Segmented Filamentous Bacteria in a Defined Bacterial Cocktail Induce Intestinal Inflammation in SCID Mice Reconstituted with CD45RB^{high} CD4+ T Cells

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Background: The aim was to analyze the influence of intestinal microbiota on the development of intestinal inflammation. We used the model of chronic inflammation that develops spontaneously in the colon of conventional severe combined immunodeficiency (SCID) mice restored with the CD45 RB^{high} subset of CD4+T cells isolated from the spleen of normal BALB/c mice.

Methods: A CD4+CD45RB^{high} subpopulation of T cells was purified from the spleen of conventional BALB/c mice by magnetic separation (MACS) and transferred into immunodeficient SCID mice. Germ-free (GF) SCID mice or SCID mice monoassociated with *Enterococcus faecalis*, SFB (segmented filamentous bacteria), *Fusobacterium mortiferum, Bacteroides distasonis*, and in combination *Fusobacterium mortiferum* + SFB or *Bacteroides distasonis* + SFB were used as recipients. SCID mice were colonized by a defined cocktail of specific pathogen-free (SPF) bacteria. Mice were evaluated 8–12 weeks after the cell transfer for clinical and morphological signs of inflammatory bowel disease (IBD).

Results: After the transfer of the CD4+CD45RB^{high} T-cell subpopulation to SCID mice severe colitis was present in conventional animals and in mice colonized with a cocktail of SPF microflora plus SFB. Altered intestinal barrier in the terminal ileum of mice with severe colitis was documented by immunohistology using antibodies to ZO-1 (zona occludens).

Conclusions: Only SFB bacteria together with a defined SPF mixture were effective in triggering intestinal inflammation in the model of IBD in reconstituted SCID mice, while no colitis was detected in GF mice or in mice colonized either with SPF microflora or monoassociated only with SFB or colonized by *Bacteroides distasonis* + SFB or *Fusobacterium mortiferum* + SFB.

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Key Words: colitis, animal model, inflammatory bowel disease, regulatory T cells, germ-free, microflora, SFB

The transfer of CD4+CD45RB^{high} T cells, a predominantly naive population from the periphery of normal BALB/c mice, to severe combined immunodeficiency (SCID) recipients (immunodeficient mice lacking T and B lymphocytes)[1] caused a severe colon inflammation.[2,3] This model of Th1-mediated colitis had a similarity to inflammatory bowel disease (IBD) in humans, where Th1 responses are also elevated in the intestine of patients with Crohn's disease. Colitis induced by the transfer of CD4+CD45RB^{high} T aggressive cells can be prevented by cotransfer of the CD4+CD45RB^{low} T cells or (CD4+CD25+) population.[4,5] A population of CD4+CD45RB^{low} or CD4+CD25+ T cells from germ-free (GF) mice had a significantly lower ability to inhibit colitis compared to the same populations from mice with normal microflora.[5] Thus, the presence of commensal bacteria in the donor mice drives T cells into the antigen-experienced/memory T cell pool.[6,7] CD4+CD25+T cells play a major role in the maintenance of oral tolerance. Besides this CD4+CD25+ population, other populations of regulatory CD4+ T cells were described: Tr1 secreting high amounts of IL-10 and Th3 secreting TGF-β.[8] Consequently, the control of inflammation during the first week after the cell transfer is the result of a balance between proinflammatory and Tr cells.[8]

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It is generally accepted that intestinal microflora plays a crucial role in inflammatory bowel disease (IBD).[9] However, the question which strain of intestinal microflora is responsible for the development of colitis still remains open. The association of GF IL-10 deficient mice (knockout mice) with *Enterococcus faecalis* leads to the development of IBD.[10] In contrast, in the IBD experimental model using SCID mice reconstituted by CD4CD45RB^{high} T cells it was shown that only monoassociation with *Helicobacter muridarum* effectively triggered the colitis, while monoassociation of GF-SCID mice with segmented filamentous bacteria (SFB) bacteria, or *Ochrobactrum anthropi, Listeria monocytogenes,* and *Morganella morganii,* was not effective.[11] Hence, not all bacterial strains have equal capacity to induce intestinal inflammation.

The major aim of our work was to determine which strain of commensal microflora is responsible for the development of colitis induced by transfer of a CD4+ CD45RB^{high} T-cell subpopulation from the spleen of syngenic conventional BALB/c mice to SCID mice. SCID mice were reared under germ-free conditions or colonized with different bacterial cocktails. In addition, we studied the expression of protein involved in the formation of ileal tight junctions (TJs), ZO-1 (zona occludens).

MATERIAL AND METHODS

Animals

SCID mice (mice with severe combined immunodeficiency, background BALB/cJHanHsd-SCID) were used as recipients of transferred cells. The mice reared in Trexler plastic isolators as GF or colonized by a defined cocktail of microflora were fed a standard granulated diet.[12] The diet and bedding material were sterilized by 2 cycles of γ radiation (5 Mrd, 30 min; Bioster, Czech Republic). FACS analysis using FITC-labeled monoclonal anti-CD3 (Serotec, UK) was employed to exclude the potential presence of T lymphocytes in SCID mice. Conventional BALB/c mice were used as the donors of transferred cells. They were reared in a conventional animal facility and regularly checked for the presence of pathogenic bacteria, viruses, and parasites.

Control of Sterility of GF Animals

To control the sterility of isolators, fecal samples from the isolator were evaluated weekly for the presence of aerobic and anaerobic bacteria, molds, and yeast. Fecal samples after colonization of SCID mice were evaluated for the presence of bacteria after 2 days, 7 days, and then every week.

