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

Markus M. Heimesaat,² Stefan Bereswill,² André Fischer,² David Fuchs, Daniela Struck, Julia Niebergall, Hannah-Katharina Jahn, Idikò R. Dunay, Annette Moter, Dorothee M. Gescher, Ralf R. Schumann, Ulf B. Göbel, and Oliver Liesenfeld³

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 microflora 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, recolonization 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)⁴ 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 microflora 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

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⁴ Abbreviations used in this paper: IBD, inflammatory bowel disease; GvHD, graft-vs-host disease; SPF, specific pathogen free; DICE, denaturing gradient gel electrophoresis; ddH₂O, double-distilled H₂O; FISH, fluorescence in situ hybridization; LPL, lamina propria lymphocyte;IEL, intraepithelial lymphocyte; p.i., postinfection; CFU, ciprofloxacin; Mtx, metronidazole; DAPI, 4′,6′-diamidino-2-phenylindole.

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the German animal protection laws. Cysts of the T. gondii ME49 strain (a gift of J. S. Remis, 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), CF (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and Mzt (1 g/L; Frenesium) to the drinking water ad libitum for 6–8 wk according to a standard protocol with mild modifications (39). To control the intestinal colonization status of mice, individual fecal samples were taken once weekly and at the time of necropsy. Samples were incubated in brain heart infusion and in thioglycolate broths (Oxoid) for at least 1 wk at 37°C and bacterial growth was monitored daily by turbidity assessment. Aliquots from turbid broths were cultivated on solid medium under aerobic and anaerobic conditions and the bacteria were identified microbiologically and biochemically as described below.

For recolonization, 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 recolonization 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 by centripetal centrifugation of 0.3-ml suspensions on 3 consecutive days. The ileal contents were pooled each day in 2.5 ml of sterile PBS and administered perorally by gavage (0.3 ml). For recolonization, 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 broths. All cultures were grown to a turbidity equivalent of 6 McFarland units (a bacterial load of 108–109 CFU/ml). L. johnsonii and E. coli were harvested by centrifugation, washed once, resuspended in 5 ml of PBS, and administered by gavage (final volume of 0.3 ml). To avoid oxidative stress, the turbid broths containing anaerobic bacteria were not centrifuged and pooled in a final volume of 5 ml and administered in the same manner. Bacterial concentrations were determined by cultivation on solid medium.

Sampling procedures and determination of small intestinal length

Mice were sacrificed with halothan (Eurim-Pharm). Tissue samples from spleen, mesenteric lymph nodes, and terminal ileum were removed under sterile conditions. Intestinal samples from each mouse were collected in 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 scoring ranging from 0 to 6 (0, normal; 1, edematous blubbing; 2, cell-free exudate into the lumen, but intact epithelium; 3, cellular shedding into the lumen; 4, complete epithelial disintegration; 5, mucosal destruction <50% of small intestine length; 6, complete destruction >50% of small intestine length, severe necroses) 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 immunohistochemistry with T. gondii antisera.

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-Alrich) for 1 h at 56°C. After heat inactivation, 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/3 min, final elongation at 72°C/3 min) of bacterial 16S rRNA genes with 20 pm cpmus primers (40) TPU1/RTUS (5′-AGATTTGGATCMTGTTGCTCAG-3′, nt 8–27 in E. coli 16S rRNA)/(5′-AAGAGGTGTTACCCANCRCA-3′, nt 1544–1522 in E. coli 16S rRNA (41)). For construction of gene libraries, the amplicons were cloned into plasmid pCR2.1-TOPO (TOPO-TO Cloning kit; Invitrogen Life Technologies). Individual 16S rRNA gene sequences (∼500 bp) were determined (CEQDTC5 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 6–8 in 16S bacterial rRNA genes were amplified (identical amplification at 95°C/45 s, 56°C/45 s, 72°C/1 min, final elongation at 72°C/2 min) from total gut content DNA with GC clamp (underlined) primer GC968F (5′-CGCCCGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTA-3′, nt 968–84 in E. coli 16S rRNA) and primer R1378 (5′-CGGTGTTGATAAGGGCGGGGAAAGT-3′, nt 1401–1397 in E. coli 16S rRNA). Amplifications were performed on a DCC-4000 thermal cycler at 94°C for 16 min in a polyacrylamide gel containing 35–60% urea/formamide. DNA band profiles were visualized by silver staining. For sequence analysis, DGGE bands were stained with SYBR green 1 (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. PCR, the amplicons were cloned and sequenced.

