Immunity
Article

The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses

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SUMMARY

Microbiota-induced cytokine responses participate in gut homeostasis, but the cytokine balance at steady-state and the role of individual bacterial species in setting the balance remain elusive. Herein, systematic analysis of gnotobiotic mice indicated that colonization by a whole mouse microbiota orchestrated a broad spectrum of proinflammatory T helper 1 (Th1), Th17, and regulatory T cell responses whereas most tested complex microbiota and individual bacteria failed to efficiently stimulate intestinal T cell responses. This function appeared the prerogative of a restricted number of bacteria, the prototype of which is the segmented filamentous bacterium, a nonculturable *Clostridia*-related species, which could largely recapitulate the coordinated maturation of T cell responses induced by the whole mouse microbiota. This bacterium, already known as a potent inducer of mucosal IgA, likely plays a unique role in the postnatal maturation of gut immune functions. Changes in the infant flora may thus influence the development of host immune responses.

INTRODUCTION

Our bodies are home to trillions of commensal bacteria, 90% of which reside in our intestine. Only a restricted number of bacterial phylotypes colonize the intestine, indicating that strong selection pressures have shaped this microbial community (Ley et al., 2006). Although dependent on their capacity to adapt to the stringent conditions in the host intestine (Giraud et al., 2008) and to use host-derived substrates (Hooper et al., 1999), intestinal commensal bacteria can in return provide benefits to their host, including the breakdown of indigestible vegetal polysaccharides, the provision of fuel for colonic epithelial cells (Backhed et al., 2005), and a barrier against invasive pathogenic bacteria. In addition, the commensal gut microbiota orchestrates the postnatal maturation of gut immune defenses (Artis, 2008; Macpherson and Harris, 2004), which in turn preserve intestinal homeostasis and host integrity (Bouma and Strober, 2003). Deciphering the dialog between the microbiota and the host immune system should facilitate an understanding of how bacteria and their hosts coevolved to maintain mutually beneficial interactions. Recent work has placed emphasis on the innate immune system, illustrating how a complex signaling network involving host epithelial cells is elicited upon colonization, promoting the production of bactericidal peptides able to contain the microbiota in the lumen while also avoiding excessive proinflammatory signals harmful for both partners (Artis, 2008). B cell response to the microflora has also been highlighted (Macpherson, 2006). Colonization of germ-free (GF) mice elicits secretory IgA responses believed to play an important role in gut homeostasis by preventing luminal bacterial overgrowth (Fagarasan et al., 2002) and bacterial translocation (Macpherson and Uhr, 2004) and by minimizing flora-induced activation of the host oxidative system (Peterson et al., 2007). Host physiological T cell responses to the microbiota are less well delineated. Colonization by the commensal gut microbiota stimulates proliferation of lymphoblasts in Peyer's patch (PP) T cell areas and T cell immigration into the lamina propria (LP) and epithelium (Guy-Grand et al., 1974; Macpherson and Uhr, 2004). How the gut microbiota shapes intestinal helper T cell response at steady-state has recently stimulated much interest given the accumulating evidence of microbial dysbiosis in allergic (Kirjavainen et al., 2002) and inflammatory (Sokol et al., 2008) bowel diseases. Contradictory data regarding the capacity of the microbiota to stimulate proinflammatory and/or regulatory T cells have also been generated (discussed in Round and Mazmanian [2009]), perhaps resulting from compositional differences in the colonizing microbiota. This hypothesis is supported by recent evidence that specific members of the gut microbiota

can orientate host T cell responses (Ivanov et al., 2008; Mazmanian et al., 2008; Sokol et al., 2008) and/or by the newly recognized plasticity of helper T cell subsets (Zhou et al., 2009). By using gnotobiotic mouse models, we have investigated in depth the impact of the microbiota on host gut responses. Intestinal colonization orchestrated a broad spectrum of proinflammatory and regulatory T cell responses, which likely balance each other to maintain local homeostasis. Strikingly, little redundancy could be demonstrated between individual microbiota members in their capacity to stimulate steady-state gut T cell responses. This function appears the prerogative of a restricted number of bacteria, the prototype of which is the segmented filamentous bacterium (SFB), a nonculturable *Clostridia*-related host-specific species, which could largely recapitulate the immune-inducing effects of a complete conventional microbiota. Unexpectedly, these results suggest that only a restricted number of microbiota members have shaped host-immune T cell interactions during evolution.

RESULTS

Intestinal Colonization Simultaneously Drives Proinflammatory and Regulatory Immune Responses

Global transcriptomic analysis of terminal ileum tissues from healthy adult C3H/HeN GF, conventional (Cv), and conventionalized (Cvd) mice colonized with whole mouse fecal flora at adult age indicated that 45% of the genes induced by the microbiota could be assigned to immune response pathways, an effect evident at both 8 and 60 days (d) postcolonization (Figure 1A). Colonization had a strong and parallel impact on *Cd3e* transcription and T helper 1 (Th1)-type gene responses including *Interferon regulatory factor 1* (*Irf1), Interleukin (Il)18, Signal transducer and activator of transcription 1 (Stat1)*, and *Interferon* γ *(Ifng)* (Figure 1B).

The nature and site of the physiological gut immune responses induced by the microbiota was further investigated with real-time quantitative polymerase chain reaction (qRT-PCR). Comparison between GF and Cv mice and kinetics studies in Cvd mice showed that intestinal colonization resulted in the coordinated induction of a large panel of genes involved in innate and adaptive T cell immune responses, maximal in the terminal ileum (Table 1). For innate immune genes, the most important differences were noted for transcripts encoding IL-12p40, inducible nitric oxide synthase 2 (NOS2), and chemokine (C-X-C motif) ligand 10 (CXCL10 or IP10). *Il1b* and *Il6* mRNA were only modestly increased while mRNA encoding chemokine (C-X3-C) receptor 1 ligand (CX3CR1-L), chemokine (C-X-C motif) ligand 1 (KC), IL-12p35, or IL-23p19 did not change (Table 1 and not shown). Analysis of T cell markers demonstrated that parallel to the increase in *Cd3e* mRNA and $CD3^+$ T cell numbers in LP (Table 1; Figure S1 available online), *Ifng* transcripts increased 30- to 40-fold in the mouse ileum upon colonization whereas *Il17* mRNA, detectable only in 2/15 tested GF mice, increased 1000- to 2000-fold. No change was observed in the transcription of *Il4* and *Il13* (not shown), but *Il10* and *forkhead box P3* (*Foxp3*) mRNA increased approximately 5- and 10-fold, respectively (Table 1). No significant changes in *retinoic acid-related orphan receptor (Ror)* γt (*Rorgt*) and *Rora* mRNA ($p = 0.7$ and $p = 0.07$, respectively) were detected parallel to the increase in *Il17* transcripts (Figure S2).

Cytokine production was next analyzed by BioPlex in the supernatants of lamina propria lymphocytes (LPL) from Cv and GF mice. Although CD3+CD28 stimulation induced very little cytokine secretion by LPL isolated from the ileum of GF animals, the same stimulation in ileal LPL from Cv mice resulted in the release of large amounts of IFN-γ, IL-17, IL-10, as well as of IL-4 and IL-13, the latter despite the lack of detectable mRNA increase (Figure 2A). Contrasting with the minor differences between GF and Cv mice in mRNA expression in the colon (Table 1), CD3+CD28 stimulation of LPL revealed the same striking difference in the capacity of colonic LPL from GF and Cv mice to produce cytokines as that observed in the ileum (Figure 2B). Taken together, these data suggested that intestinal colonization resulted in the combined induction of proinflammatory and regulatory T cells that disseminated along the whole intestine but were actively secreting cytokines only in the ileum.

Mucosal T Cell Responses Are Induced by a Restricted Number of Host-Specific Bacterial Species

To delineate which members of the microbiota are necessary to shape gut immune responses, the ileal transcriptomic responses were analyzed on d8, d20, and d60 after colonization of adult GF mice with complex or simplified microbiota. Because individual members of the microbiota such as *Bacteroides thetaiotaomicron* can reproduce the effect of the whole flora on intestinal metabolic changes (Hooper et al., 2001), we first assessed the impact of individual culturable strains representative of the two dominant bacterial phyla in the mammalian intestine, *Bacteroidetes* and *Firmicutes*, and of *Escherichia coli*, a *Proteobacteria* species that colonizes the neonatal intestine. Despite a strong degree of colonization (10^8–10^9 colony-forming units [CFU]/g of feces), monocolonization by *B. thetaiotaomicron*, *B. vulgatus*, and *E. coli* MG1655 did not modify mRNA expression for any of the studied immune genes and at all studied time points when compared to GF mice (not shown). Similar results were obtained with three distinct *Clostridium* strains (*Firmicutes*) derived from the mouse altered Schaedler's flora (ASF; not shown). These data suggested that efficient induction of mucosal responses might require a complex microbiota.