Samples were cultivated on peptone bouillon, Sabouraud bouillon, Schadller bouillon (Difco, Detroit, MI) and then on blood agar, Sabourad agar, Schadller agar in an anaerobic atmosphere (gas Anaerogen 2.5lt:DIOXO) to detect the presence of anaerobic bacteria. Furthermore, the smears were stained with Gram stain and fluorescence dyes, which stained Gram-positive bacteria yellow and Gram-negative bacteria green.[13] For this study it was necessary to eliminate the presence of *Helicobacter muridarum* in conventional SCID mice and mice colonized with SPF microflora. The detection of urease-negative *Helicobacter sp*. (forward: 5′GAAACTATCACTCTAGAGTATG-3′,reverse: 5′ TGCTCCTCATTGTATGCC-3′) was done by polymerase chain reaction (PCR). The sequences of urease-negative *Helicobacter spp*. were identical with or closely related to *H. muridarum* and *H. hepaticus*.[14] However, this strain were not detected in our colonies of conventional and SPF mice.

Colonization of GF Mice

SCID mice were colonized with different bacterial components at the age of 1 month. Colonization with SFB was performed after weaning and the transfer of CD4CD45RB^{high} T cells was done 3–4 weeks after colonization. As SFB bacteria are not cultivable by conventional bacteriological methods we used stool of GF mice monoassociated with SFB as a source of multiplying SFB. Colonization of mice with a bacterial cocktail was achieved by intragastric administration of 10^7–10^8 bacterial cells. The bacteria used for colonization studies included:

1. SPF bacterial cocktail (obtained from Hannover, Central Institute for Laboratory Animal Breeding) containing aerobes (*Bacillus species, Lactobacillus lactis, Acinetobacter species, Staphylococcus sciuri*) and anaerobes (*Lactobacillus delbrukii sp., Bulgaricus, L. fermentum, L. catenaforme, Bacteroidesdistasonis, B. thetaiotaomicron, Peptostreptococcus micros, P. asaccharolyticus).*
2. C1 bacterial cocktail containing aerobes *(Enterococcus faecalis, Micrococcus spp)* and anaerobes *(Veilonella parvula, Lactobacillus fermentum, L. delbrukii).*
3. C2 bacterial cocktail containing aerobes (*Enterococcus faecalis, Bacillus spp., Streptococcus spp.)* and anaerobes (*Veilonella parvula, Lactobacillus fermentum, Bifidobacterium longum, Fusobacterium mortiferum, F. varium).*
4. Single strains of bacteria (monoassociation): nonpathogenic *Enterococcus faecalis,* noncultivated segmented filamentous bacteria (SFB) (kindly provided by Prof. Umesaki, Japan); *Fusobacterium mortiferum* (originally from cocktail C2) and *Bacteroides distasonis* (originally from cocktail SPF, Hannover).
5. Combination single strain of bacteria + SFB bacteria: *Fusobacterium mortiferum* + SFB and *Bacteroides distasonis* + SFB.
6. Conventional microflora (not fully defined) were also used for colonizing SCID mice.

For detection of SFB bacteria and *Bacteroides* in the cecum and colon, we used fluorescence in situ hybridization (FISH) analysis using the 16S rRNA *Bac 303-FITC (Bacte-*

roides) probe (5′-CAATGTGGGGGACCTT-3′), and SFB 1008-FITC probe (sequences 5′-GCGAGCTTCCCTCAT-TACAAGG-3′).[15,16] The fecal samples were taken in Schadller bouillon and than fixed with 4% paraformaldehyde overnight. Slides were incubated overnight at 50°C with the probes in hybridization buffer.[17]

Isolation of CD4 CD45RB^{high} Population and Transfer of Cells

Suspension of spleen lymphocytes was purified by Ficoll gradient centrifugation. Positive selection of CD4$^+$ T lymphocytes was performed using Dynabeads Mouse anti-CD4 (L3T4) (Dynal A.S. Oslo, Norway). The mixture of cells was stirred with anti-CD4 Dynabeads at 4°C for 20 minutes. The CD4$^+$ fraction was then isolated by magnetic separation. CD4$^+$ fraction was detached from the beads using DETACHaBEAD Mouse CD4 (Dynal).

The separation of the CD4+CD45RB^{high} fraction of T lymphocytes was done by positive selection. CD4+ fraction of the lymphocytes was incubated with fluorescein isothiocyanate (FITC)-conjugated rat antimouse CD45RB monoclonal antibody (Pharmingen, San Diego, CA) for 25 minutes on ice. The cells were resuspended with anti-FITC microbeads (Anti-FITC MicroBeads, Mitenyi Biotec, Germany). The suspension was diluted as recommended and run over mini-MACS columns. The purity of the CD4 CD45RB^{high} population was verified using flow cytometry (Becton Dickinson, San Jose, CA). The purity of CD4+CD45RB^{high} T cells was 98%. SCID mice were reconstituted by intraperitoneal injection of 3.5×10^5 CD4+CD45RB^{high} T cells.

