Colonic microbiota alters host susceptibility to infectious colitis by
modulating inflammation, redox status, and ion transporter gene expression

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The intestinal microbiota plays a crucial role during the early
development of both systemic and local immunity. In recent
days, dysbiosis or altered bacterial communities within intes-
tinal microflora have been implicated in a wide range of
inflammatory diseases such as inflammatory bowel disease
(IBD; 31), obesity (6), diabetes (23), celiac disease (7), and
inflammatory diseases such as inflammatory bowel disease
(IBD; 31), obesity (6), diabetes (23), celiac disease (7), and
colic neoplasia (47). However, it is still debated whether
dysbiosis alters disease susceptibility or is a consequence of the
disease itself. Hence, it is not known whether specific micro-
bacterial community changes alter disease risk. It has been shown
that the microbiota composition can confer colonization resis-
tance toward pathogenic bacteria, and when the community is
disturbed, like during infection and inflammation, this can lead
to increased risks for enteric infection (26, 44). For the most
part, the factors that alter the composition of the intestinal
microbiota are elusive, although the presence of closely related
species can increase the chance of invasion of a similar species
into the gut ecosystem (42). The specific mechanisms of how
microbiota may protect against enteric disease susceptibility
are relatively undefined.

The aim of this study was to explore the potential role of the
intestinal microbiota in controlling enteric disease susceptibil-
ity by using the resistant C57BL/6 and susceptible C3H/HeOuJ

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mouse strains infected with *C. rodentium*. We crossed microflora from C57BL/6 and C3H/HeOuJ mice and then examined survival and colonic responses during *C. rodentium* infection. We found that the intestinal microflora from C57BL/6 mice transferred protective responses when their stool was gavaged into the susceptible C3H/HeOuJ mice that were subsequently infected with *C. rodentium*. Although naïve C3H/HeOuJ suffered 100% mortality during the course of infection, these mice when crossed with resistant C57BL/6 microflora were rescued and demonstrated 100% survival rates with reduced colonic damage and diminished systemic spread of *C. rodentium*. Furthermore, survival of the C3H/HeOuJ mice crossed with C57BL/6 microflora was associated with increased gene expression of inflammatory cytokines TNF-α, IFN-γ, IL-22, and macrophage inflammatory protein (MIP)-2α, along with increased prooxidant responses known to limit *C. rodentium*, along with increase prooxidant responses known to limit *C. rodentium* infection. Finally, C3H/HeOuJ mice crossed with the microflora from C57BL/6 mice had elevated gene expression of the ion transporters Dra, carbonic anhydrase (CA) IV, and CA I; these genes are known to be critical for functional ion transport during *C. rodentium* infection (5). Although C57BL/6 mice had increased total microflora compared with C3H/HeOuJ mice, the protective responses of the microflora from C57BL/6 crossed into C3H/HeOuJ mice were associated with higher levels of bacteria from the phyla Bacteroidetes. These data indicate that resistant microbiota can protect mice from colonic damage and bacterial translocation following *C. rodentium* infection, overcoming the host’s genetic susceptibility.

**MATERIALS AND METHODS**

**Mice.** Six- to 8-wk-old C57BL/6 female mice (Charles River Laboratories breeders; Wilmington, MA) were bred in-house. Age-matched female C3H/HeOuJ mice were obtained from Jackson Laboratories (Bar Harbor, ME); all were housed individually in a temperature-controlled (22 ± 2°C) animal facility with a 12-h light-dark cycle. Mice were maintained under specific pathogen-free conditions and fed a standard sterile chow (Laboratory Rodent Diet 5001, Purina Mills, St. Louis, MO) as well as tap water ad libitum throughout the experiments. All procedures involving the care and handling of the mice were approved by the University of British Columbia Committee on Animal Care Ethics and under the guidelines of the Canadian Council on the Use of Laboratory Animals.

**Fecal crossing experiment.** Mice were randomly assigned to one of six groups: C3H/HeOuJ hosts receiving C57BL/6 microflora (S^H^ + R^M^); C57BL/6 hosts receiving C3H/HeOuJ microflora (R^H^ + S^M^); C57BL/6 hosts receiving C57BL/6 microflora (R^H^ + S^M^); C3H/HeOuJ hosts receiving C3H/HeOuJ microflora (S^H^ + S^M^); C3H/HeOuJ stool donor mice (S); and C57BL/6 stool donor mice (R). The microbiota in recipient postweaned 4-wk-old mice were depleted with antibiotics [ampicillin (1 g/l, Sigma), vancomycin (500 mg/l, Abbott Laboratories), neomycin sulfate (1 g/l, Pharmacia/Upjohn), and metronidazole (1 g/l, Sidmak Laboratories)] provided in their drinking water for 4 wk. Three pellets (100 mg) of fresh stool from adult donor mice were collected and pooled in 1.2 ml sterile PBS; 100 μl of the mixture was gavaged into respective recipient mice four times over 12 days.