Surprisingly, colonization of GF mice with a fresh whole complex human fecal microbiota (humanized mice; Hum) failed to stimulate the transcription of most studied immune genes (Figures 1A, 1B, and 3A). Furthermore, when using Affymetrix to compare the numbers of genes changing in Cv mice with GF mice and mice colonized with either human or mouse flora, greatest differences were noted with human flora and GF mice, indicating that these mouse groups were most different from the Cv group (Figure 1C). Hierarchical clustering of immune system-related genes by heatmaps corroborated that Cv mice clustered with Cvd mice whereas Hum mice clustered with GF mice (Figure 1B). This result was unexpected given the degree of colonization of Hum mice (10^8–10^9 CFU/g of feces) and the large representation of the major bacterial groups initially present in the human stools as demonstrated by fluorescent in situ hybridization (FISH) analysis of fecal samples (Figure 3B). To explain the disparity between human and mouse flora effects, we postulated that the mouse flora contained specific bacterial

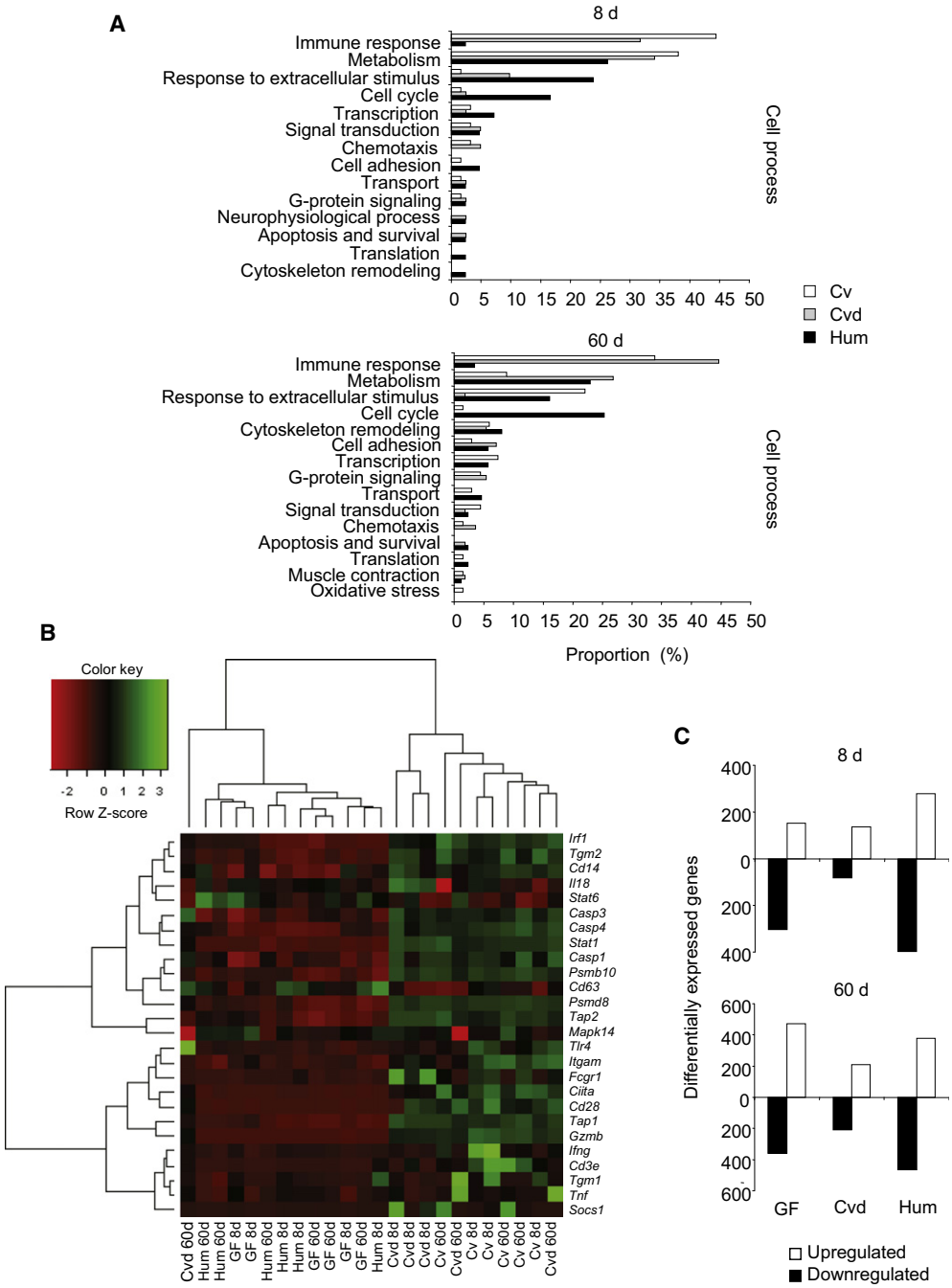

Figure 1. Conventional Mouse Microbiota Induces a Vast Repertoire of Intestinal Immune Responses

(A) RNA from ileal tissue of conventional (Cv), germ-free (GF), and mice colonized with whole mouse fecal microbiota (Cvd) or whole human fecal microbiota (Hum) for 8 and 60 days (n = 3/group) was hybridized to the Affymetrix NuGO Mouse Array (NuGO_Mm1a520177). Data were normalized with gcRMA and adjusted for multiple testing via the Benjamini Hochberg false discovery method ($p < 0.05$). Differentially expressed genes ($p < 0.05$) relative to the GF group were imported into GeneGo MetaCore analytical software to determine the significantly enriched canonical pathways in each group. Data represent the distribution (percent) in cell process categories of statistically significantly enriched pathways ($p < 0.05$).

(B) Heatmap generated from immune system-related genes significantly expressed ($p < 0.05$) between all treatments after 8 and 60 days. Columns represent individual arrays, and rows specific genes of interest. The Z-score depicts a measure of distance, in standard deviations, away from the mean. The relative value for each gene is depicted by color intensity, with green indicating higher expression and red depicting lower expression.

(C) Affymetrix microarray analysis of differentially expressed genes in GF, Cvd, and Hum mice relative to Cv (n = 3/group). Bar graphs represent number of genes higher expressed (open bar; fold change > 2, $p < 0.05$) and lower expressed (black bar; fold change < −2, $p < 0.05$) in each treatment relative to the Cv group after 8 and 60 days of colonization.

Table 1. Changes in Intestinal Gene Expression in Mice Colonized with Complex Mouse Microbiota

	Fold Increase (Relative to Germ-free)									
	Cv				Cvd			Cult		
					Ileum			Ileum		
	Duod	Cecum	Colon	Ileum	d 8	d 20	d 60	d 8	d 20	d 60
Gene	n = 8	n = 8	n = 8	n = 12	n = 5	n = 4	n = 5	n = 10	n = 8	n = 9
Nos2	nd	nd	nd	**11.4**	**4.8***	**13.3**	**11.6**	**1.8**	**1.9**	**3.9**
Cxcl10	2.1	1.6	0.7	**8.4**	**20.2**	**18.4**	**10.2**	1.2	1.7	**1.7**
Il1b	1.7	**2.4**	0.6	**3.5**	**3.8**	**6.4**	**5.0**	1.3	1.4	**1.7**
Il6	**2.0**	**1.4**	0.7	**2.4**	**4.7**	1.2	**1.8**	1.0	1.7	**1.5**
Il12p40	**4.5**	1.1	0.4	**24.5**	**21.6**	**25.9**	**31.4**	**2.1**	**4.0**	**2.9**
Cd3e	**2.9**	0.9	1.6	**10.6**	**2.8***	**6.7***	**10.5**	0.8	0.9	0.9
Tnfa	nd	nd	nd	**4.0**	**2.9**	**4.5**	**4.2**	0.7	0.9	1.2
Ifng	**5.8**	1.5	1.2	**40.2**	**28.4**	**30.3**	**31.9**	1.3	2.5	1.2
Il17	**322.3**	**87.8**	**11.0**	**1016.1**	**35.5***	**1616.4**	**1958.2**	5.4	2.5	3.0
Il10	**5.6**	**5.6**	2.3	**6.3**	**4.4**	**4.3**	**4.3**	**2.3**	**3.1**	**1.4**
Foxp3	1.9	0.6	**1.5**	**8.7**	**5.2**	**5.1**	**10.8**	0.9	2.0	**1.4**

Relative increase compared to GF mice (n = 15) of mRNA levels in duodenum (duod), ileum, cecum, and colon of Cv mice and in ileum of mice colonized with the whole mouse fecal microbiota (Cvd) or its cultured fraction (Cult, pool of two independent experiments) at d8, d20, and d60 postcolonization. Significant median fold increases with GF are in bold ($p < 0.05$).
*$p < 0.05$ between Cvd and Cv mice by a nonparametric Kruskal-Wallis test.
nd: not done.

species capable of stimulating immune activity in the gut over and above that induced by the predominant microbial groups commonly found in both human and mouse gut microbiota.

