An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses

Graphical Abstract

**Highlights**
- SFB-specific Th17 cells are primed in MLN and traffic throughout the GI tract
- IL-17A-producing Th17 cells are enriched in ileum, where SFB adheres to epithelium
- SAA1/2 from ileal epithelial cells contributes the induction of IL-17A in Th17 cells
- SFB-induced IL-22 production by ILC3 stimulates epithelial cells to make SAA1/2

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**In Brief**
Cytokines produced by Th17 cells are critical for protection and repair of mucosal barriers. IL-17A production is orchestrated by localization of specific poised Th17 cells at sites where the gut microbiome drives epithelial secretion of serum amyloid A.

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An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses

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SUMMARY

RORC+ Th17 cells are important for mucosal defenses but also contribute to autoimmune disease. They accumulate in the intestine in response to microbiota and produce IL-17 cytokines. Segmented filamentous bacteria (SFB) are Th17-inducing commensals that potentiate autoimmunity in mice. RORC+ T cells were induced in mesenteric lymph nodes early after SFB colonization and distributed across different segments of the gastrointestinal tract. However, robust IL-17A production was restricted to the ileum, where SFB makes direct contact with the epithelium and induces serum amyloid A proteins 1 and 2 (SAA1/2), which promote local IL-17A expression in RORC+ T cells. We identified an SFB-dependent role of type 3 innate lymphoid cells (ILC3), which secreted IL-22 that induced epithelial SAA production in a Stat3-dependent manner. This highlights the critical role of tissue microenvironment in activating effector functions of committed Th17 cells, which may have important implications for how these cells contribute to inflammatory disease.

INTRODUCTION

The vertebrate gastrointestinal (GI) tract is colonized by hundreds of distinct species of microorganisms that collectively maintain a mutualistic relationship with the host (Macpherson and Harris, 2004). This mutualism is critically dependent on a state of balanced immune activation, which fosters cohabitation between the host and microbiota, while providing optimal protection against opportunistic pathogens (Honda and Littman, 2012). It is now appreciated that the composition of the microbiome can contribute significantly to this immunological balance, in part through the capacity of individual bacterial or viral species to profoundly influence distinct arms of the immune response by themselves or in concert with other microbial species (Hooper et al., 2012; Virgin, 2014). For instance, Bacteroides fragilis and mixtures of various strains of Clostridia, which colonize the large intestine, have been shown to promote intestinal and systemic immune tolerance through regulatory T cells (Atarashi et al., 2013; Lathrop et al., 2011; Round et al., 2011), and segmented filamentous bacteria (SFB) induce antigen-specific T-helper-17 cells (Th17) cell differentiation in the small intestine (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Yang et al., 2014).

Th17 cells constitute a subset of activated CD4+ T cells that are characterized by the production of the cytokines interleukin (IL)-17A, IL-17F, and IL-22 (Korn et al., 2009). These cytokines act on a broad range of hematopoietic and non-hematopoietic cells to regulate central aspects of host immunity, including granulopoiesis, neutrophil recruitment, and the induction of antimicrobial peptides (Weaver et al., 2007). Th17 cells thus execute important functions in both epithelial homeostasis and host defense against various extracellular pathogens, such as Candida albicans and Pseudomonas aeruginosa (Stockinger and Veldhoen, 2007). Conversely, over-exuberant Th17 responses may promote auto-inflammatory diseases, such as Crohn’s disease, rheumatoid arthritis (RA), psoriasis, and multiple sclerosis (MS) (Furuzawa-Carballeda et al., 2007). While genetic polymorphisms significantly factor into the onset of these diseases, emerging evidence also highlights the influence that environmental factors, such as diet and microbial composition, can impose on such propensities. Accordingly, recent studies have illustrated the potential of SFB to exacerbate Th17-mediated disease in murine autoimmune models of both RA and MS (Lee et al., 2011; Wu et al., 2010), although the intermediate molecular steps connecting SFB to a distal immune response are ill defined.

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Figure 1. Site of SFB Colonization Correlates with Maximal IL-17A Induction in RORγt+ T Cells

(A) Quantitative PCR (qPCR) analysis of SFB genomic 16S in duodenum, ileum, and colon (proximal and distal segments of each) from SFB-colonized (SFB+; gavaged with SFB-rich fecal contents) or SFB-free (JAX; gavaged with SFB-free fecal contents) mice at 7 and 14 days post-gavage. Copy number of SFB 16S was normalized by copy number of mouse genomic Il-23r gene. The experiment was repeated more than three times with similar results combined. Data are represented as mean with SD.

