Lethal inflammasome activation by a multidrug-resistant pathobiont upon antibiotic disruption of the microbiota

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The mammalian intestine harbors a complex microbial community that provides numerous benefits to its host. However, the microbiota can also contain potentially virulent species, termed pathobiont, which can cause disease when intestinal homeostasis is disrupted. The molecular mechanisms by which pathobionts cause disease remain poorly understood. Here we describe a sepsis-like disease that occurs upon gut injury in antibiotic-treated mice. Sepsis was associated with the systemic spread of a specific multidrug-resistant *Escherichia coli* pathobiont that expanded markedly in the microbiota of antibiotic-treated mice. Rapid sepsis-like death required a component of the innate immune system, the Naip5-Nlrc4 inflammasome. In accordance with Koch’s postulates, we found the *E. coli* pathobiont was sufficient to activate Naip5-Nlrc4 and cause disease when injected intravenously into unmanipulated mice. These findings reveal how sepsis-like disease can result from recognition of pathobionts by the innate immune system.

The intestinal microbiota is composed of diverse microbial species that provide numerous benefits to their hosts. However, the microbiota can also contain pathobionts that can cause disease if not properly constrained. A major mechanism constraining pathobionts is believed to be competition or suppression by the healthy microbiota. Indeed, disruption of the healthy microbiota (for example, by antibiotic treatment) can elicit pathobiont virulence and disease. Although pathobionts are believed to contribute to numerous disease states, it has been challenging to identify the causal molecular mechanisms that are responsible for disease in vivo.

A key orchestrator of innate immune responses is inflammasomes, multiprotein complexes that detect infection in the cytosol and are required for activation of the caspase-1 protease, and downstream secretion of two of its substrates, the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (ref. 16). Several distinct inflammasomes have been described, including the Naip5-Nlrc4 inflammasome, which detects flagellin proteins from diverse bacteria. Recently, several reports have established a protective role for inflammasomes in regulating microbiota composition and tissue reparative and regenerative responses to intestinal injury. Although inflammasome responses in these models are protective against disease, it is also well recognized that excessive inflammation has the potential to be pathological. However, a potential pathological role of inflammasome activation in response to a disrupted intestinal flora has not yet been investigated.

Here we find that disruption of the microbiota by antibiotics predisposes mice to a lethal sepsis-like disease upon intestinal injury. Death is associated with the systemic infection with a multidrug-resistant *E. coli* pathobiont and requires activation of the Naip5-Nlrc4 inflammasome. In accordance with Koch’s postulates, intravenous injection of the *E. coli* pathobiont into normal mice recapitulates the rapidly fatal Naip5-Nlrc4–dependent sepsis. Our results reveal a molecular mechanism by which disruptions of intestinal homeostasis can result in aberrant pathobiont-induced innate immune signaling and rapid sepsis-like death.

**RESULTS**

**Antibiotics and intestinal injury results in sepsis**
To study the innate immune response to a disrupted intestinal microbiota, we established a disease model that couples antibiotic treatment with dextran sulfate sodium (DSS)-induced intestinal injury. DSS is toxic to colonic epithelial cells and typically causes a colitis-like disease characterized by severe weight loss, colonic bleeding and colonic shortening (Supplementary Fig. 1a,b). We wanted to understand how antibiotic-induced disruption of the microbiota (dysbiosis) would influence DSS-induced disease. Oral administration of a broad-spectrum antibiotic cocktail composed of ampicillin, vancomycin, neomycin and metronidazole (AVNM) to colony-born C57BL/6 wild-type mice resulted in a change in the microbiota composition and a reduction in the amount of 16S rDNA gene copy number along the intestinal tract (Supplementary Fig. 1c–e). After this initial treatment, we gave the mice AVNM plus 5% DSS and monitored survival. Consistent with previous reports, mice treated with AVNM showed faster death kinetics in response to DSS compared to wild-type littersmates that received DSS only (Fig. 1a and Supplementary Fig. 1f). Notably, ampicillin alone was sufficient to increase mortality in response to DSS treatment (Supplementary Fig. 1g,h). In contrast, mice treated with the broad-spectrum antibiotic streptomycin and DSS showed similar death kinetics to DSS-treated littersmates that were not treated with antibiotics (Fig. 1b).