**Bacterial strains and oral infection of mice.** *C. rodentium* DBS100 were cultured overnight at 37°C in 5 ml of Luria broth (LB). Mice were inoculated by oral gavage using 0.1 ml of LB containing 2.5 × 10^8 colony forming units (CFU) of *C. rodentium*. Both C57BL/6 and C3H/HeOuJ mice were infected with the same bacterial preparation 3–5 days following fecal gavage in recipients.

**Survival and body weight measurement.** Mice were monitored for mortality and morbidity throughout infection and were euthanized if they showed signs of extreme distress. Survival data are presented as the percentage of the initial mice still surviving at each time point.

**Tissue collection and pathogen load.** Mice were euthanized at day 5–6 postinfection (pi) by cervical dislocation, followed by removal of the large intestine. Distal colonic and cecal segments were immersed in RNA-later (Qiagen) for real-time quantitative PCR analysis, flash frozen in liquid nitrogen and stored at −80°C for oxidation assays, or immersed in 10% neutral buffered formalin (Fisher) and processed for histological analyses and immunofluorescence. For pathogen counts, the spleen and mesenteric lymph nodes (MLN) were removed, weighed, homogenized in 1 ml PBS, serially diluted, and plated on McConkey agar plates, which were incubated for 24–48 h at 37°C.

**Microbiota analysis.** Similar to Lupp et al. (26), fresh stool was weighed and homogenized in 1 ml PBS, and 1:10 dilution of each homogenate was stored in 3.7% formalin at 4°C until use. For SYBR green DNA staining, samples were filtered onto Anodisc 25 filters (Whatman International) with a pore size of 0.2 μm and 2.5 cm diameter. After complete drying, each sample was stained with SYBR green I nuclear acid gel stain (Invitrogen). For fluorescent in situ hybridization (FISH), samples were filtered onto a polycarbonate membrane filter (Nucleopore Track-Etch Membrane, Whatman International) and dehydrated on the filter by soaking in 50, 80, and 100% ethanol. After air drying, the filter was incubated overnight at 37°C in the dark with hybridization solution (0.9 M NaCl, 0.1 M Tris pH 7.2, 30% formamide, 0.1% SDS) containing 250 ng each of the general eubacterial probe (EUB338: 5′-GCT GCC TCC GTG AGG AGT-3′) fluorescently labeled with far red, Bacteroidetes (Cytophaga-Flavobacter-Bacteroides) probe (CFF286: 5′-TCC TCT CAC ACG CCC TAC-3′) fluorescently labeled with Texas red, and γ-Proteobacteria probe (GAM42a: 5′-GCC TTA CAT CGT TT-3′) fluorescently labeled with FITC. After incubation, the filters were washed in hybridization solution followed by wash buffer (0.9 M NaCl, 0.1 M Tris pH 7.2). Filters were dried and mounted on glass slides by use of ProLong Gold Antifade (Invitrogen) and viewed with a Zeiss Axio-Imager 2 microscope operating through Axiosview software. Six to 10 fields per disc were randomly chosen and the number of cells was counted and averaged. Total microbiota load was calculated and percentages of Bacteroidetes and γ-Proteobacteria were calculated based on the number of positively probe eubacteria.

**Histopathological scoring.** To assess colonic pathology, we used a scoring system previously described (3) and adapted (13, 14). Paraffin-embedded colonic and cecal tissue sections from mice were fixed in formalin with hematoxylin and eosin and examined by three blinded observers. For submucosal edema, submucosal leukocyte infiltration, goblet cell depletion, and crypt hyperplasia, 5–10 fields-of-view per section in each group were assessed at ×400 magnification. Infected groups were assigned a score relative to the control group average. Scores were calculated for submucosal edema (0, <100%; 1, 100–200%; 2, 200–400%; 3, 400–800%; 4, >800%), leukocyte infiltration (0, <100%; 1, 100–200%; 2, 200–400%; 3, 400–800%; 4, >800%), goblet cell depletion (0, >100%; 1, 50–100%; 2, 21–50%; 3, <20%), crypt hyperplasia (0, <100%; 1, 100–150%; 2, 150–200%; 3, >200%), and epithelial integrity (0, no change; 1, 1–10 epithelial cells shedding per crypt; 2, >10 epithelial cells shedding per crypt; 3, crypt ulceration; 4, crypt destruction). The scores of the controls were subtracted from their respective groups to obtain an accurate score of the pathological changes observed.

