ONLINE METHODS

Samples. We conducted a two-stage GWAS using independent case-control sets (**Supplementary Table 7**). Samples from individuals with ulcerative colitis were collected from Kyushu University and 25 affiliated hospitals: Kyushu University Hospital (n = 129), Fukuoka University Chikushi Hospital (n = 106), Fukuoka University Hospital (n = 85), Saiseikai Kumamoto Hospital (n = 11), Yamaguchi Red Cross Hospital (n = 20), Matsuyama Red Cross Hospital (n = 113), Fukuoka Red Cross Hospital (n = 16), Chihaya Hospital (n = 19), Kyushu Central Hospital (n = 6), Hakujuji Hospital (n = 12), Health Insurance Nogata Chuou Hospital (n = 19), Kokura Medical Center (n = 15), Japan Seamen's Relief Association Moji Hospital (n = 2), Shimonoseki City Central Hospital (n = 6), Kama Red Cross Hospital (n = 3), Sasebo Chuo Hospital (n = 45), Nippon Steel Yawata Memorial Hospital (n = 33), Kimura Hospital (n = 13), Saiseikai Yahata General Hospital (n = 12), Kyushu Dental College Hospital (n = 1), Hamanomachi Hospital (n = 48), Kamata Hospital (n = 1), Kamori Clinic (n = 3), Kaita Hospital (n = 2), Sakura Hospital (n = 1) and Osamura Clinic (n = 31). Consequently, 752 individuals with ulcerative colitis were recruited under the standardized diagnostic criteria and were randomly divided into two sets. We used 376 samples from individuals with ulcerative colitis for the first stage and 376 for the second stage. Age, sex and clinical parameters did not differ between these two sets (**Supplementary Table 8**). For the control subjects, we used 934 volunteers recruited at the Midosuji and other related Rotary Clubs for the first stage (mean age, 52 ± 15 years old). Controls for the second stage were selected from participants in the Hisayama health survey[30] conducted between 2002 and 2003, after excluding subjects with a history of ulcerative colitis (mean age, 57 ± 10 years). The age and sex of subjects, and the geographical location of the recruitment centers, are shown in **Supplementary Table 7** and **Supplementary Figure 3**, respectively.

For the replication study, 265 individuals with ulcerative colitis and 665 healthy volunteers were recruited at Tohoku University Hospital from May 1998 to January 2006 (replication set 1). Individuals with ulcerative colitis for replication set 2 were recruited at Sapporo Medical University Hospital and seven affiliated hospitals (n = 64), Sapporo Kosei General Hospital (n = 249) and Teine Keijinkai Hospital (n = 63). Healthy controls for replication set 2 (n = 376) were recruited at the Department of Public Health, Sapporo Medical University.

Diagnosis of ulcerative colitis in all subjects was made by expert gastroenterologists in accordance with clinical, radiological, endoscopic and histological features based on established uniform criteria[31]. Namely, diagnosis of ulcerative colitis requires (i) chronic or recurrent symptoms of bloody (and mucous) stool; (ii) macroscopic appearance by endoscopy or barium enema of continuous mucosal inflammation affecting the rectum in continuity with some or all of the colon; (iii) microscopic features on biopsy including the presence of widespread and diffuse mucosal distortion, diffuse transmucosal lymphocytic inflammation, cryptitis and crypt abscesses; and (iv) no suspicious findings of Crohn's disease, indeterminate colitis, infections, or other acute or chronic non-IBD conditions on small bowel radiograph, ileocolonoscopy, biopsy or stool examination. When endoscopic or histopathological findings of the individuals were divergent or inconclusive for diagnosis of ulcerative colitis or Crohn's disease, we regarded those subjects to have indeterminate colitis and excluded them from analysis. In terms of disease extent, proctitis was defined as inflammatory changes limited to the rectum. Left-sided colitis was defined as inflammatory changes up to the splenic flexure, and changes beyond the splenic flexure were defined as extensive colitis. Ulcerative proctitis is a milder ulcerative colitis subphenotype and is sometimes regarded as a separate phenotype because of its better prognosis. However, the clinical symptoms and histopathological findings of ulcerative proctitis are the same as those of other subphenotypes. In addition, the impact of the susceptibility loci identified in this study was not influenced by subphenotype. As a result, we included ulcerative proctitis in this study as a ulcerative colitis subphenotype.

The ethical committees of Kyushu University, Tohoku University, Sapporo Medical University and RIKEN approved this study, and written informed consent was obtained from all subjects.

Genotyping and data-quality filters. For the first-stage screening, 376 cases and 934 controls were genotyped by using an Illumina HumanHap550v3 Genotyping BeadChip. After excluding three cases with a call rate of < 0.98, we applied SNP quality control (call rate ≥ 0.99 in cases and controls, Hardy-Weinberg $P \geq 1 \times 10^{-6}$ in controls) and 513,923 autosomal SNPs that passed the quality filters were analyzed in the first stage. The genotype data of the 934 controls are available at the JSNP database. Of the SNPs analyzed in the first stage, we selected the top 12,000 SNPs with a minor allele frequency of ≥ 0.1 in either cases or controls after considering the statistical power required to detect associations for SNPs in the second stage. In the second stage, we genotyped an additional 376 ulcerative colitis cases and 1,128 controls by using an Affymetrix GeneChip Custom 10K array. After excluding 31 controls with a call rate of < 0.95, all cluster plots were checked by visual inspection by trained staff, and SNPs with an ambiguous call were excluded. As a result, 9,665 out of 10,635 SNPs assayed were analyzed in the second stage. We used a multiplex PCR-based Invader assay[32] for the replication study (including a replication study of previous GWAS) and fine mapping. Six cases and 15 controls were excluded from analysis owing to a low call rate in replication set 1. The concordance rate between genotypes determined by the Illumina HumanHap550v3 BeadChip and those determined by the Affymetrix GeneChip Custom 10K array among 94 duplicated samples was 0.9997. The concordance rate of 13 SNPs selected for the replication study was 0.999 between the Affymetrix GeneChip Custom 10K array and the multiplex PCR-based Invader assay. The reproducibility of the multiplex PCR-based Invader assay was 0.998 on the basis of duplicate assays of five SNPs among all samples analyzed.

Fine mapping and resequencing. We performed fine mapping for four ulcerative colitis–associated loci using all samples in the first and second sets. Haploview was used to select tag SNPs with a pairwise r^2 of > 0.90 and a minor allele frequency of ≥ 0.05 on the basis of HapMap JPT data. Resequencing of candidate regions was performed in 94 ulcerative colitis cases by using an ABI3730 Genetic Analyzer.

