# Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria

Ivaylo I. Ivanov,[1,10] Koji Atarashi,[3,10] Nicolas Manel,[1,11] Eoin L. Brodie,[4,11] Tatsuichiro Shima,[7,11] Ulas Karaoz,[4] Dongguang Wei,[5] Katherine C. Goldfarb,[4] Clark A. Santee,[4] Susan V. Lynch,[6] Takeshi Tanoue,[3] Akemi Imaoka,[7] Kikuji Itoh,[8] Kiyoshi Takeda,[3] Yoshinori Umesaki,[7] Kenya Honda,[3,9,*] and Dan R. Littman[1,2,*]

[1]Molecular Pathogenesis Program, The Kimmel Center for Biology and Medicine of the Skirball Institute
[2]Howard Hughes Medical Institute
New York University School of Medicine, New York, NY 10016, USA
[3]Laboratory of Immune Regulation, Graduate School of Medicine, WPI Immunology Frontier Research Center, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
[4]Center for Environmental Biotechnology, Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[5]Carl Zeiss SMT, Inc., Nanotechnology Systems Division, One Corporation Way, Peabody, MA 01960, USA
[6]Division of Gastroenterology, Department of Medicine, University of California San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA
[7]Yakult Central Institute for Microbiological Research, Yaho 1796, Kunitachi, Tokyo 186-8650, Japan
[8]Department of Veterinary Public Health, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[9]Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, 332-0012 Japan
[10]These authors contributed equally to this work
[11]These authors contributed equally to this work
*Correspondence: honda@ongene.med.osaka-u.ac.jp (K.H.), littman@saturn.med.nyu.edu (D.R.L.)
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## SUMMARY

The gastrointestinal tract of mammals is inhabited by hundreds of distinct species of commensal microorganisms that exist in a mutualistic relationship with the host. How commensal microbiota influence the host immune system is poorly understood. We show here that colonization of the small intestine of mice with a single commensal microbe, segmented filamentous bacterium (SFB), is sufficient to induce the appearance of CD4^{+} T helper cells that produce IL-17 and IL-22 (Th17 cells) in the lamina propria. SFB adhere tightly to the surface of epithelial cells in the terminal ileum of mice with Th17 cells but are absent from mice that have few Th17 cells. Colonization with SFB was correlated with increased expression of genes associated with inflammation and antimicrobial defenses and resulted in enhanced resistance to the intestinal pathogen *Citrobacter rodentium*. Thus, manipulation of this commensal-regulated pathway may provide new opportunities for enhancing mucosal immunity and treating autoimmune disease.

## INTRODUCTION

The vertebrate intestine is typically colonized by hundreds of distinct species of microorganisms that have a mutually beneficial relationship with the host. Intestinal microbiota are known to influence the development and balance of the host immune system and have been implicated in prevention of damage induced by opportunistic microbes, in repair of damage to the mucosal barrier and in influencing systemic autoimmune diseases (Backhed et al., 2005; Macpherson and Harris, 2004; Rakoff-Nahoum and Medzhitov, 2006). CD4^{+} T cells acquire distinct functional properties in response to signals conveyed by commensal and pathogenic microbe-activated cells of the innate immune system (Seder and Paul, 1994). T helper type 1 (Th1) and Th2 cells control intracellular microorganisms and helminths, respectively (Abbas et al., 1996; Glimcher and Murphy, 2000), whereas the induced regulatory T (iTreg) cells suppress excessive immune responses (Gavin and Rudensky, 2003). Th17 cells secrete interleukin-17 (IL-17), IL-17F, and IL-22 and have significant roles in protecting the host from bacterial and fungal infections, particularly at mucosal surfaces. Th17 cells also have potent inflammatory potential, and thus are key mediators of autoimmune disease (Aujla et al., 2007; Bettelli et al., 2007). Th17 and Treg cells are both dependent on transforming growth factor β (TGF-β) for their differentiation and are defined by the expression of the lineage-specific transcription factors RORγt and Foxp3, respectively (Fontenot et al., 2003; Hori et al., 2003; Ivanov et al., 2006; Khattri et al., 2003; Mangan et al., 2006; Veldhoen et al., 2006). At appropriate concentrations of TGF-β and IL-6, antigen-activated CD4^{+} T cells upregulate RORγt and express Th17 cell cytokines (Zhou et al., 2008).

Th17 cells are most abundant at steady state in gut-associated tissues, particularly the small intestinal lamina propria (SI LP) (Ivanov et al., 2008; Ivanov et al., 2006), where they accumulate only in the presence of luminal commensal microbiota (Atarashi et al., 2008; Hall et al., 2008; Ivanov et al., 2008).

Germ-free (GF) mice, which lack Th17 cells in the SI LP (and also in the colon), acquired them after colonization with conventional microbiota. Treatment of newborn mice with antibiotics, particularly vancomycin, resulted in marked reduction in the number of Th17 cells in the SI LP. Most strikingly, C57BL/6 (B6) mice obtained from different commercial vendors displayed marked differences in the proportion of Th17 cells in the SI LP (Ivanov et al., 2008). Thus, mice from the Jackson Laboratory had very low numbers of SI LP Th17 cells compared to mice of the same strain obtained from Taconic Farms. Transfer into GF mice of intestinal contents of Taconic B6 mice, but not Jackson B6 mice, induced Th17 cell accumulation, and Jackson mice acquired Th17 cells within weeks of cohousing with mice from Taconic Farms. GF mice colonized only with a defined cocktail of bacteria (Altered Schaedler Flora [ASF]) lacked intestinal Th17 cells (Ivanov et al., 2008). These results demonstrated that the induction of Th17 cells in the SI LP is controlled not by the presence of bacteria per se, but by the composition of the intestinal microbiota and, presumably, the presence of specific bacterial taxa. Intriguingly, Treg cells, which, like Th17 cells, are abundant in the intestine, were increased in proportion in the SI LP in GF mice, and their numbers were inversely correlated to the proportion of Th17 cells. Signals derived from microbiota may thus influence the differentiation potential of multipotent CD4$^+$ T cells in the lamina propria (Zhou et al., 2008).

Here, we report that specific members of the commensal microbiota known as segmented filamentous bacteria (SFB), with the candidate name *Arthromitus*, are potent inducers of Th17 cells in the SI LP of mice. SFB, spore-forming gram-positive bacteria most closely related to the genus *Clostridium*, have been reported to colonize the intestines of numerous species, including humans (Davis and Savage, 1974; Klaasen et al., 1993a). They typically adhere tightly to epithelium in the ileum, where their abundance has been noted to correlate with reduced colonization and growth of pathogenic bacteria (Garland et al., 1982; Heczko et al., 2000). SFB were present in large numbers in conventionally raised B6 mice from Taconic Farms but were undetectable in the same strain of mice obtained from the Jackson Laboratory. Introduction of SFB, but not other bacteria, into Th17 cell-deficient mouse models induced IL-17 and IL-22 expression in CD4$^+$ T cells in the SI LP. Upon colonization, SFB induced a proinflammatory gene program that was similar to that induced in Jackson B6 mice cohoused with Taconic animals, suggesting that SFB are major modulators of immune responses in conventional mice. SFB colonization induced production of serum amyloid A (SAA) in the terminal ileum, and SAA acted on lamina propria dendritic cells (LP DCs) to promote Th17 cell differentiation in vitro. SFB colonization resulted in reduced growth of an intestinal pathogen, suggesting that intestinal commensal microbes can contribute to Th17 cell-mediated mucosal protection.

## RESULTS

### Comparative Analysis of the Intestinal Microbiota of Jackson and Taconic B6 Mice

To identify the bacterial species that induce Th17 cells in the small intestine, we compared the bacterial content in B6 mice purchased from Taconic Farms and Jackson Laboratory. We previously showed that transfer of cecal contents could induce Th17 cells in recipient mice (Ivanov et al., 2008). Because we find more Th17 cells in the small intestine than in the large intestine (LI), we surmised that the Th17 cell-inducing bacterial species are present also in the small intestinal microbiota. Indeed, colonization of GF and Jackson B6 mice with the contents of the small intestines of Taconic B6 mice induced numbers of Th17 cells similar to those in specific-pathogen-free (SPF) Taconic B6 mice (Figure 1A). We therefore chose to investigate the bacterial composition of the small intestine in detail. To provide an in-depth profile of the bacterial communities present in the small intestine of Taconic and Jackson mice, we analyzed these samples using the 16S ribosomal RNA (rRNA) PhyloChip (Brodie et al., 2006), a high-density microarray with over 300,000 probes targeting the sequence polymorphisms in the 16S rRNA gene, permitting detection of approximately 8500 bacterial taxa. Overall 1164 taxa were detected across all samples, 509 ± 32 taxa were detected in Jackson B6 mice, and 828 ± 82 taxa were detected in Taconic B6 mice (Figure S1 available online). Our previous analysis by fluorescence in situ hybridization had demonstrated a correlation between a probe for the Cytophaga-Flavobacter-Bacteroides (CFB) phylum and the presence of Th17 cells in Taconic mice (Ivanov et al., 2008). However, closer analysis of recently published 16S rRNA sequences revealed that this probe also matches perfectly to a number of non-CFB taxa, including bacteria in the Firmicutes, Actinobacteria, and Verrucomicrobia phyla. The PhyloChip analysis demonstrated that, indeed, the overall representation of the major bacterial phyla, including CFB, was not statistically different between the two mouse strains (Figure S2).