Although DSS typically induces colitis, we found that AVNM–plus DSS-treated colony-born mice did not show the hallmark symptoms...
of colitis (for example, weight loss, colonic inflammation and shortening and poor stool pellet formation, as seen in mice treated with DSS alone; Fig. 1c–e and data not shown). Rather, AVNM- plus DSS-treated mice showed bleeding in the small intestine and hypothermia (Fig. 1f, g and Supplementary Fig. 2a), as well as multiple organ damage and elevated serum concentrations of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and IL-6 (Fig. 1h and data not shown). These results suggest that AVNM-induced changes to the microbiota trigger a systemic disease distinct from colitis in response to intestinal injury. Hypothermia and multiple organ damage are associated with sepsis in mice25,26. In addition, we found that antibiotic-treated mice showed significantly higher colonization of culturable bacteria in the lung (P < 0.05) and the liver (P < 0.05) compared to non-antibiotic-treated mice (Fig. 1i and Supplementary Fig. 2b, c).

To determine whether the sepsis-like disease was specific to mice from our facility, we analyzed disease progression in AVNM- plus DSS-treated C57BL/6 specific pathogen-free (SPF) mice from Jackson Laboratory (Jackson mice) and Taconic Farms (Taconic mice). AVNM- plus DSS-treated Taconic mice developed symptoms associated with a sepsis-like disease, characterized by hypothermia, and increased mortality compared to Taconic mice treated with only DSS. Furthermore, AVNM attenuated DSS-induced colitis symptoms in Taconic mice (Supplementary Fig. 3a, b and data not shown). In contrast, disease in AVNM- plus DSS-treated Jax mice appeared to develop similarly to that in Jax mice treated with only DSS (Supplementary Fig. 3c–e and data not shown). Thus, it seems that SPF mice from different facilities show distinct disease symptoms in response to treatment with AVNM plus DSS.

**Antibiotic-induced expansion of drug-resistant E. coli**

Several nonexclusive models could explain how alterations in the microbiota can trigger disease. For example, antibiotic treatment could eliminate members of the microbiota that elicit protective host responses24. In addition, antibiotics could result in the overgrowth of AVNM-resistant pathogens. Consistent with the latter possibility, after 7 d of AVNM treatment, colony-born wild-type mice harbored an expanded population of AVNM-resistant bacteria along the intestinal tract (Fig. 2a). We observed a similar expansion of an AVNM-resistant population in Taconic mice but no detectable levels of culturable AVNM-resistant bacteria in Jax mice (Supplementary Fig. 3). Furthermore, we found that the AVNM-resistant bacteria colonized extraintestinal tissues of colony-born wild-type mice treated with AVNM plus DSS (Fig. 2b, c and Supplementary Fig. 2). Thus, sepsis-like disease correlated with the intestinal expansion and extraintestinal colonization of AVNM-resistant bacteria.

We obtained an AVNM-resistant isolate from our mice that we identified as an E. coli O21:H+ by16S rDNA sequencing, biochemical characterization and serotyping (Fig. 2d, e and data not shown). An expansion of intestinal Enterobacteriaceae species in response to...
antibiotic treatment is consistent with previous reports. AVNM-resistant E. coli was also recovered from the lung, liver, spleen and kidney of AVNM- plus DSS-treated colony-born mice (Fig. 2f and Supplementary Fig. 2e,f). To determine whether E. coli was the predominant systemic AVNM-resistant species in our mice, we performed 16S rDNA cloning and sequencing analyses of livers and spleens from AVNM- plus DSS-treated colony-born mice. Although this approach identified clones representing multiple taxa, Escherichia coli was the most abundant taxon identified, consistent with our culturing experiments. In addition, E. coli was the only taxon recovered from all tissues from AVNM- plus DSS-treated mice analyzed (data not shown). We also identified AVNM-resistant E. coli in the intestines of Taconic mice (Supplementary Fig. 3).

E. coli O21:H+ was present in the microbiota of unmanipulated mice (Fig. 2c) but was not abundant, suggesting it was not able to compete efficiently for intestinal colonization with other members of the flora in the absence of antibiotics. Consistent with this hypothesis, we found that 1 week after cessation of AVNM treatment the aberrant expansion of E. coli O21:H+ was suppressed (Supplementary Fig. 4a), and such mice no longer developed sepsis in response to DSS (Supplementary Fig. 4b,c).

