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Gram-Negative Bacteria Aggravate Murine Small Intestinal Th1-Type Immunopathology following Oral Infection with *Toxoplasma gondii*¹

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Oral infection of susceptible mice with *Toxoplasma gondii* results in Th1-type immunopathology in the ileum. We investigated gut flora changes during ileitis and determined contributions of gut bacteria to intestinal inflammation. Analysis of the intestinal microbiota revealed that ileitis was accompanied by increasing bacterial load, decreasing species diversity, and bacterial translocation. Gram-negative bacteria identified as *Escherichia coli* and *Bacteroides*/*Prevotella* spp. accumulated in inflamed ileum at high concentrations. Prophylactic or therapeutic administration of ciprofloxacin and/or metronidazole ameliorated ileal immunopathology and reduced intestinal NO and IFN-γ levels. Most strikingly, gnotobiotic mice in which cultivable gut bacteria were removed by quintuple antibiotic treatment did not develop ileitis after *Toxoplasma gondii* infection. A reduction in total numbers of lymphocytes was observed in the lamina propria of specific pathogen-free (SPF), but not gnotobiotic mice, upon development of ileitis. Relative numbers of CD4⁺ T cells did not differ in naive vs infected gnotobiotic or SPF mice, but infected SPF mice showed a significant increase in the frequencies of activated CD4⁺ T cells compared with gnotobiotic mice. Furthermore, colonization with total gut flora, *E. coli*, or *Bacteroides*/*Prevotella* spp., but not *Lactobacillus johnsonii*, induced immunopathology in gnotobiotic mice. Animals recolonized with *E. coli* and/or total gut flora, but not *L. johnsonii*, showed elevated ileal NO and/or IFN-γ levels. In conclusion, Gram-negative bacteria, i.e., *E. coli*, aggravate pathogen-induced intestinal Th1-type immunopathology. Thus, pathogen-induced acute ileitis may prove useful to study bacteria-host interactions in small intestinal inflammation and to test novel therapies based on modulation of gut flora. *The Journal of Immunology*, 2006, 177: 8785–8795.

Inflammatory bowel diseases (IBD) are characterized by chronic intestinal inflammation with acute episodes (1, 2). Ulcerative colitis is restricted to the colon, whereas Crohn’s disease more frequently affects the small intestine including the terminal ileum. Intestinal bacteria trigger large bowel inflammation in IBD (2–7) and graft-vs-host disease (GVHD) after bone marrow transplantation (8, 9). IBD patients display immunoreactivity against bacterial Ags (10–12) and intestinal immunopathology is accompanied by accumulation of *Escherichia coli* or *Bacteroides* spp. at inflamed tissue sites (13–16). In experimental colitis (17–24), inflammation was suppressed in germfree animals or animals treated with antibiotics. However, our knowledge on the role of gut microbiota in ileitis is currently limited (25). Although a multitude of animal models has allowed analyses of bacteria-host interactions in the large intestine, models for small intestinal pathology are scarce (25–28). Terminal ileitis developing spontaneously in the SAMP1/YitFc mouse has been characterized immunologically, but the role of gut bacteria remains to be investigated (29–31).

Within 8 days after peroral infection with *Toxoplasma gondii*, susceptible C57Bl/6 mice develop severe ileal inflammation, resulting in necroses of mucosal villi and complete tissue destruction (32, 33). Ileitis is caused by a Th1-type immunopathology, characterized by a CD4⁺ T cell-mediated increase in proinflammatory mediators including IFN-γ, TNF-α, and NO. Activation of inducible NO synthase by IFN-γ and TNF-α is critical for intestinal pathology. Thus, *T. gondii*-induced ileal immunopathology resembles inflammatory responses operative in acute phases of human IBD (34), but the contribution of commensal gut bacteria to intestinal inflammation has not been studied so far. Therefore, we characterized intestinal microbiota changes during ileitis and investigated whether antibiotic treatment may prevent or ameliorate ileal inflammation as currently discussed for IBD in humans (35–38). The impact of distinct bacterial species on small intestinal immunopathology was studied by defined colonization of gnotobiotic mice, created by complete removal of cultivable gut bacteria using quintuple antibiotic treatment.

