Lymphotoxin Beta Receptor Signaling in Intestinal Epithelial Cells Orchestrates Innate Immune Responses against Mucosal Bacterial Infection

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INTRODUCTION

The epithelial layer serves not only as a natural barrier against microbial invaders, but is also involved in host defense through its ability to sense mucosal pathogens and mobilize immune cells. However, the pathways that mediate the crosstalk between immune cells and intestinal epithelial cells during mucosal bacterial infection are poorly understood. Citrobacter rodentium (C. rodentium) is a natural mouse extracellular enteric pathogen that mimics human enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic Escherichia coli (EHEC), all of which use attaching and effacing lesion formation, initially on gut epithelial cells, as a major mechanism of tissue targeting and infection (Mundy et al., 2005). Therefore, this is an ideal model to dissect how immune cells interact with gut epithelial pathogens. Both the innate and adaptive immune systems are involved in control of C. rodentium infection. The adaptive immune components, including CD4+ T cells, B cells, and C. rodentium-specific antibodies, have been shown to play an essential role in containing and eradicating the infection (Bry and Brenner, 2004; Maaser et al., 2004; MacDonald et al., 2003; Uren et al., 2005; Vallance et al., 2003). Accordingly, recombination activating gene 1 deficient (Rag1−/−) mice lacking both T and B cells fail to clear C. rodentium infection and eventually die by 3 weeks after infection (Bry and Brenner, 2004; Vallance et al., 2003). However, there are several innate immune mechanisms in the gut that help to control the infection, such as signals originating from Toll-like receptors (TLRs), that bridge innate and adaptive immunity (Gibson et al., 2008; Lebeis et al., 2007).

Membrane-bound lymphotixin (LT) (LTα1LTβ2), and LIGHT (TNF superfamily member 14 [TNFSF14]), are members of the TNF family of cytokines. Both LT and LIGHT are primarily expressed on lymphocytes and each can deliver signals through LTβ receptor (LTβR) (Browning, 2008; Ware, 2005). In contrast, LTβR is primarily expressed on epithelial, stromal, and myeloid cells, but not lymphocytes (Browning, 2008; Ware, 2005), suggesting that it may participate in the communication between lymphocytes and surrounding epithelial and stromal cells. Indeed, LTβR signaling has been shown to be critical for protection against the mucosal pathogen C. rodentium (Spahhn et al., 2004); however, the mechanisms underlying the protective role of LTβR remain predominantly unknown. Most studies have focused on the critical role of LT in the development and maintenance of secondary lymphoid organs and in immune homeostasis (Browning, 2008; Fu and Chaplin, 1999; Ware, 2005). In particular, it has been shown that LT, primarily from B cells, controls the development and maintenance of the lymphoid microstructure of the spleen to support antibody responses (Fu et al., 1998; Gonzalez et al., 1998; Tumanov et al., 2002).

A recent study identified interleukin-22 (IL-22) as an important cytokine for mediating innate protection against C. rodentium infection (Zheng et al., 2008). Both lymphoid tissue inducer-like (LTI-like) cells and a mucosal subset of NK cells that express the NKP46 surface marker (NK-like cells) are able to secrete IL-22 and thus are candidates for mucosal innate defense (Cella et al., 2009; Satoh-Takayama et al., 2008; Takatori et al., 2009; Vivier et al., 2009). These two cell types express the nuclear hormone receptor retinoic acid receptor-related orphan receptor
gamma t (RORγt) which is required for their development. Intriguingly, these cell types can also express membrane LT (Cupedo et al., 2009; Luci et al., 2009; Tsuji et al., 2008); however, whether LT on RORγt+ cells is required for host defense against mucosal infection remains unknown.

Both LT and LIGHT are upregulated on T cells after antigen stimulation and involved in Th1 cell- and Th17 cell-mediated defense against mucosal infection remains unknown. The integrity of the colonic epithelial layer was severely affected in Ltb−/− mice (Figure 1C). This suggests systemic dissemination of C. rodentium. The integrity of the colonic epithelial layer was severely affected in Ltb−/− recipients compared with WT recipients (Figures 1E and S1E). These results suggest a critical role for LTβR signaling on radio-resistant cells for protection. In contrast, LtbR−/− > WT chimeras showed a less severe phenotype: mice lost a substantial amount of weight 11 to 15 days after infection, displayed increased bacterial titers in feces, and spleen, and exhibited a disorganized colonic epithelial layer (Figure 1). However, 40% of these mice were able to recover and survive the infection (Figures 1A and 1B). Thus, LTβR signaling on bone marrow-derived cells also participates in the control of C. rodentium infection.