As streptomycin-treated mice did not show rapid sepsis-like death in response to DSS (Fig. 1b), we hypothesized that our AVNM-resistant E. coli isolate is sensitive to streptomycin. Indeed, we found that our E. coli O21:H+ isolate is sensitive to streptomycin and is eliminated from mice by streptomycin treatment (Fig. 2d). Taken together, our data clearly demonstrate that the sensitivity of mice to AVNM plus DSS and development of sepsis is associated with intestinal overgrowth and extraintestinal colonization of AVNM-resistant E. coli O21:H+. 

Figure 2 Expansion and extraintestinal colonization of a multidrug-resistant E. coli in response to intestinal injury in dysbiotic mice. (a) Levels of AVNM-resistant bacteria in the intestinal tract of AVNM-treated and water-treated wild-type male and female littersmates 7 d after treatment initiation. *P < 0.05 by Student's t test and (n = 4) for both conditions. Error bars represent s.d. (b,c) Levels of AVNM-resistant bacteria in the lung (b) and liver (c) of AVNM-treated and water-treated wild-type male and female littersmates 3 d after DSS treatment initiation. *P < 0.05 by Student's t test and (n = 4) for both conditions. Error bars represent s.d. (d) Representative images of disc diffusion assays and quantification of the zone of inhibition to determine the susceptibility of an E. coli O21:H+ isolate to AVNM or streptomycin. E. coli K12 was used as a control. Error bars indicate s.d. ND, not detected. (e) Intestinal amounts of total AVNM-resistant bacteria and AVNM-resistant E. coli O21:H+ in AVNM-treated wild-type male and female mice (n = 4) compared to littersmates that received a water control (n = 4). SI, small intestine. Error bars represent s.d. (f) Lung and liver amounts of total AVNM-resistant bacteria and AVNM-resistant E. coli O21:H+ in AVNM-plus DSS-treated wild-type male and female mice (n = 4) compared to littersmates that received DSS only (n = 4). Error bars represent s.d. (g) Intestinal amounts of AVNM-resistant E. coli and streptomycin-resistant E. coli in AVNM- or streptomycin-treated wild-type male and female mice compared to wild-type littersmates that received a water control. Unmolested mice (n = 4); AVNM-treated mice (n = 4); streptomycin-treated mice (n = 4). Error bars represent s.d. (h) Intestinal amounts of AVNM-resistant E. coli in single-housed female Jax mice and Jax mice co-housed with female colony-born (CB) mice after 7 d of AVNM treatment. Jax single-housed (n = 3); Jax co-housed (n = 3); CB co-housed (n = 3). Error bars represent s.d. (i) Temperature and survival of AVNM- plus DSS-treated female single-housed Jax mice and Jax mice co-housed with female colony-born mice. For survival, P = 0.0236 by log-rank analysis. Data represent two combined experiments; Jax single-housed (n = 11); Jax co-housed (n = 11); CB co-housed (n = 11); DSS only (n = 5). For a-c, e and f, red horizontal dashed line represents the limit of detection, and black filled circles represent individual mice below the limit of detection. ND, not detected.
Because Jax mice do not seem to be colonized with AVNM-resistant E. coli O21:H+, we could use them to determine whether this bacterium and its associated disease phenotype are transmissible. We housed Jax mice with colony-born mice and treated them with AVNM for 7 d, after which the Jax mice showed AVNM-resistant E. coli colonization levels comparable to those in the colony-born mice. In contrast, single-housed Jax mice had no detectable AVNM-resistant E. coli (Fig. 2h). Upon induction of intestinal injury by DSS, AVNM-treated Jax mice housed with colony-born mice showed symptoms of sepsis-like disease and mortality, similar to colony-born mice, and unlike single-housed Jax mice (Fig. 2i). These results indicate that E. coli O21:H+ and its associated sepsis phenotype are transmissible.

**Systemic E. coli infection is sufficient to induce sepsis**

To determine whether our E. coli O21:H+ isolate is sufficient to cause sepsis-like disease in wild-type mice, we orally infected AVNM-treated Jax mice with E. coli O21:H+. Oral administration of E. coli O21:H+ to AVNM-treated Jax mice resulted in high levels of intestinal colonization (Supplementary Fig. 4d). Furthermore, AVNM- plus DSS-treated, E. coli O21:H+-colonized Jax mice developed hypothermia that was associated with reduced colitis symptoms compared to non-antibiotic plus DSS-treated Jax mice that received an oral PBS challenge (Supplementary Fig. 4e).