The depth of coverage and phylogenetic breadth of the PhyloChip allowed us to assay the microbial community at multiple phylogenetic levels, while its sensitivity permitted detection of less abundant organisms even in dominated communities (De-Santis et al., 2007). Comparative analysis of 766 bacterial taxa detected in at least three out of four replicates from either strain of mice demonstrated that the relative abundance of 479 taxa was significantly different (p ≤ 0.05) between the two mouse strains, with 372 taxa having greater abundance in Jackson mice and 107 taxa overrepresented in the Taconic group. However, of the 479 significantly different taxa, most differences were subtle, with only 52 being above 5-fold (17 greater in Taconic and 35 greater in Jackson), and only two taxa were >25-fold more abundant. These were identified as members of the *Lactobacillaceae* and *Clostridiaceae* families—*Lactobacillus murinus* ASF361 and a segmented filamentous species of the candidate genus *Arthromitus* (Figure 1B). Both were of significantly greater (p < 0.001) relative abundance in Taconic mice (∼94-fold for *Lactobacillus murinus* and ∼40-fold for *Candidatus Arthromitus*); however, since both were below the PhyloChip threshold of detection in the Jackson mice, these fold changes should be considered a minimum. For both taxa, overrepresentation of close phylogenetic relatives was not observed, suggesting a species-specific increase in relative abundance (Figure 1B).

*Lactobacillus murinus* ASF361 is a component of the ASF (Dewhirst et al., 1999). ASF is used by Taconic Farms as a basal inoculum introduced into all Taconic rederived strains, but is not
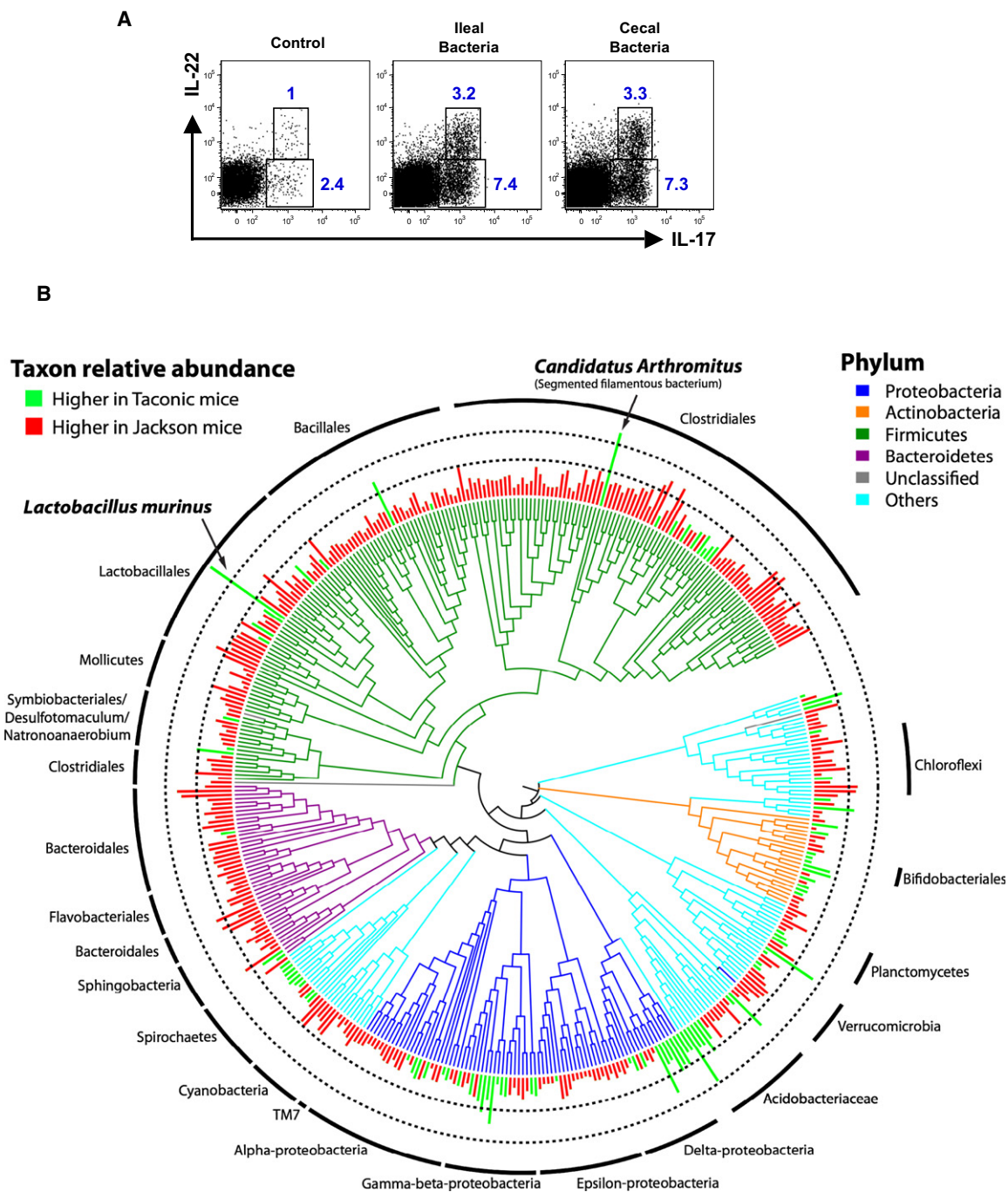
**Figure 1. Comparative Analysis of the Microbiota in the Terminal Ileum of C57BL/6 Mice from Jackson Laboratory versus Taconic Farms**

(A) Lumenal bacteria from both cecum and terminal ileum can induce Th17 cell differentiation upon transfer into Jackson B6 mice. Jackson B6 mice were gavaged with water (control) or with intestinal luminal contents from cecum or terminal ileum of Taconic B6 mice. LPL from small intestine were isolated 10 days later and analyzed for intracellular cytokines. Representative plots from one experiment with three mice per group. Plots gated on TCRβ$^+$CD4$^+$ LPL.

(B) Phylogenetic tree based on 16S rRNA gene sequences of bacterial taxa detected in the terminal ileum showing significantly different relative abundances (PhyloChip fluorescence intensity) between the suppliers, Taconic and Jackson. Branches of the tree are color coded according to phylum while green and red bars display taxa with significantly greater relative abundance in Taconic and Jackson mice, respectively. The inner and outer dotted rings represent intensities corresponding to 5-fold and 25-fold differences in 16S copy number. The two taxa with the greatest difference between Taconic and Jackson mice, *Lactobacillus murinus* (∼94-fold difference) and *Candidatus Arthromitus* (∼40-fold difference) are noted by arrows.

intentionally introduced into Jackson Laboratory animals. Because of these differences, we previously tested whether *L. murinus* ASF361, in the context of ASF, induces Th17 cell differentiation. Colonization of germ-free mice with ASF, including *L. murinus* ASF361, did not induce any Th17 cells in the SI LP (Ivanov et al., 2008). We therefore concluded that *L. murinus* ASF 361 is not involved in the induction of Th17 cell differentiation.

### Presence of SFB Correlates with the Presence of Th17 Cells

We next examined the representation of *Candidatus Arthromitus* in Th17 cell-sufficient and Th17 cell-deficient mice. *Arthromitus* is an unofficial candidate genus name for the group of so-called segmented filamentous bacteria (Snel et al., 1995). SFB are yet to be cultured, commensal, gram-positive, anaerobic, spore-forming bacteria that are resident in the terminal ileum under steady-state conditions (Davis and Savage, 1974). SFB have a characteristic long filamentous morphology, are comprised of multiple segments with well-defined septa, and often span the length of several villi. They colonize the gastrointestinal tract of mice at weaning time and adhere tightly to epithelial cells (Koopman et al., 1987). SFB are present in many vertebrate species, including rodents (Davis and Savage, 1974), fish, chicken, dogs, and primates (Klaasen et al., 1993a; Ley et al., 2008). A phylogenetic tree based on an alignment of the available SFB 16S rRNA gene sequences according to their sequence origin is presented in Figure S3. SFB are known to actively interact with the immune system (Klaasen et al., 1993b). Colonization of germ-free animals with SFB leads to stimulation of secretory IgA (SIgA) production and recruitment of intraepithelial lymphocytes (IELs) to the gut (Talham et al., 1999; Umesaki et al., 1999). Mice lacking the activation-induced cytidine deaminase (AID) required for antibody diversification had outgrowth of SFB in their small intestine (Suzuki et al., 2004).

