Microbiota-induced IL-1β, but not IL-6, is critical for the development of steady-state $T_{H17}$ cells in the intestine

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$T_{H17}$ cells are a lineage of CD4$^+$ T cells that are critical for host defense and autoimmunity by expressing the cytokines IL-17A, IL-17F, and IL-22. A feature of $T_{H17}$ cells at steady state is their ubiquitous presence in the lamina propria of the small intestine. The induction of these steady-state intestinal $T_{H17}$ ($sT_{H17}$) cells is dependent on the presence of the microbiota. However, the signaling pathway linking the microbiota to the development of intestinal $sT_{H17}$ cells remains unclear. In this study, we show that IL-1β, but not IL-6, is induced by the presence of the microbiota in intestinal macrophages and is required for the induction of $sT_{H17}$ cells. In the absence of IL-1β–IL-1R or MyD88 signaling, there is a selective reduction in the frequency of intestinal $sT_{H17}$ cells and impaired production of IL-17 and IL-22. Myeloid differentiation factor 88–deficient (MyD88$^{-/-}$) and germ-free (GF) mice, but not IL-1R$^{-/-}$ mice, exhibit impairment in IL-1β induction. Microbiota-induced IL-1β acts directly on IL-1R–expressing T cells to drive the generation of $sT_{H17}$ cells. Furthermore, administration of IL-1β into GF mice induces the development of retinoic acid receptor–related orphan receptor γt–expressing $sT_{H17}$ cells in the small intestine, but not in the spleen. Thus, commensal–induced IL-1β production is a critical step for $sT_{H17}$ differentiation in the intestine, which may have therapeutic implications for $T_{H17}$–mediated pathologies.

$T_{H17}$ cells are a selective lineage of CD4$^+$ T helper cells that are critical for host defense and autoimmunity by expressing the proinflammatory cytokines IL-17A, IL-17F, and IL-22 (McGeachy and Cua, 2008; Ouyang et al., 2008; Korn et al., 2009). The induction of $T_{H17}$ cells during inflammatory conditions such as experimental autoimmune encephalomyelitis (EAE) requires cytokines such as IL-1β, IL-6, IL-23, and TGF-β1 (Korn et al., 2009). In addition to their presence during inflammatory responses, a population of T cells that expresses retinoic acid receptor–related orphan receptor γt (Rorγt), which is a $T_{H17}$–specific transcription factor, is also found at steady state ($sT_{H17}$) in the small intestine lamina propria (LP; Ivanov et al., 2006; Ivanov et al., 2008), where they accumulate only in the presence of luminal commensal microbiota (Atarashi et al., 2008; Hall et al., 2008; Ivanov et al., 2008). Recently, one member of this bacterial community, the Clostridia–related segmented filamentous bacteria (SBF), was shown to be capable of inducing the generation of $T_{H17}$ cells, as well as other subsets of $T_{H17}$ cells (Ivanov et al., 2009). It has been suggested that the induction of ATP (Atarashi et al., 2008) and serum amyloid proteins (Ivanov et al., 2009) by commensal bacteria are required for promoting the generation of intestinal $T_{H17}$ cells. However, the host signaling pathway linking the microbiota to the induction of intestinal $sT_{H17}$ cells at steady state remains to be fully elucidated.