To determine whether the E. coli O21:H+ isolate is sufficient to cause disease in wild-type mice with a normal (non-antibiotic-treated) microbiota, we intravenously injected normal colony-born and Jax C57BL/6 mice with live or dead E. coli O21:H+ and monitored disease. Mice were highly susceptible to a systemic challenge of live but not heat-killed bacteria, as indicated by increased mortality (Fig. 3a). In addition, systemic live E. coli O21:H+ infection was sufficient to recapitulate the specific pathology associated with AVNM plus DSS treatment (Fig. 3b,c). Furthermore, live E. coli-infected mice showed tissue colonization patterns and levels similar to those observed in AVNM- plus DSS-treated wild-type mice (Figs. 1i, and 3d and Supplementary Fig. 2b,c). Thus, in fulfillment of Koch’s postulates, a live systemic infection with E. coli O21:H+ is sufficient to recapitulate the mortality and morbidity associated with AVNM- plus DSS-induced sepsis.

**E. coli O21:H+ harbors virulence factors**

E. coli O21 strains have been isolated from patients with extraintestinal infections including bacteremia and sepsis. We therefore hypothesized that the E. coli pathobiont encodes virulence factors mediating its pathogenicity in wild-type mice. Consistent with this hypothesis, disease was attenuated in mice intravenously infected with a nonpathogenic E. coli K12 strain (MG1655) as compared to our E. coli O21:H+ isolate (Fig. 3e,f), suggesting that detection of viable, nonpathogenic strains of E. coli by the immune system is not sufficient to trigger sepsis-like disease in wild-type mice.

To identify virulence-associated determinants, we sequenced the genome of our E. coli O21:H+ isolate and aligned the reads to reference E. coli genomes (Supplementary Table 1). The fliC gene, encoding flagellin (H antigen), was most similar to that of H21 isolates (Supplementary Fig. 5a). In addition, alignment to the E. coli O157:H7 strain Sakai reference genome revealed that our O21:H+ isolate harbors a cluster of genes with similarity to the ETT2 type III secretion system (Supplementary Fig. 5b). Many septicmic
Naip5-Nlrc4 inflammasome mediates AVNM/DSS-induced sepsis

As pathogenic E. coli were previously reported to activate inflammasomes containing Naip and Nlrc4 proteins, we hypothesized that the Naip5-Nlrc4 inflammasome might mediate disease in our sepsis model. Indeed, we found AVNM- plus DSS-induced disease progression was highly attenuated in Naip5−/− mice (Fig. 4a-e). Notably, AVNM-treated Naip5−/− mice showed similar levels of AVNM-resistant E. coli overgrowth in the intestinal tract as wild-type fostermates (wild-type pups that nursed on the mother of mutant pups) before DSS treatment, implying that inflammasome deficiency did not exert its protective effects by modulating bacterial growth (Fig. 4f and Supplementary Fig. 6a,b). Moreover, the organs of AVNM-plus DSS-treated Naip5−/− mice were colonized with similar levels of AVNM-resistant E. coli as wild-type mice (Fig. 4g and Supplementary Fig. 6c,d). These data indicate that the absence of Naip5-Nlrc4 function protects AVNM- plus DSS-treated mice from developing sepsis at a step after extraintestinal dissemination of E. coli O21:H+. As Naip5−/− mice eventually succumb to AVNM- plus DSS-induced sepsis, it is likely that additional host factors also contribute to disease.

The Naip5-Nlrc4 inflammasome generally detects flagellin translocated to the host cell cytosol via type III or IV secretion systems. Because E. coli O21:H+ encodes a type III secretion system and a functional flagellin (FlhC) (Fig. 5a and Supplementary Fig. 5) similar to that of other pathogenic E. coli species, we hypothesized that the cytosolic presence of flagellin from this pathobiont can activate the Naip5-Nlrc4 inflammasome. We tested this hypothesis using a previously described retroviral 'lethality' assay in which flagellin is expressed from a retroviral promoter directly in host cells, allowing for analysis of the effects of flagellin in the absence of other bacterial factors. We retrovirally transduced wild-type, Naip5−/− and Naip5−/− bone marrow-derived macrophages with FlhC-IRES-GFP, control GFP or control Legionella pneumophila flagellin (flaA-IRES-GFP). Activation of Naip5-Nlrc4 by flagellin results in cell death, as indicated by the loss of GFP-positive cells. As previously reported, cells expressing L. pneumophila flaA-IRES-GFP could only be recovered from Naip5−/− macrophages (Fig. 5b,c). Detection of FlaA by Naip6 probably accounts for the residual responsiveness of Naip5−/− cells. However, we were able to recover cells expressing E. coli O21:H+ flaC-IRES-GFP from both Naip4−/− and Naip5−/− macrophages but not wild-type macrophages (Fig. 5b,c). These data suggest that cytosolic flagellin derived from E. coli O21:H+ activates the Naip5-Nlrc4 inflammasome and triggers host cell death.

