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J Immunol 2007; 178:1809-1818; ;
doi: 10.4049/jimmunol.178.3.1809
<http://www.jimmunol.org/content/178/3/1809>

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Commensal Bacteria Exacerbate Intestinal Inflammation but Are Not Essential for the Development of Murine Ileitis¹

Giorgos Bamias,² Akira Okazawa,² Jesus Rivera-Nieves, Kristen O. Arseneau, Sarah A. De La Rue, Theresa T. Pizarro, and Fabio Cominelli³

The pathogenesis of Crohn's disease has been associated with a dysregulated response of the mucosal immune system against intraluminal Ags of bacterial origin. In this study, we have investigated the effects of germfree (GF) conditions in the SAMP1/YitFc murine model of Crohn's disease-like ileitis. We show that the bacterial flora is not essential for ileitis induction, because GF SAMP1/YitFc mice develop chronic ileitis. However, compared with disease in specific pathogen-free (SPF) mice, ileitis in GF mice is significantly attenuated, and is associated with delayed lymphocytic infiltration and defective mucosal expression of Th2 cytokines. In addition, we demonstrate that stimulation with purified fecal Ags from SPF, but not GF mice leads to the generation of IL-4-secreting effector lymphocytes. This result suggests that commensal bacteria drive Th2 responses characteristic of the chronic phase of SAMP1/YitFc ileitis. Finally, adoptive transfer of CD4⁺ cells from GF, but not SPF mice induces severe colitis in SCID recipients. These effects were associated with a decreased frequency of CD4⁺CD25⁺Foxp3⁺ T cells in the mesenteric lymph nodes of GF mice compared with SPF mice, as well as lower relative gene expression of Foxp3 in CD4⁺CD25⁺ T cells in GF mice. It is therefore apparent that, in the absence of live intraluminal bacteria, the regulatory component of the mucosal immune system is compromised. All together, our results indicate that in SAMP1/YitFc mice, bacterial flora exacerbates intestinal inflammation, but is not essential for the generation of the chronic ileitis that is characteristic of these mice. *The Journal of Immunology*, 2007, 178: 1809–1818.

The intestinal tract consists of the largest compartment of the immune system in close proximity to the most extensive and varied bacterial population found in the body (1). The diverse nature of the gut-associated immune system enables it to constantly respond to bacterial Ags and products from the commensal flora (2). This reactivity is believed to underlie the “activated” immunophenotype of gut mucosal lymphocytes, often referred to as “physiological” inflammation (3). At the same time, several control mechanisms prevent injury of the bowel under normal conditions by minimizing the reactivity of effector cells, including the function(s) of regulatory lymphocytes and cytokines (4–6). It has been shown that both the effector and regulatory compartments of the mucosal immune system require the presence of bacterial flora for their proper development (7, 8).

Under pathological conditions, a dysregulated mucosal immune response to intraluminal Ags in genetically predisposed individuals may lead to chronic intestinal inflammation, such as is observed in Crohn's disease (CD)⁴ in humans. This dysregulation may result

either in an overly aggressive effector response or may represent a primary defect in the regulatory component of the mucosal immune system. Irrespective of the underlying defect, converging lines of evidence support the notion that the intraluminal Ags that act as the trigger are derived from the commensal bacterial flora (9). CD is primarily localized to the intestinal compartments that contain the highest concentration of bacteria. Manipulation of the bacterial flora with antibiotics or probiotics is beneficial in certain subsets of patients (9). In addition, cellular and humoral responses against common bacterial Ags have been observed in patients with CD (10–12). More compelling evidence for an association between CD and the bacterial flora comes from studies in animal models of colitis (13). In fact, several studies have shown that colitis does not usually develop when animals are kept under germfree (GF) conditions (13). These observations have led to a general acceptance of the hypothesis of “no bacteria, no gut inflammation.”

We have extensively characterized the SAMP1/YitFc (SAMP) mouse, a unique model of spontaneous intestinal inflammation that has many similarities to CD (14, 15). This mouse originates from the SAMP1/Yit parental strain, initially described by Matsumoto et al. (16). After extensive brother-sister mating at the University of Virginia, novel phenotypic characteristics emerged that resulted in the designation of a separate SAMP1/YitFc strain (15).

Similar to CD, disease in SAMP mice is localized to the terminal ileum, with histological features that include transmural, discontinuous and granulomatous inflammation, involving both active and chronic inflammatory elements. In addition, a subset of mice develops perianal manifestations. Ileitis in SAMP mice responds to administration of corticosteroids or Abs against TNF (17). The initiation of ileitis in SAMP involves an induction phase that precedes the histological injury and bears the characteristics of a Th1-mediated condition (18). In contrast, the maintenance phase that follows the development of chronic ileitis displays a mixed

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Received for publication July 18, 2006. Accepted for publication November 3, 2006.

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¹ This work was supported by a Research Fellowship Award from Crohn's and Colitis Foundation of America (to G.B.), U.S. Public Health Service/National Institutes of Health Grants DK-42191, DK-44540, and DK-55812 (to F.C.), and the University of Virginia Digestive Health Research Center (DK-67629).

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⁴ Abbreviations used in this paper: CD, Crohn's disease; GF, germfree; SPF, specific pathogen free; FA, fecal Ag; MLN, mesenteric lymph node; TIS, total inflammatory score; Treg, T regulatory.

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Th1/Th2 phenotype. The role the bacterial flora plays in the pathogenesis of ileitis in SAMP mice has yet to be fully elucidated. Nevertheless, it appears that intestinal bacteria influence disease progression in SAMP mice, because treatment with broad-spectrum antibiotics prevents and ameliorates ileitis (19).

In this study, we report that bacterial flora is not essential for the establishment of disease in these mice. Nevertheless, commensal bacteria accelerate the progression and exacerbate the severity of ileitis. At the cellular level, we show that mucosal lymphocytes isolated from specific pathogen-free (SPF) mice proliferate and secrete cytokines in response to stimulation with fecal Ags (FA). In addition, we demonstrate that bacterial flora may be critical for the development of mucosal Th2 responses. In contrast, SPF-derived FA were essential for the development of IL-4-secreting effector lymphocytes. Finally, we provide evidence that regulatory pathways may be compromised in the absence of commensal flora, as evidenced by lower relative gene expression of *Foxp3* in $CD4^+CD25^+$ T cells in GF mice compared with SPF mice, as well as a decreased frequency of $CD4^+CD25^+Foxp3^+$ T cells in the mesenteric lymph nodes (MLNs) of GF mice.

Materials and Methods

Animals

SAMP and AKR mice were maintained under SPF conditions at the University of Virginia. SCID (C3H.Snm.C-Prkdcscid/J) mice were purchased from The Jackson Laboratory. GF SAMP mice were generated and maintained at Taconic Farms as described previously (20). In brief, mice were aseptically derived from SAMP mothers by caesarian section and kept thereafter in a class 1 GF isolator. The isolator was tested each month in accordance with Taconic's standard operating procedures for microbial monitoring of class 1 axenic isolators. Sterility was confirmed by inoculation of fecal pellets into different culture media, including TSA with 5% sheep blood, *Brucella* agar (for anaerobes) and nutrient agar w/dextrose (for fungi). No aerobic or anaerobic bacterial contaminants have been found in the GF isolator since its build date the week of 24 February 2003. At the appropriate time points, GF mice were shipped in GF isolators to the animal facility at the University of Virginia. Upon arrival mice were kept overnight in the isolators. The following morning, mice were euthanized and their intestinal organs were removed, rinsed with PBS, fixed in 10% formalin solution, and processed for further studies. All protocols were approved by the Animal Care and Use Committee of the University of Virginia.