Estimation of gene copy numbers. To estimate gene copy number in the FCGR gene cluster, we conducted a quantitative real-time PCR assay (TaqMan assay, Applied Biosystems). We designed at least two probes that encompassed each gene by using Primer Express 1.5. These TaqMan probes were labeled with FAM dye at the 5′ end, linked by a nonfluorescence quencher, and tagged with MGB dye at the 3′ end. As a reference gene, we used the TaqMan Copy Number Reference Assay RNase P (Applied Biosystems). All TaqMan assays were performed by following reported protocols, and copy number calculation was conducted by the $\Delta\Delta Ct$ method[33]. We assumed that samples with a median ΔCt value were two-copy samples and used them as a calibrator. All samples were examined in quadruplicate, and the average copy number values were used in the scatter plot analysis. The primer and probe sequences of all assays are available from the authors on request. We calculated confidence in the predicted copy numbers, and samples with a prediction confidence of ≤ 0.95 were removed from further analyses (total call rate, 99.8%).

Statistics. For general statistical analysis, we used R statistical environment version 2.7.1 or PLINK. To draw the LD map, we used Haploview software. To select SNPs for the second stage, single-marker analysis in the first stage was carried out by allele frequency, dominant and recessive models using Fisher's exact test. The possible influence of population stratification was investigated by EIGENSTRAT software using all of the 442,793 autosomal SNPs located at the non-MHC region with a call rate of ≥ 0.99 and a minor allele frequency of ≥ 0.01. We also included genotypes obtained from four populations of the HapMap project for estimation of the population structure. For estimation of the population substructure, we included the genotypes of JPT and CHB of the Hapmap project only. To identify spurious associations resulting from more subtle stratification of the case and control populations, we also calculated the genomic control inflation measure[16] and plotted the observed distribution as compared with the expected distribution of P values (quantile-quantile plots). Single-marker analysis of each set was carried out by using the Cochran-Armitage trend test. P values for first and second set of the GWAS were corrected by the method of genomic control. Combined analysis of the data from different case-control sets was conducted by the inverse variance method using λ_{GC}-corrected P values in the first and second

set. Heterogeneities across the population were estimated formally by using Cochran's *Q* test. To test the association between ulcerative colitis and the copy number of genes in the FCGR locus, logistic regression analysis was used in all subjects for whom complete genotyping and copy number were available.

URLs. JSNP, http://snp.ims.u-tokyo.ac.jp/; R statistical environment, http://www.cran.r-project.org/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/.

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Cell

Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria

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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 (DeSantis 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 ($p \leq 0.05$) 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

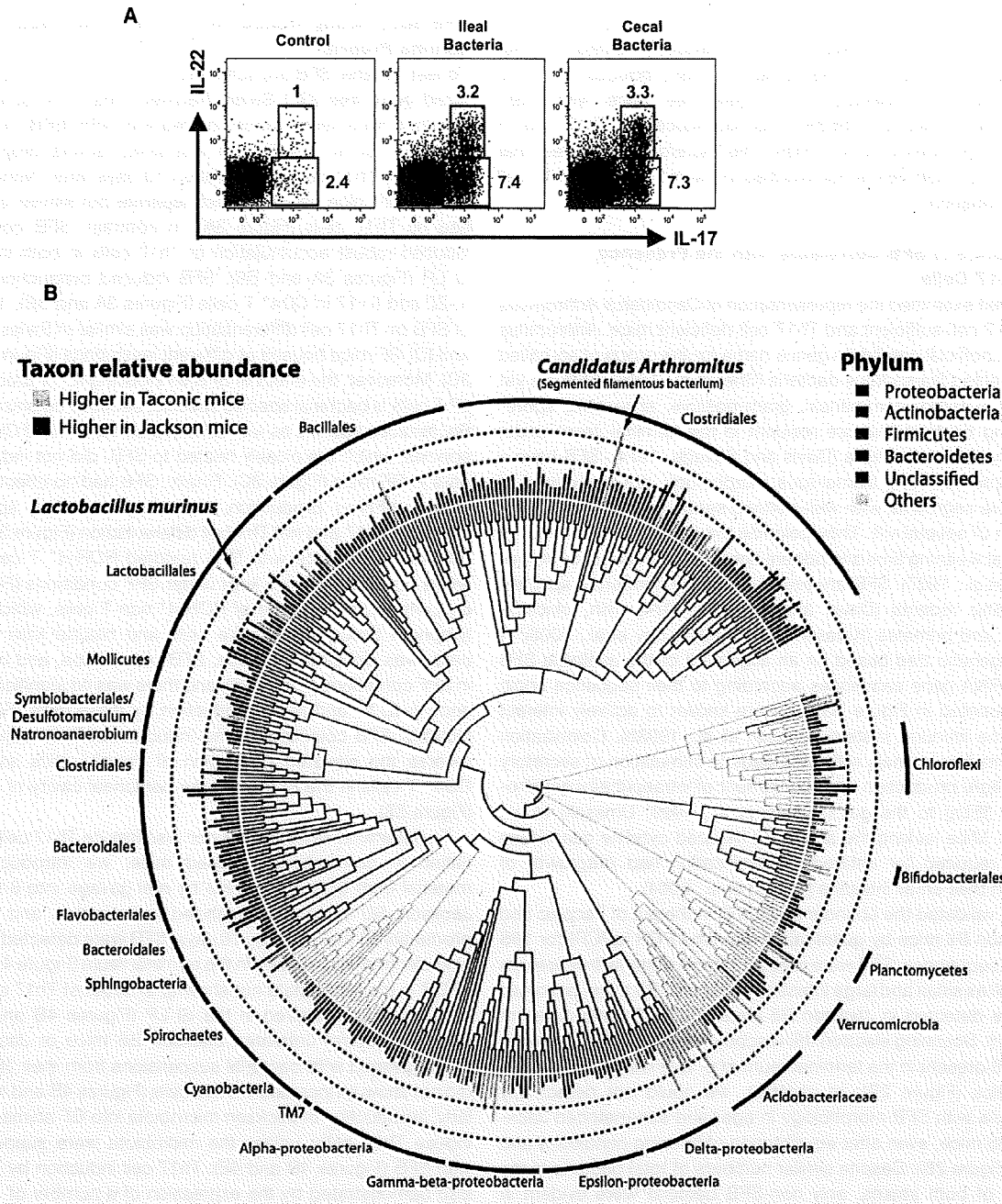

Figure 1. Comparative Analysis of the Microbiota in the Terminal Ileum of C57BL/6 Mice from Jackson Laboratory versus Taconic Farms

(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 $TCR\beta^{+}CD4^{+}$ 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 $ROR\gamma t^+$ T cells to the levels observed in mice kept under SPF conditions (Figure 3E). By contrast, the number of $ROR\gamma t^+$ 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