(B) Number of RORγt+ CD4+ T cells in intestinal lamina propria (LP) or MLN of SFB-colonized (SFB+; n = 7) or SFB-free (JAX; n = 7) mice on 7 and 14 days post-gavage. CD4+ T cells were isolated from the indicated tissues, and Foxp3– RORγt+ CD4+ T cells were quantified by fluorescence-activated cell sorting (FACS).

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SFB colonization of the small intestine promoted global transcriptional changes in host epithelia, including the induction of antimicrobial peptides and stress response genes, such as serum amyloid A (SAA1 and SAA2) (Ivanov et al., 2009). SAA is typically induced in response to infection and acute injury and can promote inflammation, in part through elicitation of proinflammatory cytokine production and recruitment of granulocytes, monocytes, and T lymphocytes (Uhlar and Whitehead, 1999). The effect of SAA on the immune response is context driven (Cray et al., 2009; Eckhardt et al., 2010; Ivanov et al., 2009), much like that of Th17 cells. Insofar as SFB and Th17 cells are intertwined, the question of whether SAA impacts aspects of Th17 biology remains to be addressed.

Th17 cells along with several other innate-like cell lineages, including specific subsets of γδ T cells (γδ17) and type 3 innate lymphoid cells (ILC3), are regulated by the transcription factor RAR-related orphan receptor gamma (RORγt) (Chien et al., 2013; Ivanov et al., 2006; Spits and Di Santo, 2011). However, in contrast to the requirement for antigen recognition in the context of major histocompatibility complex (MHC) to drive Th17 cell activation, γδ17 and ILC3 effector functions are elicited independently of antigen presentation. For example, the proinflammatory cytokine IL-23 triggers rapid IL-17 and IL-22 secretion by γδ17 cells and ILC3s, respectively, upon ligation of the highly expressed IL-23 receptor (IL-23R). As γδ17 cells and ILC3s often reside in proximity to exposed mucosal surfaces, their activation typically precedes antigen-specific T cell differentiation and recruitment (Martin et al., 2009; Sutton et al., 2009). Whether this has bearing on the function of newly recruited T cells is unclear.

We utilized models of acute SFB colonization to investigate the mechanism of Th17 cell induction in a spatiotemporal context. We found that, following early induction of SFB-specific RORγt+ Th17 cells in the mesenteric lymph nodes, there was distribution of such cells throughout the length of the gut, from duodenum to colon, but IL-17A expression was largely confined to the terminal ileum, the site of SFB attachment to epithelium (Ivanov et al., 2009). We have identified an SFB-triggered circuit in which ILC3 secretion of IL-22 is critical for local epithelial production of SAA1 and SAA2, which act directly on poised γδ T cells from intestinal LPs and MLN in IL-17A reporter (Il17agfp/gfp) mice at 14 days post-SFB colonization. (C) IL-17A (GFP+) production in Foxp3− RORγt+ CD4+ T cells from intestinal LPs and MLN in IL-17A reporter (Il17agfp/gfp) mice at 14 days post-SFB colonization. IL-17A-producing Th17 cells were monitored by FACS after staining with anti-GFP antibody without re-stimulation. (D) The proportion of IL-17A producing Th17 cells among Foxp3− RORγt+ CD4+ T cells following SFB colonization. Each dot represents a single mouse, and the orange bar indicates average of relative gene expression of each group. Data were from at least two independent experiments (JAX; n = 7, SFB8; n = 9). N.S., not significant (p > 0.05); *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001. See also Figure S1.
Figure 2. Role of Epithelial SAA1/2 in Ileal Th17 Cell Production of IL-17A

(A) Translating ribosome affinity purification (TRAP) assay for identification of the cell type expressing SAA1/2. Four individual lines of TRAP mice, conditionally expressing a GFP tagged ribosome L10 fusion protein in intestinal epithelial (Villin-Cre), T (CD4-Cre), myeloid (CD11c-Cre), or liver (Albumin-Cre) cells were generated. After 2 weeks of SFB colonization, ribosomes in small intestinal lysates collected from each line were enriched by GFP pull-down. Actively translating SAA1/2 mRNAs were quantified by qRT-PCR. Black and white bar indicates TRAP signal from SFB-free animals and light blue, dark blue, purple, and orange bars indicate TRAP signal from SFB-positive animals. White bar indicates TRAP signal from tissue harvested from JAX animals at 6 hr following intraperitoneal administration of LPS.