RESULTS

LTβR on Both Radio-Resistant and Bone Marrow-Derived Cells Controls C. rodentium Infection

LTβR signaling plays a protective role in host defense against the mucosal pathogen C. rodentium, given that all LTβR-deficient mice succumb to infection whereas all wild-type mice survive (Spahne, 2004 and Figure S1 available online). The severity of gut inflammation and tissue injury correlated well with the degree of bacterial load in the host tissues and feces (Figure S1). Because of multiple defects, especially the lack of gut-associated lymphoid tissues in Ltb−/− mice (Browning, 2008; Fu and Chaplin, 1999; Ware, 2005), it was necessary to dissect the cellular components or signaling pathways that are essential for protection. To define which LTβR-expressing cells are critical for the control of C. rodentium infection, we performed reciprocal bone marrow transfer experiments between WT and Ltb−/− mice. Mice were orally infected with C. rodentium 5 weeks after bone marrow transfer. Ltb−/− recipients that received bone marrow from either WT or Ltb−/− mice lost weight substantially during the second week after infection and died within two weeks after infection (Figures 1A and 1B). WT > Ltb−/− and Ltb−/− > Ltb−/− chimera mice show a severe colonic pathology 8 days after infection. H&E staining of representative colons from indicated mice is shown. The panel shows the original magnification × 20. Scale bars represent 100 μm. Data represent means ± SEM (A, C, and D).

(C–E) Data represent one of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, N.S., not significant. See also Figure S1.
LT\textsuperscript{b}R on Gut Epithelial Cells and Hematopoietic-Derived Cells Coordinate to Protect the Host

\textit{Ltbr}\textsuperscript{−/−} mice display multiple defects in the development and maintenance of secondary lymphoid organs, and such defects can account for the reduced clearance of bacteria. Because LT\textsuperscript{b}R is highly expressed on intestinal epithelium (Browning and French, 2002), we next sought to determine whether the absence of LT\textsuperscript{b}R signaling in gut epithelial cells alone, rather than defective secondary lymphoid organs and tissues, was responsible for the observed phenotype of \textit{Ltbr}\textsuperscript{−/−} mice. Therefore, we generated mice deficient in LT\textsuperscript{b}R only in intestinal epithelial cells (Figure S2). LT\textsuperscript{b}R-floxed mice were crossed with Villin-Cre transgenic mice (Madison et al., 2002) to generate intestinal epithelial cell-specific, LT\textsuperscript{b}R-deficient (\textit{Vil-Ltbr}\textsuperscript{−/−}) mice. Efficient deletion of the \textit{Ltbr} gene was found in epithelial cells from both the small intestine and colon (Figure S2D and data not shown). These mice were then used for studying the role of LT\textsuperscript{b}R on epithelial cells and the interplay between epithelial cells and LT\textsuperscript{b}R immune cells. \textit{Vil-Ltbr}\textsuperscript{−/−} mice showed a deficiency in clearing \textit{C. rodentium} infection, and displayed 15–20 times higher bacterial titers in the spleen and feces compared to WT mice at days 10 and 14 after infection (Figure 2A). Thus, LT\textsuperscript{b}R signaling in gut epithelial cells contributes to host defense against a mucosal bacterial pathogen.

Intriguingly, although Vil-Ltbr\textsuperscript{−/−} mice displayed an increased pathology in the colon, most of the mice survived the infection raising the possibility that LT\textsuperscript{b}R signaling in other cell types may also contribute to the severity of disease. To define whether LT\textsuperscript{b}R signaling in bone marrow-derived cells cooperates with LT\textsuperscript{b}R signals in gut epithelial cells, we transferred bone marrow from \textit{Ltbr}\textsuperscript{−/−} mice to Vil-Ltbr\textsuperscript{−/−} mice. Impressively, \textit{Ltbr}\textsuperscript{−/−} → Vil-Ltbr\textsuperscript{−/−} bone marrow chimera mice showed severe colon pathology and weight loss, and all died by day 12 after infection (Figure 2B and data not shown). Thus, LT\textsuperscript{b}R signaling in both gut epithelial cells and hematopoietic-derived cells coordinates protection of the host against mucosal bacterial cells.