IL-1R signaling is important for murine $T_{H17}$ development (van Beelen et al., 2007; Chung et al., 2009; Gulen et al., 2010) and accordingly, mice lacking IL-1R expression exhibit impaired capacity to generate $T_{H17}$ cells in the setting of EAE (Sutton et al., 2006; Chung et al., 2009). Furthermore, the IL-1β–IL-1R signaling pathway has been shown to promote IL-17 production by the unconventional γδ T cells.
T cells in the presence of commensal bacteria (Sutton et al., 2009; Duan et al., 2010). Based on analysis of intracellular staining of LP cells for Th17-associated cytokines after stimulation with phorbol ester and ionomycin, it was suggested that myeloid differentiation factor 88 (MyD88) signaling pathways that include Toll-like receptor (TLR) and the IL-1β–IL-1R signaling pathways were not important in inducing the development of normal Th17 cell responses in the small intestine (Atarashi et al., 2008; Ivanov et al., 2009). Because recent findings revealed that phorbol ester and ionomycin stimulation may exaggerate intracellular IL-17 expression in Th17 cells (Hirota et al., 2011), it remains unclear whether MyD88 signaling pathways play a role in the induction of intestinal Th17 cells in animals colonized with gut-residing bacteria. In the present study, we examined the role of IL-1R and MyD88 signaling in influencing the induction of Th17 cells in the intestinal microenvironment. Our results revealed that the microbiota induces the production of IL-1β, but not IL-6, in LP phagocytes and that stimulation of IL-1β–IL-1R signaling is necessary and sufficient in driving the generation of intestinal Th17 cells.

RESULTS AND DISCUSSION

IL-1β–IL-1R signaling promotes the development of intestinal Th17 cells

To assess the role of IL-1R signaling in the development of Th17 cells under steady-state conditions in vivo, we crossed Rorc(γt)-gfp reporter mice, which express GFP under the control of the promoter of Rorc(γt) (Eberl et al., 2004; Ivanov et al., 2006), with Il1r1−/− mice to generate Rorc(γt)-gfp reporter mice in the presence and absence of IL-1R. Although the frequency of total CD4+ T cells was not significantly different (Fig. 1A), the numbers of Rorc(γt)-gfp+ CD4+ T cells were greatly reduced in the LP of Il1r1−/− mice compared with those in wild-type littermates (Fig. 1, B and C). The impaired generation of LP Th17 cells was not the result of enhanced development of Foxp3+ T regulatory cells in the absence IL-1R signaling, given that no differences were observed in the frequency of Foxp3+CD4+ T cells between Il1r1−/− and the littermate T cells in the presence of commensal bacteria (Sutton et al., 2009; Duan et al., 2010). Based on analysis of intracellular staining of LP cells for Th17-associated cytokines after stimulation with phorbol ester and ionomycin, it was suggested that myeloid differentiation factor 88 (MyD88) signaling pathways that include Toll-like receptor (TLR) and the IL-1β–IL-1R signaling pathways were not important in inducing the development of normal Th17 cell responses in the small intestine (Atarashi et al., 2008; Ivanov et al., 2009). Because recent findings revealed that phorbol ester and ionomycin stimulation may exaggerate intracellular IL-17 expression in Th17 cells (Hirota et al., 2011), it remains unclear whether MyD88 signaling pathways play a role in the induction of intestinal Th17 cells in animals colonized with gut-residing bacteria. In the present study, we examined the role of IL-1R and MyD88 signaling in influencing the induction of Th17 cells in the intestinal microenvironment. Our results revealed that the microbiota induces the production of IL-1β, but not IL-6, in LP phagocytes and that stimulation of IL-1β–IL-1R signaling is necessary and sufficient in driving the generation of intestinal Th17 cells.

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control mice (Fig. 1D). Consistent with the reduced frequency of sT\textsubscript{H1} cells in vivo, the production of T\textsubscript{H1}-associated effector cytokines (IL-17 and IL-22) by FACS-sorted intestinal CD3\textsuperscript{+}CD4\textsuperscript{+} IL-1R\textsuperscript{−/−} cells, was also blunted after in vitro stimulation (Fig. 1 E). The impaired generation of sT\textsubscript{H1} cells observed in the Ilr1\textsuperscript{−/−} mice was not caused by the lack of intestinal colonization with SFB, as Ilr1\textsuperscript{−/−} and controls animals maintained in our specific pathogen–free (SPF) facility had a comparable number of fecal SFB as determined by quantitative real-time PCR (qPCR) of 16s rRNA gene sequences (unpublished data). In addition, the expression of IL-6 and IL-23p19 in the small intestine was unaffected by the absence of IL-1R1 signaling, as qPCR analysis revealed that the transcript levels of these genes were similar between Ilr1\textsuperscript{−/−} and wild-type littermate animals (unpublished data). The proinflammatory cytokine IL-1\textbeta is a known ligand that interacts with IL-1R1 to mediate its biological effects (Sims and Smith, 2010). Accordingly, the IL-17 responses under more physiological conditions. To do so, we sought to address the role of MyD88 in promoting sT\textsubscript{H1} cells (Fig. 1 G). To further assess the role of MyD88 in intracellular cytokine staining of LP cells stimulated with phorbol ester and ionomycin (unpublished data). In addition, the expression of the Th1-associated cytokine IFN-\gamma by total LP cells was not impaired after in vitro stimulation (unpublished data).