As a first step to delineate which components of the mouse microbiota might be necessary to efficiently induce mucosal innate and adaptive immune responses, GF mice were colonized with the cultured bacterial fraction from feces of Cv mice (Cult mice). Despite normal colonization (10^8–10^9 CFU/g of feces), induction of proinflammatory genes was very low. An increase in *Foxp3* and *Il10* mRNA expression was observed compared to GF but only in some animals (Figure 3A; Table 1). In keeping with mRNA data, the numbers of LP $CD3^+$ cells in ileal tissue sections and of effector $CD62L^-CD44^+CD4^+$ T cells in isolated LPL increased only very modestly compared to GF mice (Figure S1). Cytokine production was next analyzed in LPL isolated from ileum and colon and stimulated by anti-CD3+CD28 (Figure 2). For all cytokines tested, their release by LPL was much lesser in Cult than in Cv mice. Moreover, no significant difference with GF mice was observed for IFN-γ and IL-10 in both ileum and colon, whereas IL-4 and IL-13 production by ileal but not colonic LPL was modestly increased. An increased release of IL-17 was, however, observed, notably by ileal LPL from one Cult mouse (which also produced substantial amounts of other cytokines). Yet, median IL-17 secretion by colonic and ileal LPL was, respectively, 5- and 15-fold less than in Cv mice (Figure 2). Collectively, these results suggested that cultured flora were depleted in species able to efficiently stimulate intestinal adaptive T cell responses.

Efficient Induction of Intestinal T Cell Responses Depends on *Clostridia*-Related Sporulated Species

To assess the possible loss of specific species, the composition of the microbiota was compared by FISH in the feces of Cv mice used as donors of the culturable flora and of Cult mice on d8, d20, and d60 postcolonization. Although the flora of Cult mice remained highly diverse, a reduction in the proportion of bacteria in the *Clostridium coccoides* group was noted in both experiments (Figure 3B). A selective loss of bacteria from the *Clostridium* family was also observed when comparing the microbiota in the donor human stools and in the feces of Hum mice (Figure 3B, bottom). These results led us to hypothesize that host-specific, *Clostridia*-related bacterial species enriched in the nonculturable fraction of the flora were necessary to promote efficient maturation of the intestinal adaptive T cell responses.

Clostridia are typically sporulated bacteria enriched in the heat-resistant fraction of the microbiota. Therefore, adult GF mice were colonized with fecal heat-resistant microbiota from Cv mice (Spo mice). Colonization rose between 5×10^8 and 2×10^9 CFU/g of feces. qRT-PCR analysis showed a marked increase in ileal mRNA expression for all tested immune markers, comparable on d20 and/or d60 postcolonization with that observed in Cvd mice (Figure 4A and not shown). These results supported the hypothesis of a key role of one or several spore-forming bacteria related to the *Clostridia* class. One possible candidate bacterium was SFB, a *Clostridia*-related unculturable bacterium found in the normal intestine of all studied species, which demonstrates host-specific attachment to the ileal epithelial surface (Klaasen et al., 1992; Snel et al., 1995). SFB is a potent stimulus of the mouse intestinal IgA response (Klaasen et al., 1993; Talham et al., 1999) and induces the recruitment of intraepithelial lymphocytes (IEL) (Umesaki et al., 1995). The role of this bacterium was tested with mice colonized with SFB previously purified from the mouse ileum and kept as a single species by passage in GF mice (Klaasen et al., 1991).

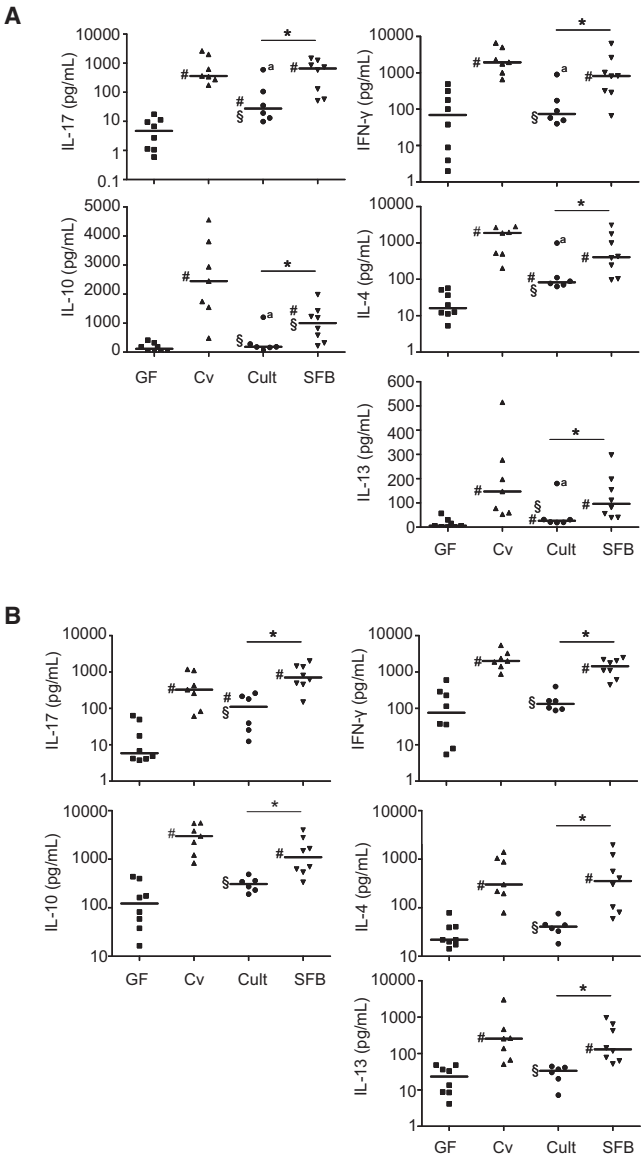

Figure 2. Cytokine Synthesis by Ileal and Colonic LPL Depends on the Composition of Intestinal Microbiota

Ileal (A) and colonic (B) LPL were isolated from GF and Cv mice and from mice colonized for 20 days with SFB or the cultured mouse microbiota (Cult). After a 72 hr stimulation by anti-CD3+CD28, cytokines were measured in culture supernatants by BioPlex. Data represent individual values and medians (n = 6–8/group). #, §, and *, $p < 0.05$ by a nonparametric Kruskal-Wallis test as compared to GF, Cv, and SFB, respectively. One Cult mouse (a) exhibited stronger ileal responses than other animals in the same group. In all groups, cytokine production by unstimulated LPL was <20 pg/mL (not shown).

SFB Is a Key Inducer of Intestinal T Cell Responses

Colonization of GF mice by SFB was first ascertained by RT-PCR with specific primers. A strong positive signal (absent in GF mice) was observed on d8 and d20 postcolonization, which decreased on d60 (Figures 4B and 4C). Analysis of cecum contents by temporal temperature gel electrophoresis indicated monocolonization by SFB (not shown). The presence of SFB was confirmed by scanning microscopy, which showed numerous bacteria with the typical rod-like and filamentous morphology anchored into ileal epithelial cells on d20 postcolonization (Figure 4D). Consistent with PCR data and previous observations (Snel et al., 1998), few adherent bacteria remained visible in the ileum on d60, although they were found in large amounts in the cecum.

The study of monocolonized mice demonstrated the major inducing role of SFB. Affymetrix analysis of all differentially expressed genes revealed closest similarity between SFB-colonized and Cv mice at d8 postcolonization (Figure S3). Analysis at this time point showed strong clustering between Cv and Cvd mice on the one hand and GF and Hum mice on the other hand, with SFB mice occupying an intermediate position (Figure 4E). The immune pathway most affected in Cv, Cvd, and SFB mice was related to bacterial interactions at mucosal surfaces and involved Toll-like receptor (TLR) signaling, induction of *RegIIIg* defensin mRNA, IFN-γ, and IL-1β driving Th1 cell events (Table S1 and Figure S4). Analysis of ileal cytokine transcripts by qRT-PCR showed a progressive increase in *Il10* and *Ifng* mRNA in SFB mice comparable to those in Cvd mice at d60 postcolonization, a striking increase in *Il17* mRNA expression similar at d8 to the one observed at d20 in Cvd mice and in mice colonized with the sporulated fraction of the microbiota (Figure 4A). Perhaps because of the reduction of adherent SFB in the ileum over time, *Il17* mRNA expression decreased on d60. This suggestion was consistent with Affymetrix data showing divergent responses between SFB and Cv mice at d60 (Figure S3). *Foxp3* mRNA increased 3.4-fold on d8 but also decreased at later time points. These changes correlated with the induction of *Cd3e* mRNA and an increase in $CD3^+$ T cells in ileal tissue sections (Figure 4A; Figure S1). Among innate markers, changes as compared to GF mice were noted for *Nos2* mRNA which increased 3.4-fold on d8 and 6-fold on d60, and *Il12p40* mRNA increased at later time points (32-fold on d60), particularly in mice with high *Ifng* mRNA expression (not shown). As previously observed in Cv mice, the increase in cytokine mRNA expression compared to GF mice was much less in the colon (Table S2).