Materials and Methods

Mice, parasites, ileitis induction, and antibiotic treatment

Mice used for experiments were 2–4 mo old and bred under specific pathogen-free (SPF) conditions. Clinical conditions as well as body weights were determined twice daily and the experiments conducted according to...
the German animal protection laws. Cysts of the T. gondii ME49 strain (a gift of J. S. Remski, Stanford University, Stanford, CA) were obtained from homogenized brains of NMRI mice that had been infected i.p. with 10 cysts for 2–3 mo. For induction of small intestinal inflammation, C57BL/6 mice were infected perorally with 100 cysts in a volume of 0.3 ml of PBS (pH 7.4) by gavage. In prophylactic and therapeutic treatment studies, administration of antibiotics was started 5 days before, or 5 days after, infection, respectively. C57BL/6 mice received PBS alone (controls) or PBS with ciprofloxacin (Cf), metronidazole (Mtz), or a combination of both (each 50 mg/kg/day) perorally by gavage twice daily. Antibiotics were withheld between 24 h before and after T. gondii infection.

Generation and defined colonization of gnotobiotic mice

To remove the commensal gut flora, C57BL/6 mice were transferred to sterile cages and treated by adding ampicillin (1 g/L; Ratiopharm), vancomycin (500 mg/L; Cell Pharm), Cl (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and Mtf (1 g/L; Frenesium) to the drinking water ad libitum for 6–8 wk according to a standard protocol as described below.

For reconstitution, gnotobiotic mice without cultivable gut microbiota received luminal ileal gut contents from mice with ileitis, E. coli, Lactobacillus johnsonii, or a mix of strict anaerobic bacteria containing Bacteroides/Prevotella spp. by gavage of 0.3–ml suspensions on 3 consecutive days. Four days before the reconstitution experiments, the antibiotic mixture was replaced by sterile drinking water. Four days after the third administration of luminal ileal gut contents or specific bacteria, mice were perorally infected with T. gondii to induce ileitis. Luminal ileal gut contents were obtained 8 days p.i. from five C57BL/6 mice on 3 consecutive days. The ileal contents were pooled each day in 2.5 ml of sterile PBS and administered perorally (gavage 0.3 ml). For reconstitution, E. coli and Bacteroides/Prevotella spp. were isolated from infected mice whereas L. johnsonii was obtained from uninfected animals. All isolates were identified by biochemical and comparative 16S rRNA sequence analysis. E. coli and L. johnsonii were grown in supplemented brain heart infusion. A mix of anaerobic bacteria containing Bacteroides uniformis, Bacteroides ovatus, Bacteroides merdae, Bacteroides thetaiotaomicron, Prevotella buccae, and Prevotella oralis was cultured in thioglycolate broth. All cultures were grown to a turbidity equivalent of 6 McFarland units (a bacterial load of 108–1010/ml). Then, E. coli were cultured in thioglycolate broths. All cultures were determined by counting of distinct colony morphotypes on Columbia blood agar. Bile esculin, McConkey, and Columbia plates (Oxoid) were incubated on a DC0 system (Becton Dickinson Vacutainer Systems) at 37°C for 16 h in a polycrylamide gel containing 35–60% urea/formamide. DNA band profiles were visualized by silver staining. For sequence analysis, DGGE bands were stained with SYBR green I (Fluka), visualized under UV light, and cut off the gel matrix. DNA was eluted by shaking in double-distilled H2O (ddH2O) overnight at 37°C. After reamplification by PCR, the amplicons were cloned and sequenced.

Cultural analysis

Luminal contents from terminal ilea (1 cm) were resuspended in PBS, weighted, and 100-μl aliquots of serial dilutions plated onto solid medium (Oxoid). Bacteria were grown at 37°C for 2 days under aerobic or for 5 days under anaerobic conditions and total numbers were determined by colony counting on Columbia blood agar. Bile esculin, McConkey, and Rogosa (Merek) medium were used for quantitative identification of enterococci, enterobacteria, and lactic acid bacteria (mainly lactobacilli), respectively. The amount of the different anaerobic bacterial groups was determined by counting of distinct colony morphotypes on Columbia blood agar. Bacteria were subcultivated and further investigated by Gram staining and by biochemical analysis with the API20E, API50 CH, and API Rapid ID 32A systems (Biomerieux). Results were expressed as CFU per gram of luminal ileal content.