(B) Quantification of SAA1/2 mRNA in intestinal epithelial cells of duodenum, ileum, and colon from SFB+ (day 7; n = 8, day 14; n = 3), pure SFB (n = 4), JAX (n = 3), or PBS-gavaged (n = 8) mice. Data were normalized using Gapdh. Similar experiments were performed three times.

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(TRAP), which was developed to identify cell type-specific transcripts in tissues containing heterogeneous cell populations (Heiman et al., 2014). TRAP Villin-Cre, TRAP CD11c-Cre, TRAP CD4-Cre, and TRAP Albumin-Cre mice expressing a conditionally induced GFP ribosome L10 fusion protein from the rpl10 locus in epithelial cells, dendritic cells, T cells, and hepatocytes, respectively, were colonized with SFB for 2 weeks or left untreated, after which GFP pull-down of tissue lysates and qRT-PCR using transcript-specific primers were performed to identify translating mRNAs in specific cell types. Using primers that amplify both SAA1 and SAA2 mRNAs, we found that both were highly transcribed and translated in epithelial cells from SFB+ mice (Figure 2A), consistent with previous reports of SAA protein expression in small and large intestinal epithelial cells (Derebe et al., 2014; Eckhardt et al., 2010). We next assessed the spatiotemporal induction of SAA1/2 mRNAs in the epithelial fraction of the intestine upon acute colonization with SFB (Figure 2B). Strikingly, SAA1/2 mRNA induction was confined to the terminal ileum and was modulated in synchrony with SFB colonization (Figures 1A and S1A). Differential SAA expression was confirmed by immunoblotting of the EDTA-epithelial cell-rich fractions from different regions of the GI tract of animals with or without SFB colonization (Figure 2C). Notably, this localization corresponds to the site of SFB attachment and the location of IL-17A-producing Th17 cells.

We next asked whether SAA1/2 contribute to functional differentiation of Th17 cells in vivo. Wild-type (WT) or SAA1 and SAA2 (DKO) mutant mice (Eckhardt et al., 2010) were crossed with Il17agfp/gfp reporter mice in an SFB-free background. Seven days after SFB+ gavage, WT and DKO littermates were colonized at similar levels (Figure S2A). On day 7, at the peak of the Th17 response, we observed in WT and DKO animals comparable proportions of Foxp3− RORγt+ CD4+ T cells in different segments of the GI tract. Within the terminal ileum, however, production of IL-17A-GFP in the poised Th17 cells was significantly reduced in DKO mice, both proportionally and on a per-cell basis (Figures 2D and 2E). In addition, IL-17A and IL-17F mRNAs in the SILP of DKO animals were significantly reduced relative to WT littermates (Figure S2A). Unlike IL-17A and IL-17F, the chemokine receptor CCR6, a marker of Th17 cells, was expressed in similar amounts in wild-type and SAA1/2DKO animals (Figure S2B), consistent with SAA1/2 contributing to a specific subset of genes in the Th17 cell program.

To exclude a possible role for SAA1/2 in T cell development or repertoire bias, we colonized WT and DKO hosts with SFB+ feces and adoptively transferred into them naive CD4+ T cells from 7B8 transgenic mice (Yang et al., 2014). One week post-transfer, we noted that comparable proportions of donor-derived cells became RORγt+ within the terminal ileum of both DKO and WT animals. However, production of IL-17A by the RORγt+ 7B8 cells upon PMA/ionomycin restimulation was markedly attenuated in DKO recipients (Figure 2F). Similar results were observed in WT and DKO recipients of T cells from 7B8 transgenic mice bred onto the IL17A-IRES-GFP reporter background (Figure S2C). Although almost all SFB-specific donor-derived T cells found in the ileum of SFB-colonized mice expressed RORγt regardless of the host genotype, we observed a reduction in total number of these cells recovered in SAA1/2 DKO animals (Figure 2F). This suggests that, in addition to a role in promoting Th17 cytokine production, SAA may promote local Th17 cell proliferation and/or retention.