Analysis of isolated LPL confirmed that colonization by SFB simultaneously induced strong Th1, Th2, Th17, and regulatory T cell responses. Thus, ileal and colonic LPL isolated from SFB mice on d20 (Figure 2) and d60 postcolonization (not shown) released amounts of IL-17, IFN-γ, IL-4, and IL-13 comparable to LPL isolated from Cv mice. A robust release of IL-10 was also observed, although secretion by ileal LPL was less than in Cv mice. Strikingly, median concentrations of cytokines released by LPL were 5- to 11-fold higher in SFB than in Cult mice and even 25-fold higher for IL-17 in the ileum (Figure 2). Flow cytometry analysis of LPL from small intestine and colon indicated that, in SFB as in control Cv mice, more than 95% of IL-17-producing cells were $CD4^+TCR\text{-}\alpha\beta^+$ lymphocytes (80%–85% of $CD3^+$ LPL). These cells, scarce or absent in GF mice, increased 30- and 100-fold, respectively, in the small intestine and colon of SFB mice at d20 (Figure 5). The majority of IFN-γ- and IL-10-producing cells were also $CD4^+$ T cells. They were present in GF mice, but their absolute numbers increased in SFB and Cv mice (Figure 5). $CD8^+TCR\text{-}\alpha\beta^+$ cells (~15% of $CD3^+$ LPL) contained rare $IL\text{-}10^+$ cells (0.5%, 1.5%, and 2.5% in GF, SFB, and Cv mice, respectively) and more numerous $IFN\text{-}\gamma^+$ cells (~15% in GF and SFB

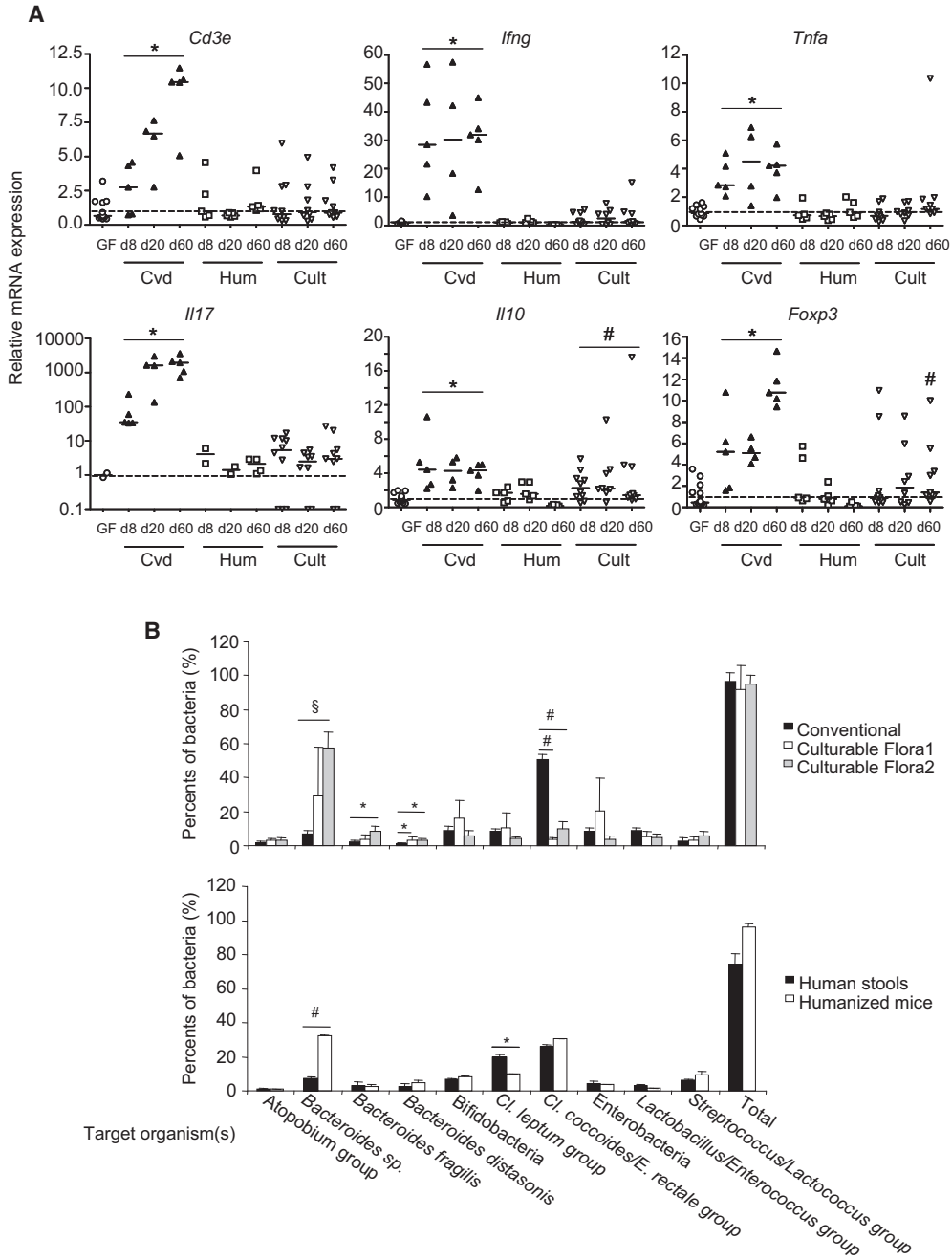

Figure 3. A Restricted Number of Host-Specific Microbial Species Induce Mouse Intestinal Adaptive T Cell Responses

(A) Ileal gene mRNA expression was analyzed by qRT-PCR on d8, d20, and d60 postcolonization in Cvd, Hum (n = 4–5/group), and Cult (n = 8–10, data pooled from two independent experiments) mice. mRNA expression is relative to GF mice (defined as 1; dotted line; n = 10). Data represent individual values and medians. *p < 0.01, #p < 0.05 by a nonparametric Kruskal-Wallis test as compared to GF.

(B) Bacterial composition in feces of Cult mice (n = 3; two independent experiments with Flora1 [open bar] and Flora2 [gray bar] are shown) and Hum mice (n = 4; open bar) on d20 was analyzed by FISH and compared with that in Cv mice (n = 4; black bar; upper panel) or original human stools (black bar; lower panel), respectively. Results in Cv mice are representative of three independent experiments. Bar graphs show mean ± SD. *p < 0.05, #p < 0.001, §p < 0.005 by a nonparametric Kruskal-Wallis test as compared to initial equilibrium in Cv feces or human stools.

mice and 25% in Cv mice). IFN-γ was also detected in 15%–25% of $CD3^{-}CD11c^{+}$ cells, but the contribution of this subset to local IFN-γ production is minor because they represent ~1% of $CD45^{+}$ LPL (not shown). Notably, a comparable proportion of $CD4^{+}CD25^{+}FoxP3^{+}$ LPL was present in the small intestine and colon of GF mice and of mice colonized with SFB for 20 days, but their numbers increased 2- to 3-fold in both sites in the later mice (Figure 5; Figure S5).