Cultural analysis

Luminal contents from terminal ilea (1 cm) were resuspended in PBS, weighted, and 10-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 (Merck) medium were used for quantitative identification of enterococci, enterobacteria, and lactic acid bacteria (mainly lactobacilli), respectively. The amounts of Gram-negative and Gram-positive bacteria were determined by blind duplicate evaluation (by M. M. Heimesaat and D. Fuchs) using quantitative colony counting 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 polyvinyl alcohol (Technovit 8100; Kulzer) as described (45). During the last embedding step, each sample was cut into two to three sections and aligned to obtain cross-sections using a rotary microtome (Medim, Type DDM 0036). Sections (3 µm) from four different areas of each sample were placed on Starfris adhesive slides (Knittel) and stored at 4°C. Before hybridization, slides were incubated at 30°C with 10 mg/ml lysozyme (Sigma-Alrich) for 10 min and then supplemented with 10 mg/ml lysostaphin (Sigma-Alrich) for an additional 10 min. After rinsing with ddH2O, sections were incubated with 20 µl of prewarmed hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.3), 0.01% SDS, 5% (v/v) formamide) containing 5 µM FISH probe EUB 338-5′-Cy3 (Biomers; Ref. 46) in a dark humid chamber at 48°C for 3.5 h. Then, slides were rinsed with ddH2O, air dried in the dark, and mounted with VectaShield Mounting Medium containing 4.6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Bacteria were visualized by epifluorescence microscopy (Axiolab 2 imaging MOT; Zeiss). Digital images were generated with an Axiocam Hrc (Zeiss) using the Axiovision 4.1 software. Z-stacks of neighboring sections were further processed using the AxioVision Deconvolution Software module (Zeiss). Three different segments (between 60 µm and 0.5 mm apart from each other) were analyzed in each specimen. At least 10 crypts per segment were analyzed.
FIGURE 1. Time course of T. gondii-induced ileal immunopathology. A, Kinetics of ileal histopathology after T. gondii infection. Ilea from four mice each were examined histologically on days 0, 3, 5, 6, 7, and 8 p.i. The grade of inflammation in the terminal ileum was evaluated according to our validated histologic scoring system (see Materials and Methods). Results (means ± SD) are representative of at least three experiments. B, Severe necrosis in ilea of T. gondii-infected mice. Sections from ilea of uninfected mice and from mice obtained at day 8 after infection with T. gondii were stained with H&E.

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 (In vitro 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 1.5% 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 Peyers' patches, intestines were cut longitudinally. Faeces and mucus were removed by washing in PBS. Lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) were isolated and analyzed by flow cytometry. Briefly, for IEL isolation intestines were 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 3400 rpm. Cells were collected from the interface of the gradient and washed in RPMI 1640 containing 10% 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/dispeps (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 × 10⁵ IEL and LPL were pretreated on ice for 10 min with 10 μl of a predetermined optimal concentration of anti-FcγRI/III receptors (2.4G2) to block non-Ag-specific binding of Abs to the FcγRI/III 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, and immunological parameters 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</td>
<td>Gram-positive</td>
</tr>
<tr>
<td></td>
<td>rods</td>
<td>rods</td>
</tr>
<tr>
<td>Without treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>$7.2 \pm 9.0 \times 10^4$</td>
<td>$5.7 \pm 6.6 \times 10^8$</td>
</tr>
<tr>
<td>Infected$^a$</td>
<td>$1.2 \pm 0.9 \times 10^{11}$</td>
<td>$5.5 \pm 4.1 \times 10^8$</td>
</tr>
<tr>
<td>Prophylactic treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (ciprofloxacin)</td>
<td>$&lt;1 \times 10^3$</td>
<td>$5.0 \pm 6.9 \times 10^7$</td>
</tr>
<tr>
<td>Infected (metronidazole)</td>
<td>$5.3 \pm 6.8 \times 10^9$</td>
<td>$2.6 \pm 2.7 \times 10^8$</td>
</tr>
<tr>
<td>Therapeutic treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (ciprofloxacin)</td>
<td>$&lt;1 \times 10^3$</td>
<td>$3.7 \pm 3.6 \times 10^7$</td>
</tr>
<tr>
<td>Infected (metronidazole)</td>
<td>$3.3 \pm 3.9 \times 10^9$</td>
<td>$5.1 \pm 6.1 \times 10^8$</td>
</tr>
<tr>
<td>Infected (ciprofloxacin +</td>
<td>$&lt;1 \times 10^3$</td>
<td>$1.1 \pm 1.3 \times 10^9$</td>
</tr>
</tbody>
</table>