Next, we treated murine naive CD4+ T cells cultured in Th17 polarizing conditions with recombinant mouse SAA1 (rmSAA1). SAA1 significantly enhanced IL-17A and IL17-F production in RORγt+ CD4+ T cells cultured in suboptimal Th17 polarizing conditions (Figures 3A–3C and S3A). Importantly, this effect was reversed when anti-mSAA blocking antibody was introduced (Figure S3A). In contrast, CCR6 expression was not changed by rmSAA1 treatment (Figure S3B). Based on our in vivo observation, we also asked whether mSAA1 exerts a direct effect on cell proliferation in addition to Th17 signature cytokine production. Labeling of naive CD4+ T cells with CFSE and intracellular Ki67 staining showed enhanced proliferation of RORγt+ Th17 cells upon treatment with rmSAA1 (Figures 3A–3C and S3C), but the higher proportions of cells expressing IL-17A and IL-17F were observed independently of the extent of proliferation (Figure 3C). Furthermore, in vitro differentiation of naive CD4+ T cells toward Th1, Th2, and iTregs was not significantly affected in the presence of recombinant mouse SAA1 (Figure S3D). Similarly, rhSAA augmented IL-17A production during human Th17 cell priming (Figure S3E). Importantly, this effect was reversed when SAA blocking antibody was introduced into culture. These results suggest that SAA acts directly on both mouse and human Th17 cells to enhance their differentiation and effector function.

To further study how SAA influences the transcriptional program of Th17 cells, we performed RNA sequencing (RNA-seq) with control and rmSAA1-treated murine Th17 cells. We identified a specific subset of the Th17 gene expression program that was specifically affected by rmSAA1 treatment (Figures 3D and S3F). Unbiased Ingenuity Pathway Analysis predicted RORγt, the key regulator of the Th17 differentiation program, as the top upstream regulatory factor sensitive to SAA perturbation in vitro (Figure 3E). ROR-element-driven luciferase assays in Th17 cells confirmed that RORγt transcriptional activity was potentiated during rmSAA1 stimulation in vitro (Figure 3F).

(C) Representative western blots of lysates from the EDTA-epithelial-rich fraction of different regions of the GI tract in animals with or without SFB colonization. (D–F) Role of SAs in intestinal LP Th17 cell differentiation following SFB colonization. (D and E) IL-17A reporter littermates in WT (black, n = 5) or Saa1/2 double-knockout (DKO) (gray, n = 5) backgrounds were colonized with SFB for 7 days. RORγt+ cells among CD4+ LP T cells and their IL-17A producing capacity were measured by FACs. MFI and cell numbers of CD4+RORγt+Foxp3−GFP+ Th17 cells were quantified. (F) Naive CD4+ T cells from SFB-specific TCR transgenic (7B8) mice were adoptively transferred into SFB-colonized WT (circles, n = 4) or Saa1/2 DKO (squares, n = 4) littermates (as indicated in diagram). Total number of donor cells recovered from ileum (far left), percentage of RORγt-expressing donor-derived cells (center left), percentage of IL-17A-expressing cells upon ex vivo re-stimulation by PMA/ionomycin (center right), and their total number (far right) were monitored by FACs at 7 days post-transfer.
N.S., not significant (p > 0.05); *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001. Data are represented as mean with SD. See also Figure S2.
Figure 3. SAA1 Promotes IL-17A Production in Polarized Th17 Cells In Vitro
(A) Schematic representation of the experimental protocol. CFSE-labeled naive CD4+ T cells from WT mice were activated under non-polarizing (Th0: anti-CD3 and anti-CD28) or Th17 polarization condition (Th17: anti-CD3, anti-CD28, IL-6, and TGF-ß). At 24 hr, RORγt+ T cells were further cultured with recombinant mouse SAA1 (rmSAA1) under Th17 polarization conditions for 48 hr.

(B) IL-17A and IL-17F production by in vitro-polarized mouse Th17 cells (CD4+ RORγt+) at 48 hr after rmSAA1 (10 µg/ml) addition. Cells were re-stimulated with PMA/ionomycin for 5 hr, and cytokine production and CFSE dilution were monitored by FACS analysis.