To further define the types of bone marrow-derived cells that contribute to protection against \textit{C. rodentium} infection, we generated macrophage- and neutrophil-specific LT\textsuperscript{b}R-deficient mice (LysM-Ltbr\textsuperscript{−/−}) by crossing \textit{Ltbr} floxed mice with LysM-Cre mice (Clausen et al., 1999) (Figure 2C and Figures S2E and S2F). Although LysM-Ltbr\textsuperscript{−/−} mice displayed increased bacterial titers in blood, and feces, they were able to survive infection (Figure 2C and data not shown). This data suggest that LT\textsuperscript{b}R signaling on macrophages and/or neutrophils contributes to bacterial clearance; however, it is not essential for the survival of mice after infection. Because the phenotypes of both Vil-LT\textsuperscript{b}R- and LysM-LT\textsuperscript{b}R-deficient mice were less severe than those of complete LT\textsuperscript{b}R-deficient mice, it is possible that cooperation of LT\textsuperscript{b}R signaling in several types of bone marrow-derived and radioresistant cells is required for complete protection against mucosal bacterial infection.

Membrane LT, but Not LIGHT, Is Essential for the Control of \textit{C. rodentium} Infection

LT\textsuperscript{b}R binds two known ligands, LIGHT (TNFSF14) and membrane LT (LT\textsuperscript{m}1\textsubscript{1}(2), and overexpression of LIGHT on T cells is known to cause gut inflammation (Wang et al., 2004; Ware, 2005). To assess which ligand is essential for the control of
membrane LT, but not LIGHT, is the major ligand for the LTβR-dependent control of C. rodentium infection.

Lymphotoxin from Adaptive T and B Cells Is Not Essential for the Control of Infection

Because T and B cells are the major LT-expressing cells within secondary lymphoid organs, and surface LT is rapidly upregulated on T and B cells after stimulation (Junt et al., 2006; Tumanov et al., 2002), we first tested whether LT-expressing T and/or B cells are required for the control of C. rodentium infection by utilizing mice with conditional inactivation of membrane LT on T cells (T-Ltb−/−), B cells (B-Ltb−/−), or simultaneously on both T and B cells (T.B-Ltb−/−) (Junt et al., 2006; Tumanov et al., 2002). Surprisingly, T-Ltb−/−, B-Ltb−/−, and even T.B-Ltb−/− mice did not lose body weight or display morbidity, and all survived C. rodentium infection (Figures 4A and 4B). Furthermore, fecal titers of C. rodentium in all three types of conditionally deficient mice were similar to those of WT mice 2 weeks after infection (Figure 4C and data not shown). The colonic epithelial cell layer was intact and showed only minimal pathology in all three conditionally deficient mice, similar to WT mice, whereas much more severe colitis was found in Ltb−/− mice (Figure 4D and data not shown). These data collectively demonstrate that membrane LT expressed on adaptive T and/or B cells does not play an important role in the control of C. rodentium infection.

Figure 3. Membrane LT, but Not LIGHT, Is Essential for the Control of C. rodentium Infection

(A and B) Ltb−/−, Tnfsf14−/−, and WT mice (n = 5/group/experiment) were orally inoculated with C. rodentium. Survival rates (A) and body weight change (B) are shown at the indicated time points (n = 9, ***p < 0.001, ***p < 0.001). (C) Histological analysis of representative colons of WT, Ltb−/−, and Tnfsf14−/− mice at day 8 after inoculation. H&E staining illustrates transmural inflammation, bacterial abscesses, submucosal leukocyte infiltration, and edema in Ltb−/− mice, but not in Tnfsf14−/− mice. The panel shows the original magnification × 20. Scale bars represent 100 μm.

Figure 4. T or B Cell-Derived Lymphotoxin Is Not Essential for Bacterial Clearance

(A and D) WT mice, Ltb−/− mice, and mice with conditional inactivation of LTβ on T, B, or T and B cells were orally infected with C. rodentium. Body weight kinetics (A), survival rates (B), bacterial titers in fecal homogenate cultures at day 14 (C), and histological analysis of representative colons (D) are shown (n = 5). All Ltb−/− mice died at day 8-10 post infection, whereas all other mice survived. H&E staining illustrates intact colon epithelial layer in T.B-Ltb−/− mice, compared to severe colon epithelial cell damage, bacterial abscesses, and inflammatory cell infiltration in Ltb−/− mice. (D) shows the original magnification × 20. The scale bars represent 100 μm. Data are representative of two independent experiments. Data represent means ± SEM (A and C).
Lymphotixin from RORγt+ Cells Is Essential for the Control of Infection

