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Host-derived nitrate boosts growth of *E. coli* in the inflamed gut

Sebastian E. Winter[1], **Maria G. Winter**[1], **Mariana N. Xavier**[1], **Parameth Thiennimitr**[1,2], **Victor Poon**[1], **A. Marijke Keestra**[1], **Richard C. Laughlin**[3], **Gabriel Gomez**[3], **Jing Wu**[3], **Sara D. Lawhon**[3], **Ina Popova**[4], **Sanjai J. Parikh**[4], **L. Garry Adams**[3], **Renée M. Tsolis**[1], **Valley J. Stewart**[5], and **Andreas J. Bäumler**[1]

[1]Department of Medical Microbiology and Immunology, School of Medicine, University of California, Davis, One Shields Ave., Davis, CA [2]Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand [3]Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX [4]Department of Land, Air and Water Resources, University of California at Davis, One Shields Ave., Davis, CA [5]Department of Microbiology, College of Biological Sciences, University of California, Davis, One Shields Ave., Davis, CA

Abstract

Changes in the microbial community structure are observed in individuals with intestinal inflammatory disorders. These changes are often characterized by a depletion of obligate anaerobic bacteria, whereas the relative abundance of facultative anaerobic Enterobacteriaceae increases. The mechanisms by which the host response shapes the microbial community structure, however, remain unknown. We show that nitrate generated as a by-product of the inflammatory response conferred a growth advantage to the commensal bacterium *Escherichia coli* in the large intestine of mice. Mice deficient for inducible nitric oxide synthase (iNOS) did not support growth of *E. coli* by nitrate respiration, suggesting that nitrate generated during inflammation was host-derived. Thus the inflammatory host response selectively enhances growth of commensal Enterobacteriaceae by generating electron acceptors for anaerobic respiration.

Over 90% of the cells in the human body are microbes, the majority of which reside in the large intestine, where they provide benefit to the host by stimulating the development of the immune system, by supplying nutrients, and by providing niche protection. The lumen of the large bowel is thought to be primarily anaerobic, with traces of oxygen being consumed by facultative anaerobic bacteria (e.g. Enterobacteriaceae), which constitute a small fraction (approximately 0.1%) of the microbial community (microbiota) (1). The vast majority of microbes in the large intestine belong to the phyla Bacteroidetes (class Bacteroidia) and Firmicutes (class Clostridia), two groups of obligate anaerobic bacteria that lack the ability to respire and instead rely on fermentation of amino acids and complex polysaccharides for growth. On the phylum level, this bacterial community structure is conserved between humans and mice (1, 2). Conditions of inflammation in the large bowel are accompanied by a microbial imbalance (dysbiosis), however, which is characterized by a marked decrease in

Correspondence to: Andreas J. Bäumler (ajbaumler@ucdavis.edu).

the representation of obligate anaerobic bacteria and an increased relative abundance of facultative anaerobic bacteria belonging to the family Enterobacteriaceae (3-12) (Fig. S1A and B).

An important component of the host inflammatory response is the generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Fig. S1C). For example, iNOS is expressed at high levels during intestinal inflammation and elevated nitric oxide ($NO^{\cdot}$) concentrations are detected in colonic luminal gas of individuals with inflammatory bowel disease (13-15). Reaction of nitric oxide radicals ($NO^{\cdot}$) with superoxide radicals ($O_2^{\cdot -}$) yields peroxynitrite ($ONOO^-$), which can either generate nitrate (NO_3^-) (16) or oxidize organic sulfides and tertiary amines to *S*-oxides and *N*-oxides (17, 18). Similarly, inflammation-derived ROS can generate *S*-oxides and *N*-oxides (17, 18). Unlike obligate anaerobic members of the gut microbiota, the facultative anaerobic Enterobacteriaceae can use nitrate, *S*-oxides, and *N*-oxides as terminal electron acceptors for anaerobic respiration. We thus hypothesized that colitis produces dysbiosis because highly oxidized by-products of intestinal inflammation (e.g. nitrate, *S*-oxides, and *N*-oxides) might enable commensal Enterobacteriaceae to edge out fermenting microbes in the gut lumen by using anaerobic respiration for energy production (Fig. S1C).