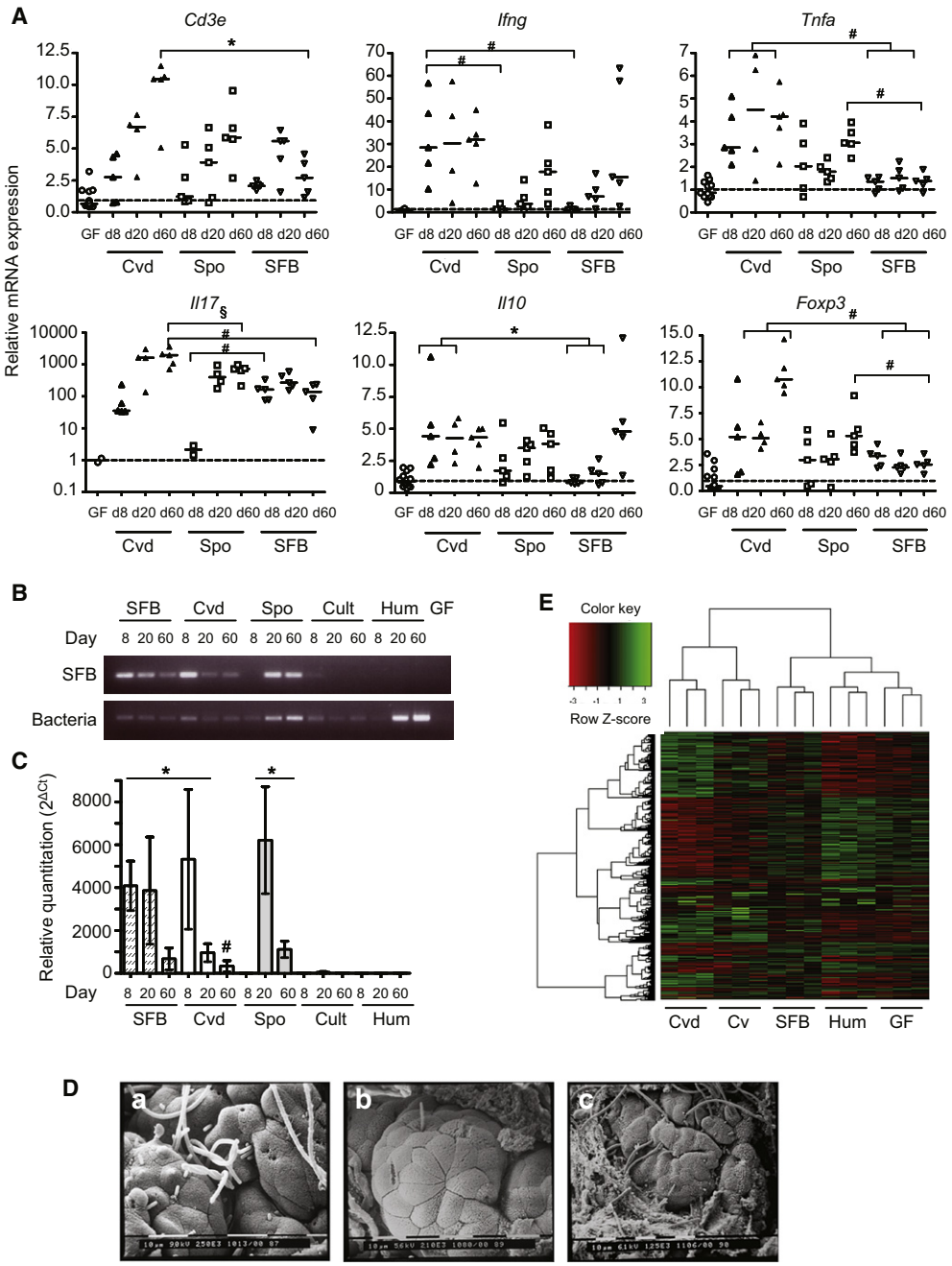

Figure 4. SFB Is Central to the Induction of Mouse Intestinal Immune Responses

(A) Ileal gene mRNA expression was analyzed in Cvd mice and in mice colonized with either the sporulated fraction of the fecal microbiota (Spo) or SFB (n = 4–5 mice/group). #$p < 0.01$, *$p < 0.02$, §$p < 0.05$ for intergroup comparison by a nonparametric Kruskal-Wallis test.

(B and C) Ileal tissues from SFB, Cvd, Spo, Cult, and Hum mice were analyzed by qRT-PCR with SFB- and total bacteria-specific 16S rRNA primers (SFB and Bacteria, respectively) at d8, d20, and d60 postcolonization and in age-matched GF mice. Results are presented as amount of target accumulated at end-point of PCR (B) and as a relative quantity assessed by the threshold cycle (Ct) method ($2^{-\Delta Ct}$) compared to GF calibrator (C). Bar graphs show mean ± SEM (n = 4–5 mice/group). *$p < 0.01$, #$p < 0.05$ as compared to GF.

(D) Scanning electron microscopy of intestine in mice colonized with SFB: (a) ileum on d20, ileum (b) and cecum (c) on d60.

(E) Heatmap generated from the list of significantly expressed genes ($p < 0.05$) between all treatments after 8 days. The relative value for each gene is expressed as in Figure 1.

Collectively, these results indicated that SFB was a critical component of the normal microbiota able to efficiently drive the coordinated maturation of intestinal T cell adaptive functions. To further demonstrate that SFB was pivotal to the immunostimulatory properties of the flora, SFB presence was assessed by specific qRT-PCR in the different models of gnotobiotic

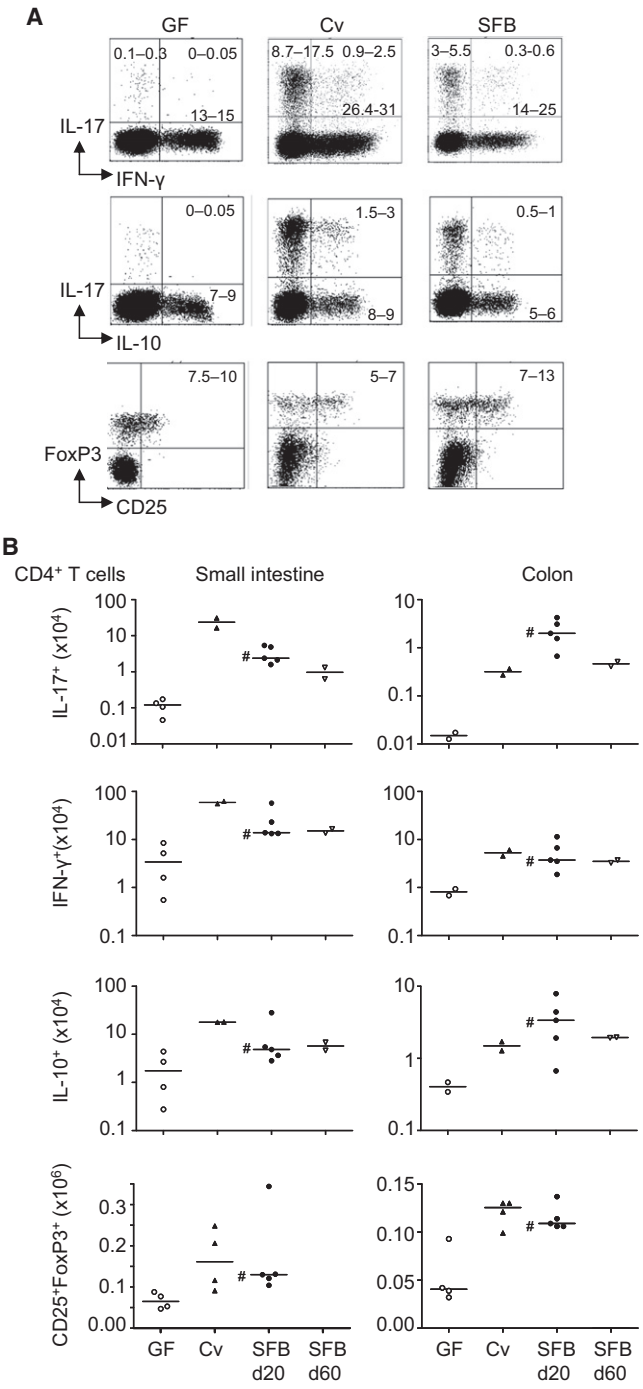

Figure 5. SFB Induces a Wide Spectrum of Intestinal CD4$^+$ T Cell Responses

(A) Small intestinal LPL from mice GF, Cv, and colonized with SFB for 20 days were stimulated for 4 hr with PMA+ionomycin in the presence of brefeldin A, and viable CD45$^+$ cells were examined for intracellular expression of IL-17, IL-10, IFN-γ, and FoxP3 and membrane expression of CD3, CD8, CD4, CD25, TCR-αβ, and CD11c. Plots are gated on CD4$^+$ T cells. Numbers in the quadrants indicate ranges of positive cell percentages (n = 2–5 mice/group).

(B) Absolute numbers of cytokine- and CD25$^+$FoxP3-positive CD4 T cells in the small intestine and colon. Individual values and medians are shown. #p < 0.05 compared with GF by a nonparametric Kruskal-Wallis test.

mice. As shown in Figures 4B and 4C, a strong signal for SFB was detected in the ileum of all gnotobiotic mice developing innate and/or adaptive T cell responses whereas no signal was observed in mice colonized with the human or the mouse culturable flora. Interestingly, in mice colonized with the sporulated flora, which show very low or no T cell response before d20, the SFB signal was undetectable on d8 and became detectable only at later time points. Altogether these results support the hypothesis of the key role of SFB in the induction of intestinal adaptive T cell responses.

SFB Is a Privileged Inducer of PP T Cell Responses

Previous studies have provided strong evidence that adaptive T cell responses to the gut microbiota are initiated in PP (Guy-Grand et al., 1974). Pioneering work by Cebra's group associated the strong induction of intestinal IgA by SFB (confirmed herein in Figure S6) with its elective capacity to stimulate germinal center B cells and CD4$^+$CD45RB^{lo} T cells in PP (Talham et al., 1999). Scanning microscopy revealed a striking adherence of SFB to the surface of PP in mice monoassociated with SFB whereas, in Cult mice, only small numbers of bacteria were observed in the vicinity of PP, most if not all of which were non-adherent and associated with the mucus (Figure 6A). Consistent with the hypothesis that adherence to the PP might be central to promoting the initiation of T cell responses, monocolonization with SFB resulted in the strong induction of mRNA encoding *Il17*, *Il10*, and *CD40 ligand* (*Cd40l*), and a more modest increase in *Ifng* mRNA in PP (Figure 6B). These changes were comparable to those observed in Cvd mice and contrasted with the lack of detectable mRNA induction in PP of Cult mice. CD3+CD28-stimulated PP lymphocytes from Cvd, SFB, and Cult mice synthesized increased amounts of IFN-γ, IL-17, and IL-10 compared to GF mice (Figure 6C). The amount of IL-10 and IL-17 produced by PP lymphocytes was, however, approximately 10-fold higher in SFB than in Cult mice and therefore comparable to those in Cvd mice. Altogether these data stressed the elective immunostimulatory effect of SFB on PP.