Aside from T and B cells, membrane LT can be expressed on innate RORγt+ cells that include LT-like cells and NKP46+ NK-like cells (Vivier et al., 2009). Both LT-like cells and RORγt+ NKP46+ cells produced LTα and LTβ in the gut lamina propria at day 5 after C. rodentium infection (Figure S3A). LT-expressing RORγt+ cells are critical for development of secondary lymphoid organs. Similar to the LT-deficient mice, Rorc−/− mice also lack lymph nodes, Peyers’ patches, and organized secondary lymphoid organs in the gut (Eberl et al., 2004; Sun et al., 2000). To define whether RORγt+ cells are essential for control of mucosal bacterial infection, we orally inoculated Rorc−/− mice with C. rodentium. Impressively, Rorc−/− mice were highly susceptible and lost weight, and all died at day 10–12 postinfection (Figures 5A and 5B). Histological evaluation of colons revealed severe disruption of the epithelial layer, multifocal necrosis, inflammation, and edema (Figure 5C). These data demonstrate the critical role of RORγt+ cells in control of early C. rodentium infection.

To define whether LT from RORγt+ cells is essential for the protection of mice against C. rodentium infection, we transferred a 1:1 mixture of bone marrow cells from Ltb−/− mice and Rorc−/− mice to lethally irradiated WT mice. Bone marrow cells from Rorc−/− mice lack RORγt+ cells, but provide LT on other cell types, whereas bone marrow cells from Ltb−/− mice lack surface LT, but provide RORγt+ cells. Therefore, recipient mice are reconstituted with all LT+ cell populations except those that lack LT on RORγt+ cells. WT mice that received a mixture of bone marrow cells from Rorc−/− and Ltb−/− mice were highly susceptible to infection and lost weight, and 75% of the mice died by day 15 after infection (Figures 5D and 5E). These mice exhibited colon shortening, increased bacterial titers in the spleen, disruption of the epithelial layer, and severe inflammation in the colon compared to control mice (Figures 5F and 5G).

To further prove the role of LT on RORγt+ cells in C. rodentium infection, we analyzed mice with specific inactivation of surface LT on RORγt+ cells (RORγt-Ltb−/− mice). All RORγt-Ltb−/− mice exhibited weight loss, displayed severe colon pathology, had increased bacterial titers in the feces and blood, and died at day 8–12 postinfection (Figures S3B–S3F). Overall, these data suggest that LT production by RORγt+ cells, but not by adaptive T and B cells, is essential for the protection of mice against C. rodentium infection.

The LTβR Pathway Controls Early Innate Immunity against C. rodentium Infection

Given that LT expressing RORγt+ cells but not LT on adaptive T and B cells was required for protection, we hypothesized that LTβR signaling by innate RORγt+ cells is essential for the early innate phase of the mucosal immune response. Therefore, to define the role of LTβR signaling in the control of early C. rodentium infection in the presence of normal gut-associated lymphoid tissues, we blocked LTβR signaling in WT mice with soluble LTβR-Ig fusion protein. Such blockade by administration of LTβR-Ig fusion protein at days −1 and 5 after infection resulted in 60% mortality (Figure 6A). In contrast, mice injected with LTβR-Ig at a later time (days 5 and 12 postinfection) all survived infection (Figure 6A). These results suggest that LTβR signaling...
is crucial in the early stage of *C. rodentium* infection in the presence of normal lymphoid tissues, probably acting before the generation of adaptive immune responses in the gut.

We next tested whether stimulation of LT\(\beta\)R signaling early in infection is sufficient for protecting mice against lethal *C. rodentium* challenge by injecting *Ltb\(^{−/−}\)* mice with agonistic LT\(\beta\)R antibody early at day \(-1, 0, 2,\) and \(4\) after infection. Impressively, whereas all untreated *Ltb\(^{−/−}\)* mice died by day 12 after infection, 75% of anti-LT\(\beta\)R-treated mice survived (Figure 6B and data not shown). Thus, early engagement of LT\(\beta\)R signals is sufficient for inducing protection against otherwise lethal infection in LT-deficient mice.