We validated the abundance of SFB in the gut of Taconic and Jackson B6 mice by quantitative real-time PCR (qPCR) for 16S rDNA sequences. SFB were present in fecal material from cecum as well as small and large intestine of Taconic B6 mice, but could not be detected in Jackson B6 mice (Figure 2A and data not shown). Scanning electron microscopy revealed a thick network of SFB present in the terminal ileum of 6- to 8-week-old Taconic B6 mice (Figure 2B). In contrast, we could not detect any bacteria with SFB morphology in age- and sex-matched Jackson B6 mice, even after equilibration of housing conditions and diet (Figure 2B). Despite similar numbers of total bacteria in the feces of both strains, only non-SFB bacteria were evident in the terminal ileum of mice from Jackson Laboratory (Figure S4). Transmission electron microscopy confirmed typical SFB morphology with well-defined segments in tight contact with the epithelial cells of ileum from Taconic but not Jackson B6 mice (Figure 2B). To confirm that SFB can be horizontally transferred, we cohoused female mice obtained from the two sources and observed Th17 cells in the lamina propria of Jackson B6 mice within 10 days (Ivanov et al. 2008) (Figure S5). qPCR analysis of fecal material and microscopy of terminal ileum confirmed the appearance of SFB in the co-housed Jackson B6 mice (Figures 2C and 2D).

### SFB Specifically Induce Th17 Cells in the Intestinal Lamina Propria

To test whether SFB are sufficient to induce Th17 cells, we colonized germ-free (GF) Swiss-Webster mice with fecal material obtained from mice monocolonized with SFB (SFB-mono mice) (Umesaki et al., 1995) and examined lamina propria CD4+ T cells for Th17 cell differentiation 10 days later. Noncolonized control GF mice housed under separate but similar conditions had no Th17 cells (Figure 3A). In contrast, SFB colonization induced robust accumulation of Th17 cells in both the SI and LI LP (Figures 3A and S6). SFB induced production of both IL-22 and IL-17 in CD4+ T cells (Figures 3A and 3B). The effect of SFB on Th17 cell differentiation was similar in Swiss-Webster and IQI GF mice housed at different institutions (Figures 3B and 3C). Moreover, the effect of SFB on inducing IL-17 production in LP T cells is bacterial species specific, because colonization with *Bacteroides* species as well as with a defined mix of *Clostridium* species, which are closely related to SFB, did not induce Th17 cells in GF mice (Figure 3C). Finally, SFB had no effect on interferon-γ (IFN-γ) production, indicating that they specifically influence Th17 and not Th1 cell differentiation (Figure 3D). Colonization of GF mice with SFB restored RORγt+ T cells to the levels observed in mice kept under SPF conditions (Figure 3E). By contrast, the number of RORγt+ non-T cells, which include lymphoid tissue inducer-like cells and natural killer (NK)-like cells, was similar in GF mice, SFB-mono mice, and mice kept in SPF conditions (Figure 3E), and there was no significant difference in IL-17 and IL-22 production by these cells (Figure S7). Notably, SFB colonization and induction of Th17 cells did not reverse the elevated proportion of Foxp3+ cells among the CD4+ T cells in the SI LP and the peritoneal cavity of GF mice. (Figure S8).

To determine whether SFB can also induce Th17 cell differentiation in conventionally raised mice, we introduced fecal material from SFB-mono mice by oral gavage into 6-week-old Jackson B6 mice and analyzed colonization and cytokine production in the SI LP. By 10 days, SFB were detected by scanning electron microscopy in the terminal ileum (Figure 4A) and by qPCR in the feces (data not shown), and robust Th17 cell differentiation was observed in the SI LP (Figures 4B and 4C). In contrast, control untreated Jackson B6 mice or Jackson B6 mice gavaged with bacterial suspensions from their littermates did not show an increase in Th17 cells (Figures 4B and 4C). Similarly, introduction of Jackson microbiota into GF animals did not induce Th17 cells, unless the microbiota were supplemented with SFB (Figures 4D and 4E). Th17 cell induction by SFB was also demonstrated by the expression of a number of Th17 cell effector cytokine messenger RNAs (mRNAs), including those for IL-17 and IL-21 (Figure 4F). We therefore conclude that SFB are members of the commensal microbiota that specifically induce the accumulation of Th17 cells in the SI LP.

### SFB Induce an Immune Response Program in the Gut

To identify specific effects of SFB, we compared the gene expression profiles in the terminal ileum of Swiss-Webster GF mice before and after colonization with SFB and in Jackson B6 mice before and after cohousing with Taconic B6 animals. Colonization of GF mice with SFB induced at least a 2-fold change in
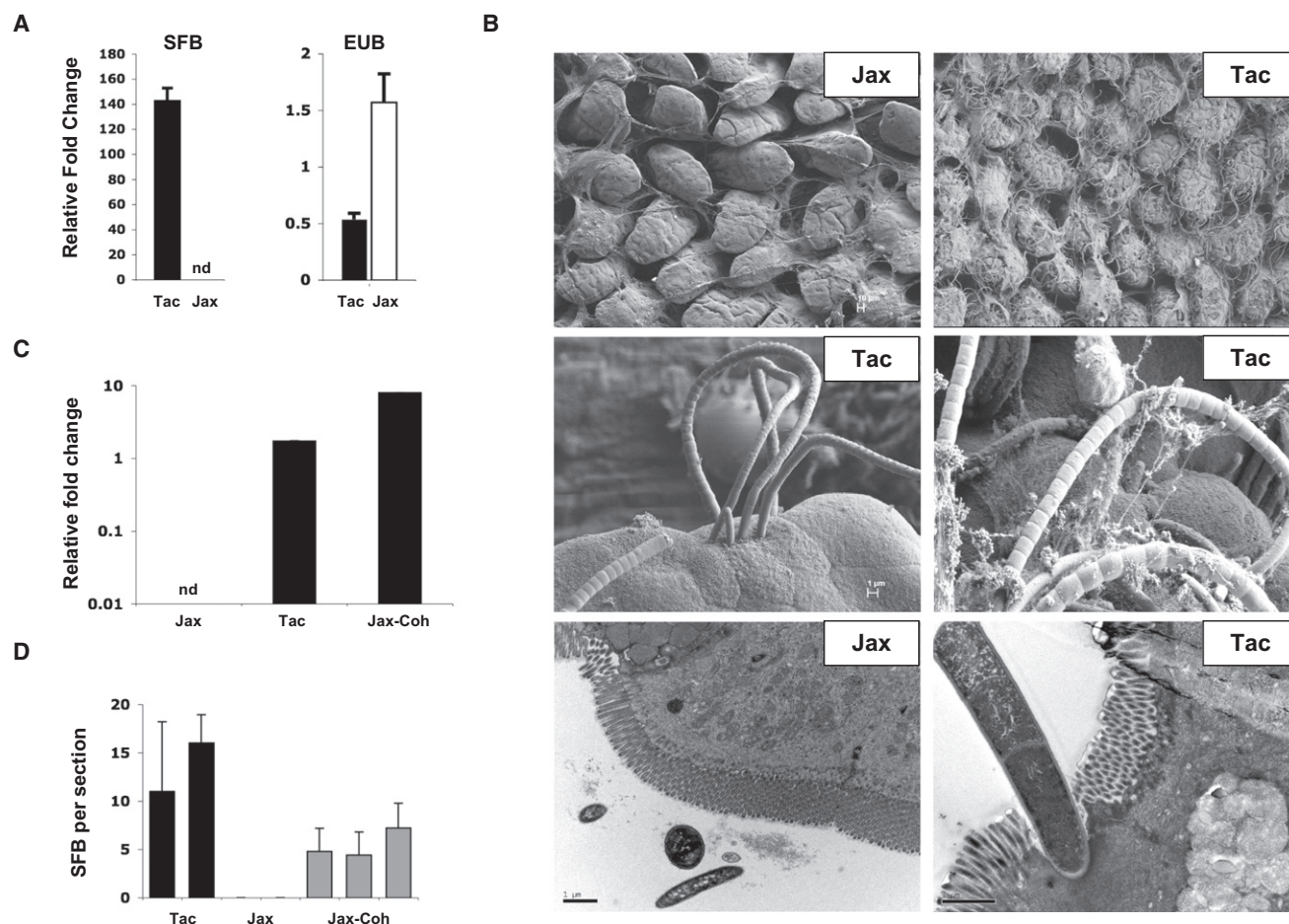
**Figure 2. Segmented Filamentous Bacteria in the Intestinal Tract of Th17 Cell-Sufficient and Th17 Cell-Deficient Mice**

(A) Quantitative PCR (qPCR) analysis of segmented filamentous bacteria (SFB) and total bacterial (EUB) 16S rRNA genes in mouse feces from Taconic (Tac) and Jackson (Jax) B6 mice. Genomic DNA was isolated from combined fecal pellets from four animals from each strain. The experiment was repeated numerous times with similar results. Error bars represent the standard deviation (SD).

(B) Scanning (SEM) and transmission (TEM) electron microscopy of terminal ileum of 8-week-old Jackson (Jax) and Taconic (Tac) C57BL/6 mice housed under similar conditions and diet for at least 1 week. Note the presence of long filamentous bacteria with SFB morphology in Taconic, but not Jackson, mice.

(C) qPCR analysis for SFB presence in Jackson B6 mice after 14 days of cohousing with Taconic B6 mice (Jax-Coh). Genomic DNA was isolated from pooled feces from three to four mice per group. Error bars represent the SD.