E. coli, a prototypic member of the Enterobacteriaceae, possesses three nitrate reductases, two *S*-oxide reductases, and three *N*-oxide reductases encoded by the *narGHJI, narZYWV, napFDAGHBC, dmsABC, ynfDEFGH, torCAD, torYZ,* and *yedYZ* operons, respectively (19). One common feature shared by these terminal reductases is the incorporation of an essential molybdenum cofactor into the active site. To test the idea that anaerobic respiration provides a growth benefit in the inflamed intestine, we generated mutants deficient for the biosynthesis of the molybdenum cofactor (*moaA* mutant) in the *Escherichia coli* strains HS and Nissle 1917 (EcN) (Fig. S2A and S2B). These *moaA* mutants were anaerobically co-cultured with the respective wild-type strains in mucin broth in the presence or absence of nitrate, DMSO (Dimethyl *S*-oxide), or TMAO (Trimethylamine *N*-oxide) (Fig. 1A and S2C). Enrichment for the *E. coli* wild-type strains occurred in the presence of nitrate, DMSO and TMAO, suggesting that anaerobic respiration can provide a growth benefit during the anaerobic growth conditions encountered in the intestinal mucus layer.

We next inoculated untreated mice (C57BL/6) or mice with chemically-induced colitis (dextran sulfate sodium [DSS]-treatment) intragastrically with an equal mixture of EcN and its isogenic *moaA* mutant (Fig. S1D). Both the wild-type strain and the *moaA* mutant colonized the intestine of mock-treated mice poorly, but similar numbers of each strain were recovered from colon contents five days after inoculation (Fig. 1B). This result suggested that in the absence of intestinal inflammation, anaerobic respiration did not provide a growth benefit for *E. coli*. DSS treatment induced inflammation in the colon and increased mRNA levels of pro-inflammatory markers in wild-type mice (Fig. S3). In contrast to mock-treated mice, the EcN wild-type strain was recovered from colon contents of DSS-treated mice in significantly higher numbers than the *moaA* mutant five days after inoculation. Similar results were observed when DSS-treated mice were inoculated with an equal mixture of the human commensal *E. coli* strain HS and an isogenic *moaA* mutant. Expression of *moaA* from a low copy-number plasmid (pMOA1) in the EcN *moaA* mutant fully restored the

phenotype to wild-type levels. Outgrowth of the EcN wild-type strain over the *moaA* mutant was also observed in the DSS colitis model when mice were precolonized with *E. coli* (Fig. S4). These findings supported the idea that anaerobic respiration provided a growth advantage upon commensal *E. coli* during intestinal inflammation.

While ROS can be generated by several NADPH oxidases, the sole source of NO during inflammation is iNOS. To determine the contribution of RNS to the growth advantage mediated by anaerobic respiration, DSS-treated mice were treated with the iNOS-inhibitor aminoguanidine hydrochloride (AG) and inoculated with a mixture of the EcN wild type and the *moaA* mutant. Consistent with the idea that RNS are a significant source for the production of terminal electron acceptors during inflammation, the growth advantage of the EcN wild type over the *moaA* mutant in the DSS-colitis model was significantly ($P < 0.01$) blunted after AG treatment (Fig. 1B). The nitrate/nitrate redox couple has a greater redox potential than the DMSO/DMS or the TMAO/TMA redox couples, which makes nitrate the preferred respiratory electron acceptor for growth of *E. coli* under anaerobic conditions (19). Therefore, we next determined if nitrate becomes available in the lumen of the inflamed intestine. To accomplish this objective, the concentration of nitrate was determined in the cecal mucus layer of mock-treated mice or mice with DSS-induced colitis (Fig. 1C). Whereas nitrate levels were at the limit of detection in mock-treated control mice, a significant ($P < 0.001$) increase in nitrate levels was observed in DSS-treated animals. AG treatment of mice with DSS-induced colitis significantly ($P < 0.05$) dampened nitrate production, thus supporting the hypothesis that nitrate is generated in the intestinal lumen as part of the host inflammatory response.