The development of intestinal sT\textsubscript{H1} responses requires MyD88, but not IL-6

Given that the adaptor molecule MyD88 plays a critical role in both TLR- and IL-1R-signaling pathways (Medzhitov et al., 1998), we reevaluated the intestinal sT\textsubscript{H1} response in Myd88\textsuperscript{−/−} mice. Consistent with previous studies (Atarashi et al., 2008; Ivanov et al., 2008), we observed normal intestinal sT\textsubscript{H1} responses in the absence of MyD88 signaling by intracellular cytokine staining of LP cells stimulated with phorbol ester and ionomycin (unpublished data). However, in light of recent findings suggesting that phorbol ester and ionomycin stimulation may exaggerate intracellular IL-17 expression in T\textsubscript{H1} cells (Hirota et al., 2011), we sought to address the role of MyD88 in promoting sT\textsubscript{H1} responses under more physiological conditions. To do so, we stimulated FACS-sorted LP CD3\textsuperscript{+}CD4\textsuperscript{+} T cells isolated from Myd88\textsuperscript{−/−} animals with antibody to CD3. Myd88\textsuperscript{−/−} mice exhibited decreased intestinal T\textsubscript{H1} cytokine responses as determined by ex vivo stimulation of CD3\textsuperscript{+}CD4\textsuperscript{+}-purified cells (Fig. 1 G). To further assess the role of MyD88 in promoting sT\textsubscript{H1} responses without ex vivo manipulation, we crossed Rorc\textsuperscript{(Ilr1-/-)} reporter mice with Mydd88\textsuperscript{−/−} mice to generate Rorc\textsuperscript{(Ilr1-/-)} reporter mice. Consistent with the results obtained with Ilr1\textsuperscript{−/−} mice in vivo and stimulation of LP CD3\textsuperscript{+}CD4\textsuperscript{+} T cells with anti-CD3 ex vivo, we found reduced frequency of CD4\textsuperscript{+} Rorc\textsuperscript{(Ilr1-/-)} cells in the intestinal LP of Mydd88\textsuperscript{−/−}/Rorc\textsuperscript{(Ilr1-/-)} mice when compared with Mydd88\textsuperscript{+/+}/Rorc\textsuperscript{(Ilr1-/-)} littermate mice (Fig. 1 H). In contrast, the frequency of CD4\textsuperscript{+} Rorc\textsuperscript{(Ilr1-/-)} cells in the intestinal LP of Il6\textsuperscript{−/−}/Rorc\textsuperscript{(Ilr1-/-)} mice was unimpaired when compared with Il6\textsuperscript{+/+}/Rorc\textsuperscript{(Ilr1-/-)} littermate mice (Fig. 1 H). Consistently, the production of T\textsubscript{H1}-associated effector cytokines (IL-17 and IL-22) by FACS-sorted intestinal CD3\textsuperscript{+}CD4\textsuperscript{+} IL6\textsuperscript{−/−} cells was comparable to that of intestinal CD3\textsuperscript{+}CD4\textsuperscript{+} T cells from Il6\textsuperscript{−/−} littermate mice (Fig. 1 I and not depicted). These observations indicate that the homeostatic development of intestinal sT\textsubscript{H1} cells does not require IL-6, but is dependent on, IL-1\textbeta–IL-1R and MyD88 signaling.