$^a$ 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 $1.6 \pm 0.8 \times 10^9$ at day 0 to $2.3 \pm 2.5 \times 10^{11}$ CFU per gram of gut content at day 8 p.i. (Table I). Aerobic and anaerobic bacteria increased from $5.8 \pm 6.6 \times 10^6$ to $1.2 \pm 0.9 \times 10^{11}$ CFU/g and $1.1 \pm 0.9 \times 10^6$ to $1.1 \pm 1.8 \times 10^{11}$ 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 $7.2 \pm 9.0 \times 10^9$ to $1.2 \pm 0.9 \times 10^{11}$ and from $<1 \times 10^9$ to $1.1 \pm 1.8 \times 10^{11}$, 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. thetaiotaomycron (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 $1.0 \pm 1.7 \times 10^7$ to $6.3 \pm 4.8 \times 10^9$ CFU/g and the

FIGURE 3. Quantification of cultivable bacteria in the healthy and inflamed ileum. Bacterial counts in ileal contents of uninfected mice (□) and from mice with severe ileitis at day 8 after T. gondii infection (■) were determined by cultivation as described (see Materials and Methods). E. coli, lactic acid bacteria (LAB, mainly lactobacilli), Bacteroides/Prevotella spp., and Eubacterium/Cladosporium spp. were identified by biochemical analysis (*, p < 0.05 compared with bacterial counts in uninfected mice). Results are representative of at least three experiments (means ± SD).
Bacteroides/Prevotella spp. increased from $<10^3$ to 6.7 ± 7.8 $\times 10^8$ 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 ~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
Briefly, mice were infected with T. gondii and treated with antibiotics. The severity of ileitis was determined histologically on day 8 p.i. in mice treated with placebo, and mice treated with either CF or Mtz survived 50% 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 significantly reduced small intestinal shortening (a parameter commonly associated with 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).

FIGURE 5. Effect of antimicrobial prophylaxis on ileitis development. A, Higher survival rates in mice prophylactically treated with antibiotics. Survival rates were determined in groups of mice treated with placebo (w/o, ◆, n = 10), CF (■, n = 19), Mtz (□, n = 16), or CF + Mtz (▲, n = 19) starting at day 5 before T. gondii infection. B, Less severe histopathology in mice treated with antibiotics. The severity of ileitis was determined histologically on day 8 p.i. in placebo-treated mice and in mice treated with CF, Mtz, or CF + Mtz (n = 5 each group) starting at day 5 before infection. The results are presented as means ± SD (*, p < 0.05; **, p < 0.01; ***p < 0.001 compared with control-treated mice).

FIGURE 6. Effect of antimicrobial therapy on established ileitis. A, Higher survival rates in mice therapeutically treated with antibiotics. The mortality of mice was determined in mice treated with placebo (w/o, ◆, n = 8), CF (■, n = 10), Mtz (□, n = 10), or CF + Mtz (▲, n = 10) starting at day 5 after T. gondii infection. B, Less severe ileal inflammation in mice treated with antibiotics. The severity of ileitis was determined histologically at day 8 p.i. in mice treated with placebo, CF, Mtz, or CF + Mtz (n = 5 each group; *, p < 0.05; **, p < 0.005 compared with control-treated mice; means ± SD).