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SFB-Induced SAA1/2 Requires IL-22 Expression and Epithelial Stat3 Signaling

We next sought to understand the upstream signaling pathway that regulates ileal SAA induction in response to SFB colonization. SFB-free C57BL/6 mice were co-housed with SFB-positive mice (Tacchino) for 2 weeks before epithelial-specific translating RNAs were assessed. RNA-seq profiling of polysome-associated mRNAs pulled-down from SFB-colonized TRAP Villin-Cre mice revealed a significant enrichment of actively translated genes that are known to be regulated by the transcription factor Stat3 (Figure S4A). To corroborate this analysis, we used immunofluorescence to probe for Stat3 activity (pStat3) in small intestinal tissue sections of mice acutely colonized with SFBβ feces. We detected pStat3 within 24 hr of SFB colonization (Figure 4A). Strikingly, SFBβ fecal-induced upregulation of SAA1/2 was abrogated almost entirely in mice with intestinal epithelial cell-specific inactivation of Stat3 (Figure 4B). Stat3 activation is elicited by multiple cytokines, including IL-10 and members of the IL-20 subfamily, of which IL-22 is the best characterized (Rutz et al., 2014). Interestingly, we detected a significant increase in IL-22 mRNA within the terminal ileum shortly after colonization with SFBβ, and this preceded the induction of epithelial SAA1/2 expression by one day (Figures 4C–4E). This result suggested that IL-22 activated Stat3, which then induced SAA1/2 expression. Indeed, treatment with neutralizing anti-IL-22 antibody prevented SAA1/2 induction following SFB colonization (Figure S4B). Consistent with this result, SAA1/2 mRNA induction by SFB was almost completely abrogated in IL-22 deficient mice (Figure 4F). In addition, IL-17A production among RORγt+ Th17 cells in ileum was partially blocked in animals receiving IL-22 blocking antibodies, similar to what was observed in SAA1/2 DKO mice (Figure S4C).

To evaluate whether IL-22 can directly induce epithelial SAA1/2 expression, we generated small intestinal epithelial (SIE) organoids as previously described (Sato and Clevers, 2013) and exposed them to recombinant murine IL-22 (rIL-22). The addition of rIL-22 to SIE organoid cultures induced SAA1/2 mRNA in a dose-dependent fashion within 2 hr (Figure 4G). Coupled with the in vivo results, these findings indicate that SFB colonization initiates an IL-22-mediated Stat3 signaling cascade in epithelial cells, resulting in localized SAA1/2 expression.

SFB Stimulates IL-23R-Dependent IL-22 Production by Activated ILC3

In the intestine, IL-22 is produced by RORγt-expressing lymphoid cells that include ILC3, Th17, and γδ17 cells (Sawa et al., 2011). To investigate the source of IL-22 that directs epithelial SAA production following SFB colonization, we compared RNA expression profiles from small intestine Th17 cells and ILC3 in mice stably colonized with SFB. Based on normalized DESeq reads, IL-22 expression was 10-fold higher in ILC3 than in Th17 cells (Figure S5A). Examination of these data sets also revealed a surface antigen, killer cell lectin-like receptor subfamily B member 1B (Klrb1b), which was uniquely expressed in ILC3. We confirmed this finding at the level of protein by flow cytometry (Figure S5B), and subsequently incorporated Klrb1b into the fluorescent cell-labeling panel to dispense with the necessity of a reporter in studying the dynamic behavior of ILC3 upon SFBβ colonization.

At day 4 of SFBβ colonization, up to 80% of ILC3s expressed the activation marker stem cell antigen-1 (Sca-1), which amounted to a 10-fold increase compared to the proportion of Sca-1+ ILC3s in mice gavaged with SFB-negative feces (Figures 5A and 5B). Unlike CCR6+ ILC3s, CCR6+ ILC3s typically populate the isolated lymphoid follicles and cryptopatches (Lügering et al., 2010). Notably, both CCR6+ and CCR6- ILC3s expressed high level of Sca-1 on their surface in response to SFB colonization. Although ILC3s increased in size in response to SFB colonization, their absolute numbers remained unchanged (Figures S5C–S5E). Intracellular cytokine staining also revealed a striking increase in both the frequency and total number of ILC3s producing IL-22 (Figure S5C). We recapitulated these findings using the fecal contents from SFB mono-associated mice, supporting the notion that SFB alone is sufficient to direct the localized ILC3 response (Figure S5F). Importantly, at this early time point, other RORγt-dependent lineages, including γδ17 and (non-SFB-specific) Th17 cells, did not produce IL-22 (Figure 5D). Furthermore, epithelial cells from Rag2 mutant mice, harboring ILC3s but lacking B and T cells, also expressed SAA1/2 in response to SFB colonization (Figure S5E).

