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Streptomycin-Induced Inflammation Enhances *Escherichia coli* Gut Colonization Through Nitrate Respiration

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ABSTRACT Treatment with streptomycin enhances the growth of human commensal *Escherichia coli* isolates in the mouse intestine, suggesting that the resident microbial community (microbiota) can inhibit the growth of invading microbes, a phenomenon known as “colonization resistance.” However, the precise mechanisms by which streptomycin treatment lowers colonization resistance remain obscure. Here we show that streptomycin treatment rendered mice more susceptible to the development of chemically induced colitis, raising the possibility that the antibiotic might lower colonization resistance by changing mucosal immune responses rather than by preventing microbe-microbe interactions. Investigation of the underlying mechanism revealed a mild inflammatory infiltrate in the cecal mucosa of streptomycin-treated mice, which was accompanied by elevated expression of *Nos2*, the gene that encodes inducible nitric oxide synthase. In turn, this inflammatory response enhanced the luminal growth of *E. coli* by nitrate respiration in a *Nos2*-dependent fashion. These data identify low-level intestinal inflammation as one of the factors responsible for the loss of resistance to *E. coli* colonization after streptomycin treatment.

IMPORTANCE Our intestine is host to a complex microbial community that confers benefits by educating the immune system and providing niche protection. Perturbation of intestinal communities by streptomycin treatment lowers “colonization resistance” through unknown mechanisms. Here we show that streptomycin increases the inflammatory tone of the intestinal mucosa, thereby making the bowel more susceptible to dextran sulfate sodium treatment and boosting the *Nos2*-dependent growth of commensal *Escherichia coli* by nitrate respiration. These data point to the generation of alternative electron acceptors as a by-product of the inflammatory host response as an important factor responsible for lowering resistance to colonization by facultative anaerobic bacteria such as *E. coli*.

H uman isolates of *Escherichia coli* are generally poor colonizers of the murine gastrointestinal tract, but robust growth in the large intestine is observed in streptomycin-treated mice (1, 2). The streptomycin-treated mouse model has been used widely to study whether the elaboration of smooth lipopolysaccharide (3, 4), attachment mediated by fimbriae (5–8), motility (9), and the utilization of certain nutrients and trace elements (10–17) are properties important for the in vivo growth of commensal *E. coli*. It also provides a model to study carbon utilization (14, 16, 18–20) and the role of Shiga-like toxin (21–26) during infection with enterohemorrhagic *E. coli*. However, the mechanism by which streptomycin treatment promotes colonization with *E. coli* remains elusive.

Marjorie Bohnhoff and coworkers first introduced the concept that microbial communities in the intestine (the microbiota) can inhibit the growth of invading pathogens by conferring “colonization resistance” after noticing that the intestinal tracts of mice pretreated with streptomycin become highly susceptible to *Salmonella enterica* serovar Enteritidis infection (27, 28). Pretreatment of mice with streptomycin enhances the luminal growth of *S. enterica* serovar Typhimurium (*S. Typhimurium*) in the murine cecum by lowering colonization resistance (29). In addition, the antibiotic exacerbates the severity of *S. Typhimurium*-induced colitis (30). The enhanced severity of colitis in streptomycin-treated mice infected with *S. Typhimurium* has been ascribed to a decreased “immune resistance,” a term coined recently to describe specific host-commensal interactions associated with colonization resistance that confer protection against mucosal damage (31). Streptomycin treatment is proposed to reduce colonization resistance by lowering growth competition between the microbiota and incoming *S. Typhimurium* (32). Interestingly, conditions that lower the resistance to colonization by commensal *E. coli* also lower immune resistance to *S. Typhimurium*-induced colitis (33), but the identities of factors that contribute to these phenomena are obscure.

Here we describe the identification and characterization of one of the mechanisms responsible for loss of resistance to *E. coli* colonization after streptomycin treatment.