Adoptive transfer

$CD4^+$ lymphocytes from the MLNs of SAMP mice (>13 wk old) were positively selected (purity >95%) on a $CD4$ column (Miltenyi Biotec). Adult (6–8 wk) MHC-matched SCID mice received 1×10^5 $CD4^+$ cells per mouse by i.p. injection. Mice were tested weekly for weight loss and rectal prolapse. Five to 6 wk posttransfer, SCID mice were euthanized, and terminal ilea and coli were processed for histological studies.

Pathological study

Histological evaluation of ileitis in SAMP or SCID mice was performed in H&E-stained sections by a single pathologist in a blinded manner. A validated scoring system was used, as described previously (18). Briefly, histological indices were evaluated for 1) active inflammation (infiltration with neutrophils), 2) chronic inflammation (lymphocytes, plasma cells, and macrophages in the mucosa and submucosa), and 3) villus distortion (flattening and/or widening of normal villus architecture). The total inflammatory score (TIS) represents the sum of the three individual components. Histological evaluation of colitis in adoptively transferred SCID mice was performed in H&E-stained sections, using a colitis scoring system calculated in a similar manner. Histological indices were evaluated for active inflammation, chronic inflammation, and transmural infiltration, and added to give the TIS.

Real-time RT-PCR for cytokine analysis

Total RNA was isolated from homogenized tissue using the RNeasy Miniprep kit (Qiagen). Reverse transcription was performed using the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer's instructions. Amplification of IL-5, IL-13, T-bet, and

GATA3, was performed as described recently (18). Target mRNA was normalized to the 18s ribosomal RNA internal control in each sample. Results were expressed as relative ratio to the lowest control sample. All samples were assayed in duplicate.

Real-time RT-PCR for *Foxp3* analysis

Total cellular RNA was extracted from 3×10^6 $CD4^+$ splenocytes or MLN cells, using the RNeasy Miniprep kit. Reverse transcription was performed using the GeneAmp RNA PCR kit, followed by real-time PCR. Amplification of β -actin and *Foxp3* was performed using the SYBR Green PCR master mix (Applied Biosystems). The primer sequences were as follows: *Foxp3* primers, 5'-CCCAGGAAAGACAGCAACCTT-3' and 5'-TTCTCACAACAGGCCACTTG-3'; β -actin primers, 5'-CAGGGTGTGATGGTGGGAATG-3' and 5'-GTAGAAGGTGTGGTGCCAGATC-3'. PCRs were performed in a total volume of 20 μ l in an iCycler iQ detection system (Bio-Rad Laboratories). Reaction conditions were 2 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Target mRNA was normalized to the β -actin RNA internal control in each sample. Results were expressed as relative ratio to the lowest control sample. All samples were assayed in duplicate.

Preparation of FA

FA were prepared using the technique described by Elson and colleagues (7). Briefly, feces were removed from the terminal ileum and cecum of GF or SPF mice, and vortexed vigorously. DNase was added and the suspension was mixed with 0.1- μ m glass beads. Disruption of the fecal material was performed in a Mini-BeadBeater (BioSpec Products) (3×150 s). Subsequently, the glass beads and insoluble material were pelleted by centrifugation twice at 14,000 rpm. The supernatants containing the FA were filter-sterilized (0.2- μ m filter), aliquoted, and stored at -80°C . Protein concentration in the FA preparations (usually 2–5 mg/ml) was determined by the colorimetric Bradford protein assay (Bio-Rad).

Cell culture

Cultures were performed in complete medium (RPMI 1640 with 10% FBS, 2 mM L-glutamine, 1×10^{-5} mol/L 2-mercaptoethanol, and 1% penicillin/streptomycin). To study the responses to stimulation with FA, splenocytes from SPF SAMP mice were depleted of $CD4^+$ and $CD8^+$ cells using magnetic beads and used as APCs. The splenocytes were incubated overnight with the appropriate amounts of FA or no FA (baseline), irradiated (3000 rads), and cocultured for 3–5 days with $CD4^+$ cells from the MLNs of SPF SAMP mice (effector population). To study the generation of effector lymphocytes, splenocytes from SPF SAMP mice were cultured under stimulation with anti- $CD3$ (2 μ g/ml; BD Biosciences) and anti- $CD28$ (10 μ g/ml; BD Biosciences), with or without the addition of FA in the cultures (primary stimulation). After 48 h, cells were recovered, washed, and expanded without stimulation in the presence of IL-2 (10 ng/ml). After 4 days, the cells were harvested and submitted to short (6 h) stimulation with anti- $CD3$ (10 μ g/ml) and anti- $CD28$ (10 μ g/ml), or PMA (50 ng/ml) and ionomycin (500 ng/ml) (secondary stimulation). The cells were used for intracellular staining and their supernatants were stored at -80°C .

Proliferation assay

APCs and effector lymphocytes (10^5 of each) were cultured in triplicate for 72 h with the indicated stimulation. Cells were pulsed with [^3H]thymidine (1 μ Ci/well) (MP Biomedicals) overnight and proliferation was estimated by measuring thymidine incorporation.

Cytokine measurement

The concentrations of TNF, IFN- γ , IL-4, IL-5, IL-13, and IL-10 in the supernatants were measured by multiplex cytokine array analysis. The Bipolar protein multiarray system was used and a mouse-plex assay was used according to the manufacturer's recommendations (Bio-Rad).

Intracellular cytokine staining

Cells were stimulated, and then staining for intracellular cytokines was performed using the Cytofix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's instructions. The percentages of $CD4^+$ lymphocytes expressing intracellular cytokines were determined by multiple-color analyses, using a FACSCalibur (BD Biosciences). All Abs were purchased from BD Pharmingen.

Flow cytometry

Isolated splenocytes or MLN cells in suspension (2×10^6 cells/condition) were labeled by 30-min incubation with the appropriate fluorochrome-tagged Abs against $CD4$ and $CD25$ (BD Pharmingen). Cells were washed