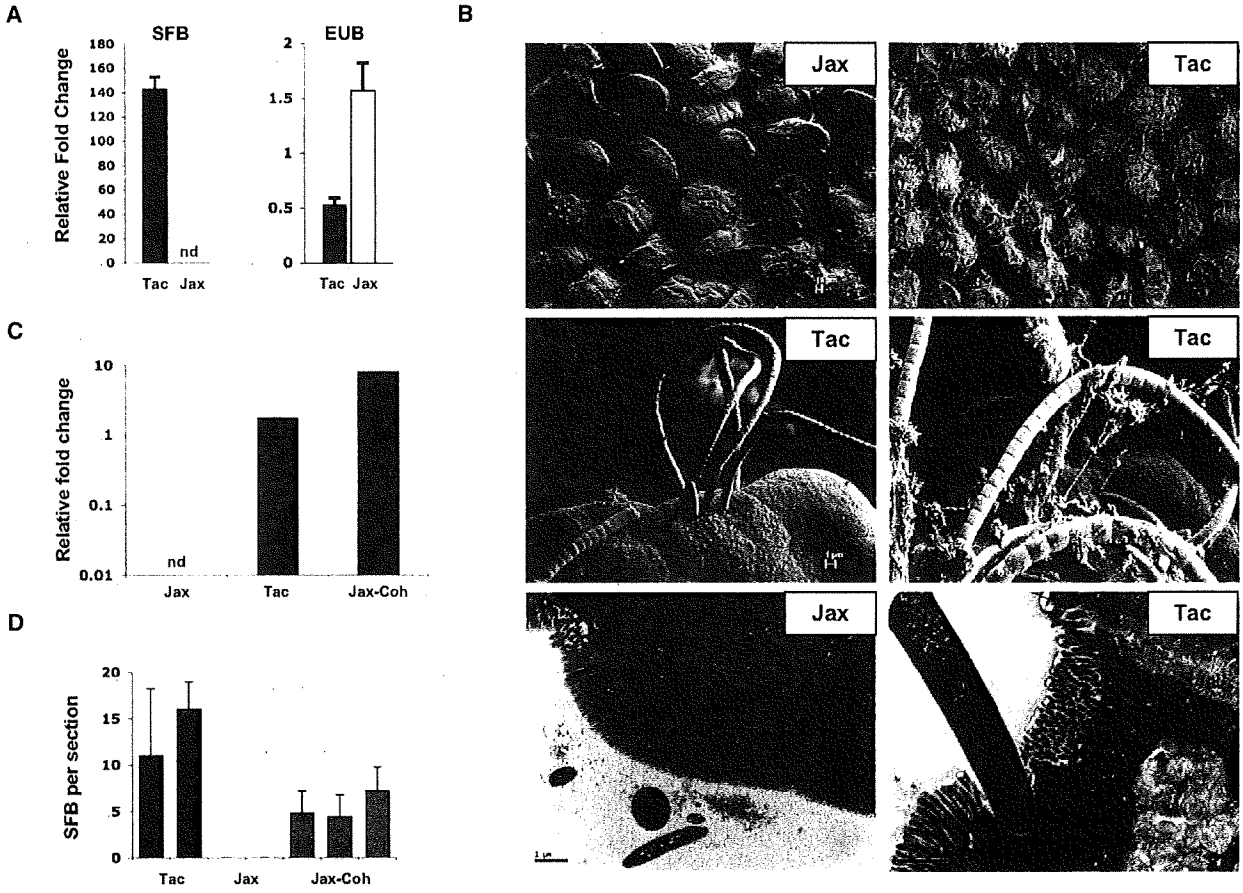

Figure 2. Segmented Filamentous Bacteria in the Intestinal Tract of Th17 Cell-Sufficient and Th17 Cell-Deficient Mice