Most previous studies focused on the role of LT\(\beta\)R signaling in the maintenance of organized lymphoid tissues and in the development of adaptive immune responses. However, our data raise the possibility that LT\(\beta\)R signaling might be important for innate responses. To further define whether LT\(\beta\)R signaling by innate ROR\(\gamma\)t cells is critical for the innate immune response during *C. rodentium* infection, we infected *Rag1\(^{−/−}\)* mice, which lack T and B cells. *Rag1\(^{−/−}\)* mice gradually lost weight and eventually died \(~3–4\) weeks after infection (Figures 6C and 6D). In contrast, *Rag1\(^{−/−}\)* mice treated with LT\(\beta\)R-Ig fusion protein lost weight very rapidly and died within 2 weeks after infection (Figures 6C and 6D). Together, these data suggest that the LT\(\beta\)R signaling pathway by innate LT expressing ROR\(\gamma\)t cells is essential for protecting mice from death during the early phase of *C. rodentium* infection in the absence of adaptive immunity.

**The LT\(\beta\)R Pathway Controls Neutrophil Recruitment to Protect against Bacterial Infection**

To define the mechanism of LT\(\beta\)R signaling during the innate immune response, we first analyzed the cellular composition of lymphoid cells in the lamina propria of *Rag1\(^{−/−}\)* mice treated with LT\(\beta\)R-Ig protein. Although the total cell number of innate ROR\(\gamma\)t and NKp46\(^{+}\) cell populations were not different between LT\(\beta\)R-Ig-treated and control mice (Figure S4A), the number of Gr1\(^{+}\)CD11b\(^{+}\) cells was dramatically reduced in the lamina propria early after infection (Figure 7A). Gr1\(^{+}\)CD11b\(^{+}\) population represented primarily neutrophils as defined by flow cytometry (CD11b\(^{+}\)Ly6C\(^{hi}\)Ly6G\(^{−}\) cells) and by anti-myeloperoxidase immunostaining (Figure 7E and Figure S4B).

To define how LT\(\beta\)R may control neutrophil recruitment to the gut, we analyzed expression of neutrophil recruiting chemokines in *Rag1\(^{−/−}\)* mice treated with LT\(\beta\)R-Ig protein. CXCL1 (KC) and CXCL2 (MIP-2) are two principal chemokines that recruit neutrophils after bacterial infection or injury (Lebeis et al., 2007; Ohtsuka et al., 2001; Rakoff-Nahoum et al., 2004). Expression of CXCL1 and CXCL2 was substantially reduced in the ceca of *Rag1\(^{−/−}\)* mice treated with LT\(\beta\)R-Ig, compared to untreated control mice (Figure 7B), and correlated with reduced numbers of neutrophils in the lamina propria at day 4 after infection (Figure 7A).

To further define whether LT\(\beta\)R signaling in intestinal epithelial cells controls early neutrophil recruitment to the colon lamina propria, we analyzed neutrophil numbers in *Vil-Ltb\(^{−/−}\)* and *Ltbr\(^{−/−}\)* mice after *C. rodentium* infection. Neutrophil numbers were greatly reduced in the lamina propria of both *Vil-Ltb\(^{−/−}\)* and *Ltbr\(^{−/−}\)* mice compared to WT mice (Figures 7C and 7E and Figure S4B). The reduced number of neutrophils and lower expression of CXCL1 and CXCL2 chemokines were also found in the colon lamina propria of ROR\(\gamma\)t-Ltb\(^{−/−}\) mice early after infection, as compared to control mice (Figures S3G–S3I). Together, these results strongly suggest that LT expression on ROR\(\gamma\)t cells activates LT\(\beta\)R signaling on intestinal epithelial cells to control neutrophil recruitment to the infection site early after mucosal infection.

Finally, to define whether neutrophils are essential for early, innate protection against *C. rodentium* infection, we depleted neutrophils in *Rag1\(^{−/−}\)* mice. *Rag1\(^{−/−}\)* mice depleted of neutrophils with specific Ly6G antibody showed accelerated weight loss, increased colon pathology, and accelerated mortality after infection, similar to LT\(\beta\)R-Ig-treated mice (Figures 7F–7J).
Thus, these data indicate that the LTβR pathway controls neutrophil accumulation at the infection site to protect against mucosal bacterial infection.