Naip5-Nlrc4 inflammasome reduces host tolerance of E. coli

To address Koch’s postulates, we infected wild-type and Naip4−/−; Naip5−/− mice intravenously with E. coli O21:H+. Systemic E. coli O21:H+ infection was significantly less lethal in the absence of...
Naip5-Nlrc4 function ($P = 0.0001$) (Fig. 6a and Supplementary Fig. 7a,b). The absence of Naip5-Nlrc4 function also alleviated the pathologies associated with E. coli O21:H+ infection, including small intestinal bleeding, multiple organ damage and hypothermia (Fig. 6b-d). Furthermore, E. coli O21:H+ infection was attenuated in Casp1$^{-/-}$ (caspase 1 deficient) and Il1b$^{-/-}$ mice (Fig. 6e and Supplementary Fig. 7c,d). Additionally, systemic administration of IL-1 receptor–specific antibody protected mice from AVNM- plus DSS-induced disease (Fig. 6f). Thus, the absence of IL-1β is sufficient to protect mice from a systemic inflammatory response induced by a systemic E. coli O21:H+ infection (Fig. 6e and Supplementary Fig. 7d). Taken together, our data clearly indicate that Naip5-Nlrc4 inflammasome signaling through IL-1β in response to a systemic E. coli O21:H+ infection leads to sepsis.

The greater susceptibility of wild-type (as compared to Nlrc4$^{-/-}$; Naip5$^{-/-}$) mice to E. coli O21:H+ infection was not associated with higher levels of extraintestinal bacterial colonization (Fig. 6g). In fact, colonization levels were similar in both genotypes.
Thus, we conclude that mice lacking Naip5-Nlrc4 fare better in response to a systemic E. coli O21:H+ infection because they show increased tolerance of the infection rather than because of negative effects on pathogen fitness.

**DISCUSSION**

We describe a mouse model of sepsis triggered by dysbiosis and intestinal injury. This model resembles the clinically significant sepsis that is a major problem in human patients undergoing combination therapies involving antibiotics and cytotoxic treatments that damage the gut epithelium. Consistent with the defining features of sepsis, we found that disease in AVNM- plus DSS-treated mice is characterized by systemic dissemination of bacteria, multiple organ damage and systemic responses such as hypothermia. A key conclusion of our study is that sepsis in our AVNM- plus DSS-treated mice is due to inflammasome activation by a specific multidrug-resistant E. coli O21:H+ pathobiont. This pathobiont exists in the normal unmanipulated flora but expands dramatically in the gut upon antibiotic treatment and reaches systemic sites following DSS-induced intestinal injury (Supplementary Fig. 7e). Systemic inoculation of this pathobiont is sufficient to induce sepsis-like disease.

Similar to our findings, a previous report demonstrated that antibiotic pretreatment rendered mice more susceptible (increased mortality) to DSS treatment. However, this report proposed that disease in AVNM- plus DSS-treated mice is not associated with systemic spread of bacteria but is instead due to a failure to receive appropriate Toll-like receptor–dependent signals needed to repair gut epithelial injury.

Consistent with this interpretation, it was found that disease could be alleviated by the oral administration of lipopolysaccharide (LPS), suggesting that Toll-like receptor signaling has a key protective role. In contrast, in our model, mice given AVNM plus DSS showed symptoms of sepsis, not colitis, and oral administration of LPS was not sufficient to prevent sepsis (Supplementary Fig. 2d).