ILC3s residing in the colon secrete IL-22 in response to IL-23 (Eken et al., 2014). To determine whether this mechanism also governs terminal ileal production of IL-22 by ILC3s upon SFB colonization, we colonized IL-23R-sufficient and -deficient mice with SFBβ feces and examined the spontaneous release of IL-22 by ILC3s. Both the frequency and absolute number of IL-22-secreting CCR6+ and CCR6- ILC3s declined sharply in IL-23R-deficient animals (Figures 6A and 6B). Consistent with this, induction of epithelial SAA1/2 mRNA was markedly blunted in IL-23R-deficient animals (Figure 6C). Altogether, these findings indicate that IL-23 signaling initiates IL-22-mediated cross-talk between ILC3s and small intestine epithelial cells, resulting in spatially restricted SAA production, which contributes to local augmentation of Th17 effector functions (Figure S6).

(C) Representative data from polarized cells (48 hr) (four experiments total). The frequencies of IL-17A- and IL-17F-producing cells during each cell division are displayed using the gates represented in (B). Data are shown as mean ± SD.
(D) RNA-seq studies from two biological replicates comparing in vitro Th17 cells cultured in 20 ng/ml IL-6 and 0.1 ng/ml TGF-β with or without 2 μg/ml rmSAA1 for 48 hr. Dots represent FPKMs of individual mRNA transcripts in the respective cell type (gray). 195 genes that were significantly different between vehicle and SAA treatment (p value <0.05, fold change >2) are highlighted in black.
(E) Ingenuity Pathway Analysis for upstream regulators of the 195 SAA-affected genes in Th17 cells. (F) A RORγt-responsive element (RORE) firefly luciferase reporter was used to monitor RORγt transcriptional activity in Th17 cells. The RORE-luciferase reporter construct was introduced by nucleofection into Th17 cells polarized for 48 hr and cultured with or without rmSAA1 (2 μg/ml), and luciferase activity was measured 24 hr later. Data were normalized by renilla reporter activity. Results from four technical repeats in each condition were averaged. Results are representative of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001. See also Figure S3.
DISCUSSION

In this report, we describe a two-step process for the functional differentiation of intestinal Th17 cells following colonization of mice with SFB. The first step is the priming and polarization of antigen-specific CD4+ T cells in the tissue-draining lymph nodes, resulting in a poised state marked by the expression of RORγt, and the second is the activation of a cytokine gene expression program in a tissue microenvironment in which epithelial cell-derived factors SAA1 and SAA2 act on poised cells. Temporally, we observed specific SFB colonization of the ileum by 24 hr post-SFB gavage. Within 5 days, SFB antigen-presentation occurred in the MLN, where a significant number of RORγt+ Th17 cells were first observed. By day 7, RORγt+ Th17 cells accumulated in the lamina propria of the intestine. During the early phase of this commensal-induced host response, IL-22 and SAA1/2 mRNA were acutely upregulated in the ileum within 3 days. Our studies revealed that activation of epithelial SAAs involved an SFB-initiated signaling pathway requiring IL-23 and ILC3-derived IL-22 (Figure S6). A similar IL-22-dependent pathway was recently described in SFB induction of Fut2, an enzyme that catalyzes fucosylation of glycans, thus providing nutritional substrates for commensals.
Figure 5. SFB Colonization Induces IL-22 Production by ILC3s

(A) Cell-surface expression of Sca-1 and CCR6 on ileal SILP ILC3s from mice at 4 days after oral gavage with JAX or SFB* fecal contents.

(B) Frequency of Sca-1-positive CCR6+ or CCR6− ILC3s in the terminal ileum from JAX- or SFB*-gavaged mice at 4 days after gavage. Data are represented as mean with SD (JAX gavaged: n = 5, SFB* gavaged: n = 6).

(C) IL-22 production in ileal SILP ILC3. Ileal LP cells from JAX- or SFB*-gavaged mice were isolated at 4 days after gavage and cultured in vitro with GolgiStop (without PMA/IONOMYCYCIN re-stimulation) for 4 hr. IL-22-producing ILC3s were analyzed by FACS, and cell numbers were calculated (right panel). Bar graph shows accumulated data as mean with SD (JAX gavaged: n = 5, SFB* gavaged: n = 6).