(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

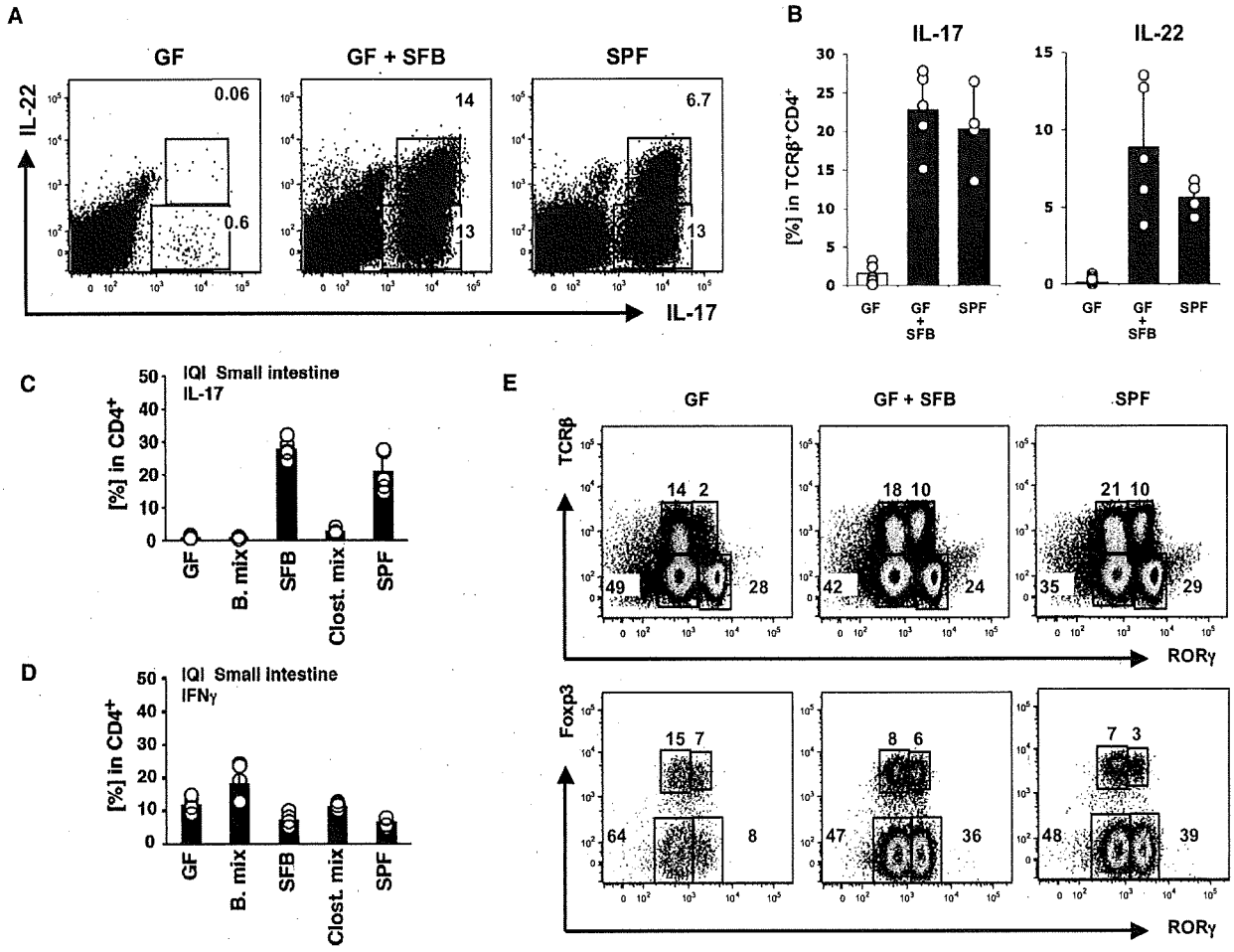

Figure 3. SFB Specifically Induce Th17 Cell Differentiation in Germ-Free Mice

(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 TCRβ$^+$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 IFNγ (D) expression in TCRβ$^+$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 RORγt expression only in CD4$^+$ T cells. RORγt expression in total SI LPL (top panels) and RORγt and Foxp3 expression in TCRβ$^+$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

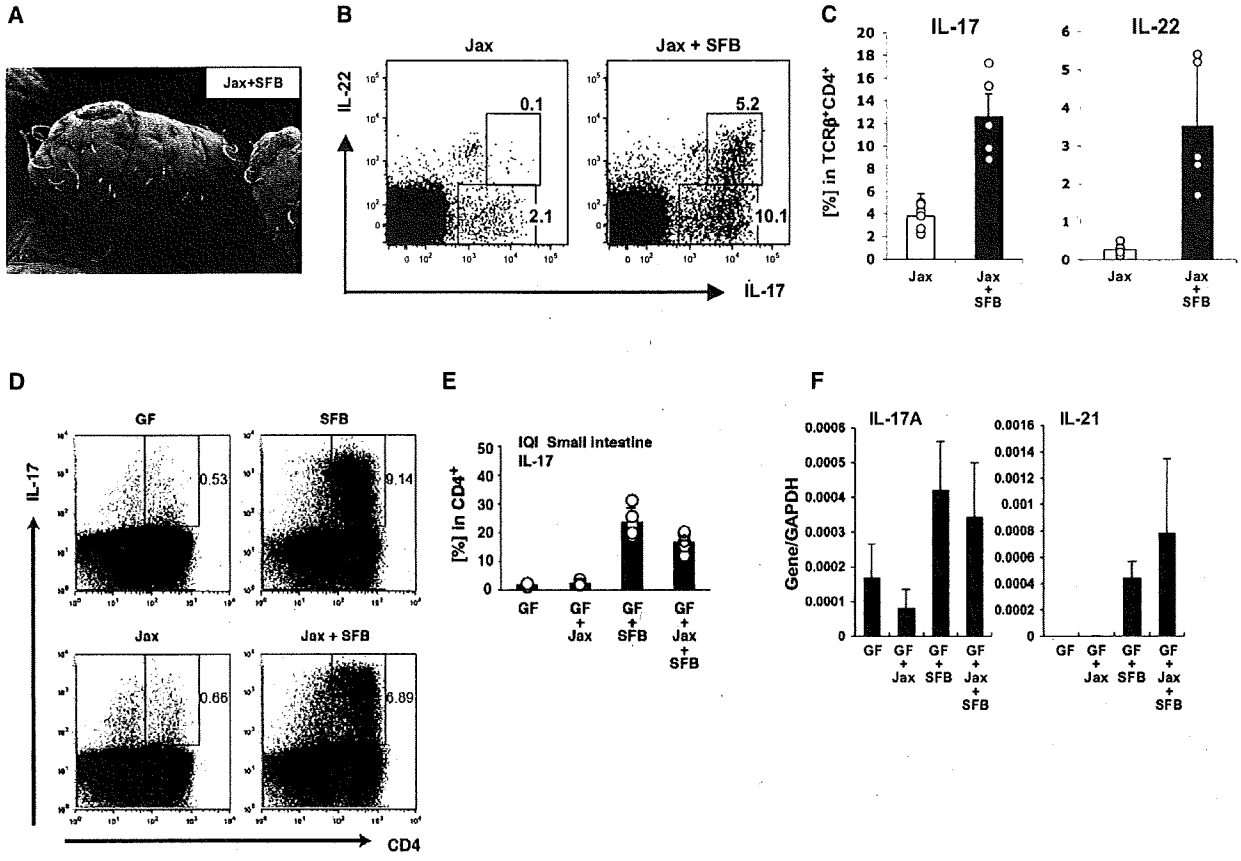

Figure 4. SFB Induce Th17 Cell Differentiation upon Colonization of Jackson C57BL/6 Mice