(D) SFB colonization of terminal ileum of Jackson B6 mice after 14 days of cohousing with Taconic B6 mice (Jax-Coh). Toluidine-blue sections were prepared from 0.5 cm piece of the terminal ileum as described in the Experimental Procedures and examined by light microscopy. Adherent bacteria with SFB morphology were counted in four to five sections from each sample. Each column represents a separate animal. Error bars represent the SD.

expression of 253 genes, while cohousing of Jackson B6 mice with Taconic B6 mice induced a similar change in 470 genes (Figure 5A). More importantly, there was a high degree of overlap between the two groups, with expression of 131 genes affected by both treatments. We could therefore distinguish three groups of genetic profiles. Group 1 includes genes whose expression was affected only in Jackson mice by cohousing, but was not statistically different after SFB colonization. This group most likely includes genes whose expression is influenced by microbiota other than SFB that differs between the mice from the different vendors, as well as strain-specific changes. Group 2 consists of genes whose expression only changed in GF mice upon colonization with SFB, but not in Jackson B6 mice

following cohousing. A subset of these genes is expected to reflect changes induced in GF animals upon general intestinal colonization with bacteria. Group 3 includes the genes with expression differences after both SFB colonization and cohousing with Taconic mice (Figure 5A) and thus contains genes specifically induced by SFB and associated with Th17 cell induction.

SFB exerted an inductive effect in the host, which was demonstrated by the finding that most (>70%) of the genes in group 3 were upregulated after SFB colonization (Figure 5B). By comparison, most genes in group 1 (>70%) were downregulated, which suggests that the rest of the Taconic microbiota has a suppressive effect that may possibly restrain the inductive effect of SFB
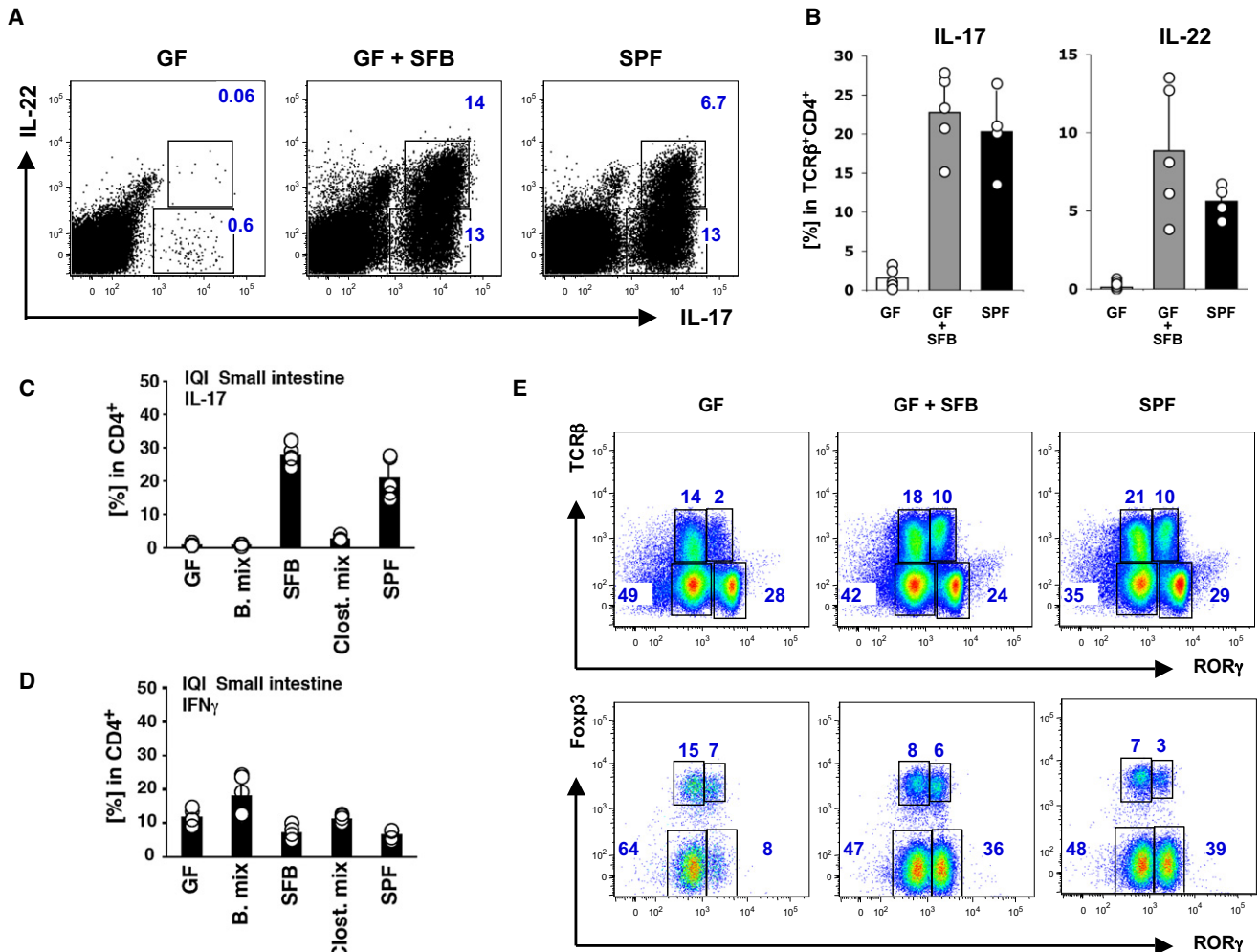
**Figure 3. SFB Specifically Induce Th17 Cell Differentiation in Germ-Free Mice**

(A and B) Six-week old Swiss-Webster (SW) germ-free (GF) mice were colonized with SFB (GF+SFB) as described in the Experimental Procedures, and small intestinal lamina propria lymphocytes (SI LPL) were isolated 10 days later. Representative plots in (A) and combined data in (B) of IL-17 and IL-22 expression in $TCR\beta^+CD4^+$ LPL. Data are from one of three separate experiments with similar results. SPF, mice raised under conventional specific pathogen-free conditions. Each circle in (B) represents a separate animal. Error bars represent the SD.

(C and D) IL-17 (C) and $IFN\gamma$ (D) expression in $TCR\beta^+CD4^+$ SI LPL from mice colonized with different commensal bacteria. IQI germ-free (GF) mice were colonized with 16 strains of Bacteroidaceae (B. mix), SFB, 46 strains of Clostridium sp mixture (Clost. mix), or microbiota from conventionally raised mice (SPF). Intracellular cytokine production in SI LP CD4 T cells was analyzed 3 weeks later by flow cytometry. Circles represent separate animals. Error bars represent the SD.

(E) SFB colonization induces $ROR\gamma t$ expression only in $CD4^+$ T cells. $ROR\gamma t$ expression in total SI LPL (top panels) and $ROR\gamma t$ and Foxp3 expression in $TCR\beta^+CD4^+$ SI LPL (bottom panels) in GF mice, GF mice colonized with SFB (GF+SFB), and conventionally raised mice (SPF).

(Figure 5B). Group 2, on the other hand, consisted of roughly equal numbers of upregulated and downregulated genes.

To evaluate changes specifically associated with Th17 cell-inducing SFB, we next concentrated on the genes in group 3. A list of the top upregulated genes is presented in Figure 5C. A GO biological pathway analysis of upregulated genes in group 3 showed that immune system pathways were among the programs most significantly induced by SFB (Figure 5D) and raised the possibility that at least some of the observed gene expression changes were mediated by Th17 cells or their effector cytokines. Because IL-17 and IL-22 have been associated with induction of antimicrobial peptides (AMPs) (Curtis

and Way, 2009; Kolls et al., 2008; Zheng et al., 2008), we compared the induction of AMP-related genes in our arrays. Multiple AMP genes were induced specifically by colonization with SFB, consistent with an upregulated Th17 cell response (Figure 5E). Upregulation of Th17 cell-associated genes (*Il17*, *Il21*, *Ccr6*, *Nos2*) and AMPs (*Reg3g*) after SFB colonization was confirmed by quantitative RT-PCR (Figures 4F and 5F).