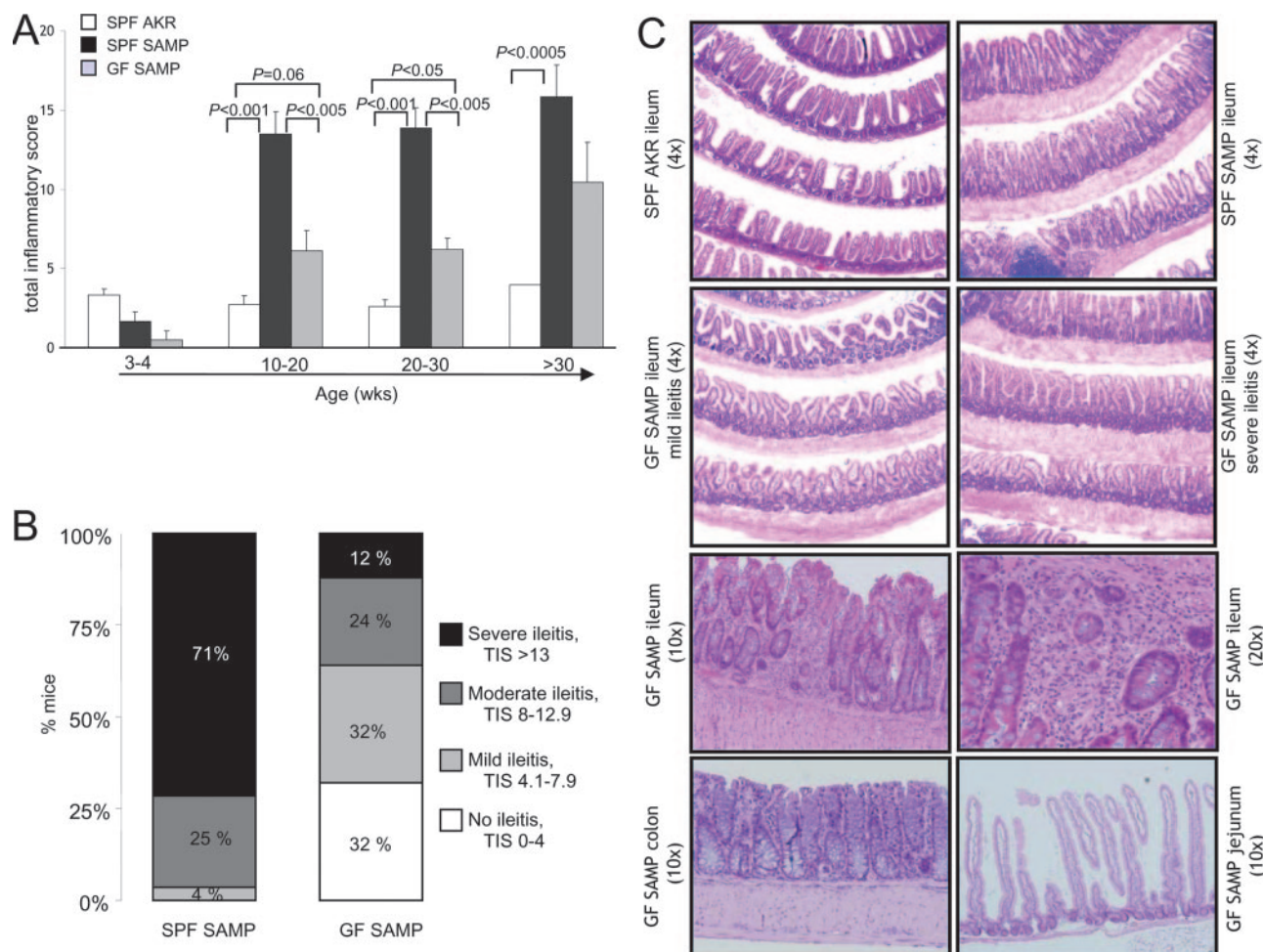


FIGURE 1. Histological assessment of ileitis in SPF and GF SAMP mice. *A*, GF SAMP (3–4-wk, $n = 4$; 10–20 wk, $n = 9$; 20–30 wk, $n = 9$; >30 wk, $n = 7$), age-matched SPF SAMP (3–4 wk, $n = 6$; 10–20 wk, $n = 9$; 20–30 wk, $n = 12$; >30 wk, $n = 7$), and AKR (3–4 wk, $n = 3$; 10–20 wk, $n = 4$; 20–30 wk, $n = 5$; >30 wk, $n = 3$) mice were euthanized and the TIS in the ileum was assessed. Data are presented as the mean \pm SEM. *B*, The distribution according to the severity of ileitis is shown for SAMP mice (GF, $n = 25$; SPF, $n = 28$) older than 13 wk of age. *C*, H&E-stained sections depict the histological characteristics of the indicated organs from GF and SPF SAMP, and AKR mice.

with cold PBS to remove excess Ab. Before staining with Alexa Fluor 647 anti-Foxp3 Ab (BioLegend) at room temperature for 30 min, the cells were fixed and permeabilized with Fix/Perm solution (BioLegend) at room temperature for 20 min. Washing and staining were performed in Perm buffer (BioLegend). After gating for lymphocytes by forward and side scatter, the percentage of cells expressing Foxp3 was determined by three-color analysis using a FACSCalibur (BD Biosciences) and CellQuest software (BD Immunocytometry Systems).

Statistical analysis

Statistical analysis was performed using the Wilcoxon rank sum test for nonparametric data. An α level of 0.05 was considered significant ($p < 0.05$).

Results

Development of ileitis is attenuated in SAMP mice under GF conditions

To investigate the role played by bacterial flora in the development of ileitis in SAMP mice, a colony of GF SAMP mice was generated at Taconic Farms. Mice were aseptically derived from SAMP mothers by caesarian section and kept in a class 1 GF isolator. The isolator was tested each month in accordance with Taconic's standard operating procedures for microbial monitoring of class 1 axenic isolators. Sterility was confirmed as described in detail in *Materials and Methods*. We then performed a time-course study during which we compared the histological appearance of the ter-

mal ileum from AKR (negative control), SPF SAMP (positive control), and GF SAMP mice at various ages (Fig. 1*A*). In line with our previous observations (14, 15), SPF SAMP mice presented with severe ileitis by 10 wk of age, without significant fluctuation thereafter. To our surprise, GF SAMP mice also developed ileitis that was detected as early as 13 wk of age (Fig. 1*A*). The severity of ileitis was considerably lower in GF than in SPF mice at all time points. Indeed, the average TIS for GF mice at 10–20 wk and 20–30 wk was 45% of the TIS for SPF mice at the same time points (10–20 wk: 6.1 ± 1.3 vs 13.5 ± 1.4 , $p < 0.005$; 20–30 wk: 6.2 ± 0.7 vs 13.9 ± 1.3 , $p < 0.005$). After 30 wk of age, the average score for the GF group was 66% of the SPF group (>30 wk: 10.4 ± 2.5 vs 15.8 ± 2 , NS).

The distribution of GF and SPF mice according to the severity of ileitis is depicted in Fig. 1*B*. Because ileitis in SAMP mice is first detected around 10 wk of age, the data are only presented for mice of that age and older. From these data, it can be seen that under SPF conditions the penetrance of ileitis in SAMP mice is 100%. Moreover, the majority of mice present with severe ileitis, because 70% have a TIS of >13. However, when SAMP mice were maintained under GF conditions, we observed several differences. First, no ileitis was observed in a substantial subset of mice (8 of 25, 32%), even at 40 wk of age (Fig. 1*B*).