Histology

Tissue samples from terminal ileum and 3 parts of colon (ascendens, transversum, descendens) were removed from SCID mice 10–12 weeks after reconstitution with CD4+CD45RB^{high} T cells. The samples were frozen in liquid nitrogen or fixed in Carnoy's solution, 4% paraformaldehyde, and then in 90% alcohol and embedded in paraffin. Five-μm paraffin-embedded sections were cut and stained with hematoxylin-eosin and by Alcian blue for mucin detection. The specimens were viewed with an Olympus BX 40 microscope equipped with an Olympus DP 12 digital camera. The diameter of the colon hypertrophy (area) of the samples and the height of each mucosal hyperplasia were measured. The measurements (10–20 per tissue) were performed with Olympus DP-Soft program. The inflammation was evaluated according to the scale described by Asseman et al[18] and Geboes et al.[19]

Immunohistochemistry

A standard immunostaining assay involving biotin-avidin-linked peroxidase detection was used for evaluating CD4+ expression. Briefly, snap-frozen samples of the colon and terminal ileum embedded in OCT (Tissue-tek, Sakura Fine Tek, Torrance, CA) were cryosectioned at 6 μm, air-dried, fixed for 5 minutes with acetone, and then stored at –20°C. Endogenous peroxidase was blocked by 3% hydrogen peroxide. The sections were immunostained with mouse monoclonal CD4 (L3T4) biotin-conjugated antibody (1:20; Pharmingen) at 4°C overnight and then rinsed 3 times in phosphate-buffered saline (PBS) and horseradish peroxidase-avidin D (1:100; Vector, Burlingame, CA). Slides immersed in 3-amino-9-ethylcarbazole (AEC: Sigma, St. Louis, MO) were rinsed and lightly counterstained with hematoxylin (Vector). Isotype-matched immunocontrols were routinely included with each experiment.

Expression of ZO-1 was evaluated using fluorescence microscopy of specimens immunostained with rabbit polyclonal antibody against ZO-1 (dilution 5 μg/mL-Z-R1, Zymed Laboratories, San Francisco, CA) at 4°C overnight. After 3 washes with PBS, secondary antibody, i.e., FITC-conjugated goat antirabbit IgG antibody (1:20) (Zymed Laboratories), was applied for 2 hours. Control sections were treated in the same way except that they were incubated with secondary antibody only.

Scanning Electron Microscopy

For scanning electron microscopy, the tissues of terminal ileum were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), dehydrated in ethanol, and dried. The samples were then sputter-coated with gold and examined with an Aquasem electron microscope in SEM mode.

Cell Isolation and FACS Analysis

Colon samples were cut into 0.5–1 cm pieces and incubated in Ca^{2+} and Mg^{2+}-free PBS containing 10% FCS and 5 mM EDTA to release mucus colonocytes and intraepithelial lymphocytes. The pieces of tissue were then digested with collagenase/dispase (100 U/mL; Sigma). Lamina propria cells were layered on a Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). The population of mononuclear cells was recovered after centrifugation (600*g*, 20 min) at the 40/100% interface. The separated mononuclear cells were stained with FITC-conjugated antibodies against CD11b (Mac-1α chain; Pharmingen) Mac-1 expressed on granulocytes, macrophages, and NK cells and analyzed by flow cytometry (Becton Dickinson).

Statistical Evaluation

The results are expressed as the mean ± SEM of the data obtained from each experimental group. Statistical analyses were performed using SigmaStat (Jandel, Sausalito, CA).

TABLE 1. Development of the Disease Correlates with Association of Live Bacteria or Bacterial Cocktails

Histological Grading							
Groups of SCID Mice	N	% Survival >12 Weeks	Sacrifice Weeks	0–1	2–3	4–5	Detection of ZO-1
Conventional	45	83	8–12	0/39	0/39	39/45	–
Germ-free	36	100	12	36/36	0/36	0/36	+
SPF +SFB	12	100	12	0/12	0/12	12/12	–
SPF	13	100	12	8/13	5/13	0/13	+
SFB	10	100	12	10/10	0/10	0/10	+
Enteroccus faecalis	7	100	12	7/7	0/7	0/7	+
Cocktail C1	8	100	12	8/8	0/8	0/8	+
Cocktail C2	9	100	12	9/9	0/9	0/9	+
Cocktail C2+SFB	5	100	12	5/5	0/5	0/5	+
Fusobacterium mortiferum	5	100	12	5/5	0/5	0/5	+
Fusobacterium mortiferum + SFB	6	100	12	6/6	0/6	0/6	+
Bacteroides distasonis	16	100	12	16/16	0/16	0/16	+
Bacteroides distasonis + SFB	8	100	12	8/8	0/8	0/8	+

Cocktail C1: containing aerobes (*Enterococcus faecalis*, *Micrococcus spp)* and anaerobes: *(Veilonella parvula, Lactobacillus fermentum, L. delbrukii.*
C2 bacterial cocktail: containing the aerobes *Enterococcus faecalis, Bacillus spp., Streptococcus spp.,* anaerobes *Veilonella parvula, Lactobacillus fermentum, Bifidobacterium longum, Fusobacterium mortiferum,* and *F. varium.*
SPF bacterial cocktail (obtained from Hannover, Central Institute for Laboratory Animals Breeding) containing aerobes: *Bacillus species, Lactobacillus lactis, Acinetobacter species*, *Staphylococcus sciuri* and anaerobes *Lactobacillus delbrukii sp., Bulgaricus, L. fermentum, L. catenaforme, Bacteroides distasonis, B. thetaiotaomicron, Peptostreptococcus micros, P. asaccharolyticus).*

RESULTS

Clinical Findings

SCID mice with conventional (CV) microflora or microflora from SPF + SFB mice developed clinical signs of colitis (diarrhea and apathy) 5–8 weeks after injection of CD4CDRB^{high} T cells. In contrast, GF SCID mice or SCID mice colonized with SPF microflora, single strain bacteria (SFB, *Enterococcus faecalis, Bacteroides distasonis, Fusobacterium mortiferum* and combination *Fusobacterium mortiferum* + SFB and *Bacteroides distasonis* + SFB) did not have any diarrhea after transfer of CD4CRB^{high} T cells at 12 weeks after transfer.