The CD11b\textsuperscript{+}F4/80\textsuperscript{+}CD11c\textsuperscript{−/low}-resident macrophage population is the main source of IL-1\textbeta in the small intestine

We next investigated which cellular subset of cells in the intestine is a potential source of IL-1\textbeta. To begin to address this question, we generated bone marrow chimera mice by reconstituting lethally irradiated wild-type recipient mice with bone marrow from wild-type or IL-1\textbeta–deficient mice. Analysis of the chimera mice revealed that IL-1\textbeta produced by hematopoietic cells is required for the induction of intestinal sT\textsubscript{H1} responses (Fig. 2 A). Thus, deficiency of IL-1\textbeta in hematopoietic cells impaired the production of the T\textsubscript{H1}-associated effector cytokines, IL-17, and IL-22, but not IFN-\gamma, by LP cells after in vitro stimulation (Fig. 2 A). Accumulating evidence suggests that LP mononuclear phagocytes are crucial for maintaining intestinal homeostasis and promoting intestinal immunity (Varol et al., 2010). Accordingly, in the intestinal LP, we identified, based on the differential surface expression of CD11c and CD11b, three distinct mononuclear phagocyte populations: CD11b\textsuperscript{+}CD11c\textsuperscript{−} (R1), CD11b\textsuperscript{+}CD11c\textsuperscript{+} (R2), and CD11b\textsuperscript{−}CD11c\textsuperscript{+} (R3; Fig. 2 B, left). Next, we examined which subsets of these intestinal phagocytes contribute to steady-state production of IL-1\textbeta. To do so, we sorted R1, R2, and R3 populations and assessed intracellular IL-1\textbeta protein levels. The R1 population of cells expressed the highest level of IL-1\textbeta protein compared to the R2 or R3 subsets (Fig. 2 B, middle). Further flow cytometry analysis demonstrated that the R1 population was CD11b\textsuperscript{+}, F4/80\textsuperscript{+}, MHCI\textbeta\textsuperscript{+}, CD11c\textsuperscript{−/low}, and CD103\textsuperscript{int} (unpublished data); thus, this population seems to belong to a previously described subset of CD11b\textsuperscript{+}F4/80\textsuperscript{+} intestinal macrophages (Kamada et al., 2005; Denning et al., 2007). Therefore, these findings indicate that the CD11b\textsuperscript{+}, F4/80\textsuperscript{+}, MHCI\textbeta\textsuperscript{+}, CD11c\textsuperscript{−/low}, and CD103\textsuperscript{int}-resident populations are the main source of IL-1\textbeta production in the LP of the small intestine.

IL-1\textbeta induction in intestinal macrophages is mediated via MyD88, but not IL-1R, signaling

The expression of IL-1\textbeta is induced by TLR ligands and IL-1\textbeta itself can also serve as a positive feedback regulator of this pathway (Dinarello, 2009). To investigate the interaction between commensal bacteria and the host immune system that promotes the induction of IL-1\textbeta, R1, R2, and R3 subsets from the LP of wild-type and Ilr1\textsuperscript{−/−} mice were sorted and the protein level of IL-1\textbeta was determined. There was no detectable difference in the level of IL-1\textbeta between wild-type and Ilr1\textsuperscript{−/−} animals (Fig. 2 B, right), indicating that IL-1\textbeta–IL-1R signaling does not regulate the expression of IL-1\textbeta in intestinal macrophages. This suggests that induction of IL-1\textbeta is
IL-1R signaling on T cells is required for the generation of intestinal sT$_{17}$ response