We next tested whether nitrate respiration bestows a growth advantage upon *E. coli* wild-type isolates. To this end, we inactivated the *narG*, *napA*, and *narZ* genes, which encode nitrate reductases, in the probiotic EcN. In contrast to the wild-type strain, the nitrate respiration-deficient *narG napA narZ* triple mutant lacked nitrate reductase activity and was outcompeted by the wild-type strain during competitive anaerobic growth in mucin broth in the presence of nitrate (Fig. S5). To determine whether nitrate respiration provides a colonization advantage in the intestine, mock-treated and DSS-treated wild-type (C57BL/6) mice were inoculated intragastrically with an equal mixture of the EcN wild type and a *narG napA narZ* triple mutant (Fig. 2). In the absence of inflammation (mock treatment, Fig. 2A, S6 and S7), both the EcN wild type and the *narG napA narZ* triple mutant were recovered in similar numbers from colonic (Fig. 2B) and cecal contents (Fig. S8A). In contrast, the EcN wild-type strain was enriched over the *narG napA narZ* triple mutant when colitis was induced by administration of DSS. Similar results were obtained using an adherent invasive *E. coli* (AIEC) isolate (LF82) that was isolated from an inflammatory bowel disease patient (Fig. 2C, 2D, S5C, and S5D). To test whether nitrate respiration provides a growth benefit in the absence of iNOS-dependent nitrate production, the competitive colonization experiment was repeated in DSS-treated, iNOS-deficient mice (i.e. mice carrying a mutation in the *Nos2* gene) and DSS-treated wild-type mice (C57BL/6) that received AG. The severity of the colitis induced by the DSS treatment was similar among all treatment groups (Fig. 2A and S7) five days after inoculation with *E. coli*. Remarkably, the EcN wild type and its nitrate respiration-deficient mutant were recovered in equal numbers from DSS-treated iNOS-

deficient mice or from DSS+AG-treated wild-type mice. Similar results were obtained using varying concentrations of DSS (Fig. S9) as well as with *E. coli* K-12 (Fig. S10). Concomitant expression of *narG* from a low-copy number plasmid (pNARG1) and restoration of the *napA* mutation to its wild-type allele (napA[restored]) in the *narG narZ napA* mutant reestablished fitness in the inflamed gut to similar levels observed with the wild-type strain (Fig. 2B and S8A). Collectively, these data suggested that reduction of host-derived nitrate by *E. coli* confers a growth advantage during gut inflammation.

To validate our findings in a second murine model of colitis, we generated mice that harbored T cells deficient for the production of the anti-inflammatory cytokine IL-10 (*Cd4 Il10* mice [$Il10^{flox/flox}$ *Cd4-cre*]), a mouse strain that developed spontaneous colitis (Fig. 2A and S7) (20). After the onset of intestinal inflammation, mice were inoculated intragastrically with an equal mixture of the EcN wild type and the *narG napA narZ* triple mutant (Fig. 2B). The nitrate respiration-proficient wild-type strain outcompeted the *narG napA narZ* mutant in the colon contents of *Cd4 Il10* mice 5 days after inoculation ($P < 0.05$). To investigate whether growth of *E. coli* by nitrate respiration can also be observed in an unrelated animal model of intestinal inflammation, bovine ligated ileal loops were inoculated with thapsigargin, a proinflammatory compound, or mock-treated (vehicle control) (Fig. 3A, B and C). At 8 hours after inoculation of loops with a mixture of EcN and the *narG napA narZ* mutant, significantly ($P < 0.05$) higher numbers of wild-type EcN were recovered from luminal fluid and mucus (Fig. 3D).

To determine whether nitrate respiration increases bacterial recovery from the inflamed intestine when mice are inoculated with a single *E. coli* strain, DSS-treated mice were inoculated either with the EcN wild-type strain or with the *narG napA narZ* mutant. Mice inoculated with EcN or the *narG napA narZ* mutant exhibited a similar severity of colonic inflammation (Fig. 3E, F, and G). Importantly, the EcN wild-type strain was recovered in significantly ($P < 0.01$) higher numbers from colon contents than the nitrate respiration-deficient mutant (Fig. 3H and S8B). Collectively, these data suggested that nitrate respiration conferred a marked growth advantage upon commensal *E. coli* in the lumen of the inflamed gut.