Intestinal Inflammation in SCID Mice Raised in Different Experimental Conditions

Tissues from SCID mice (n = 39) reared in conventional conditions (CV) after transfer of CD4+CD45RB^{high} subset of T cells developed severe inflammatory changes (grade 4–5) (Table 1). Granulomatous inflammation, consisting of infiltrates of macrophages and lymphocytes and neutrophiles in lamina propria, exfoliation of colonocytes, subepithelial mucosal edema, goblet cell, and mucin depletion were detected. Staining with anti-ZO-1 did not detect the structure of tight junctions on the ileal mucosa (Table 1).

In contrast to conventional animals, GF SCID mice (n = 36) did not develop inflammatory changes in the colon 12 weeks after transfer of CD4+CD45RB^{high} T cells (grade 0–1) (Table 1). The diameter of the whole intestine was small, with many goblet cells. The structure of TJs detected by ZO-1 Ab was consistently found on the ileal mucosa (Table 1).

The colons of SCID mice colonized with SPF microflora plus SFB bacteria (n = 12) and reared in isolators for 12 weeks after transfer of CD45RB^{high} CD4+T cells exhibited severe transmural inflammation (Fig. 1A). The lamina propria showed an increase in the number of inflammatory cells, decreased number of goblet cells, and restricted quantity of mucus (grade 4–5) (Table 1). Furthermore, immunohistochemical staining revealed marked infiltration of CD4^{+} T cells in lamina propria (Fig. 1B). ZO-1 was not detected on ileal mucosa, demonstrating that the intestinal barrier was altered (Fig. 2A).

In SCID mice colonized with SPF microflora (n = 13) mild or moderate inflammatory changes were observed in lamina propria 12 weeks after transfer of CD4+ CD45RB^{high} T cells (grade 0–3) (Table 1, Fig. 1C). Ileal TJs structures (ZO-1) were detectable (Fig. 2Ba,Bb).

Monoassociation of GF SCID mice with *Enterococcus faecalis* (n = 7), SFB (n = 10), *Bacteroides distasonis* (n = 16) and *Fusobacterium mortiferum* (n = 5) did not lead to

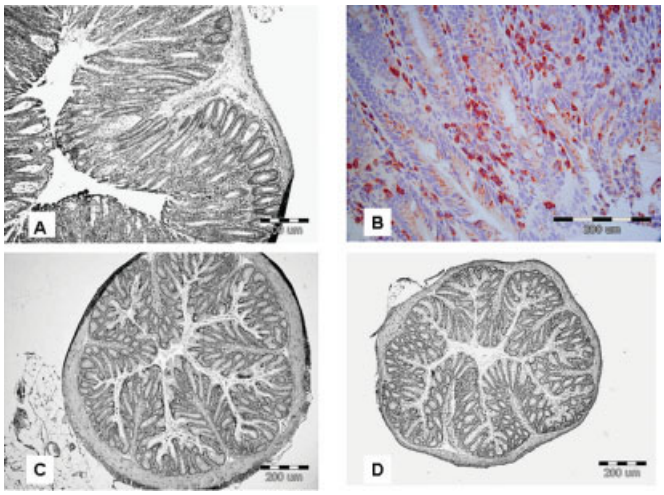

FIGURE 1. (A) Severe inflammatory changes in the colon of SCID mice colonized by SPF microflora plus SFB bacteria 12 weeks after transfer of CD4+CD45RB^{high} T cells: thick mucosa with inflammatory infiltration (H&E). (B) Immunohistochemical detection of CD4+ lymphocytes in inflammatory infiltrate present in colonic lamina propria of reconstituted SCID mice colonized by SPF microflora plus SFB bacteria. (C) The colon of SCID mice colonized with SPF microflora 12 weeks after transfer of CD4+CD45RB^{high} T cells: colon without signs of inflammation (H&E). (D) The colon of SCID mice 12 weeks after transfer of CD4+CD45RB^{high} T cells colonized with C2 cocktail: absence of inflammatory changes (H&E).

alterations of the intestinal structure. No inflammation was detected in these animals (grade 0–1) (Table 1). The structure of TJs ZO-1 was unimpaired (Table 1). No colitis was found in SCID mice colonized with of C1 (n = 8) and C2 microflora cocktail (n = 9) (Fig. 1D), and mice colonized in combination with *Bacteroides distasonis* + SFB (n = 8), *Fusobacterium mortiferum* + SFB (n = 6), or cocktail C2 + SFB (n = 5) at 12 weeks after transfer of CD4+CD45RB^{high} T cells (grade 0–1) (Table 1).

These experiments indicate that only colonization with SPF + SFB microflora leads to intestinal inflammation.

Scanning Electron Microscopy

Minimal bacterial adherence was detected in the terminal ileum of mice colonized with SPF cocktail (Fig. 3A) using scanning electron microscopy. In contrast, the terminal ileum from mice colonized with SPF + SFB bacteria at 12 weeks after injection with CD45RB^{high}CD4^{+} T cells revealed a close interaction of SFB bacteria with intestinal enterocytes (Fig. 3B). The morphology of SFB with characteristic septa was demonstrated by scanning electron microscopy (Fig. 3C).