RESULTS

Streptomycin treatment lowers immune resistance to infectious and noninfectious colitis. Pretreatment of mice with streptomy-
cin (a single dose of 20 mg/animal) enhances the susceptibility of mice to the development of S. Typhimurium-induced colitis (30) (Fig. 1A), presumably because the antibiotic lowers growth competition between the microbiota and the incoming pathogen (32). Should streptomycin enhance inflammation because it lowers microbe-microbe interactions that limit pathogen growth, the antibiotic would not be expected to increase the susceptibility of mice to the development of colitis caused by exposure to chemicals. To test this prediction, mice received a subpathological concentration of dextran sulfate sodium (DSS) in their drinking water (1%) for 8 days. Four days after being switched to DSS-containing drinking water, some mice were treated with streptomycin (a single dose of 20 mg/animal) and their ceca were collected 96 h later. Treatment of mice with streptomycin and/or 1% DSS did not result in significant weight loss (see Table S1 in the supplemental material). Drinking water containing a subpathological concentration of DSS did not induce marked gut inflammation in mice that were not treated with streptomycin (Fig. 2A), which was consistent with a previous report of a study that used this dose to identify conditions that exaggerate susceptibility to DSS (34). Surprisingly, streptomycin-treated mice that received a subpathological concentration of DSS developed mild inflammation, as indicated by a significantly ($P < 0.01$) increased histopathology score in the cecum ($P < 0.05$) (Fig. 2A). Thus, in addition to the known enhancement of the susceptibility to S. Typhimurium-induced colitis (30), these data suggest that streptomycin treatment also lowers immune resistance to DSS-induced mucosal damage. Collectively, these data suggest that streptomycin treatment makes the bowel generally more irritable. Importantly, while it is conceivable that inhibition of pathogen growth by microbe-microbe interactions, such as competition for nutrients or metabolic exclusion, explains the enhanced susceptibility of streptomycin-treated mice to the development of S. Typhimurium-induced colitis, these potential mechanisms did not provide a compelling explanation for the increased susceptibility of streptomycin-treated mice to DSS-induced colitis.

**Streptomycin treatment increases the inflammatory tone of the cecal mucosa.** Interestingly, we noted that sections of the cecal mucosa collected from mice treated with streptomycin, followed by inoculation with sterile medium (LB broth), exhibited mild
inflammatory changes, unlike those from mice inoculated with sterile medium alone \((P < 0.05)\) (Fig. 1A). These inflammatory changes included mild diffuse inflammatory infiltrates in the lamina propria and occasional focal infiltrates in the mucosa (Fig. 1B). To further investigate possible inflammatory changes induced by streptomycin treatment, we next analyzed the composition of cells present in the cecal mucosa by using flow cytometry.

Mice were treated with streptomycin (a single dose of 20 mg/animal) and sterile medium (LB broth) or with sterile medium alone, and a single-cell suspension was generated from the cecum 96 h later. After doublet elimination and exclusion of dead cells (dead/live aqua staining), live cecal cells were gated for a population that was negative for the T cell marker CD3 (cluster of differentiation 3), the B cell marker B220, and the NK/NKT cell marker NK1.1. Inflammatory phagocytes present in the CD3\(^{-}\)B220\(^{-}\)NK1.1\(^{-}\) population were identified as cells expressing CD11B, a chain of CR3 (complement receptor 3), and Ly6C (lymphocyte antigen 6 complex, locus C), a marker expressed by neutrophils and inflammatory monocytes but absent from tissue-resident macrophages and tissue-resident dendritic cells (Fig. 3A). Finally, CD3\(^{-}\)B220\(^{-}\)NK1.1\(^{-}\)CD11B\(^{+}\)Ly6C\(^{+}\) phagocytes were further differentiated into neutrophils and monocytes on the basis of the expression of the neutrophil marker Ly6G (Fig. 3B). The Ly6G/Ly6C markers are superior to Gr-1 (granulocyte differentiation antigen 1) for the identification of neutrophils/monocytes, and previous work suggests that the mouse macrophage marker F4/80 is not required for the identification of these subsets (35).