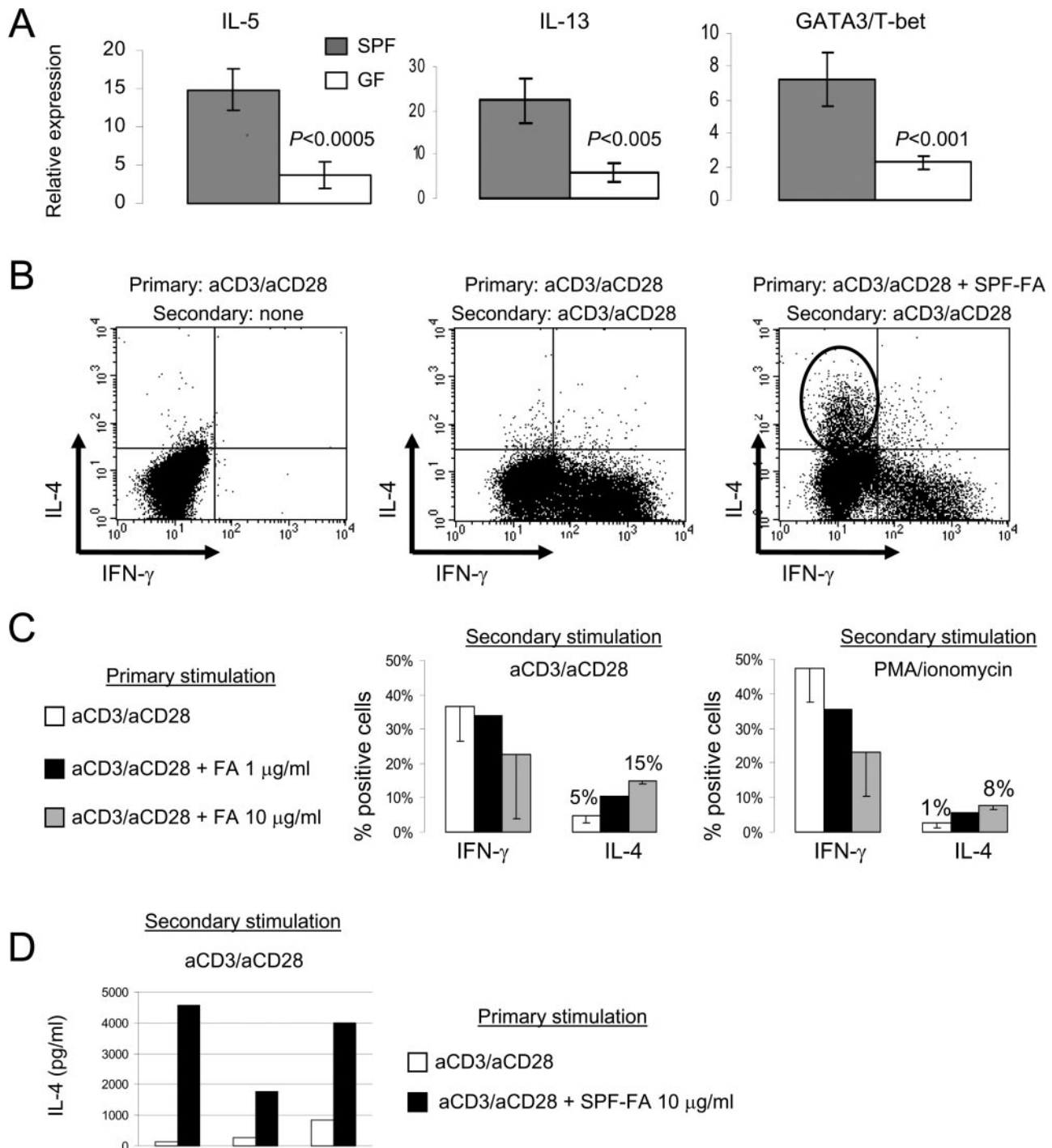


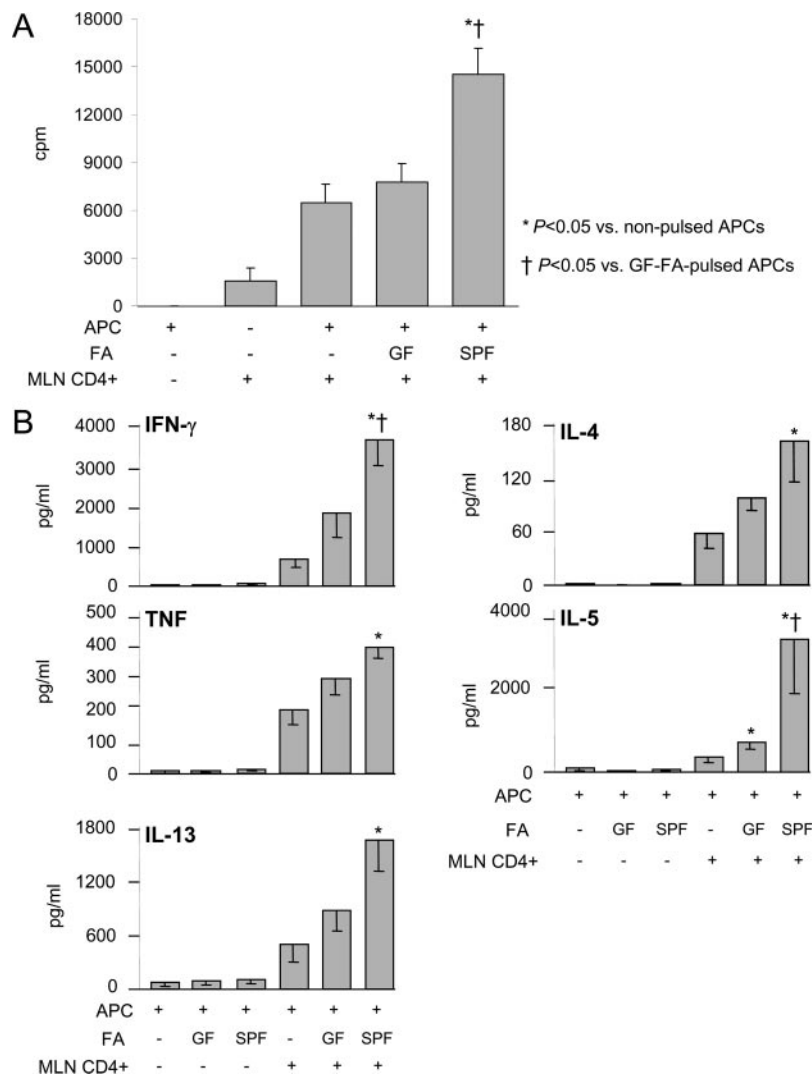
FIGURE 2. Effects of bacterial flora on the development of mucosal Th2 responses in SAMP mice. **A**, Total RNA was extracted from ileal tissue of GF ($n = 13$) and SPF ($n = 15$) SAMP mice >13 wk old. mRNA was quantified by real-time RT-PCR. The GATA3:T-bet ratio represents the ratio of GATA3 and T-bet mRNA after normalization to the 18s ribosomal RNA. Data are expressed as the mean \pm SEM. **B**, Splenocytes from SPF SAMP mice were cultured in the presence or absence of SPF-FA (primary). After expansion with IL-2, cells were cultured with anti-CD3/anti-CD28 or PMA/ionomycin (secondary). CD4⁺ lymphocytes that produced IL-4 or IFN- γ were detected by intracellular staining. The circled area depicts the IL-4-positive population. **C**, The percentage of CD4⁺ cells producing IL-4 or IFN- γ is shown. Results are pooled from three independent experiments. Data are presented as mean \pm SEM. **D**, The concentration of IL-4 in the supernatants of the secondary cultures was measured by ELISA. Three independent experiments are shown.

Second, approximately half of the mice with histologically evident disease (8 of 17) developed only mild ileitis. Nevertheless, 36% of all the GF mice examined (9 of 25) developed disease of comparable severity to the SPF mice (moderate or severe).

We also studied the relative contribution of the active (polymorphonuclear) and chronic (lymphocytes and monocytes) inflam-

matory elements to the histological injury. In SPF mice, both active and chronic inflammation develop rapidly and are fully established by 10 wk of age (data not shown). In contrast, in GF mice the polymorphonuclear component appears to develop faster, with an active inflammatory index that is 46% of that in SPF mice at 10–20 wk, 57% by 20–30 wk, and reaches 63% of the severity in SPF mice by 30 wk. In comparison, the chronic inflammatory

FIGURE 3. Reactivity of mucosal lymphocytes from inflamed SPF SAMP mice to stimulation with FA. FA were prepared from GF and SPF SAMP mice as described in *Materials and Methods*. CD4⁺ cells from MLNs represent the effector population, whereas syngeneic splenocytes act as APCs. APCs were pulsed with FA overnight, irradiated, and cocultures were performed as indicated. **A**, Proliferation of effector lymphocytes after 72 h was estimated by measuring the [³H]thymidine incorporation for the last 18 h of the culture. **B**, The concentration of the indicated cytokines at 120 h was measured by a Bioplex assay. Data are expressed as the mean \pm SEM.



index in GF mice remains low from 10–20 wk (33% of the SPF score) and 20–30 wk (36%). However, the chronic inflammatory index increases after 30 wk of age, reaching 59% of the severity in SPF mice (data not shown).