**Immunofluorescence and TUNEL staining.** Staining of tissues was performed as previously described (4), using primary antibodies that recognize myeloperoxidase (MPO, NeoMarker), F4/80 (Serotec), in-ducible nitric oxide synthase (iNOS, Upstate), and the translocated bacterial effector Tir (provided by Dr. W. Deng, Biotechnology Laboratory, University of British Columbia, Vancouver, BC, Canada) followed by secondary Alexa568- or Alexa488-conjugated goat anti-rabbit or anti-rat IgG antibodies (Molecular Probes) and ProLong Antifade.
Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). The mean numbers of MPO + or F4/80 + cells were counted by a blinded observer under fluorescence in a ×200 magnification field of view. Cell death was measured by using the Trevigen TACS 2 TdT-Blue Label In Situ Apoptosis detection kit (Invitrogen). Tissue samples were deparaffinized, labeled, and mounted as per manufacturer’s instructions. Deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick-end labeling (TUNEL)-positive cells were counted by a blinded observer under bright-field illumination at ×200 magnification. The mean number of TUNEL-positive cells for each group was the total number of TUNEL-positive cells counted in 10 longitudinally sectioned crypts. Tissues were visualized via a Zeiss AxioImage 2 microscope operating through Axioview software.

**RNA extraction and real-time quantitative PCR.** RNA was purified using RNeasy kits (Qiagen). cDNA was synthesized with Superscript II Reverse Transcriptase and oligo(dT) 12–16mer (Invitrogen) followed by quantitative PCR on a MJ Mini-Opticon Real-Time PCR System (Bio-Rad) using IQ SYBR Green Supermix (Bio-Rad) and GAPDH, TNF-α, MIP-2α, IFN-γ, IL-22 (52), CA IV and CA I (32), and Dra (41). Quantification was determined by use of GeneExMacro OM 3.0 software (Bio-Rad) and is represented as the averaged fold expression relative to the uninfected C57BL/6 control.

**Western blotting.** Following euthanization of mice, colonic tissues were homogenized and centrifuged, and supernatants were boiled in loading dye. Samples (50 μg protein) were loaded per lane in SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by standard procedures. Membranes were incubated with anti-iNOS, superoxide dismutase (SOD)1, SOD2, and β-actin antibodies raised in rabbit (Santa Cruz Biotechnology), followed by secondary goat anti-rabbit horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology) and visualized by using an ECL detection kit (Amersham) with the Chemigenius detection system with use of GeneTools software (Syngene) for densitometric analysis.

**Oxidized and reduced glutathione quantification.** Colonic glutathione (GSH) content was measured by use of a commercially available kit (Cayman). Cecal tissue segments were homogenized and subjected to deproteinization with 5% metaphosphoric acid to remove protein thiol groups, snap frozen in liquid nitrogen, and stored at −80°C. GSH determination was performed within the next 48 h. GSH was reacted with 5,5'-dithiobis-2-nitrobenzoic acid to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide GS-TNB produced is recycled back to GSH by glutathione reductase. The rate of TNB production is directly proportional to the total concentration of GSH in the sample. Oxidized glutathione (GSSG) was estimated by use of 4-vinylpyridine. The difference between total GSH and GSSG amounts gave the value of the reduced GSH. Protein assays were performed according to the Bradford method by using a nondeproteinated portion of the samples. Ratios are represented as infected values normalized to values obtained from uninfected control mice.

**Statistical analysis.** The results are expressed as means ± SE from three to eight mice per group per experiment. For comparisons of multiple groups, one-way analysis of variance with Tukey’s post hoc test was performed. For comparisons of two groups, Mann-Whitney U-test was performed. All analyses were performed by using GraphPad Prism 5 where P < 0.05 was considered significant.