Remarkably, streptomycin treatment significantly \((P < 0.01)\) increased the fraction of live cecal cells that expressed markers of inflammatory monocytes \((i.e., CD3^{-} B220^{-} NK1.1^{-} CD11B^{+} Ly6C^{+} Ly6G^{-} cells)\) (Fig. 3C). Furthermore, streptomycin treatment was associated with a significant \((P < 0.01)\) increase in the fraction of neutrophils \((i.e., CD3^{-} B220^{-} NK1.1^{-} CD11B^{+} Ly6C^{+} Ly6G^{+} cells)\) present within the live cecal cell suspension (Fig. 3B and D). In contrast, the number of resident phagocytes \((i.e., CD3^{-} B220^{-} NK1.1^{-} CD11B^{+} Ly6C^{-} Ly6G^{-} cells)\) present in the live cecal cell population remained unchanged after streptomycin treatment (Fig. 3E). We next repeated the experiment to monitor inflammatory changes at later time points after streptomycin treatment. The results revealed that the infiltrates of inflammatory monocytes (Fig. 3F) and neutrophils (Fig. 3G) were relatively short-lived, because the fraction of both cell types in live cecal cell suspensions returned to background levels within 15 days after streptomycin treatment.

Collectively, our results suggested that a single dose of streptomycin increased the inflammatory tone of the cecal mucosa temporarily, which was characterized by an infiltrate with neutrophils and inflammatory monocytes that peaked a few days after antibiotic treatment.

Streptomycin induces colitis in mice treated with a subpathological concentration of DSS. We next analyzed phagocyte populations in mice treated with a subpathological concentration of DSS by using flow cytometry, because this method provides a higher resolution for the characterization of mild inflammatory changes than histopathology scoring does (Fig. 2A). Streptomycin-treated mice (a single dose of 20 mg/animal) receiving a subpathological concentration of DSS \((1\%)\) harbored significantly \((P < 0.05)\) greater fractions of inflammatory monocytes (Fig. 2B) and neutrophils (Fig. 2C) in their live cecal cell population than did mice receiving streptomycin alone or mice receiving a subpathological concentration of DSS alone. These results further corroborated our observation that streptomycin treatment lowers immune resistance to DSS-induced mucosal damage (Fig. 2A).

Streptomycin-treated mice exhibit a marked increase in mucosal Nos2 expression. To start investigating whether there is a mechanistic link between streptomycin-induced inflammation and reduced resistance to \(E. coli\) colonization, we determined whether streptomycin treatment (a single dose of 20 mg/animal) altered the expression of inflammatory markers in the cecal mucosa by quantitative real-time PCR (Fig. 4). Streptomycin treatment resulted in a modest increase in mRNA levels of \(Kc\) (also known as \(Ccx1\)) (Fig. 4A), which encodes the neutrophil chemotactrant KC (keratinocyte-derived cytokine), and \(Mip2\) (also known as \(Ccx2\)) (Fig. 4B), which encodes the neutrophil chemotactrant MIP2 (macrophage inflammatory protein 2), but...
these differences did not reach statistical significance. There was a small but significant ($P < 0.05$) increase in mRNA levels of Mcp1 (also known as Ccl2) (Fig. 4C), which encodes MCP-1 (monocyte chemoattractant protein 1), and Mcp2 (also known as Ccl8) (Fig. 4D), which encodes MCP-2, after streptomycin treatment. The small increases in the expression of genes that encode chemoattractants that were observed after streptomycin treatment were consistent with the mild infiltrate of neutrophils (Fig. 3B and D) and inflammatory monocytes (Fig. 3C) detected in live cecal cell suspensions.