Indeed, dysbiosis in the AVNM-plus-DSS model may both reduce the levels of beneficial microbes and result in an expansion of pathobionts. The apparent discrepancy between our work and the prior study may be explained by differences in microbial flora in the mice, as well as by methodological differences. For example, the previous study administered drinking water supplemented with 2% DSS to mice for a total of 7 d, which produces milder damage that mice are able to repair. In our model, we used a continuous dose (until the end of the experiment) of 5% DSS. Nevertheless, as systemic infection of normal mice with E. coli O21:H+ is sufficient to recapitulate the AVNM-plus-DSS disease model, we favor the hypothesis that pathology in our mice is driven primarily by an E. coli pathobiont.

Previous studies have demonstrated a protective role for the inflammasome in mediating pathogen resistance and in inducing tissue repair. Our study demonstrates that excessive or systemic inflammasome-mediated recognition of a bacterium can lead to pathology and death. In our model, inflammasome activation results in IL-1β-driven immunopathology that the host cannot tolerate (Supplementary Fig. 7). The dependence on IL-1β distinguishes our sepsis model from others, including LPS endotoxemia (Supplementary Fig. 7). Blockade of IL-1 signaling has long been considered a potential therapeutic treatment for human sepsis, but it has met with little success in clinical trials. This is probably because sepsis is a complex disease involving diverse microbes and host immune pathways. Our results highlight the importance of experimentally defining the relevant pathogenic pathways for different microbial causes of sepsis and identifying the causative agents of sepsis in humans.

Antibiotic-resistant E. coli present a challenge to antimicrobial medical interventions. Our finding that mice lacking inflammasome components show increased tolerance of systemic E. coli infection may have key therapeutic implications. Tolerance (not to be confused with immunological tolerance) is a defense strategy that allows a host to endure an infection without influencing microbial load. Tolerance contrasts with resistance, which protects a host by decreasing pathogen burden. In contrast to antimicrobials (which work to decrease pathogen burden), therapeutic interventions to increase tolerance could protect the host without imposing negative effects on pathogen fitness. A key benefit of therapeutic modulation of host tolerance pathways is that pathogens are not predicted to evolve antagonistic traits to such therapeutics. Thus, we propose that the inflammasome may be an alternative therapeutic target for patients showing pathology from antibiotic-resistant pathobionts.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

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**AUTHOR CONTRIBUTIONS**

J.S.A. and R.E.V. conceived of the study, designed experiments and wrote the manuscript. J.S.A. directed the study and performed all experiments. N.J.T. did all mouse intravenous injections and performed the retroviral lethality assay.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Naip5−/− mice were generated as described previously44, Nlrc4−/− mice were from S. Mariathasan and V. Dixit28. We crossed Naip5−/− and Nlrc4−/− mice to each other to generate double-knockout mice. Caspt−/− mice were a gift from A. Van der Velden and M. Starnbach28. Il1b−/− mice were from the Zychlinsky lab at Max Planck Institute. Gene-targeted mice were on a C57BL/6 background. Wild-type C57BL/6 mice were originally obtained from the Jackson Laboratories, but all colony-born mice used were bred in our mouse facility for at least 10 generations. All mice were specific pathogen free, maintained under a 12-h light-dark cycle (7 a.m. to 7 p.m.) and given a standard chow diet (Harlan irradiated laboratory animal diet) ad libitum. For experiments involving littermate mice, we crossed Naip5+/−;Nlrc4+/− mice to generate Naip5−/−;Nlrc4−/− and Naip5+/−;Nlrc4−/− littersmates. For fostermate mice, we placed wild-type pups with the mutant litter within 48 h of birth to nurse on the Naip5−/−;Nlrc4−/− mother. Pups were weaned at 21 d of age. Mouse experiments were approved by the University of California–Berkeley Animal Care and Use Committee.

Survival assays. We used time to moribund for all survival assays. We identified moribund mice as those mice showing ataxia severe enough such that the mice could not recover (unable to move when gently touched or experiencing trouble self-correcting when placed on their sides). Moribund mice were euthanized in compliance with the Animal Care and Use Committee at the University of California–Berkeley.

DSS colitis model. We transferred 6-week-old littermates to new cages and gave them drinking water supplemented with 5% DSS (w/v) (molecular weight 36,000–50,000 Da; MP Biomedicals) continuously for the duration of the experiment. We measured disease severity by monitoring survival (time to moribund) and weight daily, and colon length was measured at 6 d after DSS treatment initiation.