**Serum Amyloid A Is Induced by SFB Colonization and Influences Th17 Cell Differentiation**

The top upregulated transcript upon SFB colonization of GF mice encoded an isoform of SAA—*Saa1*, a member of the family
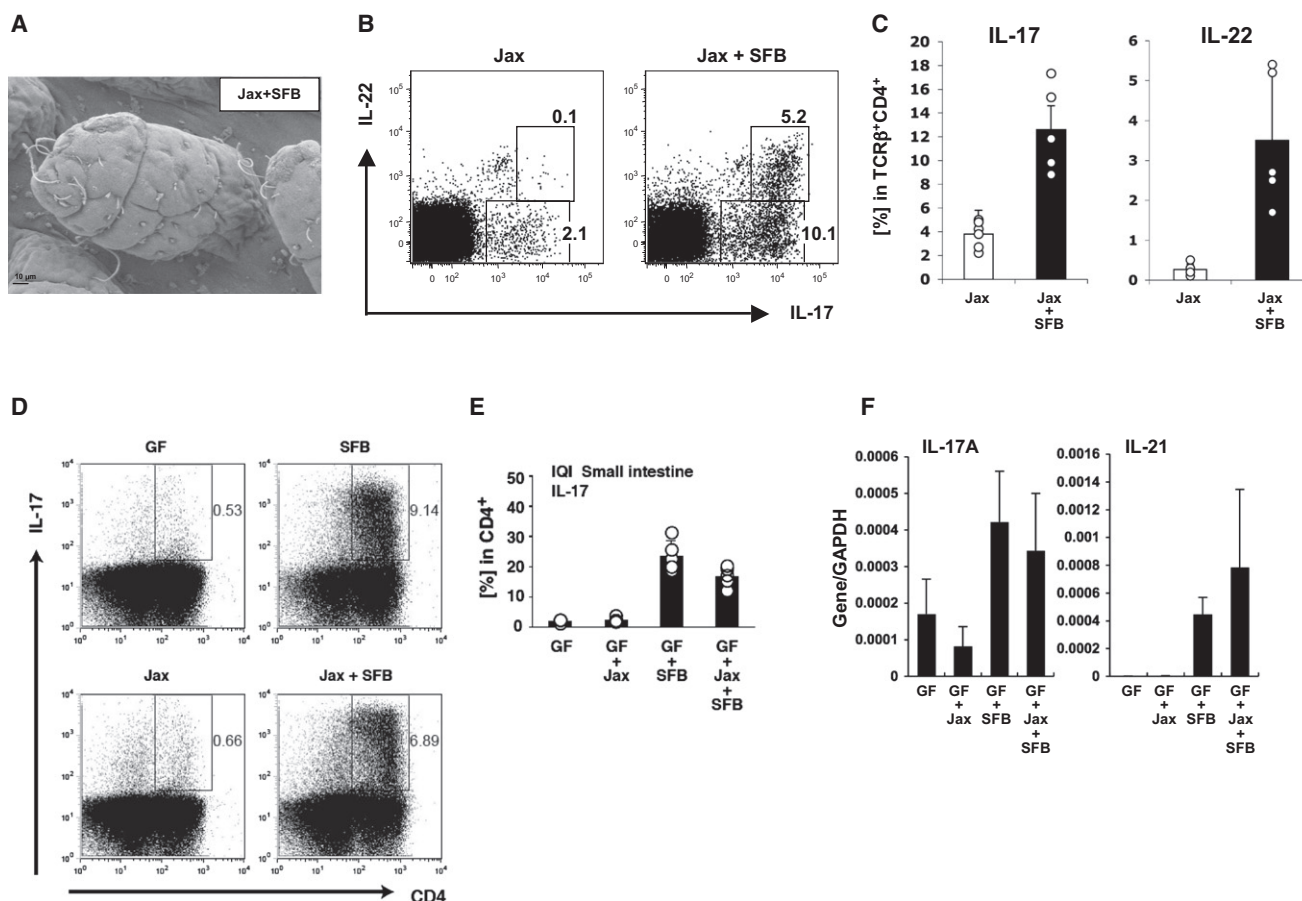
**Figure 4. SFB Induce Th17 Cell Differentiation upon Colonization of Jackson C57BL/6 Mice**

(A) Colonization of the terminal ileum by SFB 10 days after transfer of fecal homogenates from SFB-mono mice into Jackson B6 mice.

(B and C) IL-17 and IL-22 expression in TCRβ$^+$CD4$^+$ SI LPL in Jackson B6 mice colonized with SFB (Jax+SFB) compared to controls (Jax). Data from one of two experiments. Error bars represent the SD.

(D and E) Jackson microbiota induces Th17 cells only when complemented with SFB. Germ-free IQI mice were colonized with SFB, Jackson microbiota isolated from fecal pellets by itself (Jax), or a mixture of both (Jax + SFB). Th17 cell proportions were analyzed in the LP 3 weeks later by flow cytometry. Plots in (D) are gated on total lymphocytes. Data in (E) represent the percentage of IL-17$^+$ cells in the CD4$^+$ gate. Error bars represent the SD.

(F) RT-PCR for Th17 cell effector cytokines in total RNA from terminal ileum of the mice in (E). Error bars represent the SD.

of acute-phase response proteins induced during infection, tissue damage, or inflammatory disease (Uhlar and Whitehead, 1999). This transcript was also upregulated upon cohousing of Jackson B6 mice with Taconic B6 animals (Figure 5C). Transcripts for the other SAA isoforms, *Saa2* and *Saa3*, were also among the most highly upregulated genes upon colonization with SFB or cohousing (Figure 5C).

Real-time PCR confirmed that all three SAA isoforms were induced in the terminal ileum of GF mice upon colonization with SFB or SFB plus Jackson microbiota, but not by Jackson microbiota alone (Figure 6A). Recent studies have demonstrated that SAA may act as a cytokine that induces IL-8, TNFα, and IL-1β in neutrophils and IL-23 in monocytes (Furlaneto and Campa, 2000; He et al., 2006). We therefore investigated the effect of SAA on Th17 cell differentiation in vitro. Addition of recombinant SAA to cocultures of naive CD4$^+$ T cells and LP DCs induced a Th17 cell differentiation program in a concentration-dependent manner, including Th17 cell effector cytokines

and RORγt (Figure 6B). In addition, SAA induced production of IL-17 in CD4$^+$ splenic OT-II T cells cocultured with LP DCs in vitro (Figure S9). Addition of SAA to cultures containing only T cells, without DCs, did not induce Th17 cell cytokines (Figure 6B), and SAA induced production of IL-6 and IL-23 by LP DCs in vitro (Figure S10). We conclude that SFB colonization results in the production of SAA, which in turn acts on gut DCs to stimulate a Th17 cell-inducing environment.

## SFB Colonization Reduces Growth of an Intestinal Pathogen

We next examined the effect of Th17 cell-inducing microbiota and SFB on oral infection with *Citrobacter rodentium*, an intestinal pathogen whose clearance by the host requires an immune response dependent on IL-23, IL-22, and RegIIIγ (Mangan et al., 2006; Torchinsky et al., 2009; Zheng et al., 2008). Jackson B6 mice that had been cohoused with Taconic B6 mice and hence were colonized with SFB were significantly more resistant to
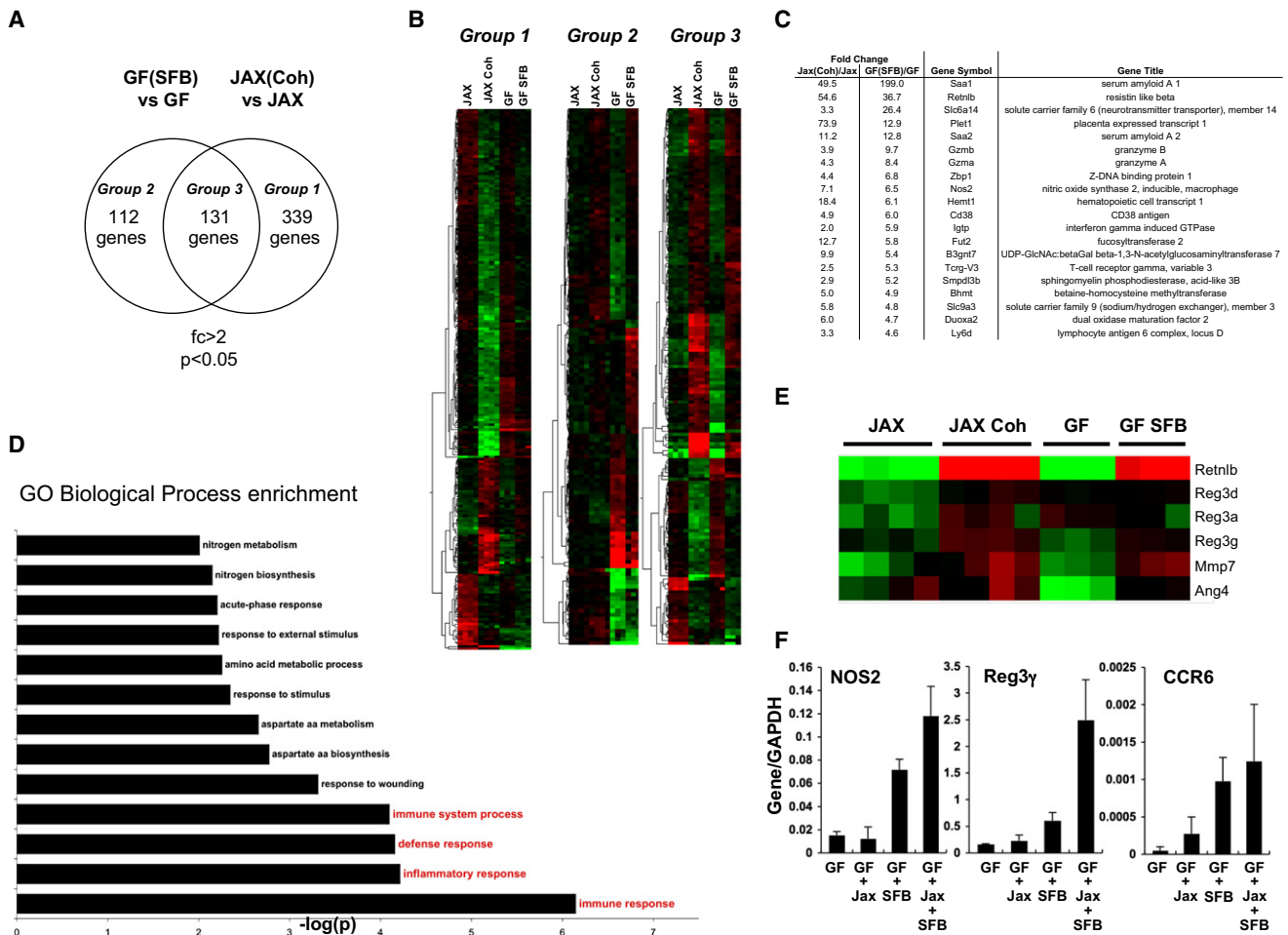
**A**