Next, we investigated the expression of other genes whose mRNA levels are markedly increased during intestinal inflammation (36, 37), including Lcn2, which encodes the antimicrobial protein lipocalin-2, and Nos2, which encodes inducible nitric oxide (iNOS). Consistent with the modest increase in mRNA levels observed for other markers of inflammation, we observed a small (3.8-fold) but significant ($P < 0.01$) increase in the expression of Lcn2 in the cecal mucosa at 1 day after streptomycin treatment (Fig. 4E). Remarkably, levels of Nos2 mRNA were, on average, 85.6-fold elevated at 96 h after streptomycin treatment ($P < 0.01$) (Fig. 4F). This marked increase in the abundance of Nos2 mRNA was of particular interest, because iNOS has recently been implicated in enhanced nitrate production in the intestinal lumen, thereby enhancing the growth of E. coli through anaerobic nitrate respiration (38). Thus, analysis of the expression of genes that encode inflammatory markers suggested a possible mechanistic link between the streptomycin-induced increase in Nos2 mRNA levels (Fig. 4F) and the reduced resistance of mice to E. coli colonization.

**Streptomycin treatment enhances the growth of E. coli by nitrate respiration.** To test the hypothesis that a streptomycin-induced increase in Nos2 mRNA levels fuels the growth of E. coli in the murine large intestine by nitrate respiration, we used the commensal E. coli strain Nissle 1917. E. coli possesses three nitrate reductases encoded by the narGHJI, narZYYV, and napFD-AGHBC operons (39). Inactivation of the narG, napA, and narZ genes in E. coli Nissle 1917 resulted in loss of nitrate reductase activity (Fig. 5A). To mimic the growth conditions in the lumen of E. coli Nissle 1917, we used the E. coli Nissle 1917 mutant strain E. coli Nissle 1917 ΔnarcGHJIΔnarcZYYVΔnapFDΔAGHBC ΔnarZ, which lacks the ability to reduce nitrate to nitrite.

![Diagram of gut microbiota](image-url)

**FIG 3** Streptomycin treatment elicits infiltrates of inflammatory monocytes and neutrophils in the cecal mucosa. (A) Gating strategy for analysis of cecal cell suspensions. After doublet elimination (top right), live cells were gated (top left) and CD3$^+$ B220$^+$ NK1.1$^+$ cells were eliminated by using a dump channel (bottom left). CD3$^+$ B220$^+$ NK1.1$^+$ cells were then analyzed for CD11B and Ly6C expression (bottom right). (B) CD3$^+$ B220$^+$ NK1.1$^+$ CD11B$^+$ Ly6C$^+$ phagocytes were separated into Ly6G$^+$ cells (inflammatory monocytes) and Ly6G$^+$ cells (neutrophils) (C to G). Bars represent the geometric means ± the standard errors of the numbers of inflammatory monocytes (C and F), neutrophils (D and G), and resident phagocytes (E) expressed as percentages of the total number of live cecal cells (C to E). The statistical significance of the difference between groups is indicated at the top of each graph. NS, not statistically significantly different. For panels A to E, the ceca of groups of mice ($n = 9$) were collected 4 days after streptomycin treatment, and for panels F and G, the ceca of groups of mice ($n = 3$) were collected 4 (black bars), 10 (gray bars), or 15 (white bars) days after streptomycin treatment.
the large bowel, mucin broth (i.e., hog mucin dissolved in a buffered solution) was inoculated with an equal mixture of streptomycin-resistant *E. coli* Nissle 1917 (CAL225) and a nitrate respiration-deficient *narG napA narZ* triple mutant (CAL222) and incubated overnight anaerobically. The two strains were recovered in equal numbers after anaerobic growth in mucin broth, suggesting that inactivation of the *narG*, *napA*, and *narZ* genes did not impair the growth of *E. coli* in the absence of nitrate. However, the nitrate respiration-deficient *narG napA narZ* triple mutant was outcompeted by the wild-type strain during competitive anaerobic growth in mucin broth supplemented with nitrate (\(P < 0.001\)) (Fig. 5B). These and previous data (17) suggest that nitrate respiration confers a fitness advantage upon *E. coli* under growth conditions that mimic those encountered in the large intestine.