**RESULTS**

**Intestinal microflora from C57BL/6 mice protect C3H/HeOuJ mice from lethal C. rodentium-induced colitis.** To explore the possibility that the intestinal microbiota contributes to differences in host susceptibility in the mice susceptible (S; C3H/HeOuJ) and resistant (R; C57BL/6) to C. rodentium-induced colitis, we performed microflora-crossing experiments. Microflora from postweaned recipient mice were depleted with antibiotics and gavaged with fecal matter either from their littermates or from the genetically unrelated mice. This procedure resulted in four groups of mice: C3H/HeOuJ hosts crossed with C3H/HeOuJ microflora (S0+S0); C3H/HeOuJ hosts crossed with C57BL/6 microflora (S0+R0); C57BL/6 hosts crossed with C57BL/6 microflora (R0+R0); and C57BL/6 hosts crossed with C3H/HeOuJ microflora (R0+S0). Subsequently, these mice were infected with C. rodentium and monitored for morbidity and mortality. Normally, S mice suffer 100% mortality, in this experiment dying by day 6 pi and R0+S0 suffered similar morality (Fig. 1A). Strikingly, all the S mice carrying R microflora (S0+R0) survived the typical lethal colitis similar to all R mice (blue diamonds colocalized with all R mice at the 100% survival line; Fig. 1A). S microflora crossed into R mice (R0+S0) did not induce mortality and was similar to R0+R0 displaying 100% survival rates during infection (Fig. 1A). Normally, S mice show significantly more tissue damage during C. rodentium infection compared with R mice (50) and also shown in this study (S and S0+S0 vs. R and R0+R0, Fig. 1C). In contrast, survival of S0+R0 mice was associated with less colonic tissue damage compared with S and S0+S0 mice at day 6 pi (Fig. 1, B–C). In this experiment, S and S0+S0 mice displayed maximal histopathological damage at day 5–6 pi with hyperplasia, submucosal cell infiltration, bleeding, loss of epithelial integrity, and mucodepletion, scoring ~8 out of 18 overall. In contrast, S0+R0 mice showed significantly less colonic damage, with an overall histopathological score of ~4, which was comparable to R and R0+R0 infected mice (Fig. 1, B and C). In particular, S0+R0 mice were able to maintain an intact epithelium with little bleeding, similar to R mice during infection (Fig. 1B). Interestingly, ~30% of R0+S0 mice had greater colonic damage compared with R0+R0 mice at day 6 pi (Fig. 1B), but this was not significant when reflected in averaged total scores (Fig. 1C). Overall, these results indicate that S mice colonized with R microflora are protected against a lethal and damaging C. rodentium infection, whereas R mice colonized with S microflora undergo a variable increase in susceptibility to acute colitis. This suggests that R microflora is protective against C. rodentium-induced colitis, whereas S microflora does not confer protection during infection.

**Intestinal microflora from C57BL/6 mice protect against systemic bacterial disease.** Since S mice are susceptible to increased systemic pathogen load (50), we examined whether S0+R0 mice were protected from lethal colitis by decreasing the number of C. rodentium that could translocate across the epithelium and into systemic tissues. Mice infected with C. rodentium were euthanized and their spleens and MLN were examined for pathogen load by homogenizing tissues and plating on McConkey media for CFU. Results showed that S mice carried significantly higher pathogen levels in the MLN and spleen than R mice (Fig. 2A), similar to Vallance et al. (50). As expected, pathogen levels were high for both S0+S0 and S mice and low for both R0+R0 and R mice. Following infection, S0+R0 mice demonstrated significantly reduced pathogen loads, corresponding with the reduced colonic damage scores (Figs. 1 and 2). In contrast, R0+R0 mice carried
significantly higher pathogen loads both systemically (Fig. 2A) and locally, as seen by specific antibody staining of *C. rodentium* in colonic tissue sections (Fig. 2B). These data show that S mice colonized with R microflora (*S*<sup>H</sup>+*R*<sup>M</sup>) or colonized with each other’s microflora (*R*<sup>H</sup>+*S*<sup>M</sup> or *S*<sup>H</sup>+*R*<sup>M</sup>) were infected with *C. rodentium* and assessed for lethal colitis. A: S mice crossed with R microbiota (*S*<sup>H</sup>+*R*<sup>M</sup>) survived infection similar to R mice unlike S infected mice, which suffered 100% mortality. The colons from the *S*<sup>H</sup>+*R*<sup>M</sup> infected mice displayed less damage (B) microscopically (×200) with (C) significantly higher total damage scores than S mice carrying native microflora (S and *S*<sup>H</sup>+*S*<sup>M</sup>). Bars represent mean scores of damage assessed from colonic tissues from mice carrying microflora from R (■) or S (□) mice. Asterisks denote significantly greater damage in S and *S*<sup>H</sup>+*S*<sup>M</sup> compared with *S*<sup>H</sup>+*R*<sup>M</sup> or R and *R*<sup>H</sup>+*R*<sup>M</sup>; *p* < 0.05; 1-way ANOVA with Tukey’s post hoc test. p.i., Postinfection; ns, not significant.