(D) IL-22 production in each indicated cell type isolated from the ileal LP of SFB-free (JAX; green line) and SFB-colonized (SFB*; magenta line) mice, analyzed at 4 days after gavage. (A–D) The experiment was repeated at least three times with similar results.

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and contributing to barrier protection (Bry et al., 1996; Goto et al., 2014; Pickard et al., 2014).

Although we identified a requirement for the ILC3-dependent pathway during the early induction of epithelial SAA1/2, we cannot rule out that SFB attachment to the epithelial cells, which is accompanied by reorganization of the cortical actin network, contributes to or is required for SAA induction. Indeed, Atarashi et al. (2015 [this issue of Cell]) showed that rat SFB failed to attach to ileal epithelium and to induce SAA1/2 in mice, yet induced ILC3 IL-22 production. Thus, multiple signals may be required for epithelial cell induction of SAA1/2 or, alternatively, a single IL-22 signal may be sufficient, but its delivery to epithelial receptors may be compromised in mice colonized with rat SFB.

**SAAs in Homeostasis and Autoimmune Diseases**

SFB is considered a commensal microbe, as there is no evidence that it induces pathology despite its adhesion to the epithelial surface and its induction of host adaptive and innate immune responses. By virtue of its activation of epithelial Stat3, SFB may reinforce the terminal ileal firewall, which harbors the greatest bacterial load in the small intestine (Sekirov et al., 2010). In the large intestine, Stat3 was found to be required for SFA production (Hall et al., 2011). Therefore, in the context of SFB colonization, the elevation of SAA1/2 and Th17 cells may be considered a homeostatic mechanism that contributes to barrier integrity in the small intestine.

SAAs are significantly upregulated in the joints and serum of rheumatoid arthritis patients (O’Hara et al., 2000) and are correlated with disease progression (Chambers et al., 1983). In mouse models, Th17 cells are also known to contribute to multiple autoimmune diseases (Genovese et al., 2010; Huh et al., 2011; Lepkies et al., 2009), and whether SAAs or differences in its binding to cofactors mediate these processes remain to be elucidated. As microbiota-specific helper T cells, including SFB-specific Th17 cells, circulate widely following their polarization in the gut-associated lymphoid tissues (Hand et al., 2012; Yang et al., 2014), it is possible that they contribute to autoimmune disease systemically through induction of their effector functions at sites where inflammatory mediators such as SAAs are elevated.

Another possibility is that under normal circumstances SAAs are produced in the joints of rheumatoid arthritis patients (O’Hara et al., 2000) and are correlated with disease progression (Chambers et al., 1983). In mouse models, Th17 cells are also known to contribute to multiple autoimmune diseases (Genovese et al., 2010; Huh et al., 2011; Lepkies et al., 2009), and whether SAAs or differences in its binding to cofactors mediate these processes remain to be elucidated. As microbiota-specific helper T cells, including SFB-specific Th17 cells, circulate widely following their polarization in the gut-associated lymphoid tissues (Hand et al., 2012; Yang et al., 2014), it is possible that they contribute to autoimmune disease systemically through induction of their effector functions at sites where inflammatory mediators such as SAAs are elevated.

Another possibility is that under normal circumstances SAAs may be required for the resolution of inflammation. Th17 cells often produce IFNγ in addition to IL-17A at sites of inflammation (Ivanov et al., 2006), and this is thought to contribute to pathogenicity, but they can also give rise to IL-10-producing cells that may be important during the resolution of inflammation (Gagliani et al., 2015). Therefore, it will be important to study in detail the regulation of SAA expression in tissues during the course of autoimmune progression and whether their manipulation affects Th17-mediated pathology. Importantly, since we did not observe

(B) SAA1/2 expression, analyzed by qRT-PCR, in the epithelial fraction from terminal ileum of Rag2-sufficient or -deficient mice, at 3 days after SFB (SFB*) or control (JAX) gavage. Data are from two independent experiments. Each symbol represents a single mouse, and the orange bar indicates average of relative gene expression in each group. Experiments were repeated two times with similar results and data were normalized by GAPDH mRNA and combined. *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001. See also Figure S5.
a complete loss in Th17 cytokine production in SAA1/2-deficient settings during SFB induction, we speculate that other epithelial cell-derived factors likely contribute to the regulation of the Th17 effector program, which will be of great interest to study.