We next investigated whether streptomycin treatment confers a growth advantage upon *E. coli* in the mouse large intestine. Groups of mice were inoculated with streptomycin (a single dose of 20 mg/animal) or left untreated and were inoculated 1 day later with an equal mixture of streptomycin-resistant *E. coli* Nissle 1917 (CAL225) and an isogenic nitrate respiration-deficient *narG napA narZ* triple mutant (CAL222). In the absence of streptomycin treatment, the wild type and the *narG napA narZ* mutant colonized the mouse intestine poorly and were recovered in equal numbers from the feces (Fig. 5C) and colon contents (Fig. 5D). In contrast, the wild-type strain was recovered in significantly (\(P < 0.005\)) higher numbers than the *narG napA narZ* mutant from streptomycin-treated mice 6 days after infection (Fig. 5D). These data suggested that nitrate respiration conferred a fitness advantage in the large intestines of streptomycin-treated mice but not during growth in the intestines of conventional mice. Furthermore, the overall numbers of *E. coli* bacteria recovered from the colon contents of streptomycin-treated mice were significantly (\(P < 0.01\)) higher than those recovered from untreated control mice (Fig. 5E), illustrating the colonization resistance-lowering effect of streptomycin. Nitrate respiration accounted for only part of this difference, pointing to the existence of additional mechanisms that contribute to the loss of colonization resistance after treatment with streptomycin.

Finally, we wanted to test whether there is a causal link between the streptomycin-induced increase in *Nos2* mRNA levels (Fig. 4F) and the streptomycin-induced growth of *E. coli* by nitrate respiration. To this end, we inoculated groups of *Nos2*-deficient mice with streptomycin or left them untreated and inoculated them 1 day later with an equal mixture of streptomycin-resistant *E. coli* Nissle 1917 (CAL225) and an isogenic *narG napA narZ* mutant (CAL222). Remarkably, the wild type and the *narG napA narZ* mutant were recovered in equal numbers from the colon contents of *Nos2*-deficient mice, regardless of streptomycin treatment (Fig. 5D). These data suggested that the fitness advantage conferred by nitrate respiration in streptomycin-treated mice was *Nos2* dependent, thereby providing a causal link between...
streptomycin-induced inflammation and the enhanced growth of (i.e., loss of resistance to colonization by) *E. coli*.

**DISCUSSION**

It is known that conditions of intestinal inflammation can lead to a microbial imbalance (dysbiosis) in the intestine that is characterized by a marked decrease in the representation of obligate anaerobic bacteria (i.e., members of the classes *Bacteroidia* and *Clostridia*) and an increased relative abundance of facultative anaerobic bacteria, which are commonly members of the family *Enterobacteriaceae* (reviewed in reference 40). In other words, intestinal inflammation is a known mechanism of reducing resistance to colonization by *Enterobacteriaceae*. However, since no overt inflammatory changes are detected as a consequence of treating mice with streptomycin (30, 41, 42), elimination of microbe-microbe interactions is commonly considered a more likely explanation for the associated loss of resistance to *E. coli* colonization. Specifically, streptomycin treatment is assumed to reduce the colonization resistance of mice because the antibiotic kills microbes that prevent colonization by incoming human *E. coli* isolates by metabolic exclusion or competition for nutrients, thereby freeing an otherwise occupied niche (reviewed in reference 31). This hypothesis suggests that colonization resistance is defined as the presence of a factor, namely, the presence of certain microbes that successfully compete with *E. coli*. In contrast, results presented here support the alternative view that colonization resistance is defined, at least in part, as the absence of intestinal inflammation.