Intestinal microflora from *C57BL/6* mice induce inflammatory cytokines. We next addressed why S mice colonized with R microflora have decreased localized and systemic disease, whereas R mice colonized with S microflora have increased disease during acute colitis.

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R host, the S microflora crossed into the R mice led to a significant reduction in TNF-α and MIP-2 gene expression during infection (Fig. 3B). This suggested that the loss of R microflora and/or the gain of S microflora inhibited cytokine gene expression in the colons of R mice. Overall, these results suggest that R microflora, when crossed into S mice, can induce inflammatory responses important for controlling _C. rodentium_ infection.

Intestinal microflora from C57BL/6 mice induces oxidative responses protecting against epithelial cell apoptosis. _C. rodentium_ induces iNOS expression in crypt epithelial cells in R mice, and the expression of this enzyme has been associated with oxidative stress and accelerated pathogen clearance (13, 49). To examine the possibility that R microflora can induce prooxidant responses we examined iNOS expression, antioxidant levels, and the ratio of oxidized/reduced glutathione (GSSG:GSH) in mice (Fig. 4, A and B). We found that RH+RM and SH+RM infected mice had upregulated iNOS protein expression, suggesting that the R microflora in either host induced this defensive response during _C. rodentium_ infection (Fig. 4, A and B). The S microflora did not appear to induce a significant iNOS response in either host. Interestingly, the SH+SM infected mice had significantly higher expression of antioxidant MnSOD/SOD2 protein than SH+RM infected mice as well as R mice with either microflora (Fig. 4A). This suggested that the susceptibility of SH+SM mice to _C. rodentium_ infection may reflect high endogenous antioxidant levels that reduce the oxidative stress response important for pathogen killing. This antioxidant response appeared to be downregulated by the R microflora since MnSOD was similarly decreased in both hosts carrying R microflora and unchanged in the presence of S microflora in the R host (Fig. 4A). We also examined the cytosolic antioxidant Cu/ZnSOD (SOD1), which was not dependent on microflora since S and R mice with...
Overall, the lack of oxidative stress in the large intestines that S mice display increased colonic epithelial apoptosis. This observation confirmed previous findings that R mice displayed increased colonic epithelial cell death during infection as measured by TUNEL (Fig. 4C). We hypothesize that control of excessive epithelial cell death is important for the survival of $S^H + R^M$ infected mice since excessive epithelial cell death is associated with increased morbidity during C. rodentium infection (13–15, 50). The S microflora did not alter colonic epithelial cell death rates since $R^H + S^M$ mice were similarly low to $R^H + R^M$ mice during infection (Fig. 4C). Overall, these results demonstrate that R microflora induces prooxidant responses associated with decreased colonic epithelial cell death during infection.

**Intestinal microflora from C57BL/6 mice induces ion transporter gene expression.** Whereas this study and others have shown that S mice carry increased pathogen loads, both at localized and systemic sites (50), another important study revealed that S mice die from fatal dehydration due to defective expression of colonic ion transporters (CA IV, CA I, and Dra) during C. rodentium infection (5). The authors showed that S mice compared with R mice had impaired expression of CA IV, CA I, and Dra during infection and this resulted in decreased chloride absorption concurrent with significant water loss and the resulting mortality could be rescued with fluid treatment (5). Remarkably, we found that the microflora could modulate the expression of these genes. As seen in Fig. 5, S$^H + S^M$ infected mice show significantly reduced expression of CA IV, CA I, and Dra genes relative to the R$^H + R^M$ infected mice similar to the findings of Borenshtein et al. (5). In contrast, S$^H + R^M$ infected mice had similarly increased gene expression of CA IV, CA I, and Dra compared with R$^H + R^M$ infected mice (Fig. 5). Although we didn’t measure chloride absorption or water loss, we did note that S$^H + R^M$ infected mice had stool similar to R, R$^H + R^M$, and R$^H + S^M$ infected mice in contrast to S and S$^H + S^M$ infected mice, which often lacked solidly formed stools. Based on these observations and the findings of Borenshtein et al., our results suggest that the microflora from R mice can normalize CA IV, CA I, and Dra gene expression in the S mice to the level of R infected mice, which do not suffer from infection-induced fatal dehydration. R$^H + R^M$ and R$^H + S^M$ infected mice were similar in their expression levels of the transporter genes, indicating that the microflora from S mice did not inhibit CA IV, CA I, and Dra gene expression in R mice. Thus R microflora in S mice could rescue impaired gene expression of colonic ion transporters previously found critical for protection from excessive dehydration and associated mortality during C. rodentium infection.