DISCUSSION

The compelling role of the immune system in "tolerating" the gut microbiota has been greatly re-enforced by the increasing evidence that defects in both immune response and microbial recognition genes are central to the onset of inflammatory bowel diseases in genetically predisposed individuals (Bouma and Strober, 2003). Our study, indicating that almost 50% of the genes regulated in response to microbial colonization cluster to immune response pathways, further underscores the central role of the immune system in the dialog between the host and its microbiota. Consistent with most recent reports (Atarashi et al., 2008; Ivanov et al., 2008; Niess et al., 2008; Zaph et al., 2008), we confirm that the gut microbiota is a potent stimulus of Th17 cell differentiation. In addition, we demonstrate that intestinal colonization induces a striking range of T cell functions and simultaneously stimulates T cells of Th17 but also Th1, Th2, and regulatory cell phenotype. Previous studies have suggested a large functional redundancy in the capacity of microbial species to elicit host innate epithelial cell defenses (Artis, 2008) and secretory IgA responses (Macpherson, 2006). Our work provides

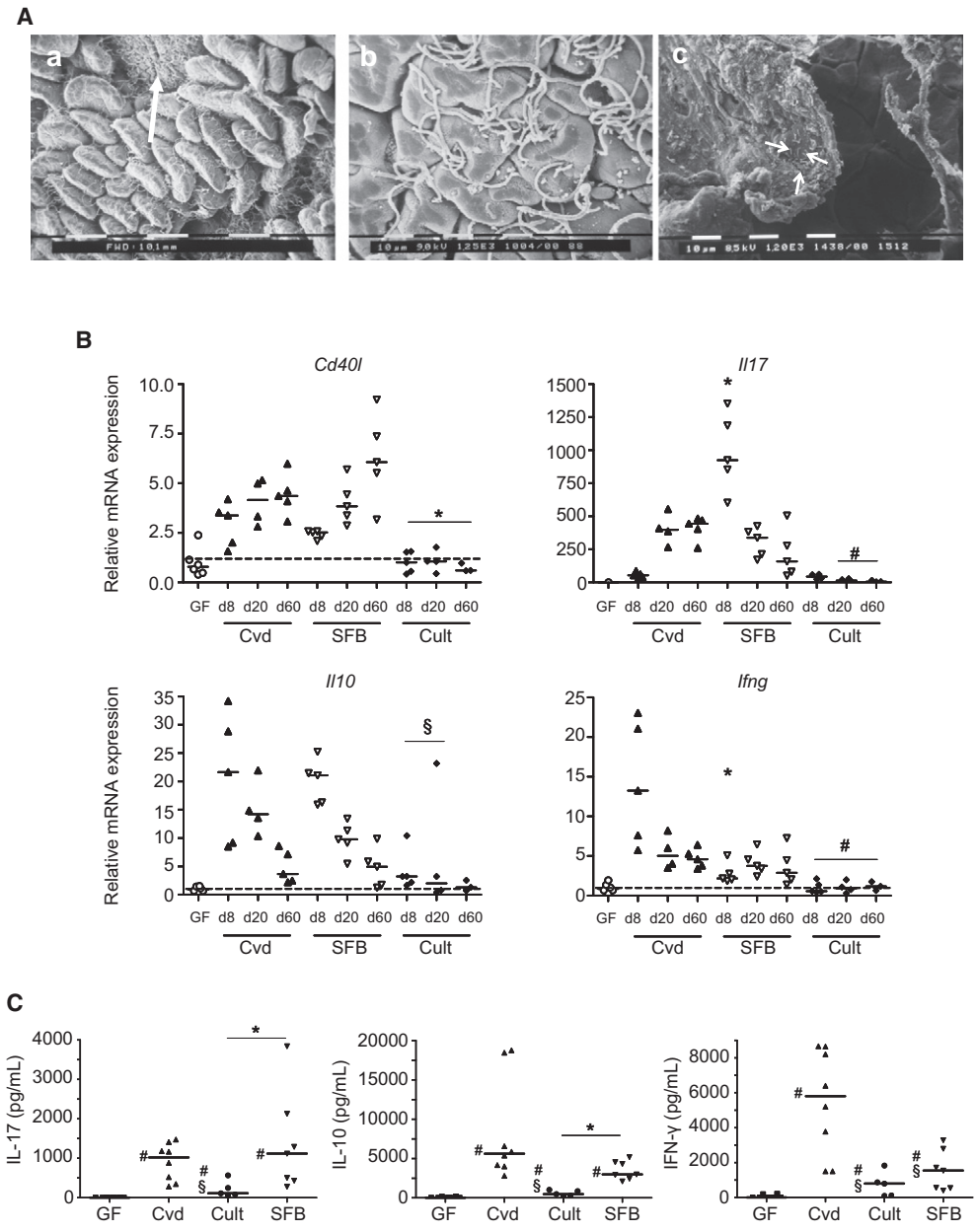

Figure 6. SFB Stimulates Peyer's Patches T Cell Responses

(A) Scanning electron microscopy of PP area in mice colonized with SFB (a and b) or Cult flora (c) for 20 days. SFB are seen adherent to PP (a; large arrow) or entering the PP epithelium (b) while bacteria from the Cult flora are seen in the mucus away from the epithelium surface (c; thin arrows).

(B) mRNA expression was analyzed by qRT-PCR in PP of Cvd, SFB, and Cult mice at indicated days postcolonization. Individual values and medians are shown. Data are expressed as in Figure 3. *$p < 0.01$, #$p < 0.02$, §$p < 0.05$ by a nonparametric Kruskal-Wallis test when compared to Cvd mice.

(C) Cells isolated from PP of Cvd, SFB, and Cult mice on d20 postcolonization or age-matched GF mice were stimulated by anti-CD3+CD28 and cytokine production assessed by BioPlex. Individual values and medians are shown. #$0.001 < p < 0.05$, compared with GF. §$p < 0.05$, *$p < 0.01$ as compared with Cv or SFB, respectively.

unexpected evidence that, in immunocompetent mice, efficient maturation of innate and adaptive gut immune T cell responses depends on selected host-specific species enriched in the unculturable *Clostridia*-related fraction of the microbiota. One prototype strain is SFB, which can alone orchestrate the wide repertoire of T cell responses inducible by a total microbiota.

Analysis of mRNA expression in the whole gut tissue was performed to obtain an instant view of local immune reactions at different intestinal sites in response to the colonizing microbiota. One surprising observation was the dominant impact of the normal complete mouse microbiota on ileal mRNA responses. Whereas the moderate duodenal response may be explained

by the low local concentration of bacteria, the weak response observed in the cecum and colon is paradoxical given the much higher bacterial density. Expansion of cytokine-producing $CD4^+$ T cells was, however, observed in the colonic LP of Cv mice, indicating that T cells primed upon bacterial exposure can migrate into the colonic LP but that they remain quiescent in physiological conditions. This may be explained by the very limited access of bacteria to the colonic surface, which is protected by an inner firmly adherent and dense layer of sterile mucus not found in the ileum (Johansson et al., 2008).

Recognizing the ileum as a good indicator of the capacity of the microbiota to stimulate maturation of mucosal effector T cell responses, we analyzed this gut segment to evaluate the respective immune-potentiating role of individual bacteria and/or of complex flora. Unexpectedly, this analysis pointed out the privileged role of host-specific unculturable *Clostridia*-related species and particularly of SFB. In striking contrast to colonization with human fecal and mouse culturable microbiota, monocolonization with SFB resulted in a rapid upregulation of a broad range of ileal transcripts. Analysis of isolated LPL confirmed that SFB alone could orchestrate the differentiation of a large spectrum of both proinflammatory and regulatory $CD4^+$ T cells that distributed into the small and large intestines. The broad effect of SFB seems at odds with recent work suggesting that distinct individual bacteria have a specific impact on IL-10 or IL-17 responses, respectively (Ivanov et al., 2008; Mazmanian et al., 2008; Sokol et al., 2008). Yet, recent studies have also emphasized the plasticity of helper T cells and shown that a common RORγt precursor can differentiate into either Th17 or regulatory T cells depending on the local concentrations of IL-6 (Lochner et al., 2008; reviewed in Zhou et al., 2009), whereas conversion of Th17 into Th1 cells can be obtained in the presence of IL-12 (Lee et al., 2009). Consistent with this hypothesis, small subsets of double-positive IL-17-IL-10 and IL-17-IFN-γ cells were detected in LPL. It is therefore conceivable that a spectrum of $CD4^+$ T cell subsets can arise from undifferentiated Th cells in response to SFB. This is not contradictory with a more specific function of other bacteria. Induction of *Foxp3* and/or *Il10* mRNA was observed in a few mice colonized with culturable flora, stressing the possibility that some culturable species alone or in association with SFB may promote the induction of regulatory T cells. Finally, our data do not exclude that some other species either culturable or nonculturable share some of the immunostimulatory properties of SFB. Thus, a substantial induction of cytokine-producing cells was observed in the ileum of one Cult mouse.