Fluorescence in situ hybridization (FISH)

Terminal ileum (1.5 cm) was tied on both ends with sterile surgical silks and cut out by transversal sectioning. Samples were fixed in PBS containing 50% (v/v) ethanol (pH 7.4) and 3.7% (v/v) formaldehyde at 4°C and embedded in polymerizing resin (Technovit 8100; Kulzer) as described elsewhere (45). During the last embedding step, each sample was cut into two to three sections parallel for histological, microbiological, immunological, and molecular analyses. The relative shortening of the small intestine was calculated by dividing the difference in the mean length of small intestines in naive control mice and the respective length of small intestine at day 8 p.i. multiplied by 100 by the mean length of small intestines in naive control mice (relative shortening in length = (mean day 0 – day 8 p.i.) / 100 mean day 0). Results were expressed as the percent shortage. There were five mice per group. Experiments were repeated three times.

Histologic scores and determination of parasite load

Histologic scores of ileitis and parasite loads were determined in tissue samples from terminal ileum immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with H&E and examined by light microscopy. Our standardized histological inflammation score ranging from 0 to 6 (0: normal; 1: edematous blunting; 2: cell-free exudate within the lumen, but intact epithelium; 3: cellular shedding into the lumen; 4: luminal epithelial disintegration; 5: mucosal destruction <50% of small intestine length; 6: complete destruction >50% of small intestine length, severe necrosis) was used for blinded duplicate evaluation (by M. M. Heimesaat and D. Fuchs). Numbers of parasitophorous vacuoles containing tachyzoites or tachyzoite Ags were determined in two areas of 1-cm length chosen at random by two independent investigators (M. M. Heimesaat and D. Fuchs) using immunohistology with T. gondii antiserum.

Molecular analysis of the ideal microflora

Luminal contents were removed from 1 cm of the terminal ilea, resuspended in PBS, and centrifuged (16,000 × g/10 min/4°C). The sediment was resuspended in 0.5 ml of lysis buffer (500 mM Tris (pH 9.0), 20 mM EDTA, 10 mM NaCl, 1% SDS) and incubated with proteinase K (2 mg/ml, Sigma-Aldrich) for 1 h at 56°C. After heating, total DNA isolated by phenol extraction served as template for PCR amplification (95°C/3 min followed by 25 cycles of 95°C/45 s, 56°C/45 s, 72°C/1 min, final elongation at 72°C/7 min) of bacterial 16S rRNA genes with 20 pm consensus primers (40) TPU/RTRUS (5′-AGAGTTTGTAGTGCTGGTCAAG3′-nt 8-27 in E. coli 16S rRNA)/(5′-AAGGGTGTTACCCNCRRCA3′-nt 1541-1522 in E. coli 16S rRNA (41). For construction of gene libraries, the amplicons were cloned into plasmid pCR2.1-TOPO (TOPO-TA Cloning Kit; Invitrogen Life Technologies). Individual 16S rRNA gene sequences (>500 bp) were determined (CEQDTCS Quick Start kit on a CEQ8000 Genetic Analysis System; Beckman Coulter) and compared with databases (NCBI-BLASTN tool (42); RDP 9.0 (43)). Genetic fingerprints were generated by denaturing gradient gel electrophoresis (DGGE; Ref. 44). Variable regions 5–6 in bacterial 16S rRNA genes were amplified (degenerate primer set at 95°C/3 min, then 25 cycles of 95°C/45 s, 64°C/1 min, 72°C/1 min, final elongation at 72°C/27 min) from total gut content DNA with GC clamp (underlined) primer GC9068F (5′-GGCCGGGGGCGCGC CCCGGGCGCGGCGGGGACGGGGGAACCGAAGACCTTCA-3′, nt 968-84 in E. coli 16S rRNA) and primer R1378 (5′-CGGTGTGTTACAAGGCGCGGGAAAG-3′, nt 1401-1378 in E. coli 16S rRNA). Amplifications were performed on a DCode system (Bio-Rad) for 16 h at a polycrylamide gel containing 35–60% urea/formamide. DNA band profiles were visualized by silver staining. For sequence analysis, DGGE bands were stained with SYBR green I (Fluka), visualized under UV light, and cut off the gel matrix. DNA was eluted by shaking in double-distilled H2O (ddH2O) overnight at 37°C. After reamplification by PCR, the amplicons were cloned and sequenced.
Determination of cytokine concentrations