Flow Cytometry Analysis

FACS analysis of lamina propria cells from colon using CD11b staining demonstrated that reconstituted SCID mice colonized with SPF microflora had fewer CD11b-positive cells (Fig. 4A) than mice colonized with SPF + SFB bacteria, i.e., severe inflammatory changes exhibited more cells stained with anti-CD11b (Fig. 4B). Mice colonized with cocktail C2 + SFB and SCID mice monoassociated with SFB had only low numbers of CD11b-positive cells, which corre-

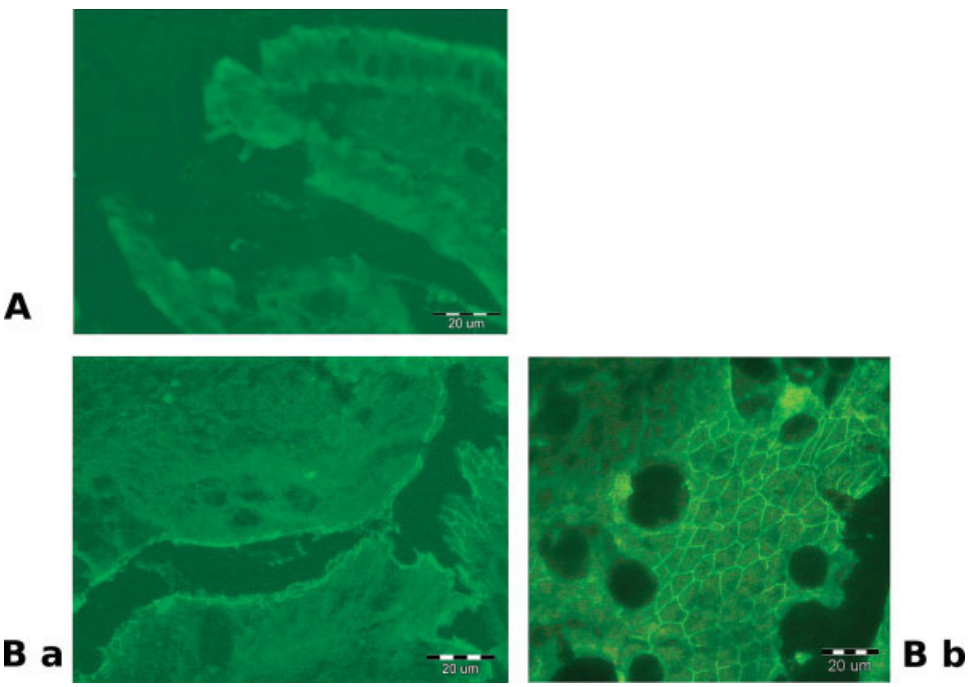

FIGURE 2. Detection of TJs structure by immunofluorescence using ZO-1 antibody. (A) Impaired TJs in terminal ileum of SCID mice colonized with SPF + SFB bacteria 12 weeks after transfer of CD4+CD45 RB^{high} of T cells: ZO-1 not detectable. (Ba) Tight junctions (TJs) in terminal ileum of SCID mice colonized with SPF microflora 12 weeks after transfer of CD4+CD45RB^{high} T cells. The vertical section: ZO-1 on the crypt and along the villi. Integrity of TJs structure preserved. (Bb) Horizontal section: staining for ZO-1.

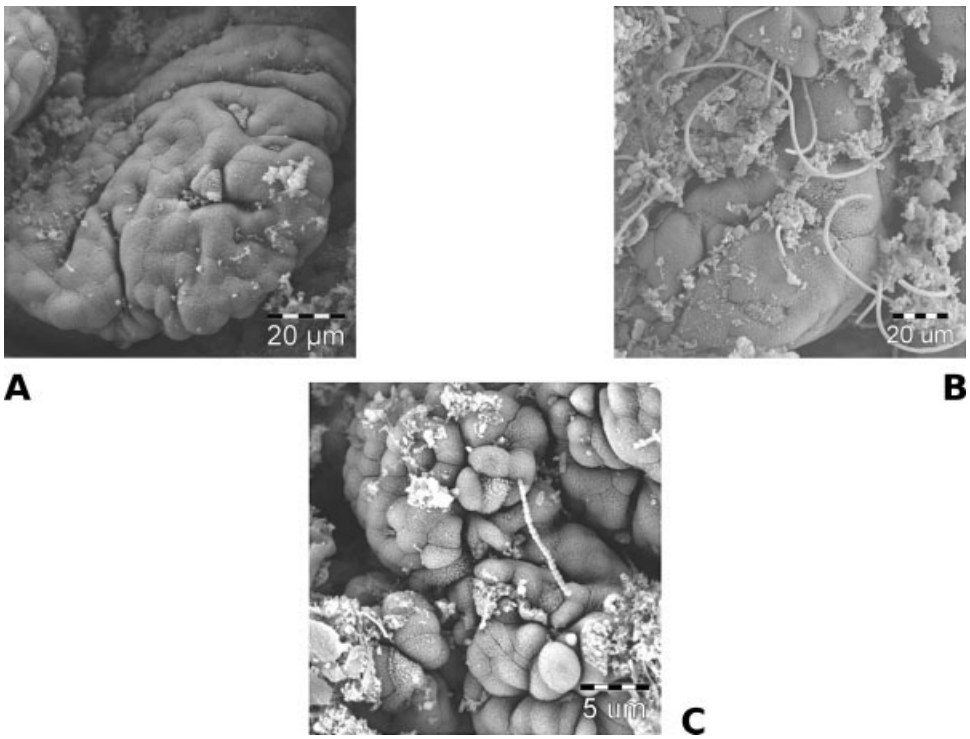

FIGURE 3. Scanning electron microscopy. (A) Terminal ileum of SCID mice colonized with SPF microflora 12 weeks after transfer of CD4+CD45RB^{high} T cells. Minimal bacterial adherence was detected. (B) Terminal ileum of SCID mice colonized with SPF + SFB microflora 12 weeks after transfer of CD4+CD45RB^{high} T cells. Interaction of SFB bacteria with enterocytes in the apical part of the ileal villi. (C) Morphological characteristics of SFB bacteria. Scanning electron microscopy of SFB bacteria with characteristic septa. The bacteria attach to ileal enterocytes of the SCID mice colonized with SPF + SFB microflora.

sponds with the absence of inflammatory changes (Fig. 4C,D).