The histological characteristics of ileitis in GF SAMP mice were similar to those previously described in SPF mice (14) (Fig. 1C). No inflammatory changes were detected in the jejunum of either GF or SPF SAMP mice (Fig. 1C). Mild inflammation was occasionally seen in the colon of GF SAMP mice.

Ags derived from the bacterial flora are essential for the generation of mucosal Th2 responses

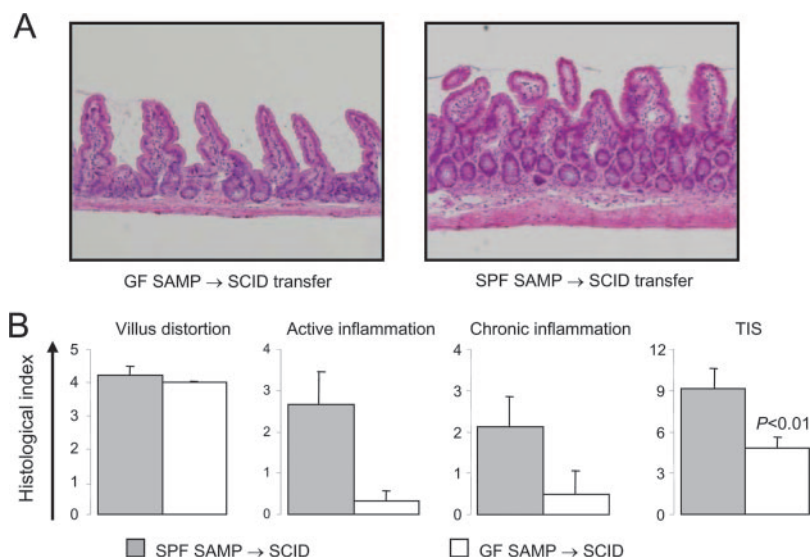
We have recently shown that the chronic phase of ileitis in SAMP mice is characterized by a marked up-regulation of mucosal Th2 responses, including higher ileal expression of IL-5 and IL-13, and an elevated ratio of GATA3:T-bet (18). We hypothesized that the bacterial flora provides the immunostimulatory signals for the up-regulation of mucosal Th2-type responses in SAMP mice. To test our hypothesis, we first compared the expression of the mRNAs for IL-5 and IL-13 in the terminal ileum of GF and SPF SAMP mice (Fig. 2A). Th2 responses were dramatically suppressed in GF mice, with significant decreases in the ileal expression of the mRNAs for IL-5 (25% of the average expression in age-matched SPF controls; $p < 0.0005$) and IL-13 (26%; $p < 0.005$). This defect in the up-regulation of Th2 responses under

GF conditions was confirmed by the significant decrease in the GATA3:T-bet ratio in GF SAMP mice compared with SPF controls ($p < 0.001$). In contrast, no considerable differences were seen in the expression of Th1 cytokines, such as IFN- γ (data not shown).

These data indicate that, in the absence of bacterial flora, the mucosal immune system is unable to mount a vigorous Th2 effector response in SAMP mice. This inability may underlie the decreased severity of inflammation under GF conditions. We therefore hypothesized that Ags derived from fecal material containing commensal bacteria (i.e., from SPF mice) may be able to induce Th2 responses. To test our hypothesis, we conducted long-term cultures of lymphocytes from SPF SAMP mice with or without stimulation with SPF-FA. We used splenocytes as the effector population, because they contain high numbers of naive lymphocytes and do not produce significant amounts of Th2-type cytokines when nonspecifically stimulated (data not shown). A primary stimulation (with or without SPF-FA) was applied, which was followed by expansion with IL-2. Finally, cells were subjected to a short stimulation with anti-CD3/anti-CD28 or PMA/ionomycin. The type and number of cytokine-secreting lymphocytes that were generated in the primary cultures were detected by intracellular cytokine staining.

When nonspecific stimulation was applied, the default pathway in both primary and secondary cultures was a Th1-type response, with large numbers of IFN- γ -positive, but not of IL-4-positive

FIGURE 4. Severity of ileitis induced in SCID mice by the transfer of lymphocytes from SAMP mice. *A*, H&E-stained sections depict the histological characteristics of the terminal ileum of SCID mice that were injected with 1×10^5 CD4⁺ cells from the MLNs of SPF or GF SAMP donors. Severe ileitis is observed in recipients of SPF donors, whereas ileitis was either not observed or was mild in recipients of GF donors. *B*, Indices for villus distortion, active and chronic inflammation, and the TIS were calculated as described in *Materials and Methods*. Data are expressed as the mean \pm SEM. $n = 9$ for recipients of SPF donor cells and $n = 6$ for recipients of GF donor cells.



lymphocytes (Fig. 2*B*). In sharp contrast, when SPF-FA were added to the primary cultures, we detected a distinct population of IL-4-positive lymphocytes in addition to the IFN- γ -positive population, indicating a mixed Th1/Th2 secondary response (Fig. 2*B*). Primary stimulation with FA resulted in a significant increase in

the number of IL-4-positive cells compared with primary stimulation in the absence of FA, irrespective of the nature of the secondary stimulation (15 ± 1 vs $5 \pm 2\%$ for anti-CD3/anti-CD28 and 8 ± 1 vs $3 \pm 1\%$ for PMA/ionomycin) (Fig. 2*C*). To further confirm that Th2 responses are induced by FA, we measured the