Ileum samples (~1 cm²) were flushed thoroughly with sterile PBS, cut longitudinally, and cultured in 24-well plates (Nunc) containing 0.5 ml of RPMI 1640 (Invitrogen Life Technologies) with penicillin and streptomycin (Biochrom). After 24 h at 37°C, supernatants were harvested and stored at −80°C. IFN-γ concentrations were determined by ELISA (BD Biosciences). NO was measured by Griess reaction (50 μl supernatant were mixed with 50 μl of 15% sulfanilamide (Roth) in 1 M HCl plus 0.15% N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich). After 10 min, the absorbance at 540 nm was measured photometrically. Nitrite concentrations were calculated from standard curves.

Isolation of lymphocytes and flow cytometry

Small intestines were obtained and washed in PBS. After removal of Peyer’s patches, intestines were cut longitudinally. Faeces and mucus were removed by washing in PBS. Lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) were isolated after IEL purification. Pieces of small intestines were washed for 10 min in RPMI 1640 and incubated shaking in PBS with 10% FCS and 5 mM EDTA at 37°C for 15 min. Supernatants were filtered through a 70-μm nylon sieve and washed in RPMI 1640 containing 5% FCS and centrifuged to pellet the cells. This process was repeated three times. Cells were resuspended and centrifuged through a 40%/70% Percoll gradient (Biochrom) for 34 min at 37°C, 8000 rpm. Supernatants were collected and filtered through a 0.2-μm nylon sieve and washed in RPMI 1640 containing 5% FCS and penicillin/streptomycin. LPLs were isolated after IEL purification. Pieces of small intestines were washed for 10 min in RPMI 1640 and incubated shaking in RPMI 1640 containing 5% FCS, collagenase/dispase (Sigma-Aldrich), and DNase I (Sigma-Aldrich) for 60 min at 37°C. Supernatants were filtered through a 70-μm nylon sieve and washed in RPMI 1640 containing 5% FCS and centrifuged to pellet the cells. Cells were resuspended and centrifuged through a 40%/70% Percoll gradient (Biochrom) for 34 min at 3400 rpm. Cells in the interface were collected and washed in RPMI 1640 containing 10% FCS and penicillin/streptomycin.

A total of 1 × 10⁶ IEL and LPL were pretreated on ice for 10 min with 10 μl of a predetermined optimal concentration of anti-FcγRIII receptors (2.4G2) to block non-Ag-specific binding of Abs to the FcγRIII receptors. Thereafter, cells were incubated on ice for 30 min with 10 μl of optimal concentrations of PE-conjugated anti-CD4 mAb (RM4-5), FITC-conjugated anti-CD8 mAb (53-6.7), PE-conjugated anti-TCR αβ mAb (H57-597), FITC-conjugated anti-TCR γδ mAb (GL3), and FITC-conjugated anti-CD69 (H1.2F3). Analysis of stained cells was performed with a FACScan (BD Biosciences). Dead cells were gated out on the basis of propidium iodide staining.

Liver lymphocytes were obtained following perfusion of mice through the vena portae using PBS. Livers were removed from the animal and homogenized through a nylon mesh sieve (70 μm). Cell suspensions were washed with PBS and centrifuged at 50 g for 1 min. Supernatants were taken. This process was repeated four times. Afterwards, supernatants were pooled, washed using RPMI 1640 containing 5% FCS and penicillin/streptomycin and centrifuged through a 40%/70% Percoll gradient. Cells were collected from the interface, washed with RPMI 1640, and used for flow cytometry analysis as described above.

Statistics

At least four animals per group were used to determine histological, microbiological, immunopathological, and parameter of inflammation. Statistical parameters were determined using the Student t test. Probability (p) values <0.05 were considered significant. Experiments were repeated at least three times as indicated.