Our data suggest that streptomycin reduces colonization resistance, at least in part, by generating a mild inflammatory response that supports the growth of *E. coli* through nitrate respiration, thereby generating a new niche rather than clearing an existing niche of competitors. Inflammation induces the expression of iNOS (43), an enzyme that generates NO (44). NO can react with superoxide radicals produced during inflammation to yield peroxynitrite (ONOO⁻⁻) (45), which can be converted to nitrate (NO₃⁻) (46). Nitrate respiration enhances the growth of commensal *E. coli* in mice with DSS-induced colitis. In contrast, iNOS-deficient mice with DSS-induced colitis or normal mice that lack intestinal inflammation do not support the growth of *E. coli* by nitrate respiration (40). These data suggest that a by-product of intestinal inflammation is the generation of alternative electron acceptors, such as nitrate, that support the growth of *E. coli* and other members of the family *Enterobacteriaceae* by anaerobic res-
pilation (38, 47, 48). Anaerobic respiration enables facultatively anaerobic bacteria to utilize nonfermentable carbon sources, thereby sidestepping the competition with obligate anaerobic bacteria that rely on the fermentation of carbohydrates and amino acids for growth (49). Through this mechanism, anaerobic respiration provides *E. coli* with a fitness advantage during growth in the inflamed intestine (38). Conversely, the absence of this fitness advantage in the healthy gut is manifested as resistance to *E. coli* colonization.

The unexpected identification of intestinal inflammation as one of the mechanisms by which streptomycin treatment reduces colonization resistance puts the spotlight on a previously unknown consequence of using this antibiotic. That is, how does streptomycin treatment induce the mild inflammatory changes in the cecal mucosa that ultimately drive bacterial growth by nitrate respiration? An increased inflammatory tone of the intestinal mucosa following antibiotic therapy is not without precedent, as mice exhibit increased macrophage and NK cell infiltration in the intestinal mucosa after oral metronidazole treatment (42). However, the elucidation of underlying mechanisms is complicated because factors that confer immune resistance to intestinal inflammation are not identical to those that enhance resistance to colonization by *Enterobacteriaceae*. For example, the abundance within the microbial community of bacteria belonging to the family *Porphyromonadaceae* (phylum *Bacteroidetes*) correlates with protection against S. Typhimurium-induced colitis (i.e., with increased immune resistance) but does not affect the growth of the pathogen in the intestinal lumen (i.e., it does not alter colonization resistance) (50). Our finding that streptomycin lowers immune resistance to DSS-induced colitis is of interest because factors that confer immune resistance to DSS are unknown. Indeed, immune resistance provides protection against pathogens that rely on the fermentation of carbohydrates and amino acids for growth (49). Through this mechanism, anaerobic respiration provides a fitness advantage during growth in the healthy gut. Inflammation enhances *E. coli* gut colonization.

Interestingly, transfer of a normal complex microbiota can restore the resistance of mice to colonization by S. Typhimurium (52). Consistent with a possible role for microbiota-induced mucosal responses in mediating colonization resistance, intestinal bacteria and their metabolic by-products are known to directly impact the repertoire and activity of intestinal immune cells (53–57). However, previous metagenomic studies did not succeed in establishing a correlation between reduced colonization resistance induced by antibiotic treatment and a decreased abundance of specific bacterial species or family within the microbial community (32, 41, 50, 52). As a result, the identification of microbiota-host interactions underlying colonization resistance remains an important challenge for future research.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** S. Typhimurium strain IR715 (58) is a fully virulent, nalidixic acid-resistant derivative of wild-type isolated ATCC 14028 (American Type Culture Collection). Bacteria were cultured overnight aerobically at 37°C in Luria-Bertani (LB) broth or on LB plates (15 g/liter agar) with the appropriate antibiotics at the following concentrations: nalidixic acid, 0.05 mg/ml; carbenicillin, 0.1 mg/ml; kanamycin, 0.1 mg/ml.

**Plasmid construction.** *E. coli* strains used for mouse infections contained a streptomycin resistance marker to facilitate recovery. To this end, a streptomycin resistance cassette (Ω) was isolated by digesting plasmid pHp45E (59) with the restriction enzyme BamHI (New England Biolabs). The Ω cassette was then inserted into the BamHI site of pWSK29 or pWSK129 (60) via ligation to yield plasmid pCAL62 or pCAL61, respectively. Plasmid pCAL62 was transformed into wild-type *E. coli* Nissle 1917 (61) to create CAL225, and plasmid pCAL61 was transformed into the isogenic napA narZ narG mutant (SW930) (38) to yield CAL222.

