: 1mm for top panel, and 200μm for lower panel. **G.** Colitis histopathology score. n=5, *p<0.05. Data represent means ± s.e.m.

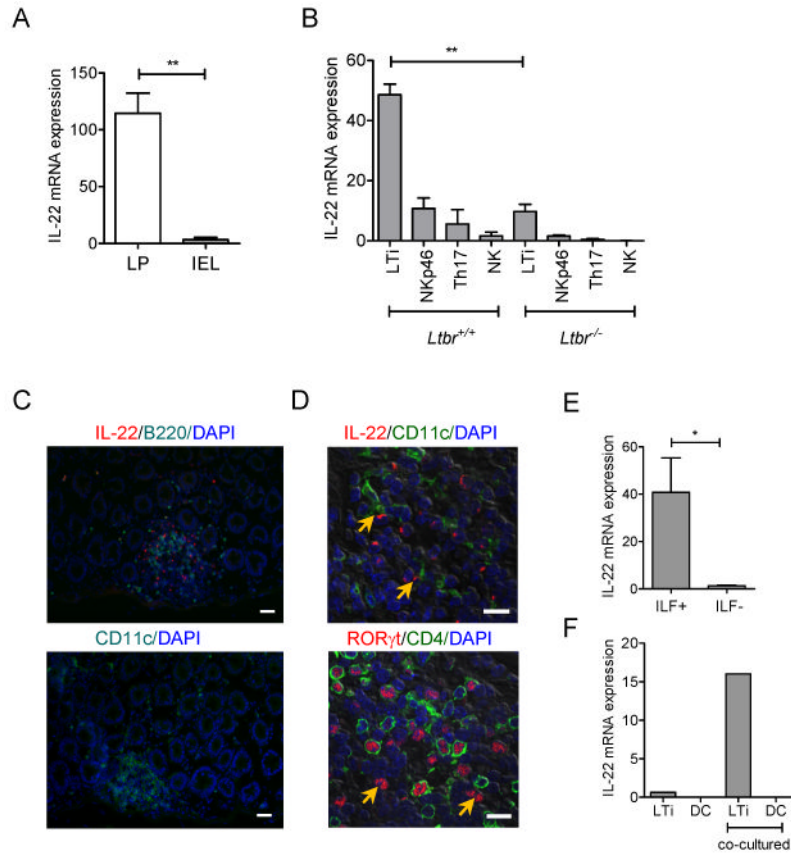


Figure 3. IL-22 is produced in lymphoid follicles by innate $ROR\gamma^t^+$ cells

A. IL-22 is predominantly expressed in lamina propria. Colon lamina propria (LP) and intraepithelial lymphocyte (IEL) cell populations from WT mice were purified at day 5 post infection. IL-22 expression was measured by real-time PCR. Data normalized to *hprt* expression. Data represent means \pm s.e.m. $n=5$ mice, $**p<0.01$. **B.** LTi cells are predominant IL-22 producing cells in the lamina propria. Colon LP innate cell populations from $ROR\gamma^t-GFP^{+/-}/Ltbr^{+/+}$ and $ROR\gamma^t-GFP^{+/-}/Ltbr^{-/-}$ mice at day 5 post infection were purified by flow cytometry. IL-22 expression was measured by real-time PCR. Sorted cell populations are indicated as: LTi: $CD45^+CD3^-GFP^+NK1.1^-NKp46^-$, NKp46: $CD45^+CD3^-GFP^+NKp46^+$, NK: $CD45^+CD3^-GFP^-NK1.1^+$, Th17: $CD45^+CD3^-GFP^+NK1.1^-NKp46^-$. Data combined from two experiments, means \pm s.e.m, $n=4$, $**p<0.01$. IL-22 data were normalized to *hprt* expression. **C.** IL-22 expressing cells are predominantly located in lymphoid follicles. WT mice were orally infected with *C. rodentium* and colon sections at day 5 post infection were stained with indicated antibodies. Nuclei were stained with DAPI. Bars: $20\mu m$. **D.** IL-22 producing cells interact with DC in lymphoid follicles. Colon sections of mice at day 5 post infection were stained with indicated antibodies and analyzed by confocal microscopy. Nuclei were stained with DAPI. Arrows indicate contact of DC (green) with IL-22 producing cells (red) on top panel, and $ROR\gamma^t^+CD4^-$ cells on bottom panel. Bars: $10\mu m$. **E.** IL-22 is expressed predominantly in isolated lymphoid follicles. WT mice were orally infected with *C. rodentium*, and colon tissue collected at day 5 post infection. Isolated lymphoid follicles (ILF+) and surrounded tissue (ILF-) were microdissected under stereo microscope, and IL-22 expression measured by real-time PCR. IL-22 data were normalized to *hprt* expression. $*p<0.05$, $n=3$ mice. **F.** Co-culture of LTi cells with DC promotes IL-22 production by LTi cells. Lamina propria LTi cells from naïve $ROR\gamma^t-GFP^{+/-}$ mice were co-cultured *in vitro* for 18h with lamina propria DCs from WT mice at day 5 post infection.