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 mouse models. Mice were 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 colonized with E. coli displayed moderate histopathologic changes in their ileum 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. 8, 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 \(\gamma\delta\) 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., cholinolones (59), amelioration of ileitis in gnotobiotic mice as well as the induction of ileitis in monoclonalized 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 as compared with controls; furthermore, a reduction in immunopathology in TLR4−/− mice as well as aggravation of intestinal pathology in gnotobiotic mice following treatment with purified E. coli lipid A point toward an essential role of LPS in our model system (M. Heimesaat, A. Fischer, H.-K. Jahn, J. Niebergall, M. Fréndénberg, M. Blant, O. Liesenfeld, R. R. Schumann, U. B. Göbel, and S. Bereswill, submitted for publication). The detection of bacteria in inflamed subepithelial tissue layers by FISH demonstrated that T. gondii-induced ileal inflammation is accompanied by substantial mucosal barrier defects resulting in bacterial translocation. The finding that live E. coli, but not Bacteroides/Prevotella spp., were detected by culture in >80% of the mesenteric lymph nodes and in 70% of the spleens from mice with severe ileitis (data not shown) suggests that following translocation gut bacteria potentiate tissue destruction and intestinal inflammation by direct cell contact and mediator release (33, 68). Such interactions are further supported by decreased IFN-γ and NO levels after antibiotic treatment and by the elevated intestinal NO levels in gnotobiotic animals monoclonized with E. coli. The low histopathology and tissue destruction displayed by T. gondii-infected but uncolonized gnotobiotic animals demonstrated that induction of immune responses in the course of T. gondii-driven ileitis essentially depends on the presence of gut bacteria. In a small percentage of treatment/recolonization groups, a decrease in ileal pathology (alternatively, development of pathology in colonized mice) was not directly paralleled by a reduction (increase, respectively) in NO and IFN-γ levels. We assume that detection of cytokines levels by ELISA in ileal tissue does not have the discriminatory power of other outcome measures (i.e., histopathology) to indicate changes following antibiotic treatment or recolonization; we have previously observed such a lack of correlation between cytokine levels and histopathological changes (69). Similarly, mortality also did not in all cases correlate with changes in histopathology.

The presence of gut bacteria appears to increase numbers of activated CD4+ T cells in the lamina propria of SPF mice. Interestingly, frequencies as well as absolute numbers of all CD4+ T cells did not differ in naive vs infected SPF mice. We assume that these cells are most likely lost due to activation-induced cell death before day 7 after infection and therefore do not show increased frequencies in our analysis. In line with our findings, Minns et al. (70) recently reported that TLR9 is required for Th1-type immunopathology following oral infection with T. gondii. Infected TLR9−/− mice showed decreased total T cell as well as decreased IFN-γ-producing T cell frequencies in their lamina propria compared with wild-type mice. Because TLR9 is associated with ligation of unmethylated bacterial CpG DNA, the report by Minns et al. (70) and the results reported here point toward a role for the interaction of gut bacteria, i.e., E. coli, with lamina propria cells in the induction of parasite-induced ileitis.

Reduced proinflammatory mediator levels after antimicrobial treatment were observed in SAMP1/YitFc mice with ileitis (60) and the low inflammatory potential of lactobacilli in ileitis is well in line with similar observations in experimental colitis (21, 36, 71) and in GvHD (72).

Taken together, the results presented here support the integrated view that infection with T. gondii most likely causes a breakdown of intestinal physiological and barrier functions followed by accumulation of Gram-negative bacteria in the ileum, bacterial translocation, and bacteria-mediated aggravation of inflammation via Th1-type cytokine and/or proinflammatory mediators. The fact that similar processes occur in human IBD highlights T. gondii-driven ileitis as a valuable model to determine the role of gut bacteria and their products in small intestinal inflammation, to determine the bacterial factors contributing to inflammation and to analyze the efficacy of novel therapeutic strategies. The profound contributions of the ileal microflora to inflammation indicate that the modulation of the intestinal flora by antibiotics represents a promising strategy for prophylaxis or therapy of small intestinal inflammation.

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Disclosures
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References