Previous studies in gnotobiotic adult mice have demonstrated the potent inducing effect of SFB on the intestinal IgA response (Talham et al., 1999) as well as on the activation and recruitment of $CD8\alpha\beta^+$ TCR-$\alpha\beta^+$ IEL (Umesaki et al., 1995). Our present data unravel a unique function of SFB in the induction of intestinal $CD4^+$ T cell responses. Microarray data also suggest that SFB can elicit *RegIIIg*, a microbicidal lectin produced by enterocytes and Paneth cells that contributes to host innate protection against Gram-positive bacteria (Vaishnava et al., 2008). Altogether, these results suggest a unique role of SFB in coordinating the postnatal maturation of gut immune functions. Colonization of adult GF mice is unlikely to fully recapitulate colonization of newborn mice. Yet, SFB is detected strongly attached to the ileum of rodent species at time of weaning, coincident with the disappearance of IgA provided by maternal milk and when the postnatal maturation of the gut adaptive immune system is initiated (Klaasen et al., 1992). These observations support the notion that the immunostimulatory effects of SFB can operate during physiological colonization from birth.

The mechanisms underlying the prominent role of SFB in the induction of mucosal adaptive immune responses remain to be elucidated. Strikingly, scanning electron microscopy demonstrated that SFB, in contrast to bacteria present in the culturable flora, developed tight interactions with the luminal surface of PP. Extending previous observations (Talham et al., 1999), we showed that colonization by SFB induced a robust stimulation of PP almost comparable to that observed in Cvd mice and much stronger than that induced by the culturable flora. The elective capacity of SFB to adhere and presumably enter PP may be instrumental in promoting intestinal adaptive immune responses. Thus, a recent study in mice indicates that the capacity of invasive *Salmonella* to drive a specific, presumably T cell-dependent, IgA response relies solely on their ability to enter into PP (Martinoli et al., 2007). Along similar lines, the efficient induction of pathogen-specific T cells in response to *Salmonella* required the activation of the specialized subset of $CCR6^+$ dendritic cells present in the dome of PP (Salazar-Gonzalez et al., 2006). The host specificity in SFB attachment (Klaasen et al., 1992) may thus explain the lack of mRNA cytokine induction in response to a whole human flora. Altogether, these data support the hypothesis of a specific receptor(s) or adherence factor(s), the identification of which may have to await sequencing of this unculturable bacterium. The additional contribution of innate immune receptors is, however, highlighted by the transcriptional upregulation of genes encoding innate cytokines and of *RegIIIγ*, induced in Paneth cells via MyD88-dependent signals (Vaishnava et al., 2008). Moreover, the gene pathway analysis performed on the ileal tissues points out the activation of TLR2 and TLR1 signaling. The specificity of SFB-induced immune responses remains elusive. In preliminary experiments, LPL did not proliferate or produce cytokines in response to sonicates of SFB purified from the cecal content (data not shown). Such extracts may not be appropriate to assess T cell responses. Yet, a striking contrast between the high amounts of total IgA induced by SFB and their very low specificity (~1%) has been reported, suggesting a prominent polyclonal effect of SFB (Talham et al., 1999).

Another unsolved question concerns the selective pressures that have led to the persistence of SFB in the intestine of all studied species so far. Avoidance of proinflammatory responses is a strategy expected to be beneficial both for the host and for their commensal bacteria. Yet some bacteria may utilize the host inflammatory responses to eliminate competitors and promote microniche colonization (Stecher et al., 2007). SFB may operate in this way within the gut ecosystem. Clearly, the degree of inflammation induced by SFB in an immunocompetent host is too low to result in intestinal damage but may provide an important advantage to protect the host. In this context, it is noticeable that individuals with either T cell or IFN-γ deficiencies are at risk of developing severe local or systemic infections (Bustamante et al., 2008). Production of moderate amounts of IFN-γ and IL-17 may help optimizing local protective bactericidal

mechanisms and reducing infection risk in immunocompetent hosts. Conversely, RegIIIγ and the strong IgA response induced by SFB may provide feedback mechanisms that limit the population size of SFB and avoid excessive local proinflammatory responses (Fagarasan et al., 2002; Suzuki et al., 2004). Interestingly, no colitis was observed in SCID mice reconstituted with $CD45RB^{hi}$ $CD4^+$ T cells and colonized with either SFB alone or a specific pathogen-free flora, but inflammation was triggered upon cocolonization (Stepankova et al., 2007). SFB might thus become pathogenic in genetically predisposed hosts depending on the local bacterial environment and perhaps participate in inflammatory bowel diseases, notably Crohn's disease.

In conclusion, the maturation of intestinal T cell responses in an immunocompetent host appears the prerogative of a restricted number of commensal species able to develop host-specific interactions. A prototype strain is SFB, which strongly adheres to the ileal mucosa and to PP. That only restricted members of the normal flora can stimulate mucosal T cell responses supports the notion that changes in the infant flora may influence the development of host immune responses and thereby the susceptibility to inflammatory and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

All procedures were carried out in accordance with European guidelines. Cv and GF C3H/HeN mice were bred at the INRA facilities in Jouy-en-Josas. GF and gnotobiotic mice were maintained in plastic isolators and fed ad libitum on a commercial diet (R03-40; UAR) sterilized by γ-irradiation (40 kGy).

Colonization Experiments

GF female 8- to 9-week-old mice were gavaged twice at a 24 hr interval with 0.5 ml of: fresh anaerobic cultures of *Escherichia coli* MG1655, *Bacteroides thetaiotaomicron* (ATCC 29148), *Bacteroides vulgatus* L16 (UEPSD collection), or ASF-derived *Clostridium* strains 502, 500, and 356 (Taconic), fecal homogenate from SFB monoassociated mice (Klaasen et al., 1991), a 10^{-2} dilution of fresh feces from Cv mice (Cvd) or from a healthy human donor (Hum), and a 10^{-7} dilution of a fresh mouse fecal homogenate cultured for 4 days at 37°C on brain heart infusion agar plates (BHI; Difco) (Cult). The sporulated fraction (Spo) was prepared by heating the 10^{-2} dilution of fresh Cv mouse feces at 70°C for 10 min. All samples were prepared in an anaerobic chamber. Gnotobiotic mice were sacrificed on d8, d20, and d60 postcolonization in parallel to age-matched Cv and GF controls.

Colonization was monitored by microscopic examination of the feces and/or bacterial counts after culture on nonselective BHI medium. FISH analyses were performed with a series of bacterial probes on paraformaldehyde-fixed fecal suspensions as described (Lan et al., 2007). For SFB monitoring, DNA was extracted from frozen ileal biopsies and 16S rRNA amplified by qRT-PCR via specific primer pairs for SFB or total bacteria on an ABI Prism 7300 Sequence Detection System (Applied-Biosystems) according to a protocol adapted from Suzuki et al. (2004). As RT-PCR efficiency, determined on serial 10-fold dilutions of DNA samples, was close to 100%, the amount of SFB was quantified with a comparative threshold cycle (Ct) method ($2^{-\Delta Ct}$) with a GF calibrator.

Microarray Hybridizations and Data Analysis

Ileal tissue was lysed in Trizol (Invitrogen) and total RNA was extracted and cleanup with the RNeasy kit and RNase-free DNase I (QIAGEN) digestion based on the manufacturer's protocol. RNA integrity was determined by Agilent 2100 Bioanalyzer (Agilent Technologies). Five micrograms of total RNA was reverse transcribed to cDNA and then transcribed into biotin-labeled cRNA with the One-Cycle Target Labeling Kit (Affymetrix). Hybridization was made to the NuGO Mouse Array (NuGO_Mm1a520177) on a GeneChip Fluidics Station 450 (Affymetrix). Chips were scanned with an Affymetrix GeneChip Scanner 3000 and Image quality analysis performed with Gene Chip Operating Software.

Microarray analysis was performed with the software packages R (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org). The data were normalized with gcRMA and subsequently adjusted for multiple testing by the Benjamini and Hochberg false discovery method ($p < 0.05$). Heatmaps were generated with the R-package *gplots* with gene subsets created from the list of significant genes. For in-depth functional analysis of the microarray data, all differentially expressed genes ($p < 0.05$) were imported into MetaCore analytical software (GeneGo) to generate pathway maps based on p values calculated with hypergeometric distribution.

Quantitation of Gene Expression with Real-Time PCR

RNA was purified from intestinal tissues and PPs as described above. qRT-PCR was performed with either TaqMan gene expression assays with TaqMan Universal PCR master mix (Applied Biosystems) or mouse-specific primers with SYBR-Green PCR master mix (Applied Biosystems) for *Ifng*, *Foxp3*, *Il12p40*, *Nos2*, *Cxcl10*, and *Cxcl1* (sequences available on demand). cDNA samples were assayed in duplicate and gene expression levels for each sample were normalized relative to TATA-box Binding Protein or to Transferrin Receptor with ΔCt calculation.