IL-1R1 is ubiquitously expressed on both hematopoietic and nonhematopoietic cells (Dinarello, 2009; Sims and Smith, 2010). To assess the importance of IL-1R1 signaling in various intestinal cellular populations in promoting the generation of sT$_{17}$ cells, we generated bone marrow chimera mice. Reconstitution of lethally irradiated recipients, regardless of genotype, with Ilr1$^{-/-}$ bone marrow recapitulated the defective sT$_{17}$ response observed in intact Ilr1$^{-/-}$ animals (Fig. 3 A). Thus, deficiency of IL-R1 in hematopoietic cells impaired the production of the T$_{17}$-associated effector cytokines (IL-17 and IL-22) by LP cells after in vitro stimulation (Fig. 3 A). The requirement of an intact IL-1R1 signaling pathway in the hematopoietic compartment using bone marrow chimeric mice does not distinguish whether or not direct IL-1R1 signaling on T cells promotes the induction of intestinal sT$_{17}$ cells. To this end, we adoptively transferred Ilr1$^{-/-}$ or Ilr1$^{-/-}$Rorc$^t$-gfp$^+$ cells into SPF Rag1$^{-/-}$ recipients (Fig. 3 B, left). Reconstituting Rag1$^{-/-}$ recipients with either Ilr1$^{-/-}$ or Ilr1$^{-/-}$Rorc$^t$-gfp$^+$ CD4$^+$ T cells resulted in a similar recruitment and expansion of CD4$^+$ T cells to peripheral organs and frequency of GFP-expressing CD4$^+$ T cells in the spleen and liver (Fig. 3 B, middle). However, despite normal recruitment to and expansion in the intestinal LP, the absence of IL-1R1 on CD4$^+$ T cells selectively impaired the ability of the donor cells to acquire Rorγt expression specifically in the intestinal microenvironment (Fig. 3 B, right). Consistent with diminished numbers of Rorγt-expressing CD4$^+$ T cells present in the intestinal LP of Ilr1$^{-/-}$ T cell reconstituted Rag1$^{-/-}$ recipients, in vitro production of IL-17 was also

![](https://example.com/image.png)

mice and the level of spontaneous IL-6 production in overnight cultured supernatant was determined by ELISA. Representative results are shown from one of at least two to three independent experiments. Experiments in panels C and D are representative of two experiments using pooled cells from $n = 2–3$ mice.
negatively impacted by the lack of IL-1R1 expression on T cells (Fig. 3 C). Thus, these results emphasize that maintaining IL-1β responsiveness in T cells in the intestine is necessary for the development of sTh17 cells.

Administration of IL-1β is sufficient to induce the maturation of splenic CD3+CD4+Rorγt-negative cells into intestinal Rorγt-expressing sTh17 cells in GF mice

We next asked whether administration of IL-1β was capable of inducing the presence of sTh17 cells in the LP of GF animals that are largely devoid of these cells (Atarashi et al., 2008; Hall et al., 2008; Ivanov et al., 2008). To assess this, GF mice were injected with rIL-1β or PBS as a control and the presence of sTh17 cells and production of sTh17-associated cytokines were assessed in the small intestine. As expected, the production of sTh17-associated cytokines IL-17 and IL-22 by LP cells was low in GF mice treated with PBS (Fig. 4 A). Importantly, IL-17 and IL-22 production by LP cells was markedly increased in GF mice treated with exogenous IL-1β when compared with mice treated with PBS (Fig. 4 A). To further interrogate the role of IL-1β in the induction of intestinal sTh17 response, GF wild-type animals were first infused with FACS-purified Thy1.1+/1.2+CD3+CD4+ Rorγt-gfp-negative cells derived from SPF wild-type donors and then treated with PBS or exogenous rIL-1β (Fig. 4 B, top). Administration of rIL-1β selectively induced the donor CD4+ reporter cells to acquire Rorγt expression in the intestinal microenvironment, but not in other peripheral organs, such as the spleen (Fig. 4 B, bottom). These data indicate that the presence of IL-1β is sufficient to induce the differentiation of intestinal sTh17 cells in the absence of microbiota.