The picture emerging from this study is that nitrate generated as a by-product of the host inflammatory response can be utilized by *E. coli*, and likely by other commensal Enterobacteriaceae, to edge out competing microbes that rely on fermentation to generate energy for growth. Obligate anaerobic microbes in the intestine compete for nutrients that are available for fermentation, but cannot utilize non-fermentable nutrients (e.g. fermentation end products.). The ability to degrade non-fermentable substrates likely enables *E. coli* to sidestep this competition, which explains the fitness advantage conferred by nitrate respiration in the inflamed gut. Through this mechanism, inflammation contributes to a bloom of nitrate-respiration proficient Enterobacteriaceae, providing a plausible explanation for the dysbiosis associated with intestinal inflammation (3-12). This general principle might also influence the dynamics of host-associated bacterial communities outside the large bowel, as nitrate respiration confers a fitness advantage in the oxygen-poor and nitrate-rich environment of the cystic fibrosis airway (21).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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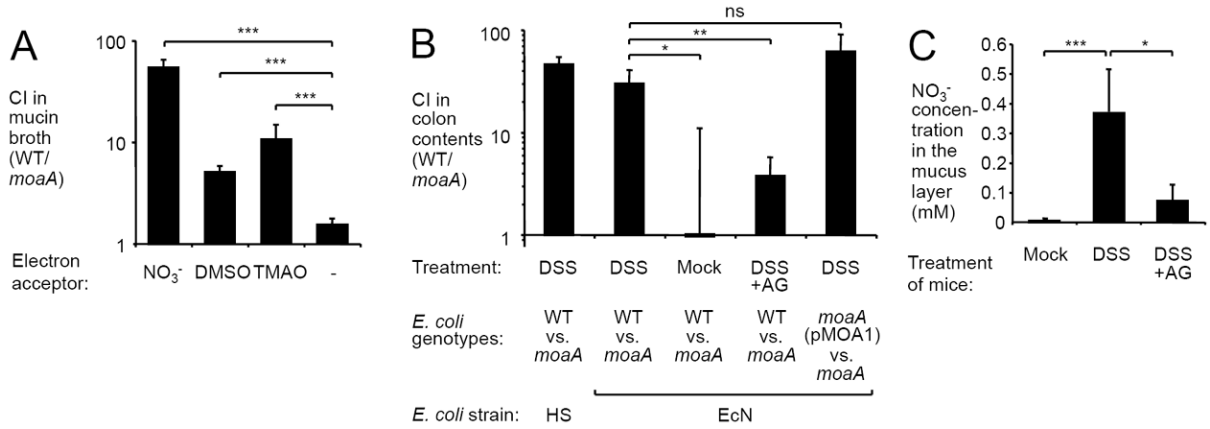

Figure 1. Anaerobic respiration enhances luminal growth of *E. coli* during DSS-induced colitis

(A) Competitive index (CI) of the EcN wild type (WT) and the *moaA* mutant after anaerobic growth in mucin broth supplemented with 40 mM of the indicated electron acceptors ($N = 3$). (B) Mock-treated mice (Mock), DSS-treated mice (DSS) or mice treated with DSS and AG (DSS+AG) were inoculated with the indicated mixtures of *E. coli* strains and the CI in colon contents determined 5 days after inoculation. A plasmid (pMOA1) carrying the cloned *moaA* gene was used to complement the *moaA* mutant (*moaA*). *N* is given in Fig. S3C. (C) Concentration of nitrate (NO_3^-) determined in the cecal mucus layer of mock-treated mice ($N = 4$), DSS-treated mice (DSS, $N = 3$) or mice treated with DSS and AG (DSS+AG, $N = 4$). Bars represent geometric means ± standard error. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant (Student's *t*-test).