After co-culture, LTi and DC cells were separated by flow cytometry and IL-22 measured by real-time PCR. Data normalized to b-actin. One of two independent experiments with similar results is shown.

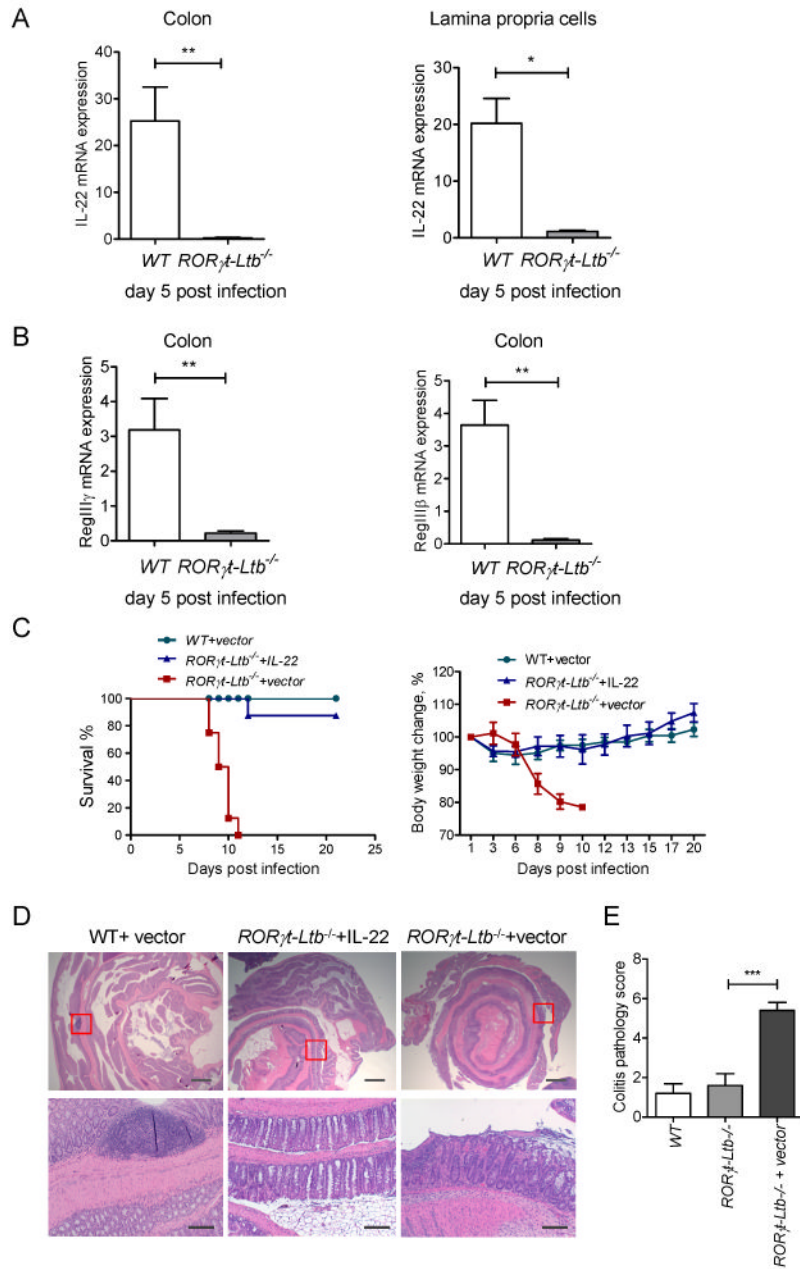


Figure 4. LT expression on ROR γ t $^{+}$ cells is essential for control of IL-22 production and protection of mice against *C. rodentium* infection
A-B. LT expression on ROR γ t $^{+}$ cells controls IL-22 protection pathway. WT and ROR γ t-Ltb $^{-/-}$ mice were orally infected with *C. rodentium*. Expression of IL-22 in colon and purified lamina propria cells (A), and antimicrobial proteins RegIII γ , and RegIII β (B) was measured by real-time PCR at day 5 post infection. Data represent means \pm s.e.m. One out of three independent experiments with similar results is shown. n=5 mice, *p<0.05, **p<0.01. Expression data were normalized to *hprt* expression. **C.** IL-22 expression is sufficient to rescue ROR γ t-Ltb $^{-/-}$ mice from lethal *C. rodentium* infection. WT and ROR γ t-Ltb $^{-/-}$ mice were intravenously injected with IL-22 expressing plasmid or control vector at 6h after *C. rodentium* infection. n=10/group. Data combined from two experiments with similar results. Survival and body weight change are shown. **D.** Representative hematoxylin and eosin

staining of colon at day 9 post infection. Red boxes in top panel are shown at higher magnification at lower panel. Bars: 1mm for top panel, and 200 μ m for lower panel. **E.** Colitis histopathology score for mice in panel A. n=5, ***p<0.001. Data represent means \pm s.e.m. One of two experiments with similar results is shown.