(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

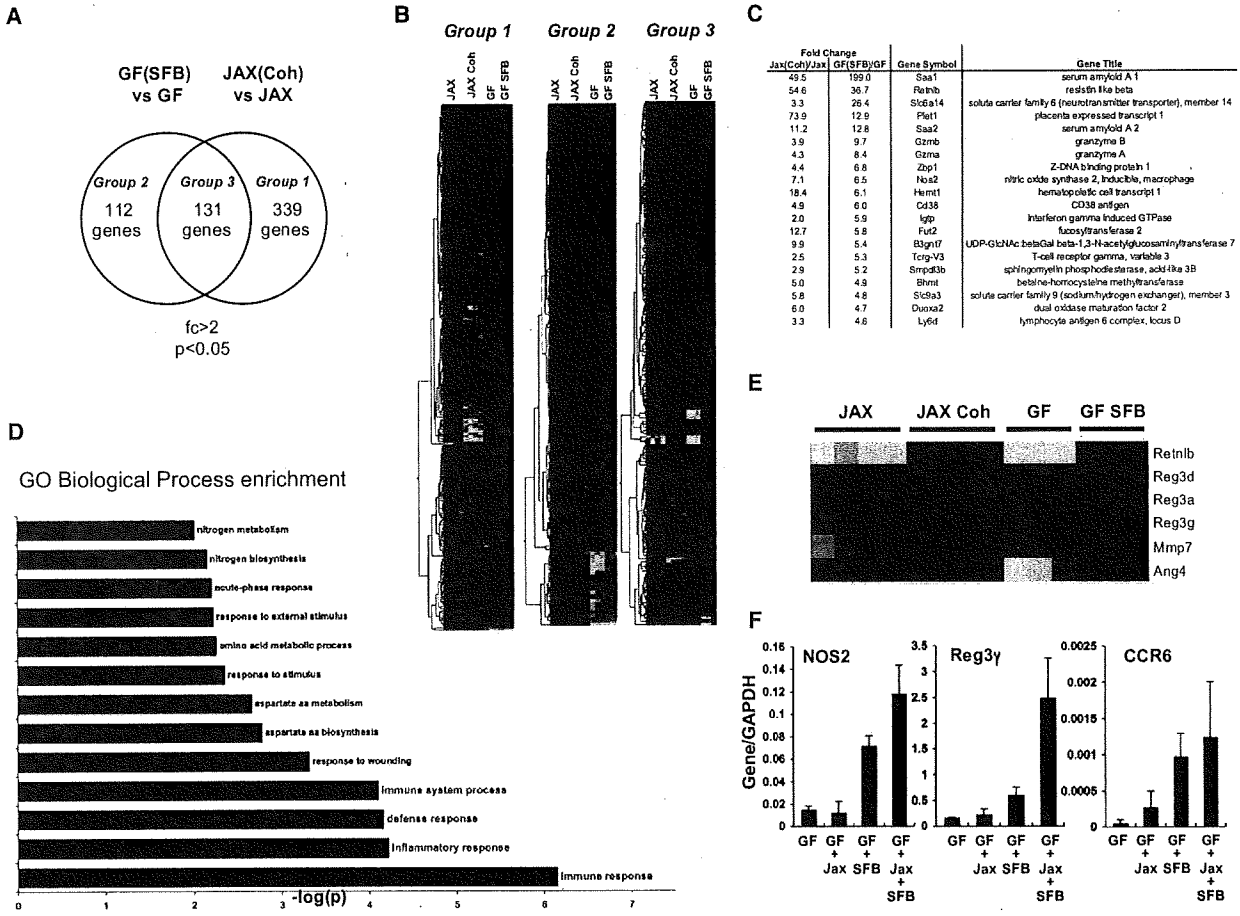

Figure 5. Transcriptional Programs Induced by SFB Colonization

(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.

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;

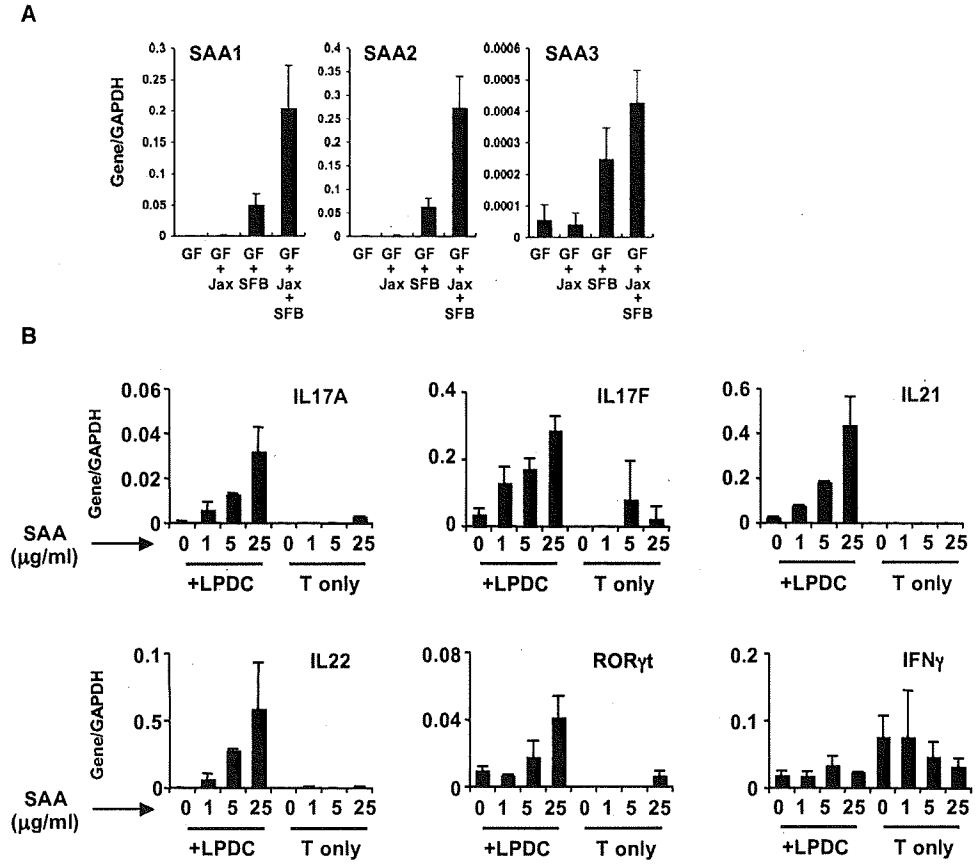

Figure 6. SFB Colonization Induces SAA Expression that Influences Th17 Differentiation

(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

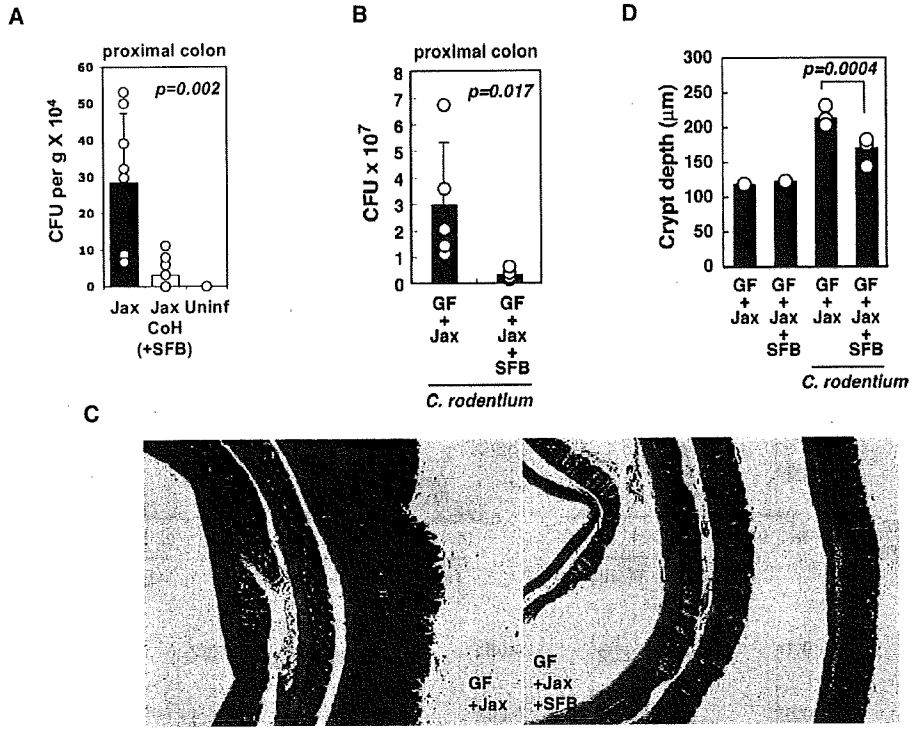

Figure 7. SFB Colonization Confers Protection from Infection by *Citrobacter rodentium*

(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 ΔCt 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 ~20 cycles and for EUB ~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 μl 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 2×10^5 cells/well for 4 days with MACS-purified LP $CD11c^+$ cells (1×10^5/ well) and 1 μg/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 μl 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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BASIC—ALIMENTARY TRACT

DNA Hypermethylation Contributes to Incomplete Synthesis of Carbohydrate Determinants in Gastrointestinal Cancer

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See editorial on page 305.