Results

Ileitis is accompanied by intestinal microflora changes

Histopathology of the ileum of T. gondii-infected C57BL/6 mice revealed that mild inflammation developed 3–5 days postinfection.
The acute phase of inflammation (days 6–8) was accompanied by cellular shedding, massive tissue destruction, and necroses (Fig. 1). Monitoring of intestinal bacterial communities by PCR-based DGGE demonstrated that the acute stage of ileitis was accompanied by a profound loss in bacterial diversity (Fig. 2A) and a shift in the flora toward Enterobacteriaceae (Fig. 2B). Sequence analysis of 16S rRNA gene fragments in DGGE bands revealed that lactobacilli, bifidobacteria, clostridia and so far uncharacterized Bacteroides/Prevotella species of the Porphyromonadaceae family, which dominate the flora in healthy mice, disappear during infection (Fig. 2B). This was confirmed by analysis of 16S rRNA gene libraries constructed from ileal contents of mice (n = 3 each) with 40 individual sequences analyzed and without (121 individual sequences analyzed) ileitis. In the ileum of healthy mice, Firmicutes (Lactobacillales/Clostridiales) and Bacteroidetes (Bacteroidales/Prevotellales spp.) predominated exhibiting frequencies of 67 and 28%, respectively. L. johnsonii (36.6%) was the most abundant Lactobacillus species, followed by Lactobacillus murinus (20%), Lactobacillus reuteri (9.9%), Lactobacillus intestinalis (3.3%), and other taxonomically uncharacterized Lactobacillus spp. (30.2%). Proteobacteria (Desulfovibrionales, Burkholderiales) and Actinobacteria (Coriobacteriales, Bifidobacteriales) represented 5% of clones. In contrast, clone libraries from mice with inflamed ilea contained 16S rRNA genes exclusively from Enterobacteriaceae (96.6%) and Bacteroides spp. (3.4%). Thus, molecular analyses indicate that the development of immunopathology is accompanied by profound changes in the bacterial flora.

Table I. Bacterial concentrations in ileal contents of mice after antibiotic treatment

<table>
<thead>
<tr>
<th>Treatment Group (Antibiotics)</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram-negative rods</td>
<td>Gram-positive rods</td>
</tr>
<tr>
<td>Without treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>7.2 ± 0.9 10⁴</td>
<td>5.7 ± 6.6 10⁸</td>
</tr>
<tr>
<td>Infected</td>
<td>1.2 ± 0.9 10¹¹</td>
<td>5.5 ± 4.1 10⁸</td>
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<td></td>
<td></td>
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<tr>
<td>Prophylactic treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (ciprofloxacin)</td>
<td>&lt;1 10³</td>
<td>5.0 ± 6.9 10⁷</td>
</tr>
<tr>
<td>Infected (metronidazole)</td>
<td>5.3 ± 6.8 10⁹</td>
<td>2.6 ± 2.7 10⁸</td>
</tr>
<tr>
<td>Infected (ciprofloxacin + metronidazole)</td>
<td>&lt;1 10³</td>
<td>1.1 ± 1.3 10⁷</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Therapeutic treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (ciprofloxacin)</td>
<td>&lt;1 10³</td>
<td>3.7 ± 3.6 10⁷</td>
</tr>
<tr>
<td>Infected (metronidazole)</td>
<td>3.3 ± 3.9 10⁹</td>
<td>5.1 ± 6.1 10⁸</td>
</tr>
<tr>
<td>Infected (ciprofloxacin + metronidazole)</td>
<td>&lt;1 10³</td>
<td>1.1 ± 1.3 10⁷</td>
</tr>
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</table>

*Mice with severe ileitis at day 8 after T. gondii infection; n = 5 each group.

Escherichia coli and Bacteroides/Prevotella spp. dominate the intestinal flora in acute ileitis