**Competitive-growth assays.** For competition assays, bacteria were grown in mucin medium containing 0.25% type II porcine mucin (Sigma-Aldrich), 40 mM morpholinepropanesulfonic acid buffer, trace elements (62), and magnesium sulfate (265 mg/liter) dissolved in sterile water. Either sterile water as a negative control or 40 mM sodium nitrate (Sigma-Aldrich) was added to the medium immediately prior to inoculation with a 1:1 ratio of CAL225 and CAL222 at a total concentration of 10^9 CFU/ml. Bacteria were allowed to grow anaerobically for 16 h at 37°C (Bactron I anaerobic chamber; Sheldon Manufacturing, Cornelius, OR), and then the ratio of the two strains was determined by spreading serial 10-fold dilutions on LB agar plates containing the appropriate antibiotics. In *vitro* competition assays were performed in triplicate with cultures inoculated from different colonies. The competitive index was calculated by dividing the number of bacteria carrying the wild-type allele by the number of bacteria with the respective mutant allele and corrected by the ratio of these strains in the inoculum.

**Nitrate reductase activity assay.** Overnight cultures of *E. coli* strains were diluted 1:50 in fresh LB broth supplemented with 40 mM sodium nitrate. Cultures were statically incubated for 3 h at 37°C, and the relative nitrate reductase activity was measured as described previously (63). Briefly, the nitrate reductase assay measures the reduction of nitrate to nitrite with methyl viologen as the electron donor. Nitrate is added to the reaction medium containing lysed bacterial cells, and nitrite (from nitrate reductase activity) is measured on the basis of its formation of a colored azo compound, which is quantified with a spectrophotometer. Assays were performed in triplicate with different colonies.

**Animal experiments.** All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California, Davis, and performed according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Female 10- to 12-week-old C57BL/6 mice (The Jackson Laboratory) or Nos2-deficient mice (stock no. 2609; The Jackson Laboratory) were used for mouse experiments. In brief, mice were inoculated intragastrically with streptomycin (0.1 ml of a 200-mg/ml solution in distilled water) as described previously (30). For infected groups, 24 h later, mice were inoculated intragastrically with either sterile LB broth or with bacteria (0.1 ml containing approximately 1 × 10^10 CFU/ml). At the indicated time points after infection, mice were euthanized and samples of their ceca were collected for the isolation of mRNA, for histopathological analysis, or for collection of cells. For bacteriologic analysis, cecal contents, colon contents, or fecal pellets were collected at the indicated time points and homogenized and serial 10-fold dilutions were spread on agar plates containing the appropriate antibiotics. For low-level DSS experiments, mice were given 1% DSS salt (catalogue no. 160110; MP Biomedicals) in their drinking water continuously for 8 days as described previously (34). DSS was replaced with fresh DSS solution every 2 to 3 days during this time. Mice were then inoculated with streptomycin at day 4 of DSS treatment as described above. Mouse body weights were determined daily.

**Quantitative real-time PCR.** Transcript levels of murine genes in RNA isolated from the cecal mucosa were determined as described previously (36). For quantitative analysis of mRNA levels, 1 μg of RNA from each sample was reverse transcribed in a 50-μl volume (TagMan reverse transcription reagent; Applied Biosystems), and 4 μl of cDNA was used for each real-time reaction. Real-time PCR was performed with Sybr green (Applied Biosystems) and a 7900HT fast real-time PCR system. The data were analyzed by a comparative cycle threshold method (Applied Biosystems). Increases in cytokine expression in infected mice were calculated relative to the average level of the respective cytokine in four
control animals from the corresponding time point. A list of the genes analyzed in this study with the respective primers is provided in Table 1.

**Histopathology.** Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding as described previously (36), sectioned at 5 μm, and stained with hematoxylin and eosin. A veterinary pathologist scored inflammatory changes by blind sample analysis. Neutrophil and monocyte counts were determined by high-magnification (×400) microscopy, and numbers of cells in 10 microscopic fields were averaged for each animal.