**Intestinal microflora from C3H/HeOuJ and C57BL/6 mice differ in composition and total bacterial loads.** In the healthy mammalian colon, Bacteroidetes is one of the main bacterial phyla whereas Proteobacteria are relatively scarce (9, 24). To determine what differences in the intestinal microbiota from R mice could account for the protective host responses, we compared numbers of bacteria from the phyla Bacteroidetes and $\gamma$-Proteobacteria in stool samples of S and R mice. We used FISH analysis with probes specific for bacteria within Bacteroidetes and $\gamma$-Proteobacteria, as well as the eubacterial domain (all bacteria) to generate percentages for each group. We found that S mice had significantly fewer Bacteroidetes compared with R mice whereas $\gamma$-Proteobacteria were not significantly different (Fig. 6A). Additionally, we examined the

![Fig. 3. Intestinal microbiota from C57BL/6 mice protects C3H/HeOuJ mice by inducing inflammatory responses during C. rodentium-induced colitis. C57BL/6 (R) and C3H/HeOuJ (S) mice colonized with their own flora (R$^H + R^M$ or S$^H + S^M$) or colonized with each other’s flora (R$^H + S^M$ or S$^H + R^M$) were infected with C. rodentium and assessed for colonic inflammatory responses (A). R microflora induces expression of proinflammatory cytokines. Distal colonic tissues from uninfected and infected mice carrying microflora from R (solid bars) or S (open bars) mice were assessed for transcript expression by real-time quantitative PCR (qPCR). Bars represent the averaged fold expression relative to the uninfected R mice. Asterisks denote significantly increased change of IFN-γ, TNF-α, IL-22, and macrophage inflammatory protein-2α (MIP-2α) from S$^H + R^M$ compared with S$^H + S^M$ tissues or significantly decreased expression of TNF-α and MIP-2α from R$^H + S^M$ compared with R$^H + R^M$ tissues. B: distal colonic tissues were stained for the presence of F4/80-positive macrophages and MPO-positive neutrophils. Quantification of MPO-positive and F4/80-positive cells from immunofluorescence images shows increased neutrophil recruitment in S$^H + R^M$ colon sections; *P < 0.05, 1-way ANOVA with Tukey’s post hoc test.

![Fig. 4. Relative expression of TNF-α, IFN-γ, IL-22, MIP-2α, and TNFR-α 6 d.p.i. compared with uninfected mice (A). Microflora from C3H/HeOuJ (S) and C57BL/6 (R) mice were colonized with each other’s flora. After colonization, R and S mice were infected with C. rodentium. R microflora induces increased epithelial apoptosis compared with R mice during C. rodentium infection (50). Whereas S mice had the highest epithelial cell death rate, the microflora from R mice was able to decrease cell death significantly in the colons of S mice during infection (Fig. 4C). Overall, these results demonstrate that R microflora induces prooxidant responses associated with increased colonic epithelial cell death during infection.
overall bacterial load using the nucleic acid stain SYBR green, since differences in bacterial load have been implicated in intestinal immune dysfunction (45). We found that S mice carried significantly lower total bacterial loads compared with R mice (Fig. 6A). Overall, these results indicate that the S mice have fewer Bacteroidetes in concert with an overall decreased bacterial load compared with R mice.