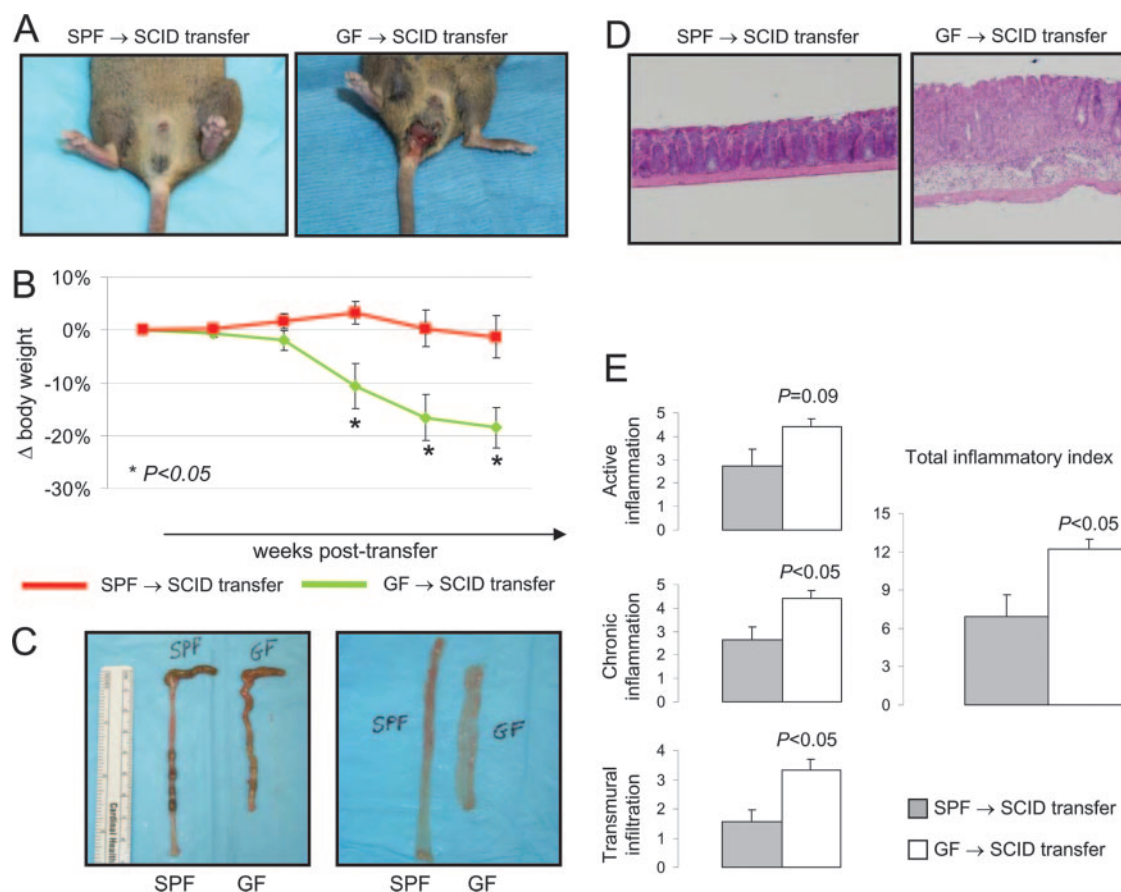


FIGURE 5. Severity of colitis induced in SCID mice by the transfer of CD4⁺ lymphocytes from SPF or GF SAMP mice. *A–C*, SCID mice that had received donor cells from GF SAMP mice showed frequent rectal prolapse, significantly decreased weight loss, and shortened and thickened coli. *D*, H&E-stained sections depict the histological characteristics of the colon of SCID mice that were injected with 1×10^5 CD4⁺ cells from the MLN of SPF or GF SAMP donors. Severe colitis is observed in recipients of GF donors, whereas mild colitis was induced in SCID mice that had received cells from SPF donors. *E*, Indices for transmural inflammation, active and chronic inflammation, and the TIS were calculated as described in *Materials and Methods*. Data are expressed as the mean \pm SEM. $n = 9$ for recipients of SPF donor cells and $n = 6$ for recipients of GF donor cells.

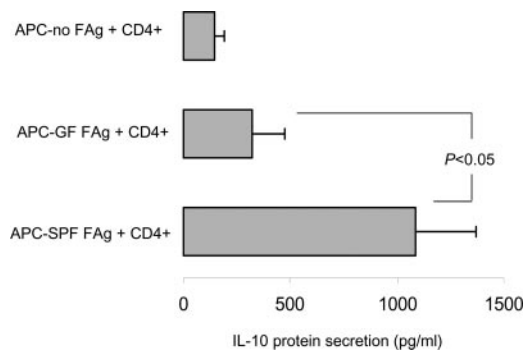


FIGURE 6. IL-10 secretion in response to stimulation with GF-FA or SPF-FA. FA were prepared from GF and SPF SAMP mice. The effector cells were CD4⁺ cells from MLNs of SAMP mice, and syngeneic splenocytes were used as APCs. The APCs were pulsed overnight with GF-FA, SPF-FA, or no-FA, irradiated, and cocultures were performed as indicated. The concentration of IL-10 was measured by a Bioplex assay. Data are expressed as the mean \pm SEM.

concentration of IL-4 in the supernatants of the secondary cultures. There was a clear increase in the levels of the IL-4 protein that were secreted when FA were included in the primary cultures (Fig. 2D).

Mucosal lymphocytes from SAMP mice show immunoreactivity against Ags from commensal bacteria

We then hypothesized that immunoreactivity against FA takes place in SAMP mice in vivo. To test our hypothesis, we studied the responses of freshly isolated mucosal (i.e., MLN) CD4⁺ cells to FA presented by APCs. We also investigated the responses to FA from GF mice (i.e., in the absence of commensal bacteria). Our study showed that CD4⁺ cells from SAMP mice proliferated in response to SPF-FA but not GF-FA (Fig. 3A), indicating that they recognize Ags that originate from live intraluminal bacteria. In addition to proliferation, stimulation of mucosal CD4⁺ cells with SPF-FA led to increased cytokine secretion. In line with our in vitro studies, freshly isolated MLN lymphocytes secreted a mixed pattern of Th1/Th2 cytokines when stimulated with FA. The most profound increases were in IFN- γ (202% increase; $p = 0.041$), IL-5 (441% increase; $p = 0.0043$), and IL-13 (191% increase; $p = 0.065$) (Fig. 3B). Interestingly, stimulation of CD4⁺ cells with

GF-FA also resulted in some degree of cytokine production in our studies, but to a lesser extent compared with SPF mice.

Adoptive transfer of GF CD4⁺ lymphocytes induces severe colitis in SCID recipient mice

We have previously demonstrated that injection of CD4⁺ lymphocytes from the MLNs of SAMP into MHC class II-matched SCID mice induces severe ileitis but mild colitis in the recipient mice (14). In this study, we used this adoptive transfer system to directly test the effects of GF conditions on the immunological properties of the mucosal lymphocytes. We transferred CD4⁺ cells from either SPF or GF SAMP donors and compared the effects on the induction of intestinal inflammation in SCID recipients.

The capacity of CD4⁺ lymphocytes from the MLNs of GF SAMP mice to induce ileitis in SCID recipients was significantly decreased (Fig. 4A). SCID mice that received cells from GF donors displayed decreased indices for both active (0.3 ± 0.2 vs 2.7 ± 0.8 in SPF transferred cells) and chronic inflammation (0.5 ± 0.5 vs 2.1 ± 0.8) (Fig. 4B). In fact, there were signs of active inflammation in only two of six (26%) SCID recipients of GF donors, whereas only one of six (13%) had chronic inflammatory cells in the small intestinal lamina propria. Overall, the TIS was significantly lower in the group that received cells from GF donors (4.8 ± 0.7 vs 9 ± 1.4 , 50% decrease; $p = 0.0082$) (Fig. 4B).

In contrast to their inability to induce substantial ileitis, CD4⁺ cells from GF mice induced severe colitis in the recipient mice. By 5 wk after the transfer, all mice that had received cells from GF donors showed clinical signs of severe colitis. In particular, all recipients of GF donor cells (100%) had marked rectal prolapse, in comparison with only three of nine mice (33%) that received cells from SPF donors (Fig. 5A). Mice that received cells from GF donors suffered from significantly greater weight loss, a marker of severe colonic inflammation, compared with the SPF recipient group (Fig. 5B). There was also increased incidence of shortening and thickening of the large bowel in the recipients of GF donor cells (Fig. 5C). At the histological level, SCID mice that received cells from GF mice showed increases in all parameters of inflammation (transmural infiltration, GF recipients: 3.3 ± 0.3 , SPF: 1.6 ± 0.4 , $p < 0.05$; active inflammation, GF: 4.4 ± 0.3 , SPF: 2.7 ± 0.7 , $p = 0.09$; chronic inflammation, GF: 4.4 ± 0.3 , SPF: 2.7 ± 0.5 , $p < 0.05$) (Fig. 5D). Overall, the TIS was significantly higher in the group that