Detection of SFB by Fluorescent In Situ Hybridization (FISH)

SFB are Gram-positive, spore-forming obligate anaerobes. They have not been successfully cultivated by common microbiological techniques so GF mice were used for multiplication (growth) of SFB. Using FISH, these bacteria could be detected and identified by the 1008-oligonucleotide in the cocktail microflora SPF + SFB (Fig. 5).

Morphological Evaluation of the Hypertrophy and Mucosal Hyperplasia of Colon Tissue

We measured the diameter of the colon to determine its hypertrophy as a sign of inflammation in all experimental groups. We did not find statistically significant differences between GF animals and mice monoassociated with SFB bacteria. Statistically significant increase of colonic wall thickness in CV animals and SPF + SFB colonized animals versus GF animals or animals colonized only with SFB bacteria or SPF cocktail bacteria was observed ($P < 0.001$) (Fig. 6A).

Hyperplasia of mucosa was measured in colitic mice (CV and colonized with SPF + SFB bacteria) compared to noncolitic mice (GF, colonized by SPF cocktail microflora and SFB bacteria). We did not find significant differences between groups of colitic mice CV versus colonized SPF + SFB cocktail bacteria. We found substantial, statistically significant differences between groups CV versus GF, SPF, and monocolonized with SFB bacteria only ($P < 0.001$) (Fig. 6B).

DISCUSSION

Development of Colitis Depends on the Presence of Bacterial Flora

In chronic IBD, intestinal lesions were considered the consequence of a dysfunction of the mucosal immune system. We have shown in this work that colitis did not develop in the SCID mice after transfer of CD4+CD45RB^{high} T cells when they were reared in GF conditions, or in SPF conditions with a limited diversity of intestinal microflora SPF (Han). When SCID mice were colonized with C1 (*Veilonella, Enteroccocus faecalis,* and *Lactobacilli)* and C2 microflora cocktail *(Veilonella, Enteroccocus faecalis, Lactobacilli,* and *Fusobacterium varium, F. mortiferum*) colitis again did not develop. In contrast, severe colitis developed when CD4+CD45RB^{high} T cells were transferred into SCID mice conventional or mice colonized with SPF cocktail microflora together with SFB bacteria. This finding confirms the results of other authors demonstrating that not all bacteria are similarly effective in giving rise to inflammation, and another mechanism may be important for the development of inflammation in the model of immunological disbalance.[20] Also, significant expansion of CD11b+ cells detected by FACS