Considering that bacteria within Bacteroidetes have been shown to induce beneficial immune responses (48) and a reduced total bacterial load could mean differences in colonization resistance, we analyzed the microbiota from the fecal transplanted mice to determine whether these microflora differences between the S and R mice had been transferred (Fig. 6B). The results showed that significantly increased levels of Bacteroidetes were present in the stool of S\(^{H}+R^{M}\) compared with S\(^{H}+S^{M}\) and similar to R\(^{H}+R^{M}\) mice. The R\(^{H}+S^{M}\) had decreased levels of Bacteroidetes present in their stool compared with R\(^{H}+R^{M}\) mice, but this was not significant. Although the total bacterial loads in stool from S\(^{H}+S^{M}\) and R\(^{H}+R^{M}\) mice were significantly different, like S and R mice (Fig. 6, A and B), the stool from S\(^{H}+R^{M}\) mice did not contain significantly more bacteria than S\(^{H}+S^{M}\) mice. These results suggested that the increased levels of Bacteroidetes, not the total bacterial load, may be playing a role in the protective responses transferred from R microflora into the S mice during C. rodentium infection.

DISCUSSION

In this study, we examined the role that the intestinal microbiota plays in host susceptibility during C. rodentium infection in mice. Our results highlight the importance of the microflora as a prognostic factor that can modulate the severity of infection and the subsequent morbidity and mortality caused...
Tukey's post hoc test.

infection, the microbiota has been implicated in a number of induced colonization and enterocolitis (43). Other than enteric Salmonella enterica reported to increase susceptibility to expression.

atory and oxidant responses and rescued ion transport gene expression.

mortality, similar to S mice, during infection. Although this needs further clarification, components of the microbiota are in constant communication with our innate immune receptors to maintain intestinal homeostasis (34), driving the expansion of B and T cells in Peyser's patches and mesenteric lymph nodes, especially CD4+ T cells, including Foxp3-expressing T regulatory cells (19). Specifically, it has been shown that early colonization with Bacteroides fragilis can downregulate lipopolysaccharide responsiveness in infancy, revealing the importance of this particular species in inducing immune tolerance during early life (39).

Colonization with fewer beneficial microbes, like a reduction in Bacteroidetes, may influence enteric disease susceptibility through deregulated immune responses. In this study, we found that R mice carried more Bacteroidetes bacteria and a larger overall bacterial load compared with S mice. It is possible that the S mice were more susceptible to infection due to differences in total microbiota load; however, this is unlikely since after recolonization both the S$_{H11001}^{+}$ and the R$_{H11001}^{+}$ had similar levels of bacteria. Thus it is more likely that the protective effects of the R microflora were conferred, at least in part, by increasing the numbers of Bacteroidetes in the S mice. Although this needs further clarification, Bacteroides sp. have been shown to be important for intestinal homeostasis and systemic immune responses (48). Additionally, we observed that R microflora increases colonic oxidative stress during infection, as measured by the increase in oxidized to reduced GSH ratio, induction of iNOS, and reduced antioxidant Mn-SOD/SOD2 protein expressions. In several models of inflammation, iNOS is responsible for production of large amounts of nitric oxide (NO). When antioxidants like MnSOD/SOD2 are low, excess free radicals including superoxide (O$_{2}^{-}$) either form hydrogen peroxide (H$_2$O$_2$) or combine with NO to form peroxyxinitrite (ONOO$^-$), which is extremely potent as an oxidant and has strong bactericidal activity against E. coli (20). Excess H$_2$O$_2$ produced during such prooxidant conditions also oxidizes available reduced GSH to its oxidized form (GSSG), thus altering cellular redox status.

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Differences in microbiota composition have been recently reported to increase susceptibility to Salmonella enterica-induced colonization and enterocolitis (43). Other than enteric infection, the microbiota has been implicated in a number of other enteric diseases including IBD. The intestinal microbiota from IBD patients display decreased diversity (1, 29), with phyla-level reductions in Bacteroidetes and Firmicutes (11) and increases in Proteobacteria (18, 27, 36, 40). Additionally, the microflora from IBD patients has been shown to be unstable in both active disease and remission, in contrast to the microflora from healthy adults, which are relatively stable over time (36). Although microflora stability could ensure continued gut functions in healthy individuals, in other individuals whose intestinal communities contain fewer beneficial microbes or increased pathogenic microbes, the microbiota could be an initial factor that increases inherent disease risk. For example, dysbiotic microbiota has been shown to induce colitis in mice (12). Dysbiosis could contribute to enteric disease where specific populations of the microbiota have reduced protective features with subsequent alterations in immune responses (e.g., biased Th1 and Th17 responses and loss of immunological tolerance) or have more pathogenic properties promoting increased mucosal adherence/invasion activating immune cells in a detrimental manner (31). Although the mechanisms by which microbes regulating intestinal immunity are for the most part elusive, components of the microbiota are in constant communication with our innate immune receptors to maintain intestinal homeostasis (34), driving the expansion of B and T cells in Peyser’s patches and mesenteric lymph nodes, especially CD4+ T cells, including Foxp3-expressing T regulatory cells (19). Specifically, it has been shown that early colonization with Bacteroides fragilis can downregulate lipopolysaccharide responsiveness in infancy, revealing the importance of this particular species in inducing immune tolerance during early life (39).