Background & Aims: **It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants. The aim of this study was to clarify the cause of cancer-associated abnormal glycosylation in gastrointestinal (GI) cancers.** ***Methods:*** **We compared the expression levels of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa and in gastric and colorectal cancer cells. To examine the possibility that DNA hypermethylation contributed to the down-regulation of these genes, we treated GI cancer cells with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase.** ***Results:*** **The silencing of some of these glyco-genes, but not up-regulation of certain molecules, was observed. The Sd^a carbohydrate was abundantly expressed in the normal GI mucosa, but its expression was significantly decreased in cancer tissues. When human colon and gastric cancer cells were treated with 5-aza-dC, cell surface expression of Sd^a and the transcription of *B4GALNT2*, which catalyzes the synthesis of the Sd^a, were induced. The promoter region of the human *B4GALNT2* gene was heavily hypermethylated in many of the GI cancer cell lines examined as well as in gastric cancer tissues (39 out of 78 cases). In addition, aberrant methylation of the *B4GALNT2* gene was strongly correlated with Epstein-Barr virus-associated gastric carcinomas and occurred coincidentally with hypermethylation of the *ST3GAL6* gene.** ***Conclusions:*** **Epigenetic changes in a group of glycosyltransferases including *B4GALNT2* and *ST3GAL6* represent a malignant phenotype of gastric cancer caused by silencing of the activity of these enzymes, which action may eventually induce aberrant glycosylation and expression of cancer-associated carbohydrate antigens.**

It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants.[1] Many glycosyl epitopes such as sialyl Tn, Tn, T, and sialyl Lewis x/a ($sLe^{x/a}$) have been reported to be cancer-associated antigens. Some of them show statistically significant correlations between the degree of their expression in cancer tissues and the postoperative prognosis of patients with many types of human cancers.[2–4] In addition, $sLe^{x/a}$ determinants are known to serve as ligands for E-selectin, which is inducibly expressed by endothelial cells, in hematogenous metastasis of cancers.[5,6] A long-standing debate is which is more important in understanding cancer-associated carbohydrate antigens, "neo-synthesis" or "incomplete synthesis." To verify the former hypothesis, the levels of many glycosyltransferases involved in "neo-synthesis" of tumor-related glycosyl epitopes and their mRNA expression have been studied; however, no conclusive results have been obtained to date.[7–9]

On the other hand, there is a group of carbohydrate determinants that is less expressed in cancer tissues when compared with their level in normal tissues. Because their structures are commonly more complicated, the concept of "incomplete synthesis," that the synthesis of complex carbohydrate determinants in nonmalignant cells might be impaired upon malignant transformation, has been proposed as an important cause of cancer-associated abnormal glycosylation.[10] The blood group Sd^a carbohydrate antigen serves as a typical example among the latter

Abbreviations used in this paper: 5-aza-dC, 5-aza-2′-deoxycytidine; COBRA, combined bisulfite restriction analysis; DNMT, DNA methyltransferase; EBV, Epstein-Barr virus; FUT, fucosyltransferase; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GALNT, *N*-acetylgalactosaminyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; mAb, monoclonal antibody; HP, *Helicobacter pylori*; Sd^a-β1, 4GalNAcT, β1,4*N*-acetylgalactosaminyltransferase, which forms Sd^a carbohydrate determinants; ST, sialyltransferase; type II precursor, Galβ1,4GlcNAc-R.

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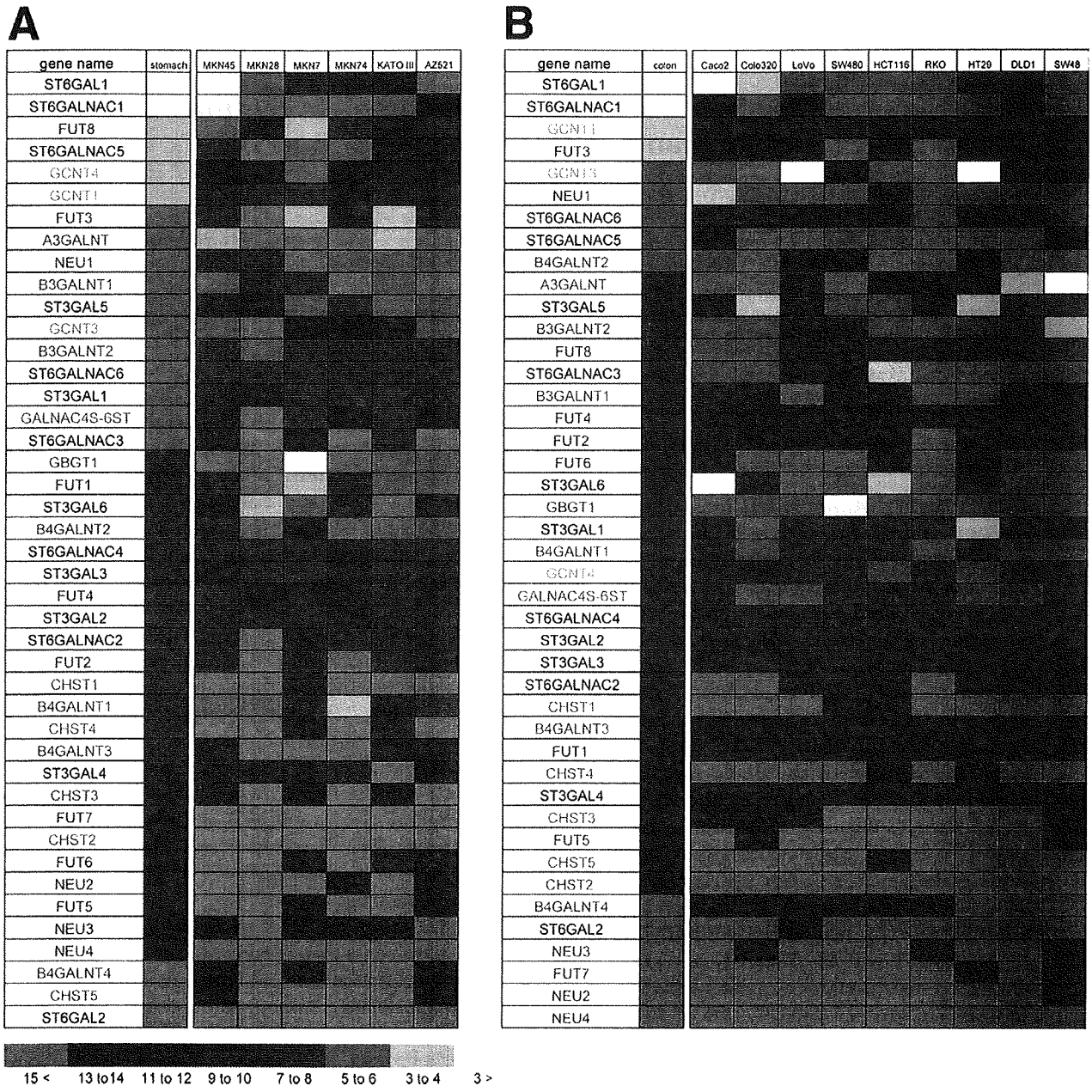