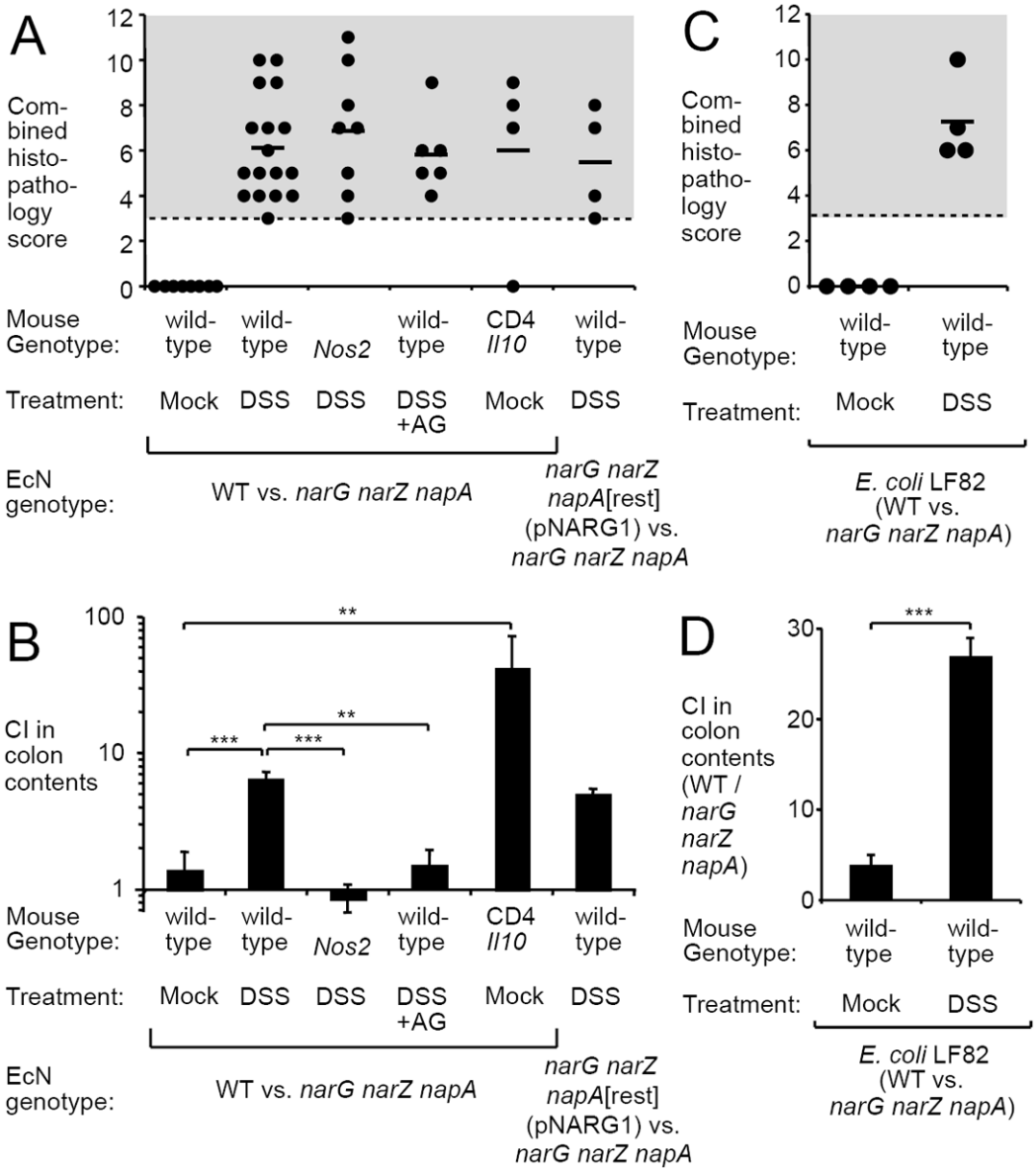

Figure 2. Wild-type *E. coli* outcompetes a nitrate respiration-deficient mutant during colitis

Mock-treated (Mock), DSS-treated (DSS) or DSS+AG-treated (DSS+AG) wild-type mice, *Nos2*-deficient mice (*Nos2*) or mice harboring T cells deficient for the production of IL-10 (*Cd4 Il10* mice) were inoculated with the indicated mixtures of *E. coli* strains. WT, *E. coli* wild type; *narG narZ napA, E. coli* nitrate respiration-deficient mutant. The *narG narZ napA* mutant was complemented by introducing a functional chromosomal *napA* allele and a plasmid (pNARG1) carrying the cloned *narG* gene (*narG narZ napA*[rest] [pNARG1]). Pathological changes in the colon (A and C) and the competitive index (CI) recovered from colon contents (B and D) were determined 5 days after inoculation. (A and C) Combined histopathology score in the colon. Each dot represents data from an individual animal. Experiments were performed with EcN (A-B) or *E. coli* LF82 (C-D). (B and D) Bars represent geometric means ± standard error. **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test). *N* is given in panels A and C.