GF(SFB)    JAX(Coh)
vs GF       vs JAX

*Group 2* | *Group 3* | *Group 1*
112 genes | 131 genes | 339 genes

fc>2
p<0.05

**B**

*Group 1*    *Group 2*    *Group 3*



**C**

| Fold Change | | Gene Symbol | Gene Title |
|---|---|---|---|
| Jax(Coh)/Jax | GF(SFB)/GF | | |
| 49.5 | 199.0 | Saa1 | serum amyloid A 1 |
| 54.6 | 36.7 | Retnlb | resistin like beta |
| 3.3 | 26.4 | Slc6a14 | solute carrier family 6 (neurotransmitter transporter), member 14 |
| 73.9 | 12.9 | Plet1 | placenta expressed transcript 1 |
| 11.2 | 12.8 | Saa2 | serum amyloid A 2 |
| 3.9 | 9.7 | Gzmb | granzyme B |
| 4.3 | 8.4 | Gzma | granzyme A |
| 4.4 | 6.8 | Zbp1 | Z-DNA binding protein 1 |
| 7.1 | 6.5 | Nos2 | nitric oxide synthase 2, inducible, macrophage |
| 18.4 | 6.1 | Hemt1 | hematopoietic cell transcript 1 |
| 4.9 | 6.0 | Cd38 | CD38 antigen |
| 2.0 | 5.9 | Igtp | interferon gamma induced GTPase |
| 12.7 | 5.8 | Fut2 | fucosyltransferase 2 |
| 9.9 | 5.4 | B3gnt7 | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7 |
| 2.5 | 5.3 | Tcrg-V3 | T-cell receptor gamma, variable 3 |
| 2.9 | 5.2 | Smpdl3b | sphingomyelin phosphodiesterase, acid-like 3B |
| 5.0 | 4.9 | Bhmt | betaine-homocysteine methyltransferase |
| 5.8 | 4.8 | Slc9a3 | solute carrier family 9 (sodium/hydrogen exchanger), member 3 |
| 6.0 | 4.7 | Duoxa2 | dual oxidase maturation factor 2 |
| 3.3 | 4.6 | Ly6d | lymphocyte antigen 6 complex, locus D |

**E**

JAX    JAX Coh    GF    GF SFB



Retnlb
Reg3d
Reg3a
Reg3g
Mmp7
Ang4

**D**

GO Biological Process enrichment



nitrogen metabolism
nitrogen biosynthesis
acute-phase response
response to external stimulus
amino acid metabolic process
response to stimulus
aspartate aa metabolism
aspartate aa biosynthesis
response to wounding
immune system process
defense response
inflammatory response
immune response

-log(p)

**F**



NOS2    Reg3γ    CCR6

Gene/GAPDH

GF | GF+Jax | GF+SFB | GF+Jax+SFB

**Figure 5. Transcriptional Programs Induced by SFB Colonization**

(A) Venn diagrams showing the overlap between genes affected by either SFB colonization of SW GF mice only (group 2), introduction of Taconic microbiota into Jackson B6 mice by cohousing (group 1), or both (group 3). Total RNA was prepared from terminal ileum of the corresponding mice after 10 days of colonization and Affymetrix gene chip analysis was performed as described in the Experimental Procedures.

(B) Heat-map analysis of the three groups in (A). Each line represents a single Affymetrix probe and each column a single mouse. Green, probes that were at least 2-fold downregulated; red, probes that were at least 2-fold upregulated.

(C) Top upregulated genes in group 3 in (A) arranged by fold change in GF+SFB mice.

(D) Biological processes specifically induced by SFB (genes in group 3 in A). Gene ontology analysis was performed as described in the Experimental Procedures.

(E) Changes in antimicrobial peptide related genes upon SFB colonization and Th17 cell induction by cohousing. Each column represents an individual mouse.

(F) RT-PCR analysis of selected genes, induced by SFB colonization. IQI GF mice (GF) were colonized with fecal homogenates from SFB-mono mice (SFB), Jackson B6 mice (Jackson), or a mixture of both (Jackson+SFB). Total RNA from terminal ileum was prepared 3 weeks later, and RT-PCR was performed as described in the Experimental Procedures. Error bars represent the SD.

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growth of *C. rodentium* than were noncohoused mice, as demonstrated by recovery of infectious units from the wall of the colon (Figure 7A).

To assess specifically the ability of SFB to provide protection, we colonized GF IQI mice with Jackson microbiota with or without SFB for 14 days. The mice were then infected orally with *C. rodentium* and pathogen colonization and disease were assessed at day 8 after infection. Although some infection and disease were observed in both experimental groups, the presence of SFB in the gut prevented infiltration of the pathogen into the colonic wall (Figure 7B). In addition, SFB colonization ameliorated colonic inflammation as demonstrated by reduced

epithelial hyperplasia and colon shortening in its presence (Figures 7C, 7D, and S11, and data not shown). We thus conclude that the presence of SFB as a component of the commensal microbiota increases mucosal protection to infection with *C. rodentium*.

**DISCUSSION**

**Commensal Intestinal Bacteria and Regulation of Th17 Cell Differentiation**

Commensal intestinal bacteria influence multiple metabolic and physiological functions of the host (Backhed et al., 2005;
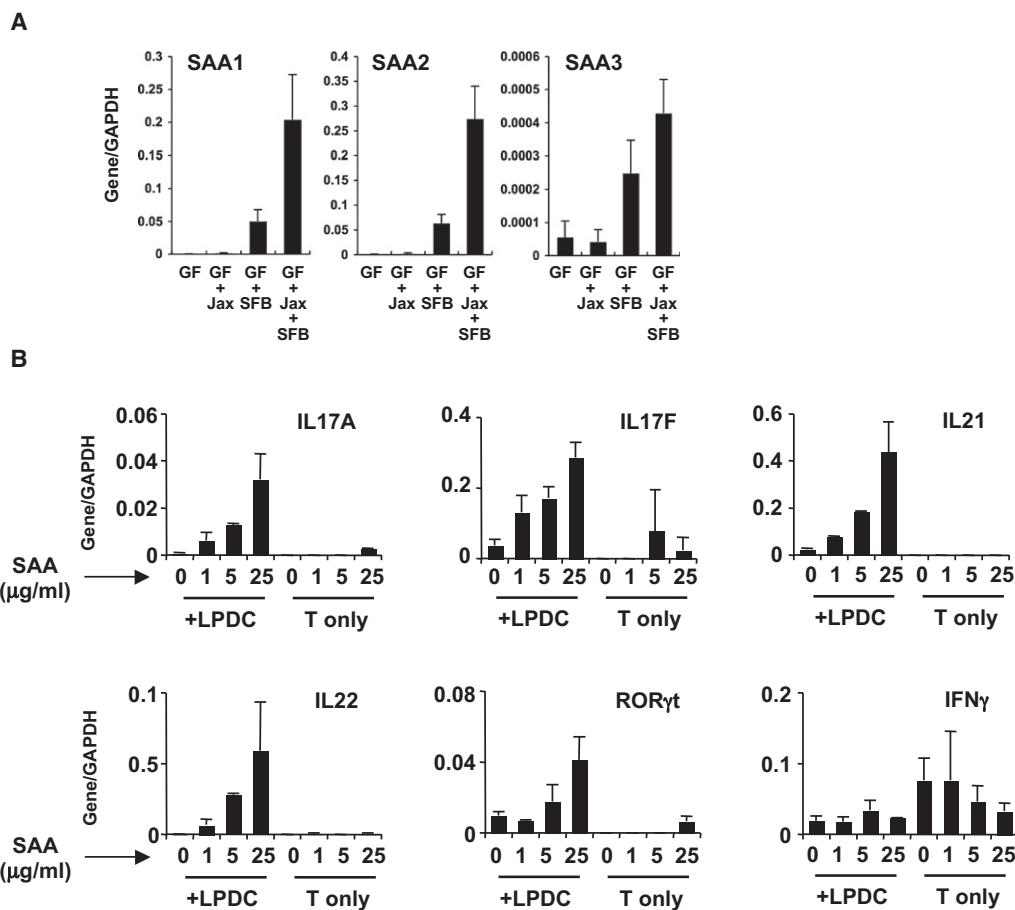
**Figure 6. SFB Colonization Induces SAA Expression that Influences Th17 Differentiation**

(A) Relative mRNA expression levels of SAA1-3 genes by real-time RT-PCR in the terminal ileum of IQI GF mice (GF) colonized with fecal homogenates from SFB-mono mice (SFB), Jackson B6 mice (Jackson), or a mixture of both (Jackson+SFB).

(B) Splenic naive CD4+ T cells were cocultured with or without LP CD11c+ cells in the presence of an anti-CD3 antibody with the indicated concentration of recombinant Apo-SAA for 4 days. T cells were collected and restimulated with PMA and ionomycin, and real-time RT-PCR was performed. Results were normalized to expression of GAPDH mRNA. The data are representative of four independent experiments with similar results.