**DISCUSSION**

Most studies of LTβR signaling focus on its role in the organization of lymphoid tissues and in the development of adaptive immune responses as lymphoid tissues and adaptive immunity coevolved. Instead, our data suggest that LTβR signaling is important for innate responses. The impaired Th1 cytokine production and DC function in LTβR-deficient mice were previously thought to be responsible for the high susceptibility of Ltbr−/− mice to oral C. rodentium infection (Spahn et al., 2004). Unexpectedly, we found that LT from innate RORγt+ cells but not from adaptive T and B cells was essential for protection. Consistently, lymphocyte-deficient Rag1−/− mice become more susceptible after LTβR blockade. Furthermore, LTβR signaling in gut epithelial cells and innate cells is required for the early defense against C. rodentium infection, independently of the adaptive immune responses, but dependent upon neutrophils and innate RORγt+ cells. These results support a model wherein LT-expressing RORγt+ cells instruct intestinal epithelial cells, via LTβR signals, to mobilize the innate immune response against microbial infection.

How epithelial cells may coordinate with innate and adaptive immune cells during mucosal infection is poorly understood. The LT-LTβR pathway in the gut provides an interesting model to dissect such interactions. LTβR is expressed, or can be induced, on both bone marrow-derived cells, such as neutrophils, macrophages, DCs, and radioresistant cells, including intestinal epithelial cells and other stromal cells (Browning and French, 2002; Ware, 2005). Although the role of LTβR in the production of homeostatic chemokines in secondary lymphoid tissues is well-known, the role of LTβR signaling in the gut in the context of mucosal infection remains largely unexplored.
LTβR-dependent Control of Gut Innate Immunity

LTβR can be produced by both RORγt+ LT-like cells and CD3−NKp46− cells in the gut of naïve mice (Luci et al., 2009; Tsuji et al., 2008). We detected both LTα and LTβ transcripts in both RORγt+ LT-like cells and RORγt− NKp46− cells in the colonic lamina propria early after C. rodentium infection. Our data suggest that the increased mortality of LTβR-Ig-treated mice is not due to impaired migration of these cell populations to the lamina propria after infection, but more likely due to the lack of LT activity by those cells. Using Rag1−/− mice and timing of LT blockade, we have shown LT from innate cells is essential for the protection at an early, but not late (>day 5) phase of infection. Furthermore, analysis of mixed bone marrow chimeras and mice with specific inactivation of LT on RORγt+ cells revealed the essential role of LT+RORγt+ cells in mucosal innate protection. However, which population, RORγt+ LT-like cells or RORγt− NKp46− cells, is more important for protection remains to be determined.

Bacterial invasion of the mucosa is often followed by infiltration of neutrophils that provide early, innate defense against infection (Appelberg, 2007; Lebeis et al., 2007). We found that a lack of LTβR signaling prevented effective recruitment of neutrophils to the infection site early after infection, and this was followed by increased bacterial counts and severe tissue injury. This effect is not simply due to aberrantly organized lymphoid structures in Ltbr−/− mice (data not shown). Our data suggest that, in addition to gut epithelial cells, LTβR signaling in other radiosensitive stromal cells may contribute to protection, given that the phenotype of Ltbr−/− mice was less severe than that in WT > Ltbr−/− chimeras. Identification of additional LTβR-expressing cells that contribute to protection will help to further define the role of LTβR in regulation of mucosal immune defense homeostasis.

LTβR can be engaged by at least two known ligands: membrane LT and LIGHT (Wang et al., 2009; Ware, 2005). Both ligands have been implicated in mucosal immune homeostasis (Spanh et al., 2004; Wang et al., 2004). Our previous study showed that expression of LIGHT on T cells in LIGHT-transgenic mice or in a RORγt−/− adoptive transfer model promotes autoimmune inflammation in the gut (Wang et al., 2004). Interestingly, in this study we found a normal response to C. rodentium infection in Tnfsf14−/− mice, as compared to Ltbr−/− mice. The reason for this difference is currently unclear, but it is possible that additional defects in the development of gut-associated lymphoid organs and impaired generation of DCs may be responsible for the severe phenotype of Ltbr−/− mice. Although both ligands were shown to be expressed on RORγt+ cells in the gut (Luci et al., 2009), different kinetics or expression amounts of LIGHT and LT during infection could be responsible for the distinct phenotypes of bacterial clearance in LT- and LIGHT-deficient mice.