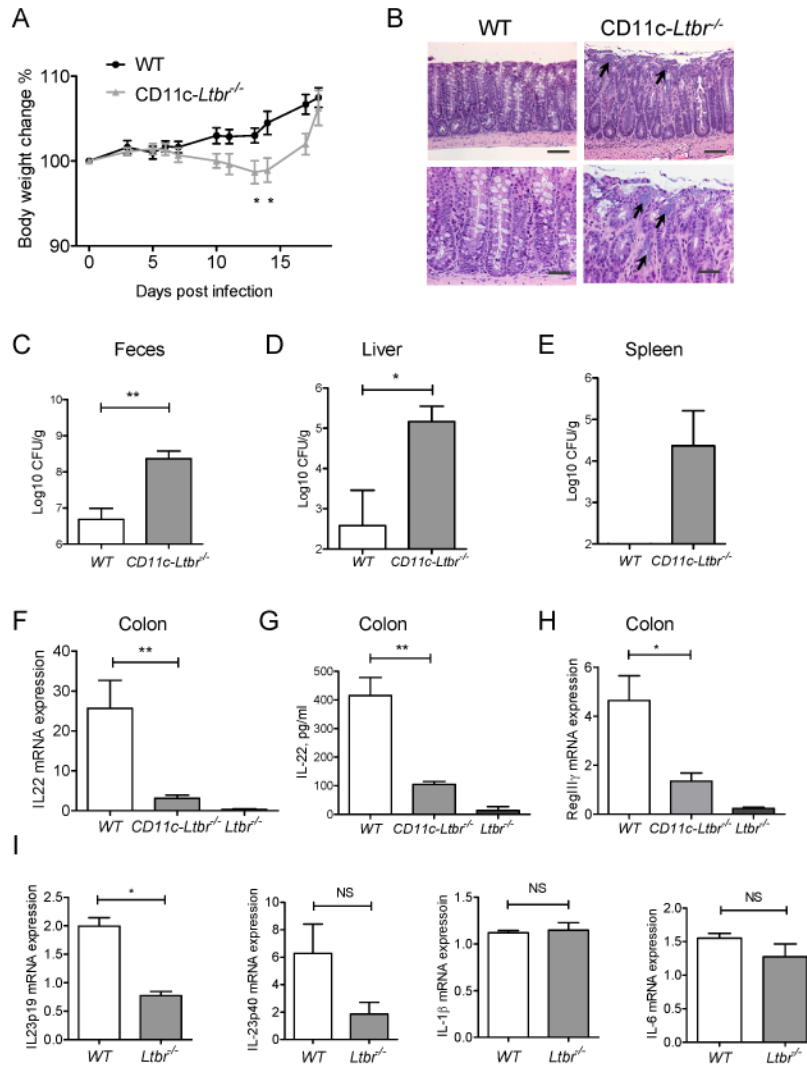


Figure 5. LTβR signaling in DCs is required for IL-22 production

WT, CD11c-Ltbr^{-/-}, and Ltbr^{-/-} mice were orally infected with *C. rodentium*. Body weight change (A) and representative hematoxylin and eosin staining of colon at day 12 post infection (B). Arrows indicate bacterial lesions. Bars: 50μm (top panels), and 20 μm (lower panels). Bacterial titers were measured in feces (C) on day 14, and liver (D), and spleen (E) on day 10 post infection. F. IL-22 expression in colon at day 5 post infection. G. IL-22 levels in colon supernatants at day 5 post infection. H. Expression of RegIIIγ mRNA in colon at day 5 post infection. C-H. Data represent means ± s.e.m. n=5 mice, *p<0.05, **p<0.01. Represents one of three independent experiments with similar results. Real-time PCR data were normalized to *hprt* expression. I. LTβR signaling in DCs regulates IL-23 production. CD11c⁺CD11b⁺ DCs from colon lamina propria of WT and Ltbr^{-/-} mice were purified