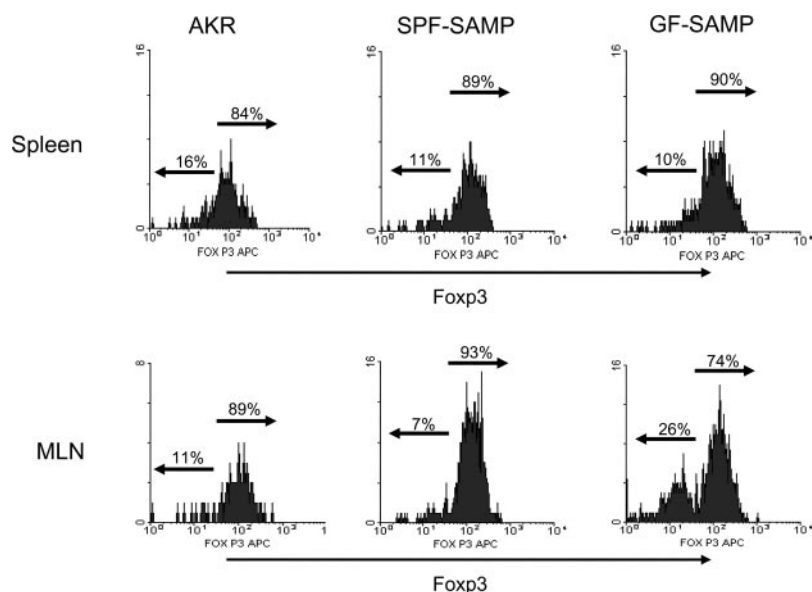
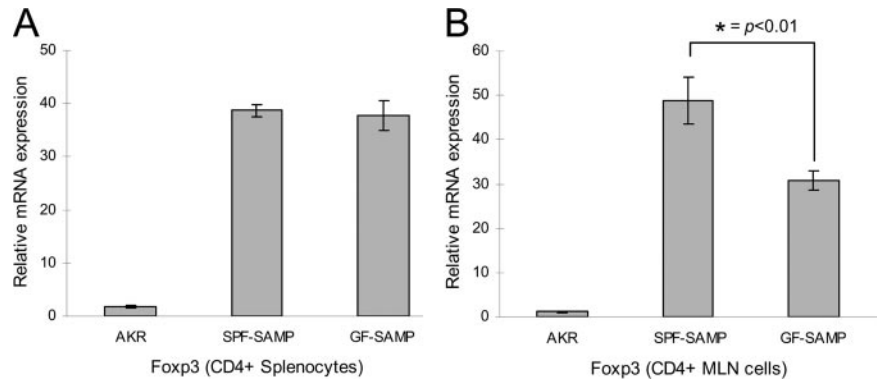


FIGURE 7. Flow cytometric analysis of splenocytes and MLN cells in SPF and GF SAMP mice. Cells were labeled with CD4-FITC-, CD25-PE-, and Foxp3-allophycocyanin conjugated mAbs. CD4⁺CD25⁺ cells were gated and Foxp3 expression was analyzed by flow cytometry. Results are from a representative experiment using pooled lymphocytes from three mice per group repeated twice.

FIGURE 8. Quantification of relative Foxp3 mRNA levels in AKR, SPF, and GF SAMP mice. Total RNA was extracted from CD4⁺ splenocytes (A) or CD4⁺ MLN cells (B). For each group CD4⁺ lymphocytes were pooled from two mice. cDNA samples were quantified by real-time PCR, and the relative expression of Foxp3 in each sample was normalized to the relative expression of β -actin. The results were expressed as the mean \pm SEM of three separate experiments.



received cells from GF mice (GF: 12.2 ± 0.8 , SPF: 6.9 ± 1.6 , $p < 0.05$, 170% increase) (Fig. 5E).

Regulatory mucosal pathways are defective in GF SAMP mice

The colitis that develops in the adoptive transfer model has traditionally been linked to an imbalance between pathogenic proinflammatory factors and disease-preventing regulatory elements. The latter include regulatory lymphocytes, as well as cytokines such as IL-10 and TGF- β 1. We therefore hypothesized that the induction of severe colitis after adoptive transfer of CD4⁺ cells from GF donors could result from a defect in the T regulatory (Treg) cell population and their ability to secrete regulatory cytokines. To test this hypothesis, we first compared the secretion of IL-10 after stimulation with FA originating from GF or SPF mice. Our results (Fig. 6) clearly show that stimulation with SPF-FA induced secretion of IL-10 (814 ± 248 pg/ml) at levels that were significantly higher than both the levels at baseline (101 ± 41 pg/ml; $p < 0.05$) and the levels secreted upon stimulation with GF-FA (187 ± 90 pg/ml; $p < 0.05$). In fact, stimulation with GF-FA did not induce any significant increase in IL-10 secretion over baseline.

Secondly, we analyzed cells from the spleens and MLNs of AKR, and GF and SPF SAMP mice for the Treg cell markers CD25⁺ and the Foxp3 protein. The data presented in Fig. 7 show that a lower proportion of CD4⁺CD25⁺ cells in the MLNs of GF mice express intracellular Foxp3 protein (74.4%) compared with the MLNs of AKR (89.4%) and SPF SAMP mice (93.4%; Fig. 7A). In addition, CD4⁺CD25⁺ cells isolated from the MLNs of GF mice showed a significantly lower relative expression of Foxp3 mRNA compared with the MLNs of SPF mice ($p < 0.01$; Fig. 8A). These effects appear to be specific to the MLN compartment, because no differences were observed in the proportion of CD4⁺CD25⁺Foxp3 cells isolated from splenocyte populations of GF SAMP (89.7%) compared with AKR (84%) and SPF SAMP mice (89.3%; Fig. 7B). Similarly, no differences in Foxp3 mRNA expression were observed in CD4⁺ splenocytes obtained from GF and SPF SAMP mice (Fig. 8B).

Discussion

The currently accepted hypothesis in mucosal immunology is that commensal bacteria are essential for the development of chronic intestinal inflammation. This concept is primarily supported by the consistent absence of intestinal inflammation in animal models of colitis when the bacterial flora is completely removed (13, 21). In contrast, this study demonstrates that chronic ileitis can develop in SAMP mice under GF conditions. Indeed, the ileitis that developed in GF SAMP mice was histologically similar to the disease seen in SPF animals. This indicates that the development of intestinal inflammation under GF conditions is a true characteristic of this

mouse strain, and not a nonspecific effect of the GF status. This is further supported by the absence of ileitis in normal C57BL/6 mice that were raised GF in the same facility (data not shown), and the fact that no significant inflammatory changes were observed in other intestinal regions of GF SAMP mice. Nevertheless, our studies confirm the important role played by the commensal flora in triggering and exacerbating chronic intestinal inflammation in this model.

The differences observed in SAMP mice compared with other models of colitis that do not develop disease in GF conditions were unexpected. Nonetheless, this phenomenon may be explained by the specific immunogenetic characteristics of the SAMP mouse (22). First, SAMP mice present with terminal ileitis rather than colitis. The ileum contains the most developed compartment of the mucosal immune system, and may therefore display a lower threshold for immunological activation compared with the colon, where stimulation by live bacteria appears to be indispensable for the expression of colitis. Second, experimental colitis results from single causative factors, such as administration of chemicals, deletion or insertion of specific genes, or manipulation of particular cell types. Such single pathway manipulations may easily be inactive in the absence of the immunostimulation provided by the bacterial flora. In contrast, ileitis in SAMP mice is truly spontaneous and represents a polygenic trait (23). In addition, there are multiple and complex immunological abnormalities, which may vary in their dependence on the presence of commensal flora. Indeed, several of the findings from the current study support this hypothesis. Active neutrophilic inflammation develops earlier in GF SAMP mice, whereas the chronic lymphocytic response was significantly delayed. In addition, there was selective suppression of the intestinal Th2 cytokines IL-5 and IL-13, whereas no effect was seen on others, i.e., IFN- γ . Finally, we have recently shown that SAMP mice display an epithelial cell defect that occurs before the development of histological injury (24). Because this defect is also present in GF mice (25), it therefore occurs independently of colonization by commensal flora.