Error bars represent the SD.

Turnbaugh et al., 2006), but they also have profound effects on the host immune system (Cash et al., 2006; Macpherson and Harris, 2004). For example, most rodent colitis models are dependent on the presence of microbiota (Elson et al., 2005; Sartor, 2008), whose products can also influence systemic immune responses (Mazmanian et al., 2005; Turnbaugh et al., 2006). The effects of intestinal bacteria on the immune system are considered to be the result of stimulation of innate immune "pattern recognition receptors," but we are limited in our understanding of how individual bacteria influence the type and location of immune responses in gnotobiotic models or in the presence of other commensal microorganisms (Kim et al., 2005; Macpherson and Harris, 2004; Umesaki et al., 1999). A notable exception was the recent demonstration that a polysaccharide product from the commensal bacterium *Bacteroides fragilis* can specifically induce systemic Th1 and mucosal regulatory T cell responses and protect mice from pathogen-induced colitis (Mazmanian et al., 2005, 2008).

We recently discovered that the homeostasis of effector helper T cell populations in the gut is dependent on the composition of intestinal bacteria (Ivanov et al., 2008). Th17 cell induction was not controlled simply by the presence of high numbers of diverse bacterial species that activate major bacterial pattern-recognition pathways. Thus, colonization of mice with several defined bacterial species as well as with diverse microbiota from Jackson Laboratory B6 mice did not induce Th17 cell differentiation, in sharp contrast to the induction observed with bacteria from Th17 cell-sufficient Taconic Farms B6 mice (Ivanov et al., 2008). The identity of microorganisms that induce Th17 cells and the signaling mechanisms involved had remained important unresolved issues.

In this study, we have identified SFB as the first commensal bacterium that can induce accumulation in the gut of CD4+ T cells with a defined effector function. Colonization with a number of other species, including members of the SFB-related *Clostridiaceae* family, failed to induce Th17 cells. Our results are
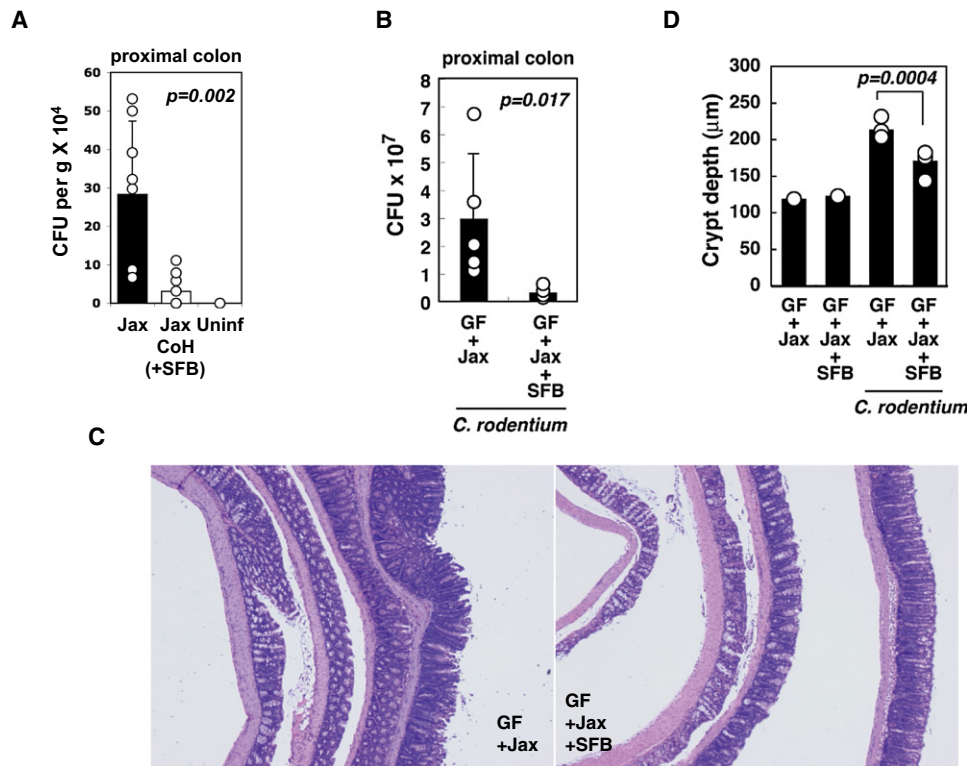
**Figure 7. SFB Colonization Confers Protection from Infection by *Citrobacter rodentium***

(A) Jackson B6 mice (Jax) were cohoused with Taconic B6 mice (Jax CoH) for 10 days to induce colonization with SFB and Th17 cells. Both groups were infected with ∼1 × 10$^9$ CFU *C. rodentium*/mouse, and pathogen colonization of the colon was examined at day 8 of infection (n = 9/group). Uninf, uninfected controls. (B–D) IQI GF mice colonized with Jackson microbiota (Jax) or Jax+SFB were orally infected with 2 × 10$^9$ CFU *C. rodentium*, and colons were harvested at day 8 of infection (n = 5/group). *C. rodentium* CFUs in proximal colon (B), histopathology (C), and crypt length (D) in the distal colon (H&E) are shown. Data represent means ± SD, and circles represent separate animals. Similar effects of SFB on *C. rodentium* colonization were observed in a separate experiment with C.B17 mice colonized with ASF with and without SFB.

most consistent with a mechanism that requires unique features of a specific commensal species to trigger Th17 cell differentiation and/or accumulation in the lamina propria. Pathways activated by common bacterial patterns and shared by large classes of bacteria appear to be dispensable or redundant, as both MyD88/TRIF double-deficient animals and RIP-2 mutant mice still possessed mucosal Th17 cells (Atarashi et al., 2008; Ivanov et al., 2008) (Figure S12). We previously reported that adenosine 5′-triphosphate (ATP) derived from commensal bacteria led to the differentiation of Th17 cells in the colonic LP (Atarashi et al., 2008). However, the ATP concentrations in the ileal and colonic luminal contents of SFB-mono mice were lower than those in mice gavaged with SPF feces (data not shown). Thus, SFB-mediated Th17 cell differentiation is likely to occur through a mechanism independent of TLR, NOD, and ATP signaling.

SFB associate closely with epithelial cells in the terminal ileum. This interaction was reflected in the host genes induced after SFB colonization. Multiple epithelial cell-specific genes, as well as inflammatory response host genes, were upregulated by the bacteria. Among these were the three inducible or "acute-phase" isoforms of SAA (A-SAA). SAA is highly induced during both acute and chronic inflammation. A-SAA expression is induced in hepatocytes in the liver and in macrophages and

other cells in extrahepatic sites, including the intestine, by bacterial products and inflammatory cytokines, such as IL-6 and IL-1β (Uhlar and Whitehead, 1999). In addition to its role in the acute-phase response, SAA can induce IL-23 production by monocytes at concentrations that are orders of magnitude lower than the peak plasma concentration during an acute-phase response (He et al., 2006). In accordance with this, SAA induced transient production of IL-23 by LP DCs in vitro. We further demonstrated that SAA can act on LP DCs in vitro to induce Th17 cell differentiation, suggesting that an acute phase inflammation-like response, including induction of A-SAAs, is responsible for the SFB-mediated accumulation of Th17 cells in the intestine. Although the signaling pathways induced by A-SAA are currently unknown, it most likely acts on DCs and contributes to the establishment of a Th17 cell-inducing cytokine environment.

## SFB and Th17 Cell-Mediated Protection from Pathogenic Microorganisms

The identification of SFB as Th17 cell inducers in the intestine may have important implications for a better understanding of how components of the commensal microbiota contribute to host protection from microbial pathogens. It is well known that treatment with broad-spectrum antibiotics can result in

outgrowth of intestinal pathogens, such as vancomycin-resistant *Enterococcus* (VRE) or *Clostridium difficile*, resulting in severe colitis. SFB colonization has been suggested to reduce replication in rabbits of enteropathogenic *Escherichia coli* (EPEC) and in rats of *Salmonella enteritidis* (Garland et al., 1982; Heczko et al., 2000). In mice, Th17 cell effector cytokines, such as IL-17 and IL-22, as well as IL-23, which is required for Th17 cell function, have been proposed to play protective roles in infections with *Salmonella* and *Citrobacter rodentium* (Curtis and Way, 2009). We found that colonization with SFB reduced the capacity of orally inoculated *C. rodentium* to grow and/or invade colonic tissue. Although we cannot at this point formally demonstrate that this protection is a direct result of Th17 cell induction, our data, taken together with results of recent studies (Kolls et al., 2008; Zheng et al., 2008), strongly suggest that SFB-induced Th17 cytokines, particularly IL-22, limit the growth of *C. rodentium*, at least in part through production of AMP's such as RegIIIγ. While IL-22, IL-23, and RegIIIγ are required for host survival after *C. rodentium* infection (Mangan et al., 2006; Zheng et al., 2008), mice lacking SFB and Th17 cells survive despite increased bacterial growth. This may be because intestinal γδ T cells, CD4+CD3− lymphoid tissue inducer (LTi)-like cells, and NK22 cells that also produce Th17 cytokines are present even in the absence of SFB and other microbiota. Contribution of these cells to SFB-independent antimicrobial defense may hence protect the host from lethal outgrowth of the pathogenic bacteria.