The blunted intestinal sTh17 response of GF animals is well-characterized (Ivanov et al., 2008); however, the reason for the impaired sTh17 response that is associated with the absence of commensal microbiota is currently unclear. We provide evidence that the microbiota induces the production of IL-1β in resident macrophages via MyD88 and IL-1β-IL-1R1 signaling is critical for the development of sTh17 cells in the small intestine. Administration of rIL-1β selectively induced the donor reporter cells to acquire Rorγt-expression in the intestinal microenvironment, but not in other peripheral organs, such as the spleen. Thus, the results suggest that the IL-1β/IL-1R axis is not only required, but also sufficient, to drive the development of sTh17 in the intestinal microenvironment.
Unexpectedly, IL-6 was not required for the development of intestinal sTh17 cells. Because IL-6 is important for the induction of Th17 cells during inflammatory conditions such as EAE (Korn et al., 2009), the results suggest differential regulation for the development of Th17 cell population which may be explained by differences in the local tissue environment or specific triggers. These results do not rule out that IL-1β acts in concert with other cytokines such as IL-23 that are known to be critical for the development of Th17 cells during inflammatory conditions in nonintestinal tissues (Korn et al., 2009). However, our results clearly indicate that IL-1β, but not IL-6, is induced by the microbiota and critical for the development of intestinal sTh17 cells in vivo.

Our findings also identified MyD88 as a critical mediator for the induction of IL-1β in intestinal macrophages while the expression of pro-IL-1β in intestinal phagocytes was not impaired in IL-1R1-deficient mice. These results suggest that MyD88 acts at two distinct steps to regulate the development of sTh17 cells in the intestine. First, MyD88 links the microbiota to pro-IL-1β induction in intestinal macrophages. This mechanism likely involves the TLR-MyD88 signaling pathway given that this appears to be the major innate immune pathway by which the intestinal microflora stimulate the production of proinflammatory cytokines in host cells (Hasegawa et al., 2010). The second step involves IL-1β/IL-R1/MyD88 signaling in CD4 T cells to drive the development of intestinal sTh17 cells. Collectively, our data suggest that interactions between commensal bacteria and TLR signaling mediated through MyD88 promote IL-1β production in intestinal CD11b+ macrophages, which in turn induces the differentiation of intestinal sTh17 cells. Notably, exogenous IL-1β increased sTh17 cells in the intestine, but not in the spleen, in the absence of microbiota. We do not have an explanation for the latter results, but it suggests that the regulation of Th17 cell differentiation is more complex that originally described and varies in different tissue environments. Because Th17 cells have been suggested to play either a detrimental role in autoimmune disease (McGeachy and Cua, 2008; Ouyang et al., 2008; Korn et al., 2009) or protective role during enteric infection (Ivanov et al., 2009), further understanding the mechanism that regulate the quiescence and the reactivation of these cells may have therapeutic potential.

**MATERIALS AND METHODS**

**Animals.** SPF wild-type and Rag-1−/− mice in C57BL/6 background were originally purchased from The Jackson Laboratory. B1R−/−, IL-1B−/−, and Myd88−/− mice in C57BL/6 background have been previously described (Eigenbrod et al., 2008). B6−/− mice in C57BL/6 background were obtained from Dr. Evan Keller, the University of Michigan. Rorγt-GFP mice were obtained from The Jackson Laboratory and crossed to B1R−/−, Myd88−/−, or Il-6−/− mice to generate gene-deficient reporter mice. GF wild-type mice in C57BL/6 background were bred and maintained at the Germ-Free Animal Core Facility of the University of Michigan. GF mice were maintained in flexible film isolators and were checked weekly for GF status by aerobic and anaerobic culture. The absence of microbiota was confirmed by aerobic and anaerobic culture. The absence of microbiota was confirmed by aerobic and anaerobic culture.
verified by microscopic analysis of stained cecal contents to detect uncultur-able contamination. All animal studies were performed according to approved protocols by the University of Michigan on Use and Care of Animals.