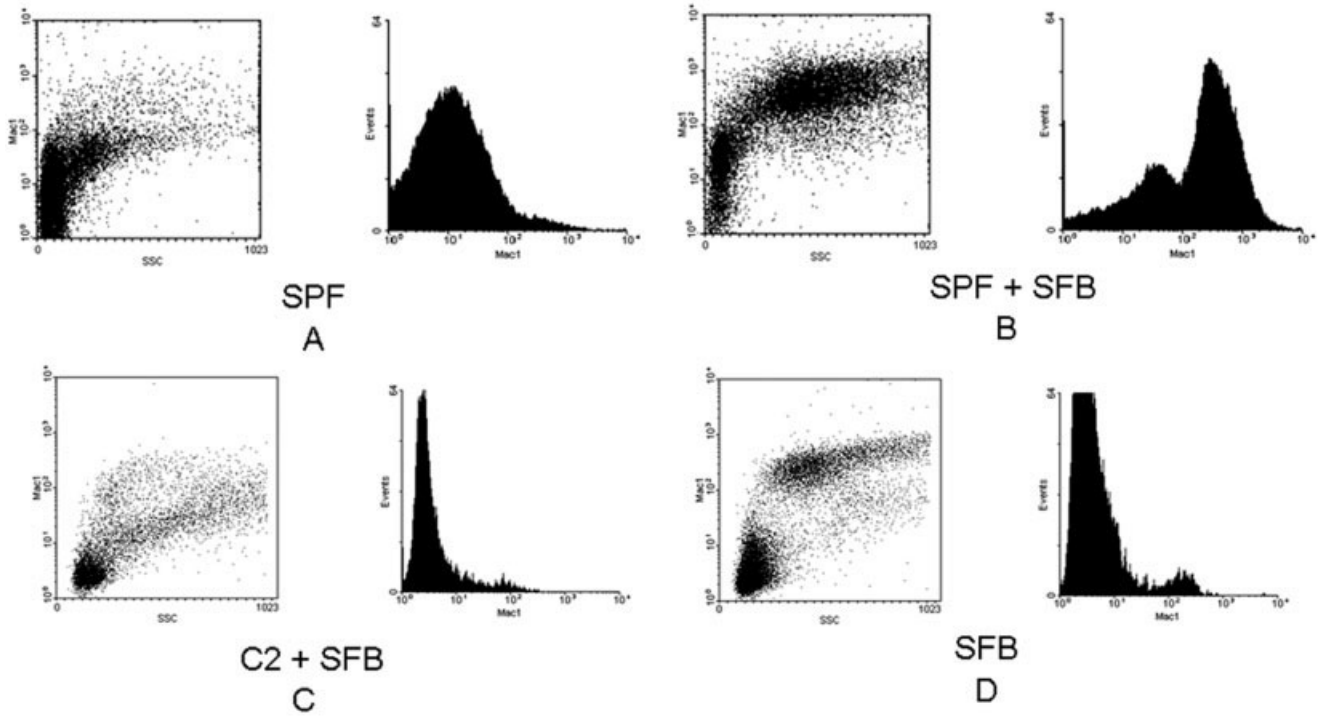

FIGURE 4. The presence of Mac1α+ cells in lamina propria (FACS analysis). (A) Lamina propria mononuclear cells of colon isolated from SCID mice colonized with SPF microflora 12 weeks after transfer of CD4+CD45 RB^{high} T cells, stained with mA-CD11b-FITC: low amount (9.0%) of stained granulocytes, macrophages, and NK cells. (B) FACS analysis of cells isolated from lamina propria of colon from SCID mice colonized with SPF + SFB microflora 12 weeks after transfer of CD4+CD45RB^{high} T cells. Staining with mA-CD11b FITC showed a large amount (66.8%) of positive cells: granulocytes, macrophages, and NK cells. (C) Lamina propria mononuclear cells of colon isolated from SCID mice colonized with C2+ SFB microflora 12 weeks after transfer of CD4+CD45RB^{high} T cells. Staining with mA-CD11b FITC showed a low amount (2.7%) of positive cells: granulocytes, macrophages, and NK cells. (D) FACS analysis of cells isolated from lamina propria of colon from SCID mice colonized with SFB bacteria 12 weeks after transfer of CD4+CD45RB^{high} T cells. Staining with mA-CD11b FITC showed a low amount (3.7%) of positive cells: granulocytes, macrophages, and NK cells.

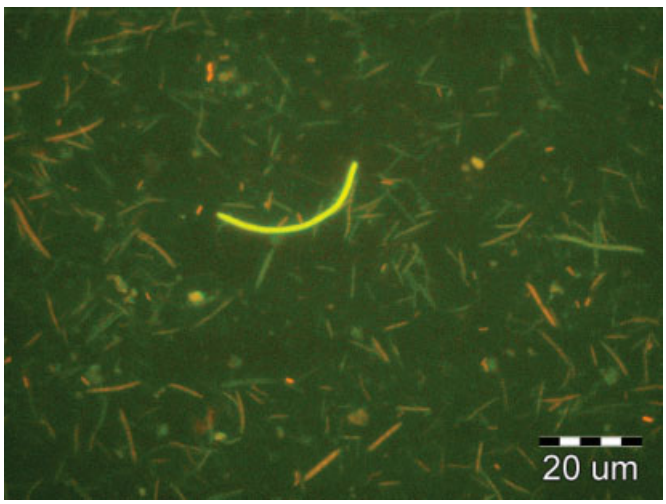

FIGURE 5. FISH of SFB in intestinal sample SCID mice colonized SPF + SFB microflora (probe SFB 1008).

analysis in lamina propria colonic cells present only in mice colonized with and SPF + SFB mixture increased the amount of innate immunity cells in intestinal wall affected by inflammation.

Microbial flora from stool samples, obtained from patients with Crohn's disease (CD), contains higher concentrations of anaerobic bacteria, among others *Bacteroides* and *Eubacterium, Peptostreptococcus,* and *Coprococcus*.[21] The evidence to date suggests that the anaerobic bacteria in the gut outnumbers the aerobic bacteria 1000–10,000 times. The main Gram-negative anaerobes are *Bacteroides* species, Gram-positive anaerobes are *Clostridium spp., Eubacterium,* and facultative anaerobes such as *Enterococcus spp.* and *Escherichia coli*.[22] Detection of intestinal microflora in IBD is complicated, since conventional bacteriology techniques recover only 30% of the total microflora.[23] Molecular biology techniques analyzing bacterial 16S rRNA improves the overall detection rate.[23,24] However, a recent report suggests that significant numbers of bacteria still remain undetected even if these molecular probes are used.[25]

Rodents have shown a high proportion of anaerobes,

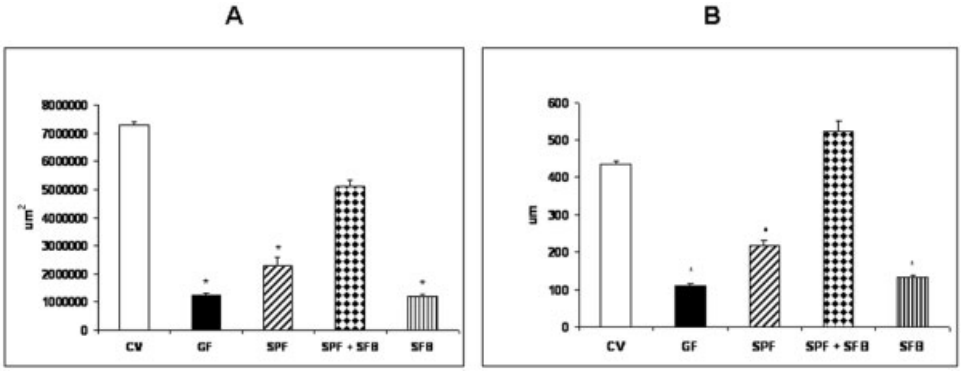

FIGURE 6. Morphometric characterization of inflammatory changes in the colon. (A) Hypertrophy of colon was detected in conventional and SPF + SFB colonized mice 12 weeks after transfer of CD4CD45RB^{high} T cells. The results are expressed as means ± SEM. Statistical significance: CV versus GF, SPF and SFB (*$P < 0.001$). (B) Hyperplasia of colon mucosa in a different group of SCID mice 12 weeks after transfer of cells CD4CD45RB^{high}. The results are expressed as means ± SEM. Statistical significance: CV versus GF, SPF and SFB (*$P < 0.001$).