Our results are also consistent with the report that a vancomycin-sensitive component of the commensal microbiota induces RegIIIγ in the mouse small intestine, thus reducing colonization by VRE and enhancing killing of the pathogen (Brandl et al., 2008). Future studies will be required to determine whether vancomycin-sensitive SFB enhance mucosal protection from pathogenic VRE and other bacteria through the upregulation of Th17 cells and antimicrobial peptides. Such studies will further test the hypothesis that specific commensal microbiota, by regulating the host immune system rather than by direct microbial competition, enhance protection from potentially harmful microbes.

### Do SFB Influence Th17 Cell-Mediated Inflammatory Disease?

Th17 cells are recognized to have significant roles in multiple mouse models of autoimmune disease, and there is accumulating evidence that they likewise contribute to human autoimmune disease pathogenesis (Hue et al., 2006; Langrish et al., 2005; Murphy et al., 2003; Yen et al., 2006). Mice with almost complete loss of Th17 cells due to the absence of RORγt are resistant to experimental autoimmune encephalomyelitis and colitis (Ivanov et al., 2006; Leppkes et al., 2009). In humans, polymorphisms in the gene encoding the IL-23 receptor are associated with both increased resistance and susceptibility to Crohn's disease and inhibition of the Th17 cell differentiation pathway has been reported to be an effective therapy for psoriasis (Duerr et al., 2006; Krueger et al., 2007).

Although Th17 cells are involved in multiple organ-specific inflammatory diseases, they are not normally present in such organs, and they are relatively scarce in secondary lymphoid tissues. However, Th17 cells are abundant in the intestinal lamina propria, and, as described in this study, their differentiation within and/or migration to this lymphoid-rich site is dependent on commensal microbes with specialized properties. There is evidence that the course of certain autoimmune diseases in humans and in animal models can be altered by treatment with antibiotics and probiotics and by restricting the complexity of the microbiota (O'Dell et al., 2006; Sartor, 2008). Indeed, in rodents, differential arthritogenic potential of different commensal microbiota components and dependence of spontaneous arthritis models on "cleanliness" of housing conditions have been reported (Severijnen et al., 1989; Simelyte et al., 2003). Moreover, K/BxN mice that have a genetic predisposition to spontaneous arthritis (Monach et al., 2008) fail to develop disease when kept in GF conditions, but do progress to arthritis when colonized with SFB (H.J. Wu, I.I.I., D.R.L., C. Benoist, and D. Mathis, unpublished data). Our results thus raise the possibility that manipulation of the number of SFB that colonize the terminal ileum may alter the course of Th17 cell-associated autoimmune diseases.

If Th17 cells involved in organ-specific autoimmunity originate in the gut, then the question arises as to what is the antigenic specificity of such cells. It is not yet known if Th17 cells in the lamina propria are specific for intestinal microbiota. If they are mostly reactive with microbial products, then it may be surprising that similar numbers of Th17 cells are observed in mice monocolonized with SFB and in mice with a broad distribution of microbiota. Th17 cells specific for bacterial products may constitute a sufficiently broad repertoire to provide subsets that are cross-reactive with self-antigen. Alternatively, intestinal Th17 cells may be broadly specific for self-antigen, rather than bacterial products, but may normally be kept in check by mechanisms of peripheral tolerance. Signals from bacteria such as SFB may provide an adjuvant effect that polarizes such self-reactive T helper cells toward the Th17 lineage without tissue damage under the immune suppressive environment in the gut. Further studies on the repertoire and antigen specificity of Th17 cells and on the role of SFB in autoimmune disease models will be necessary to resolve these issues.

SFB represent the first example of a specific component of the commensal microbiota that induces a particular helper T cell population in the lamina propria. The elucidation of additional commensal bacteria involved in this or other immune pathways and of the mechanisms employed will undoubtedly lead to further understanding of the complex host-commensal interactions that shape our immunity and will allow for tailored therapeutic manipulation of these processes.

### EXPERIMENTAL PROCEDURES

#### Mice and Bacterial Strains

B6 mice were obtained from Taconic Farms or Jackson Laboratory. Swiss-Webster germ-free and conventionally raised (SPF) mice were purchased from Taconic Farms. Germ-free IQI mice were purchased from Japan CLEA Inc. Mice monocolonized with SFB or 46 strains of Clostridia were developed previously (Itoh and Mitsuoka, 1985; Umesaki et al., 1995). For generation of *Bacteroides*-associated mice, 16 strains of *Bacteroides* (six strains of *B. vulgatus*, seven of *B. acidifaciens* group 1, and three of *B. acidifaciens* group 2), which were originally isolated from murine intestinal commensal bacteria (Miyamoto and Itoh, 2000), were cultured on Eggerth-Gangon agar (Nissui) in an anaerobic stainless steel jar and inoculated orally into germ-free IQI mice.

## PhyloChip Analysis

Six-week-old Jackson B6 and Taconic B6 mice were purchased from the corresponding vendor and housed for 3 weeks in separate microisolator cages at the NYUSOM animal facility to equilibrate housing conditions, including bedding and diet. Sample collection, processing, and PhyloChip analysis are described in detail in the Supplemental Data.

## 16S rRNA Gene Quantitative PCR Analysis

Bacterial genomic DNA was isolated from fecal pellets as described in the Supplemental Data. Quantitative PCR analysis was carried out as described in Barman et al. (2008). Primer sequences for SFB and bacterial 16S rRNA genes as well as PCR conditions were as described in Barman et al. (2008). For SFB, relative quantity was calculated by the $\Delta$Ct method and normalized by the presence of total bacteria (EUB primers), dilution, and weight of the sample and presented as relative fold change to an external sample. Typical Ct values for SFB were $\sim$20 cycles and for EUB $\sim$11 cycles. Samples that were negative after 40 cycles were considered "not detected" (n.d.).

## Gene Expression Analysis

RNA was prepared from terminal ileum as described (Ivanov et al., 2008). For microarray analysis, RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays according to the Affymetrix protocols. Data were analyzed in GeneSpring GX10. Significant genes were selected based on p values smaller than 0.05 and fold change greater than 2. For enrichment analysis of biological process ontology, probe lists were analyzed in DAVID (Dennis et al., 2003; Huang da et al., 2009) and processes were selected based on p values smaller than 0.01.

## Real-Time RT-PCR

Complementary DNAs (cDNAs) were synthesized from RNA samples prepared with a RNeasy Mini Kit (QIAGEN) with M-MLV Reverse Transcriptase (Promega). Real-time RT-PCR was performed with the ABI 7300 real-time PCR system. Serial dilutions of a standard were included for each gene to generate a standard curve and allow calculation of the input amount of cDNA for each gene. Values were then normalized by the amount of GAPDH in each sample. Primer sequences are reported in the Supplemental Data.

## Cohousing and Microbiota Reconstitution

Cohousing and microbiota reconstitutions were performed as described before (Ivanov et al., 2008). For inoculation of germ-free mice with SFB, fecal pellets were collected from SFB-mono mice using sterilized test tubes in the vinyl-isolator and were preserved frozen under dry ice until immediately before oral administration. SFB colonizations were performed by oral gavage with 300–400 $\mu$l of suspension obtained by homogenizing the fecal pellets from SFB-mono donor mice in water. Control mice were gavaged with water or homogenates prepared from their own feces.

## Cell Isolation and Flow Cytometry

Lamina propria lymphocyte (LPL) isolation and intracellular cytokine staining were performed as described before (Ivanov et al., 2008). Naive CD4$^+$ T cells were purified from spleens using a CD4$^+$CD62L$^+$ T cell isolation kit II (Miltenyi Biotec; purity 95%). Anti-mouse ROR$\gamma$ monoclonal antibody conjugated to PE was purchased from eBioscience.

## In Vitro T Cell Differentiation

Naive CD4$^+$ T cells were cultured in 24-well plates at $2 \times 10^5$ cells/well for 4 days with MACS-purified LP CD11c$^+$ cells ($1 \times 10^5$/ well) and 1 $\mu$g/ml anti-CD3 antibody (BD Biosciences) in the presence or absence of recombinant human Apo-SAA (Peprotech). The cultured cells were harvested and restimulated with PMA and ionomycin for 3 hr before analysis.

## Electron Microscopy

Electron microscopy (EM) was performed on 0.5–1 cm pieces from terminal ileum (immediately proximal to the ileal-cecal junction). Tissue processing for EM is described in the Supplemental Data. The analysis was performed with a Zeiss Supra 55 FESEM.

## C. rodentium Infection

IQI mice were inoculated with 200 $\mu$l of a bacterial suspension (1–2 $\times$ 10$^9$ CFU) by way of oral gavage. For the colony formation assays, proximal colons and mesenteric lymph nodes were harvested and homogenized, and serially diluted homogenates were plated on MacConkey agar (Difco). For histological analysis, distal colons were fixed with 4% paraformaldehyde and analyzed after hematoxylin and eosin staining. For assessment of crypt depth, only crypts visible along the entire length of the distal colon were analyzed (20–30 crypts/mouse).

## ACCESSION NUMBERS

The array results reported in this paper have been deposited in the GEO database with the accession number GSE18348.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 12 figures, and two tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01248-3.

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