**Innate Lymphoid Cell Regulation of Barrier Tissue Immunity**

Tissue-resident RORγt-expressing innate lymphoid cells and "innate-like" T cells, e.g., γδ T cells, can respond rapidly to cytokine stimulation, including IL-1β and IL-23, by producing their own cytokines, particularly IL-22. This occurs in the absence of antigen receptor stimulation in γδ T cells (Cua and Tato, 2010; Martin et al., 2009; Sutton et al., 2009). For ILC3s, microbiota-induced local production of IL-23-IL-1β, and TL1α/TNFSF15 by CX3CR1+ mononuclear phagocytes is required for their production of IL-22 and intestinal barrier protection functions (Longman et al., 2014). We do not know whether RORγt+ SFB-specific T cells that migrate to the intestinal lamina propria require TCR signaling for induction of effector cytokines, or whether SAA1/2, in the appropriate microenvironment, suffices for this purpose. It will be important, in future studies, to identify the receptor(s) for SAA1/2 on Th17 cells and characterize the signaling mechanism required for the cytokine response.

Our findings suggest that SAA1 and SAA2 serve as sentinels for the tissue microenvironment, enlisting T helper cells with poised Th17 functions to protect or heal the epithelial barrier. It will be important to determine whether the SAAs are unique in their ability to amplify the Th17 cell transcriptional program and whether other induced mediators similarly influence other functional T cell subsets. In this regard, IL-18 and IL-33 were recently shown to amplify Th1 effector responses during infection with Salmonella or Chlamydia (O’Donnell et al., 2014). Efforts to dissect the upstream pathways and secretory outputs that impinge on T cell effector functions at steady state and in the face of multiple challenges will create novel opportunities to modulate tissue immunity.

A critical remaining question is how SFB are sensed within the ileum. Atarashi et al. (2015) showed that adhesion of microbes to epithelium is a necessary step for Th17 differentiation. It will be important to learn whether distinct signals received by ileal epithelial cells from bound SFB are critical for Th17 cell induction and subsequent cytokine production. Characterization of such signals may provide insights into how intestinal microbes contribute to local and systemic inflammation.

**EXPERIMENTAL PROCEDURES**

**Mouse Strains and SFB**

C57BL/6 mice were obtained from Taconic Farms or the Jackson Laboratory. All transgenic animals were bred and maintained in the animal facility of the Skirball Institute (NYU School of Medicine) in specific-pathogen-free (SPF) conditions. IL-23 receptor-deficient mice (Awasthi et al., 2009) were provided by M. Oukka and maintained on Jackson flora. Il-23r−/− mice were obtained from Taconic Farms or the Jackson Laboratory. C57BL/6-Il17atm1Bcgen/J and Ly5.1 background (JAX; C57BL/6-Il17atm1Bcgen/J) and Ly5.1 background (JAX; B6.129S1-Stat3tm1Xyfu/J) were bred to Villin-Cre mice. Six- to 18-week-old animals were used for experiments. Fecal pellets were provided by Y. Umekawa (Yakult) to establish a colony of SFB mono-colonized mice in our gnotobiotic animal facility. All transgenic animals were maintained in the animal facility of the Skirball Institute in accordance with protocols approved by the Institutional Animal Care and Use Committee of the NYU School of Medicine.

**Acute SFB Colonization**

Fecal pellets were collected from SFB-enriched Il-23r Rag2 DKO mice (SFBe). SFB mono-associated mice (pure SFB), or SFB-free JAX B6 mice (JAX), respectively. Fresh fecal pellets were homogenized through a 100-μm filter, pelleted at 3,400 rpm for 10 min, and re-suspended in PBS. Each animal was administered one-quarter pellet by oral gavage.

**Accession Numbers**

RNA-seq data sets and Trap-seq data sets are available on GEO (GSE70599).

**Supplemental Information**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.08.061.

**Author Contributions**

T.S. and W.H. designed and performed most experiments and analyzed the data. J.A.H. performed experiments related to ILC3 activation, J.-Y.L. performed organoid culture studies, A.C. and J.G. assisted with in vivo experiments, and Y.Y. generated TCR transgenic mice and provided cells for RNA-seq analyses, W.H. and A.I.D. designed and performed Ribosome TRAP-seq studies. J.W.Z. performed studies related to IL-23R regulation of SFB-induced epithelial responses. T.S., W.H., J.A.H., and D.R.L. wrote the manuscript. D.R.L. supervised the research and participated in experimental design.

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