similar to humans, such as *Bacteroides,* Gram-positive bacilli, and spiral-shaped bacteria.[26] Colitis did not develop in SCID mice colonized with SPF microflora 12 weeks after the transfer of CD4+CD45RB^{high} T cells. The SPF cocktail and C1, C2 cocktail includes a large proportion of bacteria such as *Lactobacillus delbrukii spp., Bulgaricus, Lactobacillus fermentum, L. catenaforme* and *Lactobacillus lactis* and *Bifidobacterium longum.* All of these bacteria are known to have probiotic potential. Probiotic bacteria were previously used for the treatment of colitis in a model that develops spontaneous colitis. Colitis was prevented in IL-10-deficient mice by continuous feeding with probiotic strain *Lactobacillus plantarum*.[27] Furthermore, in humans a better quality of life was reported in patients with colitis after the application of a probiotic VSL#3 mixture containing 4 strains of *Lactobacilli (casei, plantarum, acidophilus, delbrukii spp. Bulgaricus)*, 3 strains of *Bifidobacterium (longum, breve, infantis),* and 1 strain of *Streptococcus salivarius*.[28]

SFB are not cultivable by conventional microbiological methods; however, they could by grown in GF animals. They could be detected morphologically by electron microscopy and by molecular biology methods using specific nuclear probes. SFB are nonpathogenic, G+ spore-forming bacteria, which have a characteristic long filamentous shape with dependent septa. They are described as an important part of normal flora mainly during the weaning of mice.[29–31] Using 16S rRNA analysis, the phylogenetic position of SFB has been reported as being closely related to the genus *Clostridia*.[32] The invading property of SFB followed by a phagocyte process could be the first stimulus for the immune mucosal system, leading to subsequent dysregulation of intestinal immunity. In the present study we found that a monoassociation of SCID mice with SFB has no effect on the development of experimental colitis. The same observation has been previously reported by Jiang et al[11] and Keilbaugh et al.[33] The combination of *Fusobacterium mortiferum* (originating from C2 cocktail) + SFB or *Bacteroides distasonis* (originating from the SPF cocktail) + SFB followed by transfer of Th1 cells did not induce experimental colitis in our model.

One hypothesis suggests that IBD may be caused by the alteration of the epithelial barrier. The epithelial barrier is a complex of proteins, collectively termed tight junctions (TJs), which are positioned between adjacent epithelial cells. TJs consist of numerous proteins, e.g., claudin, zonulin, and occludin. In the SCID mice that were without colitic changes after transfer of CD4CD45RB^{high} T cells, ZO-1 were detected in the enterocytes in the terminal ileum. The intestinal barrier in these animals was not altered. In contrast, in conventional SCID mice or SCID mice colonized with SPF + SFB with severe colitis, this barrier was altered since ZO-1 was not detected in the terminal ileum of enterocytes. We suppose that the loss of ZO-1 is a secondary phenomenon due to the presence of severe inflammation, probably a reaction to the presence of living bacteria and production of proinflammatory cytokines. Similar findings were recently described in patients with active CD, where barrier dysfunction due to altered TJs structure was found.[34,35]

Important for the alteration of TJs is the adherence of bacteria with increased IFNγ. In human epithelial cells the treatment T 84 cells by IFNγ caused a loss of ZO-1.[36] Regulatory CD4+CD45RB^{low}T cells inhibit colitis.[9–13] The inhibition is probably dependent mainly on immunoregulatory cytokines such as TGFβ, which play a role in suppressing inflammatory response and also in preserving the integrity of the TJs complex.[37,38] TJs can be regulated by IFNγ and TNFα, which can cause a downregulation of a component of TJs, the ZO-1. In contrast, TGFβ can block the effects of IFNγ and TGFα on the TJs complex.[39]

The translocation of bacteria from lumen toward the lamina propria is important for the pathogenesis of colitis. We have shown by electron microscopy that SFB bacteria attach (invade) to enterocytes in the terminal ileum of SCID

mice colonized with SPF + SFB after transfer of Th1 cells, but in mice colonized with SPF cocktail and other bacterial cocktails adhesion/invasion of bacteria to epithelium was not found. Interestingly, the adhesion of SFB bacteria in colitic SCID mice takes place in terminal ileum but not in colon, where mainly inflammatory changes are present. In our previous study we found signs of activation of dendritic cells as a consequence of bacterial colonization and also in terminal ileum.[40]

Animal models of human diseases are helping to elucidate the etiology and pathogenetic mechanisms of various human diseases. Intestinal inflammation could develop as a consequence of aberrant immune reactions to food components (gliadin) and/or to gut microbiota in the early postnatal period.[41] An invaluable tool for studying the role of normal microflora in the development of these diseases (IBD) is represented by experimental models reared in germ-free conditions and colonized by defined bacterial strains.[9,11] Identifying the components of commensal bacteria involved in IBD thus could help in finding tools for the regulation of the mucosal immune responses and for new types of treatment for these severe chronic diseases.

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