Surface LT is readily detected on T and B cells, especially after activation (Browning, 2008; Fu and Chaplin, 1999; Ware, 2005). To identify the critical LT-expressing cells in our model, we employed mice with conditional inactivation of membrane LT on T or B cells, given that previous studies implicated these cells as major LT producers in secondary lymphoid organs (Junt et al., 2006; Tumanov et al., 2002). Unexpectedly, LT deficiency in either T or B cells showed no phenotype. We then generated double-deficient mice that lacked LT on both T and B cells; again, these mice were able to efficiently clear C. rodentium infection, which opened the possibility that LT expression is necessary on innate immune cells such as RORγt+ cells. Innate RORγt+ cells are important for the development of lymphoid tissues in a LT-dependent fashion (Eberl et al., 2004; Sun et al., 2000); however, their role in mucosal immunity is poorly defined. To directly address the role of these cells in host defense, we have tested the sensitivity of Rorc−/− mice to C. rodentium infection. Our data suggest that RORγt+ innate cells are essential for the mucosal bacterial infection.
neutrophil recruitment to the infection site to fight the bacterial pathogen. Contact of RORγt+ cells with LTir on intestinal epithelial cells may further promote cooperation of various innate immune cells in early defense to invading pathogen before the development of sterilizing adaptive immune responses.

**EXPERIMENTAL PROCEDURES**

**Mice**  
C57BL/6 and Rag1−/− mice were purchased from Harlan Teklad. Ltbr−/−, Tnfrsf14−/−, and Ltb−/− mice were backcrossed onto C57BL/6 background. LTir−/− mice were generated with Cre-LoxP technology (see Supplemental Information for details). VilsLtb−/− and LysM-Ltb−/− mice were generated by crossing LTir−/− mice with Vils-Cre or LysM-Cre transgenic mice, respectively. RORγt−/− mice were generated by crossing Ltbr−/− mice (Tumanov et al., 2002) with RORγt−/− transgenic mice (Eberl and Littman, 2004). 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.

**Bacterial Strain and Infection of Mice**  
For induction of bacterial colitis in mice, mice were orally gavaged with 2 × 10^9 cfu C. rodentium strain DBS100 (ATCC 14028; American Type Culture Collection), as previously described (Zheng et al., 2008). In brief, mice were fasted for 8 hr before oral inoculation of C. rodentium culture in a total volume of 0.2 ml per mouse. Bacteria were prepared by shaking at 37°C overnight in LB broth. Concentration was assessed by measurement of absorbance at OD600. Bacterial culture was serially diluted and plated after each inoculation so that the colony-forming units (CFUs) analyzed could be confirmed. Body weight was assessed before and then frequently during the course of disease.

**Tissue Collection, Histology, and Colony-Forming Unit Counts**  
Colonies were dissected from the mice and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue sections were stained with H&E for tissue pathology evaluation. Fecal samples were collected and weighted, then diluted 1:10 with 10% neutral buffered formalin. Paraffin-embedded tissue sections were stained with H&E for tissue histology. Tissue collection, Histology, and Colony-Forming Unit Counts were done with the log rank (Mantel-Cox) test. Statistical Analysis.

**RNA Isolation and Real-Time Reverse Transcriptase PCR**  
RNA from cells or frozen tissues was isolated with the RNeasy Mini Kit (QiAGEN). For cDNA synthesis, RNAs were digested with DNase I and reverse transcribed with random primers with AMV Reverse Transcriptase (Promega). The concentration of the target gene was determined with the comparative CT (threshold cycle number at a cross-point between amplification plot and threshold) method and normalized to HPRT and beta-actin. cDNA were amplified with the Power Sybr Green PCR master mix (Applied Biosystems) or SsoFast EvaGreen supermix (Bio-Rad) and run on ABI 7300 cycler (Applied Biosystems) or StepOne Plus (Applied Biosystems). PCR primers and probes used as follows: for CXCL1: forward 5’-CCACCCAGTCGCTTCTC-3’, reverse 5’-CACAGACGCGAACGTCATT-3’, for CCL2: forward 5’-AAGAAACCACACGGGTAGA-3’; for LTb: forward 5’-AGGGTACACACTCAAGCTCT-3’, reverse 5’-AGAGAAGCCATGTCGGAG-3’, and for RORγt: forward 5’-TCCACTCTCCCTAAGAAGACCT-3’, reverse 5’-AGAGAAGCCATGTCGGAGAA-3’. 

**SUPPLEMENTAL INFORMATION**  
Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.immuni.2010.02.011.

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