by flow cytometry at day 5 post infection. Expression of IL23p19, IL23p40, IL-1b, IL-6 cytokines was measured by real-time PCR. Data combined from two experiments, means ± s.e.m, n=4, *p<0.05, NS-not significant. RT-PCR data normalized to *hprt* expression.

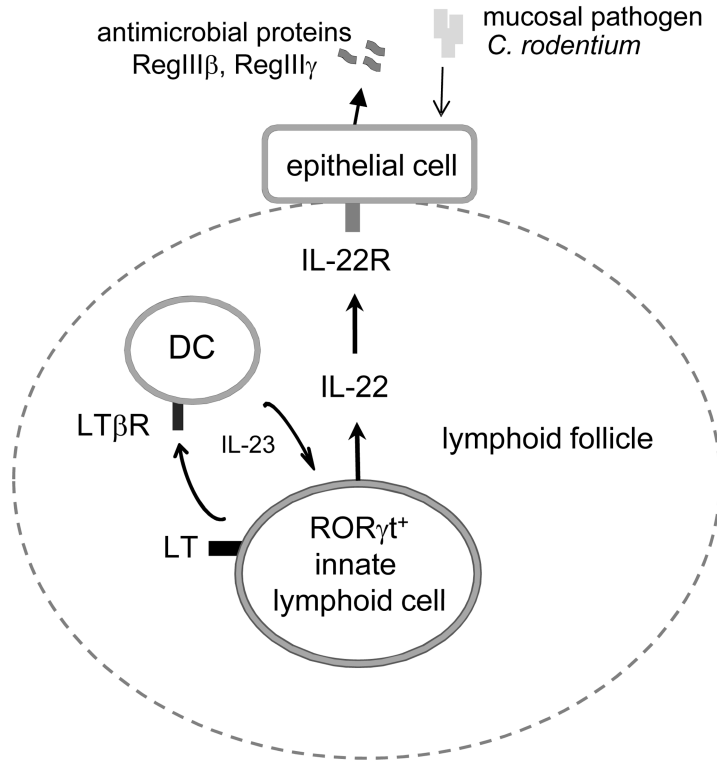


Figure 6. Proposed model to control IL-22 pathway against mucosal bacterial pathogen
 LT expression by RORγt+ ILCs is necessary for IL-22 production following invasion of mucosal bacterial pathogen. LT signaling by RORγt+ ILC promotes the development of lymphoid follicles in the gut. Lymphoid follicles provide necessary microenvironment for interaction between innate RORγt+ cells and DC. Interplay between RORγt+ ILCs and DCs promotes IL-23 production by DCs that, in turn, activates IL-22 synthesis by RORγt+ cells as a positive feedback loop. IL-22 stimulates IL-22R on epithelial cells which triggers production of antimicrobial proteins RegIIIγ and RegIIIβ to eliminate mucosal bacterial pathogen.