Because molecular analyses indicated marked changes in the composition of the bacterial flora, we next performed quantitative microbiological analyses of the cultivable ileal flora. During inflammation the total bacterial load in the ileum lumen increased from 1.6 ± 0.8 × 10⁹ at day 0 to 2.3 ± 2.5 × 10¹¹ CFU per gram of gut content at day 8 p.i. (Table I). Aerobic and anaerobic bacteria increased from 5.8 ± 6.6 × 10⁸ to 1.2 ± 0.9 × 10¹¹ CFU/g and 1.1 ± 0.9 × 10⁸ to 1.1 ± 1.8 × 10¹¹ CFU/g, respectively (Fig. 3). In mice with acute ileitis at day 8 p.i., aerobic and anaerobic Gram-negative bacteria identified as E. coli and Bacteroides/Prevotella spp. increased by 6–8 orders of magnitude from 7.2 ± 9.0 10⁹ to 1.2 ± 0.9 10¹¹ and from <1 10³ to 1.1 ± 1.8 10¹¹, respectively (Fig. 3, Table I). Of the strict anaerobic Gram-negative bacteria, Bacteroides spp. and Prevotella spp. constituted 59.1 and 40.9%, respectively. The Bacteroides population comprised B. ovatus (61.5%), B. merdae (23.1%), B. uniformis (7.7%), and B. thetaiotaomicron (7.7%). Prevotella spp. were represented by P. oralis (88.9%) and P. buccae (11.1%). In the course of inflammation, Gram-positive rods including lactobacilli and clostridia were reduced (Fig. 3, Table I), whereas levels of Enterococcus spp. (Enterococcus faecalis, Enterococcus faecium, Enterococcus gallinarum) tended to increase (not significant). Ileal overgrowth by Gram-negative bacteria started at day 6 p.i., when E. coli levels rose from 1.0 ± 1.7 × 10⁷ to 6.3 ± 4.8 × 10⁹ CFU/g and the...
Bacteroides/Prevotella spp. increased from $<10^3$ to $6.7 \pm 7.8 \times 10^6$ CFU/g within 24 h. Quantitative cultural analyses thus confirmed and substantiated the molecular analyses described above. The importance of the combined use of molecular techniques and cultivation is further supported by the fact that the cultured Bacteroides/Prevotella spp. and enterococci could not be detected by DGGE. A detailed sequence comparison of 16S rRNA gene fragments revealed that bacteria represented by the DGGE bands labeled Porphyromonadaceae (Fig. 2B) are taxonomically related to so far uncultured Bacteroides/Prevotella spp. but differ considerably from the well-defined Bacteroides species detected by cultivation and biochemical analysis.

Translocation of gut bacteria to subepithelial tissues in inflamed ileum

FISH revealed that ileal inflammation was accompanied by translocation of bacteria to subepithelial tissue sites. Bacteria were detected in the gut lumen, in close contact to epithelial cells, and in the crypts in uninfected control and in infected mice with ileitis (Fig. 4, A and B). Translocation of bacteria into the submucosa was exclusively found in focal areas of disrupted epithelium (Fig. 4, B and D) but not in areas of intact epithelium in infected mice (Fig. 4C) nor in control mice (Fig. 4A). Translocated bacteria in subepithelial tissues were identified as Enterobacteriaceae and Bacteroides spp. by FISH with specific oligonucleotide probes Ecoli1531 (47) and Bac303 (48), respectively (data not shown).

Antibiotic treatment prevents ileitis

Because increased numbers of Gram-negative bacteria and translocation to subepithelial sites were observed in infected mice, we next treated mice with antibiotics. Prophylactic treatment with Cf and Mtz starting 5 days before T. gondii infection (Fig. 5) resulted in survival of $\sim 30\%$ of mice during the acute stage of infection (Fig. 5A). Animals treated with Cf alone displayed higher survival rates than untreated or Mtz-treated mice (Fig. 5A). E. coli and Bacteroides/Prevotella spp. were eradicated after Cf and/or Mtz administration, respectively (Table I). At day 8 p.i. when all placebo-treated animals had died, >90% of animals treated with antibiotics were still alive (see Fig. 5A). Moreover, mice treated with Cf plus Mtz displayed significantly less histopathologic changes in their ilea than mono-treated mice (Fig. 5B), which showed mild to...
moderate histopathology (Fig. 5B). Antibiotic prophylaxis significantly reduced small intestinal shortening (a parameter commonly determined in the large intestine in mice with colitis). In mice with severe ileitis, the lengths of the small intestines were reduced by 19.0 ± 4.8% as compared with uninfected controls. Significantly less shortening was observed in animals treated with Cf, Mtz, or Cf plus Mtz (8.1 ± 6.1% (p < 0.05), 9.3 ± 2.1% (p < 0.01), and 9.5 ± 3.4% (p < 0.01), respectively).