Analysis of Cytokine Production by Lamina Propria Lymphocytes

After excision of PP, mouse duodeno-jejunum, ileum, and colon were washed in PBS and cut into 0.5 cm pieces incubated four times in 30 mL of PBS-3 mM EDTA (Sigma) for 10 min at 37°C and digested in 30 mL of RPMI 1640 added with 20% SVF, 100 U/mL of collagenase (Sigma), and 50 U/mL of DNase I (Sigma) for 40 min at 37°C. LPL were purified on a 40%–80% Percoll gradient run for 15 min at 2000 × g and cultured for 72 hr in 24-well plates (1.10^6 cells/well) coated overnight with anti-CD3 and anti-CD28 (5 μg/mL; BD Biosciences). Cytokines were measured in the supernatants with BioPlex assay (BioRad) according to manufacturer's instructions. Flow cytometry analysis of surface antigens coupled or not with intracellular staining of FoxP3, IL-17, IFN-γ, and IL-10 producing LPL after stimulation with 100 ng/mL phorbol 12-myristate 13-acetate and 1 μg/mL ionomycin (both from Sigma) was performed as described (Lochner et al., 2008). Absolute numbers of $CD4^+$ T cells were determined with TruCount tubes according to manufacturer's instructions (BD Biosciences). The list of antibodies is given in Supplemental Data.

Scanning Electron Microscopy

Segments from terminal ileum and mid-colon were fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer, dehydrated, and processed as described (Brandi et al., 1996).

Statistics

Statistical analysis was performed with GraphPad Prism software. The nonparametric Kruskal-Wallis test was used for comparing data; differences were considered significant for $p < 0.05$.

ACCESSION NUMBERS

Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with the accession number GSE18056.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00404-X.

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REFERENCES

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat. Rev. Immunol. *8*, 411–420.

Atarashi, K., Nishimura, J., Shima, T., Umesaki, Y., Yamamoto, M., Onoue, M., Yagita, H., Ishii, N., Evans, R., Honda, K., and Takeda, K. (2008). ATP drives lamina propria TH17 cell differentiation. Nature *455*, 808–812.

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I. (2005). Host-bacterial mutualism in the human intestine. Science *307*, 1915–1920.

Bouma, G., and Strober, W. (2003). The immunological and genetic basis of inflammatory bowel disease. Nat. Rev. Immunol. *3*, 521–533.

Brandi, G., Pisi, A., Biasco, G., Miglioli, M., Biavati, B., and Barbara, L. (1996). Bacteria in biopsies of human hypochlorhydric stomach: A scanning electron microscopy study. Ultrastruct. Pathol. *20*, 203–209.

Bustamante, J., Boisson-Dupuis, S., Jouanguy, E., Picard, C., Puel, A., Abel, L., and Casanova, J.-L. (2008). Novel primary immunodeficiencies revealed by the investigation of paediatric infectious diseases. Curr. Opin. Immunol. *20*, 39–48.

Fagarasan, S., Muramatsu, M., Suzuki, K., Nagaoka, H., Hiai, H., and Honjo, T. (2002). Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science *298*, 1424–1427.

Giraud, A., Arous, S., De Paepe, M., Gaboriau-Routhiau, V., Bambou, J.C., Rakotobe, S., Lindner, A.B., Taddei, F., and Cerf-Bensussan, N. (2008). Dissecting the genetic components of adaptation of *Escherichia coli* to the mouse gut. PLoS Genet *4*, 52–61.

Guy-Grand, D., Griscelli, C., and Vassalli, P. (1974). The gut-associated-lymphoid system: Nature and properties of the large dividing cells. Eur. J. Immunol. *4*, 435–443.

Hooper, L.V., Xu, J., Falk, P.G., Midtvedt, T., and Gordon, J.I. (1999). A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc. Natl. Acad. Sci. USA *96*, 9833–9838.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. Science *291*, 881–884.

Ivanov, I.I., Frutos Rde, L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., and Littman, D.R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe *4*, 337–349.

Johansson, M.E.V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., and Hansson, G.C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc. Natl. Acad. Sci. USA *105*, 15064–15069.

Kirjavainen, P.V., Arvola, T., Salminen, S.J., and Isolauri, E. (2002). Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? Gut *51*, 51–55.

Klaasen, H., Koopman, J., Van den Brink, M., Van Wezel, H., and Beynen, A. (1991). Mono-association of mice with non-cultivable, intestinal, segmented, filamentous bacteria. Arch. Microbiol. *156*, 148–151.

Klaasen, H., Koopman, J., Poelma, F., and Beynen, A. (1992). Intestinal, segmented, filamentous bacteria. FEMS Microbiol. Rev. *8*, 165–180.

Klaasen, H.L., Van der Heijden, P.J., Stok, W., Poelma, F.G., Koopman, J.P., Van den Brink, M.E., Bakker, M.H., Eling, W.M., and Beynen, A.C. (1993). Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice. Infect. Immun. *61*, 303–306.

Lan, A., Bruneau, A., Philippe, C., Rochet, V., Rouault, A., Herve, C., Roland, N., Rabot, S., and Jan, G. (2007). Survival and metabolic activity of selected strains of *Propionibacterium freudenreichii* in the gastrointestinal tract of human microbiota-associated rats. Br. J. Nutr. *97*, 714–724.

Lee, Y.K., Turner, H., Maynard, C.L., Oliver, J.R., Chen, D., Elson, C.O., and Weaver, C.T. (2009). Late developmental plasticity in the T helper 17 lineage. Immunity *30*, 92–107.

Ley, R.E., Peterson, D.A., and Gordon, J.I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell *124*, 837–848.

Lochner, M., Peduto, L., Cherrier, M., Sawa, S., Langa, F., Varona, R., Riethmacher, D., Si-Tahar, M., Di Santo, J.P., and Eberl, G. (2008). In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. J. Exp. Med. *205*, 1381–1393.

Macpherson, A.J. (2006). IgA adaptation to the presence of commensal bacteria in the intestine. Curr. Top. Microbiol. Immunol. *308*, 117–136.

Macpherson, A.J., and Harris, N.L. (2004). Interactions between commensal intestinal bacteria and the immune system. Nat. Rev. Immunol. *4*, 478–485.

Macpherson, A.J., and Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science *303*, 1662–1665.

Martinoli, C., Chiavelli, A., and Rescigno, M. (2007). Entry route of *Salmonella typhimurium* directs the type of induced immune response. Immunity *27*, 975–984.

Mazmanian, S.K., Round, J.L., and Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature *453*, 620–625.

Niess, J.H., Leithauser, F., Adler, G., and Reimann, J. (2008). Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. J. Immunol. *180*, 559–568.

Peterson, D.A., McNulty, N.P., Guruge, J.L., and Gordon, J.I. (2007). IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe *2*, 328–339.

Round, J.L., and Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. *9*, 313–323.

Salazar-Gonzalez, R.M., Niess, J.H., Zammit, D.J., Ravindran, R., Srinivasan, A., Maxwell, J.R., Stoklasek, T., Yadav, R., Williams, I.R., Gu, X., et al. (2006). CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. Immunity *24*, 623–632.

Snel, J., Heinen, P.P., Blok, H.J., Carman, R.J., Duncan, A.J., Allen, P.C., and Collins, M.D. (1995). Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "*Candidatus Arthromitus*". Int. J. Syst. Bacteriol. *45*, 780–782.

Snel, J., Hermsen, C.C., Smits, H.J., Bos, N.A., Eling, W.M.C., Cebra, J.J., and Heidt, P.J. (1998). Interactions between gut-associated lymphoid tissue and colonization levels of indigenous, segmented, filamentous bacteria in the small intestine of mice. Can. J. Microbiol. *44*, 1177–1182.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L.G., Gratadoux, J.J., Blugeon, S., Bridonneau, C., Furet, J.P., Corthier, G., et al. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc. Natl. Acad. Sci. USA *105*, 16731–16736.

Stecher, B., Robbiani, R., Walker, A.W., Westendorf, A.M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A.J., Buer, J., Parkhill, J., et al. (2007). *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS Biol. *5*, 2177–2189.

Stepankova, R., Powrie, F., Kofronova, O., Kozakova, H., Hudcovic, T., Hrncir, T., Uhlig, H., Read, S., Rehakova, Z., Benada, O., et al. (2007). Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. Inflamm. Bowel Dis. *13*, 1202–1211.

Suzuki, K., Meek, B., Doi, Y., Muramatsu, M., Chiba, T., Honjo, T., and Fagarasan, S. (2004). Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc. Natl. Acad. Sci. USA *101*, 1981–1986.

Talham, G.L., Jiang, H.-Q., Bos, N.A., and Cebra, J.J. (1999). Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect. Immun. *67*, 1992–2000.

Umesaki, Y., Okada, Y., Matsumoto, S., Imaoka, A., and Setoyama, H. (1995). Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and

fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. Microbiol. Immunol. *39*, 555–562.

Vaishnava, S., Behrendt, C.L., Ismail, A.S., Eckmann, L., and Hooper, L.V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc. Natl. Acad. Sci. USA *105*, 20858–20863.

Zaph, C., Du, Y., Saenz, S.A., Nair, M.G., Perrigoue, J.G., Taylor, B.C., Troy, A.E., Kobuley, D.E., Kastelein, R.A., Cua, D.J., et al. (2008). Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. J. Exp. Med. *205*, 2191–2198.

Zhou, L., Chong, M.M., and Littman, D.R. (2009). Plasticity of CD4+ T cell lineage differentiation. Immunity *30*, 646–655.