**Isolation of intestinal lymphocytes.** The isolation of intestinal lymphocytes for experiments has been described previously (64). To isolate cecal lymphocytes for measurements of in vivo cellular analysis, control and experimental mouse groups were sacrificed at the designated time points. The cecum and proximal colon were collected, and fat and connective tissue were removed. Intestinal sections were cut longitudinally from the proximal colon to the tip of the cecum. The cecal content was removed by gentle scraping with the flat edge of scissors. The sections were subsequently washed in cold 1× Hanks balanced salt solution (catalogue no. 14185; Gibco) containing 0.015 M HEPES (catalogue no. 15630; Gibco) a total of six times to remove mucus and remaining fecal matter. To isolate lymphocytes, the tissue was added to 10 ml of prewarmed (37°C) 1× RPMI (Sigma catalogue no. R1145) containing 10% fetal bovine serum, 1% penicillin-streptomycin (catalogue no. 15240-062; Gibco), 0.015 M HEPES, and 0.01 M glutamine. Additionally, collagenase digestion units (furylacryloyl-leucine-glycyl-propyl-alanine) (Liberase, catalogue no. 05401127001; Roche) at 2.9 U/ml and 300 U of DNase nase digestive units (furylacryloyl-leucine-glycyl-propyl-alanine) (Liberase, catalogue no. 05401127001; Roche) at 2.9 U/ml and 300 U of DNase I (Roche catalogue no. 04716728001) were added to the medium. The tissue was then processed with a GentleMACS tissue disruptor according to a protocol provided by the manufacturer (Miltenyi).

**Flow cytometry.** The analysis of surface marker expression in intestinal lymphocytes (C57BL/6; The Jackson Laboratory) has been described previously (64). In brief, a total of 4 × 10^6 intestinal cells were resuspended in 2 ml Dulbecco’s phosphate-buffered saline (PBS) without calcium and magnesium and stained with aqua live/dead cell discriminator (no. L.34597; Invitrogen) in accordance with the manufacturer’s protocol. Cells were then rinsed and resuspended in 50 μl fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% bovine serum albumin and 1 mM EDTA). Four microliters of the anti-CD16/32 blocking antibody (clone M1/70; BioLegend) antibodies. Cells were washed twice with FACS buffer and subsequently fixed in 4% paraformaldehyde for 1 h. Cells were then washed twice and resuspended in FACS buffer and analyzed with an LSR II flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed by using FlowJo software (TreeStar, Inc., Ashland, OR). Gates were set on singlets and then on live cells. Subsequent gates were based on fluorescence minus one and unstained controls.

**Statistical analysis.** To determine the statistical significance of differences between treatment groups in the animal experiments, an unpaired Student t test was used. A P value of less than 0.05 was considered to be significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00430-13/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.

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We have no conflicts of interest to declare.

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**TABLE 1 Primers used for real-time PCR to quantify mRNA levels of murine genes**

| Gene   | Primer 1        | Primer 2        |
|--------|-----------------|-----------------|
| Ccl2   | 5’-ATTGGGATCATCTTTGCTGGTT-3’ | 5’-CTCTTGCTGCTGGCTCATAGT-3’ |
| Ccl6   | 5’-GAAGGGGAGATCTTCCAGGT-3’    | 5’-CATGGGAACTGTGGTTAGTCCG-3’  |
| Lcn2   | 5’-ACATTGTGCTCAGCTCCAAAGGC-3’ | 5’-TTGTCAGAAGCCAGCGTTGAC-3’  |
| Cxcl1  | 5’-TGACCCCAAAAGGAAGGACTCAT-3’ | 5’-AGGCAAACTTTTTTGACGCCG-3’  |
| Cxcl2  | 5’-AGTGAACCTGGCTGCTCATGGC-3’  | 5’-CCTCTTTAGTTCAACTTGGTTAGG-3’ |
| Nos2   | 5’-TGGGTTCTGTTCTACTCCAGGG-3’  | 5’-AGGTCCGGTTGAACGGGATTG-3’  |
| Gapdh  | 5’-TGTAGACCACGTAGTTGAGTCA-3’  |                  |
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