To determine whether antimicrobial therapy (starting on day 5 p.i.) also ameliorates small intestinal inflammation, mice were treated with the antimicrobial agents. Approximately 20% of mice treated with Cf plus Mtz combination therapy survived acute infection (Fig. 6A). Mice treated with either Cf or Mtz survived 3 days longer than untreated mice. Mice treated with either Cf or Mtz alone or in combination showed significantly less ileal inflammation on day 8 p.i., as compared with controls (Fig. 6B). Prophylactic treatment with Cf plus Mtz was more effective than the therapeutic administration of the same combination (p < 0.05). The decrease of mortality and tissue damage by both schemes was paralleled by reduced intestinal inflammatory responses (Fig. 7), as demonstrated by lower NO (Fig. 7A) and IFN-γ (Fig. 7B) levels in ilea from treated vs control mice. These results indicate that the profound increase in Gram-negative bacteria in the ilea of infected mice contributes to ileal immunopathology and early death in susceptible mice. Interestingly, both prophylactic and therapeutic treatment ameliorated the severity of immunopathology. Numbers of T. gondii parasitophorous vacuoles in the ileum did not differ between controls and mice treated with either antimicrobial regimen (data not shown).

Development of ileitis in gnotobiotic mice
To further dissect the contribution of gut bacteria to ileitis and to avoid possible parasite-related or immunomodulatory influences of antibiotics, we investigated the development of ileitis in gnotobiotic mice that had received the same treatment but differed in the colonization status of the gut. Approximately 80% of gnotobiotic animals survived acute infection and up to 75% survived for >4 wk (Fig. 8A). In strong contrast, gnotobiotic mice reconstituted with ileal flora obtained from mice with ileitis all died by day 9 p.i. as did conventional SPF mice (Fig. 8A). The survival rates correlated with inflammatory ileal changes. Although gnotobiotic mice showed no signs of ileal inflammation, SPF animals and gnotobiotic mice reconstituted with ileal gut content of diseased mice developed severe histopathology (Fig. 8B). Monitoring of ileitis in gnotobiotic animals reconstituted with either E. coli, Bacteroides Prevotella spp., or L. johnsonii revealed that the potential of individual bacteria to induce inflammation varies profoundly. Mice monocolonized with E. coli displayed moderate histopathologic changes in their ilea on day 8 p.i., but did not survive beyond day 13 p.i. (Fig. 8A, A and B). Histopathologic changes in mice colonized with Bacteroides/Prevotella spp. did not differ from those in mice monocolonized with E. coli, but up to 22% of mice survived until 4 wk p.i. Gnotobiotic mice monoassociated with L. johnsonii did not develop ileitis. More than 80% of mice survived until day 9 p.i., and 4 wk p.i. up to 37.5% of these mice had survived (Fig. 8A, A and B). Immunopathology induced by colonizing mice with gut flora or E. coli obtained from mice with ileitis was associated with elevated intestinal NO (Fig. 8C) and IFN-γ.
levels (Fig. 8D) whereas colonization with L. johnsonii did not alter cytokine levels compared with untreated gnotobiotic mice. Taken together, these results indicate that Gram-negative bacteria, i.e., E. coli, contribute to intestinal immunopathology, most likely by increasing local proinflammatory (Th1-type) responses.

**SPF mice show increased frequencies of activated CD4+ T cells in the small intestinal lamina propria and liver**

Because CD4+ T cells in the lamina propria are key mediators of ileitis development, we analyzed lamina propria and intraepithelial cells in naive and infected gnotobiotic compared with SPF mice. Upon infection, neither gnotobiotic nor SPF mice increased relative percentages of CD4+ T cells in the lamina propria (21.2 ± 4.5 vs 23.6 ± 3.5 in naive vs infected gnotobiotic mice (Fig. 9B) and 22.6 ± 2.4 vs 23.6 ± 4.2 in naive vs infected SPF mice (Fig. 9A)). However, frequencies of activated CD4+ T cells did not differ in naive (19.4 ± 0.1) and infected (19.6 ± 1.9) gnotobiotic mice (Fig. 9B). Whereas frequencies of CD8+ T cells did not differ in naive vs infected SPF mice, frequencies of CD8+ T cells increased in SPF mice upon infection (Fig. 9C). The absolute numbers of mononuclear cells remained stable in gnotobiotic mice upon infection (6.9 ± 5.1 × 10^6 vs 5.6 ± 2.4 × 10^6 in naive vs infected gnotobiotic mice) whereas absolute numbers of cells decreased markedly in infected compared with naive SPF mice (8.4 ± 2.7 × 10^6 vs 1.1 ± 0.9 × 10^6 in naive vs infected SPF mice, respectively). These results reveal that the presence of gut bacteria increases the frequencies of activated CD4+ T cells in the lamina propria of mice following infection with T. gondii; activated cells are most likely lost in large numbers due to activation-induced cell death in SPF, but not gnotobiotic, mice.