Figure 1. Profiles of expression of glycosylation-related genes in human GI tissues and cancer cell lines. Quantitative PCR analysis was carried out on normal human stomach and gastric cancer cell lines (*A*) and on normal human colon and CRC cell lines (*B*). Human glyco-genes, encoding 8 fucosyltransferases (classified by *blue*), 8 *N*-acetylgalactosaminyltransferase genes (*red*), 3 *N*-acetylglucosaminyltransferase genes (*orange*), 14 sialyltransferase genes (*black*), 6 sulfotransferase genes (*green*), and 4 sialidase genes (*purple*) were examined. Expression levels of each gene were sorted according to ΔCt (see Materials and Methods) calibrated by using GAPDH and visualized by color as indicated by the bar below.

group. This carbohydrate determinant is abundantly expressed on glycolipids and glycoproteins in the normal gastrointestinal (GI) tract mucosa in the majority of humans; however, its expression in cancer tissue is strikingly reduced or absent.[11,12] The last step in the biosynthesis of Sd^a is catalyzed by β1,4-*N*-acetylgalactosaminyl-transferase (β1,4GalNAcT). The activity of the β1,4GalNAcT responsible for synthesizing the Sd^a determinant (Sd^a-β1,4GalNAcT) also dramatically decreases in gastric and colonic cancer tissue.[13,14] Recently we reported that forced expression of Sd^a-β1,4GalNAcT in GI cancer cells reduced their expression of $sLe^{x/a}$ carbohydrates and decreased their metastatic potential in nude mice, probably owing to competition with $sLe^{x/a}$ synthases for acceptor carbohydrate.[15] Thus, the lack of Sd^a antigens in cancer cells is functionally important;

however, very little is known about the molecular mechanism underlying the regulation of Sd^a expression.

In line with these hypotheses of "neo-synthesis" and "incomplete synthesis," we compared the expression of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa with that in gastric and colorectal cancer (CRC) cells in this study. Recently, epigenetic changes, such as DNA hypermethylation, have been recognized as one of the important mechanisms for gene inactivation.[16] In this study, we investigated the possible role of aberrant methylation in the glycosyltransferase gene promoter region in human GI cancer cells. We also examined epigenetic changes in a group of glycosyltransferases in human gastric cancer tissues and analyzed their relation to clinicopathologic features of the cases.

Materials and Methods

Cell Lines and Specimens

The gastric and colon carcinoma cell lines that were used in this study were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) or the American Tissue Type Collection (Manassas, VA). Human CRC cell line HCT116 with genetic disruption of DNMT1 (DNMT1 KO) or both DNMT1 and DNMT3b (DKO) were established as described previously.[17] The 78 gastric tumor specimens and their paired normal tissue specimens were obtained from 78 randomly selected Japanese patients. Informed consent was obtained from all patients before the samples were collected.

Reverse Transcription-Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) of glyco-genes was performed by using ABI TaqMan probes (Applied Biosystems, Foster City, CA) as described previously.[18,19] Threshold cycle numbers (Ct) were determined with Sequence Detector software and transformed by using the ΔCt method as described by the manufacturer, with *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) used as the calibrator gene. Human glyco-genes examined in this study, 8 genes encoding fucosyltransferases (*FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7,* and *FUT8*), 8 *N*-acetylgalactosaminyltransferase genes (*A3GALNT, GBGT1, B3GALNT1, B3GALNT2, B4GALNT1, B4GALNT2, B4GALNT3,* and *B4GALNT4*), 3 *N*-acetylglucosaminyltransferase genes (*GCNT1, GCNT3,* and *GCNT4*), 14 sialyltransferase genes (*ST3GAL1, ST3GAL2, ST3GAL3, ST3GAL4, ST3GAL5, ST3GAL6, ST6GAL1, ST6GAL2, ST6GALNAC1, ST6GALNAC2, ST6GALNAC3, ST6GALNAC4, ST6GALNAC5,* and *ST6GALNAC6*), 6 sulfotransferase genes (*GALNAC4S-6ST, CHST1, CHST2, CHST3, CHST4* and *GCNT5*), and 4 sialidase genes (*NEU1, NEU2, NEU3,* and *NEU4*), and TaqMan probe kits used in this study are summarized in Supplementary Table 1 (see supplementary material online at www.gastrojournal.org). Human stomach and colon total RNA (BioChain, Hayward, CA) were used as normal controls; they were prepared from normal stomachs and colon mucosae pooled from healthy subjects.

Flow Cytometry

Flow cytometry was performed with a FACScan (BD Bioscience, Franklin Lakes, NJ). Monoclonal antibody (mAb) KM694 (directed against Sd^a) was provided by Tokyo Research Laboratories (Kyowa Hakko Kogyo Co, Ltd. Tokyo, Japan).

Combined Bisulfite Restriction Analysis and Bisulfite Sequencing

We assessed gene methylation by using primers that were designed to amplify both the methylated and unmethylated alleles.[20] Bisulfite modification was carried out by using an EpiTect Bisulfite Kit (Qiagen, Tokyo, Japan). For combined bisulfite restriction analysis (COBRA), the PCR primers used for B4GALNT2 were 5′-ATTGGTTTTTYGTATAGGTGGTTG-3′ and 5′-CCRAACCRATTCCCACACTC-3′, yielding a PCR product of 174 bp. Primers for ST3GAL6[21] were 5′-GTTTGTTATATYGGGTYGTAGAAG-3′ and 5′-AATTAAAACTAACRAAAACCTAAAACT-3′ (162 bp). The products were then digested with the restriction endonuclease Hha*I* (for *B4GALNT2*) or AfaI (for *ST3GAL6*), which cleave only methylated CpG sites. For bisulfite sequencing, the PCR primers used for B4GALNT2 were 5′-GAGAGGTGAAATTTYGGGAGTA-3′ and 5′-RACTATCCACAACCCRCAATC-3′ (430 bp). For sequencing of the bisulfite-PCR product, the DNA fragment was purified and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). Clones for subsequent sequencing were randomly picked up.