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responses in the colon during infection, these same responses specifically elicit cytotoxicity toward pathogenic *C. rodentium*, evident by reduced pathogen burdens in these mice. The reduced levels of *C. rodentium* in the presence of R microflora was associated with attenuated cell death in the intestinal epithelial cells; most likely because *C. rodentium* itself induces apoptosis and this has been shown to increase mucosal ulcerations and mortality (13–15, 50). Among other pathways of protection, we also found that microflora from R mice induced a host-protective inflammatory response in the colons of SH mice. This included elevated IFN-γ, TNF-α, IL-22, and MIP-2α induction with corresponding neutrophil infiltration; responses known to be important for host defense during *C. rodentium* infection (2, 17, 37, 38). It has been shown that intestinal inflammation changes microbiota composition, enhancing pathogen growth (26, 43, 44). Although we didn’t examine microbial changes during infection, our study reveals that the microflora does contribute to host responses important for inducing inflammatory responses important for host survival to infection. Finally, the increased survival of *S*<sup>H</sup>+*R*<sup>M</sup> infected mice was associated with increased ion transporter gene expression. Previous studies found that, compared with R mice, S mice have significantly reduced expression of the ion transporters CA IV, CA I, and Dra that results in profound electrolyte loss and dehydration, contributing to the mortality of the S mice during *C. rodentium* infection (5). In support of this study, we also found that *C. rodentium* infected *S*<sup>H</sup>+*S*<sup>M</sup> mice had decreased gene expression of CA IV, CA I, and Dra. We found that this response was controlled by the microflora since R microflora could rescue impaired ion transporter gene expression in the S mice. Taken together, our data demonstrate that R microflora can induce host-protective responses important in controlling against *C. rodentium*-induced mortality including proinflammatory and prooxidant responses and ion transporter gene expression. Although R microflora controlled systemic pathogen translocation in both the S and R hosts, this did not appear to be due to increased barrier dysfunction since R and S mice had similar barrier permeability (data not shown). The cellular mechanisms of R microflora induced protection in the S host remains unknown, but it is associated with increased numbers of Bacteroidetes bacteria, or other bacterial changes we did not detect.

Although we show that R microflora confers a protective effect to the S host, the S microflora in the R host did not show a clear phenotype. This could be due to the fact that the S microbiota did not transfer as effectively to the R host for unknown reasons, evident by the lack of significantly decreased Bacteroidetes. Nonetheless, it was apparent that R hosts crossed with S microflora during infection had greater epithelial damage in the R host of some mice, but it was not through activation of the nuclear transcription factor, pSTAT3, previously found to be important in maintaining epithelial integrity (15), since pSTAT3/STAT3 levels were not significantly modulated with various microflora (data not shown). Although R hosts crossed with S microflora survived infection, these mice had increased pathogen loads, decreased cytokine expression, and reduced iNOS, suggesting that the loss of R microflora or the gain of the S microflora resulting in the impairment of some protective responses during infection.

In summary, this study demonstrates that differences in host microbiota can dramatically alter host susceptibility to *C. rodentium* and colonization patterns in the gastrointestinal tract, which results in striking effects on the clinical course of infection. These results reveal that the microbiota and the host have a truly mutualistic interaction in the gut that can control those host responses that are beneficial and promote host survival during enteric infection. We are currently exploring...
the cellular mechanisms underlying this susceptibility in mice since these finding could have important health implications if a similar form of susceptibility exists in humans. Not only is understanding the basis for host defense against A/E bacteria critical for identification of susceptible individuals and development of new therapies, but such findings may also shed light on how differences in individual host microflora contribute to the degree of intestinal protection to enteric infectious diseases. Finally, these data could have significant implications for our understanding of other enteric diseases that implicate the intestinal microbiota such as IBD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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