In the IEL compartment, we observed a remarkable increase in the frequencies of activated CD4+ T cells in gnotobiotic, but not SPF, mice (Fig. 10, A and B), suggesting that contact with gut flora results in activation of these cells. Frequencies of CD8+ T cells increased slightly in IEL in both SPF and gnotobiotic mice (Fig. 10C) frequencies of γδ T cells in the IEL compartment were higher in naive and infected gnotobiotic
compared to SPF mice (Fig. 10D). In the liver, the increase in activated CD4⁺ T cells was even more pronounced; whereas SPF mice showed an increase in the frequency of activated CD4⁺ T cells from 1.2 to 20.0% upon infection (Fig. 11A), the frequency of these cells increased from 0.7 to only 3.4% in gnotobiotic mice following infection (Fig. 11B). A decrease in the frequencies of CD8⁺ T cells in the livers of SPF and gnotobiotic mice was paralleled by a marked increase in the frequency of γδ T cells in SPF and gnotobiotic mice (Fig. 11D).

**Discussion**

The contribution of accumulating Gram-negative bacteria to intestinal inflammation and the varying inflammatory potential of gut bacteria indicates that studies focusing on the role of bacteria in IBD should be accompanied by comprehensive analyses of the intestinal microflora. However, due to the complexity of the gastrointestinal ecosystem (49) and limitations of culture-based techniques (50), valid data are scarce. In addition, only few experimental models allow analysis of small intestinal inflammation. The combination of molecular and conventional culture techniques revealed that *T. gondii*-induced ileitis is reproducibly accompanied by a pronounced loss of bacterial diversity and a rise in commensal Gram-negative gut bacteria identified as *E. coli* and *Bacteroides/Prevotella* spp.

Reduced microflora diversity (51) and elevated levels of Gram-negative bacteria were reported for areas of inflamed gut in IBD patients (13–16, 52, 53) and the same bacterial groups are suspected to trigger GvHD (8). The fact that ileal overgrowth by enterobacteria was observed earlier during liver injury, portal vein obstruction, prolonged enteral feeding, and reduced bowel motility (54–58) suggests that bacterial proliferation is most likely caused by a breakdown in small intestinal physiology. Prevention and even amelioration of ileitis by antibiotic treatment shown here demonstrates for the first time that accumulating *E. coli* and *Bacteroides/Prevotella* spp.
potentiate the severity of acute murine small intestinal immunopathology. Whereas we cannot exclude that the beneficial effects of antibiotics on immunopathology were partly mediated by immunomodulatory effects of these drugs, i.e., chinolones (59), amelioration of ileitis in gnotobiotic mice as well as the induction of ileitis in monoclonal gnotobiotic mice clearly indicate the impact of intestinal bacteria on development of ileitis. These findings are well in line with the amelioration of ileitis developing in SAMP1/YitFc mice by antibiotic treatment (29–31, 60) and the abundance of E. coli and Bacteroides/Prevotella spp. detected in experimental colitis (61, 62) and in patients with IBD (13). Furthermore, the colitogenic potential of both bacterial groups was determined earlier (18, 19, 21, 63) and the contributions of Gram-negative bacteria to the severity of intestinal inflammation were supported by successful antibiotic treatment of experimental colitis and in human IBD (22, 64, 65), as well as in GvHD (8).

We present new evidence that the length of the small intestine is a sensitive marker for the severity of small intestinal inflammation, as mice with severe ileitis lost up to 20% of their small intestine length, and reduced shortening accurately reflected ameliorated histopathology in animals treated with antibiotics.

Mechanisms by which gut bacteria trigger ileitis are not known so far. The replacement of Gram-positive bacteria by Gram-negative species in the inflamed ileum provides evidence that specific bacterial Ags such as LPS contribute to ileal inflammation. In favor of a role of LPS, we observed that mice treated with the LPS scavenger polymyxin-B (66, 67) displayed less ileal inflammation whereas we could not observe a role of LPS, we observed that mice treated with the LPS scavenger polymyxin-B (66, 67) displayed less ileal inflammation.

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References