Detection of the Epstein–Barr Virus Genome and Helicobacter pylori

To detect the Epstein–Barr virus (EBV) genome in gastric tumors, we performed real-time PCR using 2 sets of primers as described previously.[22] Consistent results were obtained with both systems. *Helicobacter pylori* (HP) infection was identified by conducting histologic review of hematoxylin and eosin-stained tissue specimens and PCR assays as described by Clayton et al.[23]

Mutation Analysis

Genomic DNA was amplified by using exon-specific primers for p53 exons 2–11 and the mutations were examined as described previously.[24]

Immunohistochemical Analysis

Frozen sections of 8-μm thickness were prepared from a surgical specimen. After blocking sections with 3% bovine serum albumin in phosphate-buffered saline and then incubating them with mAb KM694, bound mAbs were detected with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin M (Southern Biotechnology Associates, Inc, Birmingham, AL).

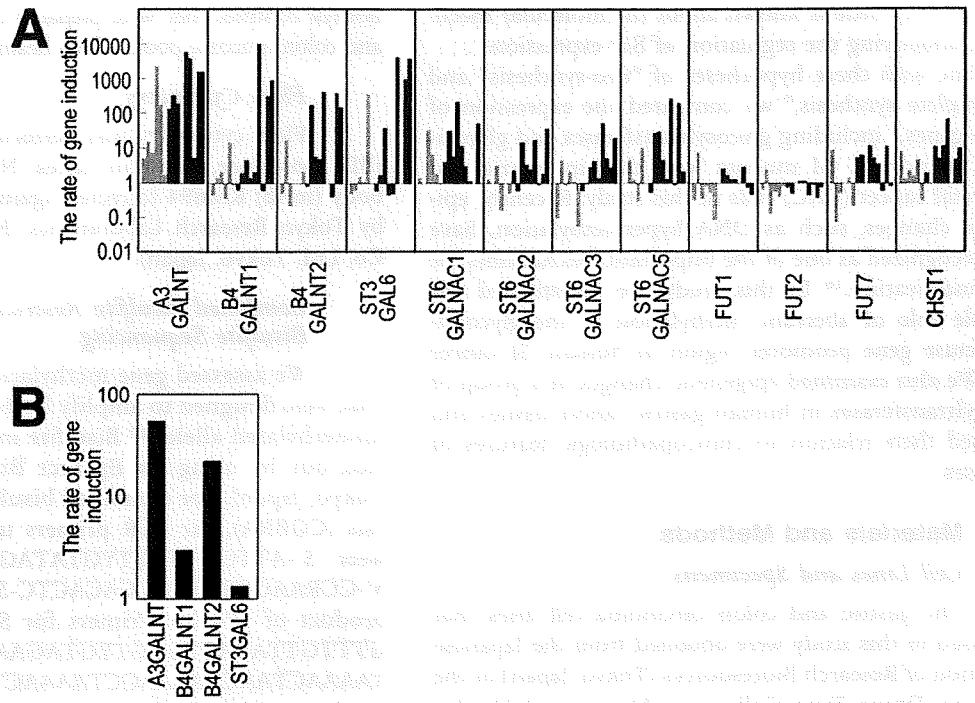

Figure 2. Expression of glycosylation-related genes in DNA methyltransferase-inhibited cells. (*A*) Six human gastric cancer (MKN45, MKN28, MKN7, MKN74, KATO III, and AZ521, as depicted in order from left to right by the *gray bars*) and 9 CRC (Caco2, Colo320, LoVo, SW480, HCT116, RKO, HT29, DLD1, and SW48, as depicted in order from left to right by the *black bars*) cell lines were treated with 2 μmol/L 5-aza-dC for 72 hours, and the expression level of each gene was then assessed by RT-PCR. The rate of induction is expressed as the ratio of treated to untreated cells. The genes that were analyzed are shown at the bottom of the bar graph. (*B*) RNA was harvested from HCT116 and DKO cells, and the expression level of each of the indicated genes was assessed by RT-PCR. The rate of induction is expressed as the ratio of induction in DKO cells to that in the parental HCT116 cells.

Statistical Analysis

Each tumor was classified based on tumor location; macroscopic type; lymphatic invasion; venous invasion (Japanese Gastric Cancer Association)[25]; pathologic tumor, lymph node, metastasis (pTMN) classification[26]; and the Lauren classification.[27] Methylation of *B4GALNT2* was compared by using the Student *t*-test for age; the Mann–Whitney *U* test for tumor size, pT status, pN status, and disease stage; and the Fisher exact test for gender, tumor location, macroscopic type, histology, lymphatic invasion, venous invasion, pM status, EBV association, HP status, p53 mutation, and methylation of ST3GAL6. The Fisher exact test was carried out by using SAS (SAS Institute Inc, Cary, NC), and other statistical analyses were made with SPSS software (version 11.0; SPSS Inc, Chicago, IL). All tests were 2-tailed, and values of $P < .05$ were considered significant.

Results

Expression of Genes Involved in the Synthesis and Modification of Carbohydrate Determinants in Human GI Tissue and Cancer Cells

It has been known that carbohydrate structures in GI cancers are quite different from those in normal GI epithelium. To clarify the cause of this abnormal glycosylation in GI cancer cells, we first examined the expression levels of 43 "glyco-genes," including 8 genes encoding fucosyltransferases, 14 sialyltransferase genes, 8 *N*-acetylgalactosaminyltransferase genes, 3 *N*-acetylglucosaminyltransferase genes, 6 sulfotransferase genes, and 4 sialidase genes (Supplementary Table 1). There was no gene whose expression was universally up-regulated in the GI cancer cell lines examined when compared with normal tissues. On the other hand, we found approximately one third of glycosyltransferase genes that were expressed in normal GI mucosa but whose expression levels were decreased in many GI cancer cell lines (Figure 1*A* and *B*). This silencing of glycosyltransferases was the major cancer-associated change detected in glyco-gene expression. To examine the possibility that DNA methylation contributed to the low expression levels of these genes, we chose 12 genes containing CpG islands in their promoter region from among cancer-associated down-regulated glyco-genes. When GI cancer cells were treated with 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, the mRNA expression of glycosyltransferases was significantly induced in many of them (Figure 2*A*). On the other hand, the expression of ≥2 glycosyltrans-