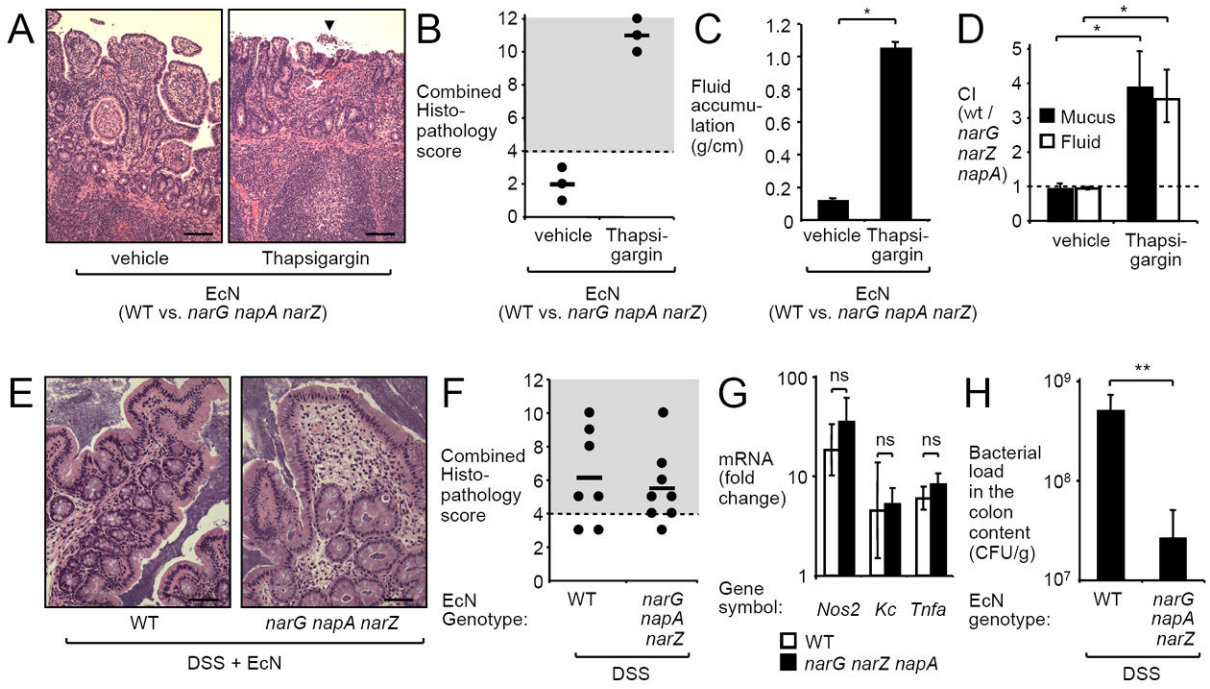

Figure 3. Nitrate respiration enhances luminal growth of EcN during inflammation

(A-D) Bovine ligated ileal loops treated with thapsigargin or mock-treated (vehicle) were inoculated with a mixture of EcN (WT) and a nitrate respiration-deficient mutant (*narG napA narZ*). Samples were collected 8 hours after inoculation. (A) Representative H&E stained ileal sections. Scale bar, 200 μm. (B) Combined histopathology score in the ileum. Each dot represents data from an individual animal. (C) Fluid accumulation in ligated ileal loops. (D) Competitive indices (CI) recovered from the luminal fluid (open bars) or mucus scrapings (closed bars). (E-H) DSS-treated mice were inoculated either with EcN (WT) or with the *narG napA narZ* mutant. Inflammation in the colon (E, F and G) and bacterial numbers recovered from colon contents (E) were determined 5 days after inouclation. (E) Representative H&E stained colonic sections. Scale bar, 100 μm. (F) Combined histopathology score in the colon. (G) Expression of *Nos2*, *Kc* and *Tnfa* in colonic RNA samples using qRT-PCR (fold-increases over mock-treated, mock-treated mice). (H) Bacterial numbers (CFU) recovered from colon contents. (C, D, G, H) Bars represent geometric means ± standard error. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significant (Student's *t*-test). *N* is given in panels B and F.