Our findings differ from the original description of the Japanese SAMP1/Yit strain, which did not appear to develop ileitis under GF conditions (16). It is possible that the relative independence of ileitis from the bacterial flora may be among the novel phenotypic features of the SAMP1 substrain at the University of Virginia. The presence of perianal manifestations in the United States but not the Japanese SAMP1/Yit mice is a good example of major phenotypic changes that can occur even between closely related substrains. A second explanation could be that the GF Japanese mice were not observed for a sufficient length of time to detect the presence of ileitis. Given the fact that disease in the Japanese strain develops later than in the SAMP1 substrain, and that ileitis in GF mice is

later in onset and slower to progress, it is possible that inflammation might also have been observed if the Japanese strain raised under GF conditions had been permitted to reach an older age.

Two hypotheses may explain the presence of ileitis in GF SAMP mice. One possibility is that disease is caused by bacterial-independent, autoimmune reactivity against small intestinal Ags. The acceleration of disease under SPF conditions could then result from a generalized nonspecific immune stimulation in the presence of colonization by bacterial flora. An alternative explanation is that the sterile diet fed to GF mice may nonetheless contain dead bacteria and bacterial proteins, in addition to food- and epithelium-derived Ags (26). Indeed, cytokine production was detected against fecal extracts from GF mice, although to a significantly lower level than against SPF extracts. Therefore, the low amounts of antigenic material present in sterilized food pellets in combination with the significant defect in intestinal permeability characteristic of SAMP mice (25) may be sufficient to induce the attenuated immune response that results in low-grade intestinal inflammation in GF SAMP mice. Nevertheless, severe disease occurs consistently only in the presence of an intact bacterial flora that provides large quantities and variable specificities of Ags. Our laboratory is currently engaged in actively pursuing both these hypotheses.

Our results emphasize the importance of the commensal flora in triggering and exacerbating chronic intestinal inflammation. Bacterial-dependent and -independent mechanisms therefore coexist during the course of ileitis in SAMP mice. First, the presence of intraluminal bacteria under SPF conditions significantly accelerates the inflammatory process and results in more severe disease. Second, mucosal lymphocytes from the MLNs of SAMP mice proliferate and produce cytokines after stimulation with SPF-FA. Such immunobacterial interactions are expected *in vivo*, because SAMP mice display an early epithelial cell defect (24, 25) that allows for a constant cross-talk between commensal bacteria and the gut immune system, which is in line with previous studies from both humans and mice (7, 10, 27). Third, our results indicate that different components of the mucosal immune system have unequal dependence on the presence of commensal bacteria. Th2 effector pathways clearly require the presence of intact flora, because IL-5, IL-13, and GATA3 are not up-regulated in the mucosa of GF SAMP mice. In addition, IL-4-secreting lymphocytes were only generated in response to stimulation with SPF-FA. Our results are in line with recent studies showing that bacterial colonization plays an important role in the regulation of a physiological balance between Th1 and Th2 cytokines (20). Finally, regulatory mucosal responses are deficient in the absence of bacterial flora, because severe colitis is induced in SCID mice by the transfer of CD4⁺ cells from GF but not SPF donor mice. These results are in line with previous reports that have demonstrated that specific microorganisms can induce regulatory elements of the mucosal immune system (8). This concept is further supported by the observation that some GF SAMP mice spontaneously developed mild colonic inflammation. However, it is possible that other regulatory cells are present in the native state, but not transferred with the CD4 T cell population.

The results of the adoptive transfer experiments using GF and SPF CD4 T cells were intriguing. In fact, GF CD4 T cells produce minimal ileitis upon transfer to recipient SCID mice, yet produce a severe colitis not observed in native SAMP mice under either GF or SPF conditions. Several hypotheses can be postulated to explain this phenomenon. First, MLN CD4⁺ T cells may show differential ability to home to the ileum and colon of SCID recipient mice according to the presence or absence of commensal flora in the donor mice. Second, it is possible that cells other than CD4 T cells

mediate ileitis in GF SAMP mice. This hypothesis is consistent with our recent studies showing a strong nonhemopoietic epithelial cell component in the pathogenesis of ileitis in SAMP mice (25). Third, a defect in CD4 Treg cells in GF mice may lead to severe colitis in the recipient mice. Our results show that the frequency of CD4⁺CD25⁺Foxp3 cells in the MLNs of GF mice was decreased compared with SPF SAMP mice. In addition, the relative expression of Foxp3 was decreased in the same cell population, suggesting that an abnormality of Tregs in SAMP GF compared with SPF mice may be responsible for the observed severe colitis. The decreased production of IL-10 in response to stimulation with FA supports this concept, suggesting that the IL-10 pathway may be defective in the absence of bacterial flora (28). These results are consistent with studies from Rakoff-Nahoum et al. (29), which suggest that commensal flora is required for protection from colonic injury by stimulation of tissue-protective factors in experimental colitis.

Our results provide additional information regarding the development of the gut-associated immune system. Indeed, data presented in this study point to the presence of a default Th1 pathway within the intestinal mucosa that exists irrespective of the presence of bacteria. Upon colonization, this default pathway may be of critical importance for the rapid and effective clearance of pathogenic microorganisms. Moreover, Th2 and regulatory responses may primarily function to prevent an uncontrolled expansion of Th1 responses, and a bystander injury to the bowel. Because such an expansion would occur in the constant presence of immunostimulatory flora, it is not surprising that the regulatory mechanisms are also driven by Ags of bacterial origin. Our observation of a significant decrease in Tregs and IL-10 production from MLNs of GF mice strongly supports this concept.

It is generally accepted that the development of CD requires breakdown of the mechanisms that are responsible for tolerance against intestinal flora. Mucosal lymphocytes from patients with CD showed increased reactivity against antigenic material that originated from the commensal flora (10–12, 30). Patients with CD could be divided into two separate groups depending on whether or not they had circulating anti-bacterial Abs (31). This difference in immunophenotype appears to have clinical and therapeutic implications. In one study, patients with serum reactivity against multiple bacterial Ags suffered from more aggressive disease with frequent localization to the small intestine, a fibrostenotic and perforating phenotype, and increased need for surgery (30, 32). Subsequent studies also reported that patients in the “high reactivity” group responded well to antimicrobial therapy but less so to steroids, whereas the opposite situation was observed for the group with no bacterial reactivity (33). These studies indicate that there may be bacterial-dependent and bacterial-independent pathways in the human condition as well. Given that perspective, the data presented in this study raise the possibility that the SAMP murine model of ileitis may be representative of a specific subgroup of patients with CD localized to the ileum, in whom bacterial-dependent mechanisms are less critical for the development of chronic intestinal inflammation.

In conclusion, we describe for the first time the development of spontaneous, chronic small intestinal inflammation in the complete absence of colonization by commensal flora. This model offers a unique opportunity to study and dissect bacterial-dependent and bacterial-independent components of chronic intestinal inflammation, as well as providing a context to address local (ileum vs colon) issues controlling homing, regulation, and microbial impact on disease susceptibility.

Acknowledgments

We thank the Histology/Imaging Core (S. Hoang, G. Harp, and Dr. J. Mize); the Immunology/Cell Isolation Core (E. Wiznerowicz); the Molecular Biology/Gene Expression Core of the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases, University of Virginia Digestive Health Research Center; and E. Hackett of Taconic Farms for maintaining the GF SAMP colony.

Disclosures

The authors have no financial conflict of interest.

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