**Cell isolation, adoptive transfer, and administration of IL-1β in vivo.** To isolate Rorc(γ-t)-gfp-/- cells for adoptive transfer, pan T cells microbeads (Miltenyi Biotec) were initially used to enrich total T cells, followed by FACs sorting of CD3*CD4* Rorc(γ-t)-gfp-/- cells. For adoptive transfer of sorted CD3*CD4* Rorc(γ-t)-gfp-/- cells into intact GF animals, wild-type Thy1.2/* Rorc(γ-t)-gfp were crossed to Thy1.1 mice (The Jackson Laboratory) and the F1 progeny were used as donors. Donor CD4 cells were distinguished from recipient CD4 cells by flow cytometric analysis for the co-expression of Thy1.1 and Thy1.2 markers. GF mice were treated every other day for 14 with either PBS or 1 µg/mouse of hIL-1β (Pepro-Tech) i.p., and then intestinal tissue was harvested for analyses. After the last round of injections, there was no evidence of bacterial contamination in the hIL-1β as determined by standard culture conditions.

**Isolation and stimulation of LP cells.** Mice were killed and intestines were removed and placed in ice-cold HBSS + 1% heat-maturated FBS + 0.1% Pen/Strep (complete HBSS). After removal of residual mesenteric fat tissue, Peyers patches were carefully excised and the intestine was flushed two times with 10 ml complete HBSS. After flushing, the intestine was opened longitudinally and cut into ~1-cm pieces. The cut pieces were washed two times at 37°C with 30 ml complete HBSS for 5 min each on a magnetic stir plate (600 rpm), followed by incubation with 50 ml 1-mM DTT for 15 min at 37°C with rotation. After DTT treatment, the tissue was then incubated twice with 30 ml 1-mM EDTA at 37°C with rotation. After each incubation, the epithelial cell layer containing the intraepithelial lymphocytes was removed by aspiration. After the last EDTA incubation, the pieces were washed three times in complete HBSS and placed in 30-ml digestion solution (complete HBSS containing Type III Collagenase and DNase I; Worthington).Digestion was performed by incubating the pieces at 37°C for 1–1.5 h with rotation. The digestion product was collected, filtered through a 70-µm cell strainer (BD), and pelleted by centrifugation. The digestion pellet was then resuspended in 4 ml of 40% Percoll (GE Healthcare) fraction and overlaid on 4 ml of 75% Percoll fraction in a 15-ml conical tube. Percoll gradient separation was performed by centrifugation for 20 min at 2,500 rpm at room temperature. LP cells were collected at the interphase of the Percoll gradient, washed once, and resuspended in either FACs buffer or T cell medium. The cells were used immediately for in vitro stimulation, flow cytometric analysis, or cell sorting. Cells purified by flow cytometry from the LP or total LP cells were cultured at the adjusted density of 10^6 cells per ml with soluble anti-CD3 (1 µg/ml) overnight, and then cytokines in supernatants (IL-2, IL-17A, and IFN-γ) were analyzed by ELISA (R&D Systems). Intracellular cytokine analysis of total LP cells was performed in cells stimulated in vitro with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich) and 5 µM ionomycin in the presence of Brefeldin A for 6 h. Surface staining was performed with a corresponding cocktail of fluore-scence labeled antibodies (eBioscience) for 30 min on ice; followed by per-meabilization with Cytofix/Cytoperm solution (BD), and intracellular cytokine staining was performed with fluorescence labeled anti-IL-17 antibody (eBioscience). Immunoblotting for IL-1β was performed using rabbit anti-mouse IL-1β antibody (R&D Systems).

**Real-time PCR analysis.** RNA samples for transcript analysis were iso-lated from the small intestine or FACs-sorted cells using Total RNA kit I (OMEGA Bio-Tek). Complementary DNAs (cDNAs) were synthesized from and prepared with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Values were then normalized by the amount of GAPDH in each sample.

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc.). Differences between two groups were evaluated using a Student's t test. Differences at P < 0.05 were considered significant.

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The authors declare no competing financial interests.

**Author contributions:** M.H. Shaw and G. Nuñez designed the research. M.H. Shaw conducted most of the experiments and analyzed data. N. Kamada performed experiments shown in Fig. 1 (G and I). N. Kamada and Y.-G. Kim performed experiments provided as “unpublished data.” M.H. Shaw and G. Nuñez wrote the manuscript.

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