Antibiotic/DSS sepsis model. At 5 weeks of age, we transferred littermates or fostermates to new cages and gave them drinking water supplemented with a combination of ampicillin (1 g L−1), neomycin (1 g L−1), metronidazole (1 g L−1) and vancomycin (0.5 g L−1), ampicillin only (1 g L−1) or streptomycin only (2 g L−1) for 7–10 d (Sigma-Aldrich). After this initial antibiotic treatment, we gave mice drinking water supplemented with the appropriate antibiotic at the above mentioned concentration plus 5% DSS (w/v) continuously over the course of the experiment. We monitored mice for signs of disease including weight loss and colonic shortening as described above, rectal temperature and serum analysis of organ damage as well as bacterial extraintestinal tissue colonization. For administration of LPS plus AVNM plus DSS, we gave mice drinking water supplemented with AVNM plus 5% DSS and 10 µg ml−1 LPS O55:B5 (Sigma) after an initial 10 d AVNM treatment.

E. coli in vivo infections. We grew E. coli overnight in LB medium shaking at 37 °C. We transferred age-matched female mice to new cages and injected them intravenously with 5 × 107, 7.5 × 107 or 1 × 108 live bacteria in PBS and gave them food and water ad libitum. We monitored body temperature and survival (time to moribund). For infections with dead bacteria, we prepared inoculums at the appropriate concentration in PBS and incubated at 65 °C for 30 min to heat-kill the bacteria. For E. coli K12 infections, we grew E. coli overnight in LB medium shaking at 37 °C. We transferred age-matched female mice to new cages and injected them intravenously with 5 × 108 bacteria in PBS and gave them food and water ad libitum.

Body temperature. We monitored body temperature using a rectal probe and microtherma thermometer (Braintree Scientific). We lubricated the probe with a water-based lubricant (Astroglide) before use.

Serum analysis. We euthanized mice and obtained blood by cardiac puncture at the indicated time points. We then aliquoted blood into BD serum separator tubes, incubated at room temperature for 20 min and centrifuged at 2.6 r.p.m. for 20 min. We stored serum at –80 °C until analysis. BUN, CPK, AST and ALT measurements were done by IDEXX Laboratories.

Bacterial culturing. For compositional analysis of the intestinal tract and extraintestinal tissues, we harvested whole tissues and homogenized them using a Polytron PT2100 homogenizer at 17,000 r.p.m. (Kinematica) in sterile thiglycolate medium. We then serially diluted the homogenate and plated on LB agar and Schaedler agar supplemented with 5% defibrinated sheep blood plates and grew at 37 °C aerobically and anaerobically for 24 h. We counted bacterial colonies and then separated on the basis of colony appearance and performed colony 16S rDNA PCR and sequencing (see below).

For cultivation of Enterobacteriales species, we harvested and homogenized intestinal and extraintestinal tissues from mice treated with AVNM plus DSS as described above. We then serially diluted the homogenates and plated them on bacterial media that support the growth of Enterobacteriales, including LB, Macconkey and EMB. We then incubated the plates aerobically at 37 °C for 24 h, after which we counted colonies, classified on the basis of colony appearance and subjected them to 16S rDNA colony PCR and sequencing (see below). We then tested isolates for antibiotic susceptibility (see below).

For CFU analysis of E. coli in mice infected with E. coli O21:H+, we harvested and homogenized intestinal and extraintestinal organs in sterile PBS, serially diluted and plated the homogenates on EMB or LB plates containing AVNM and incubated them at 37 °C for 24 h.

Colony PCR. We resuspended colonies in sterile PBS and boiled them for 10 min at 100 °C. We used this directly for PCR analysis with the universal bacterial primers 27F (5′-AGAGTTTGTATCCTTGCTGAG-3′) and 1492R (5′-GGTTACCTTGTTAAGCTT-3′)46. We performed PCR on a Bio-Rad iCycler with the following conditions: 95 °C (5 min), 30 cycles of 95 °C (30 s), annealing at 51 °C (1 min), 72 °C (2 min) and final extension at 72 °C (10 min). Reactions were then subjected to a PCR cleanup using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the 1492R primer (EliL Biopharm). We classified the sequences using the Michigan State University Ribosomal Database Project classifier function (http://rdp.cme.msu.edu/).

Antibiotic susceptibility assays. We determined E. coli O21:H+ susceptibility to AVNM and streptomycin using a disc diffusion assay. We inoculated liquid cultures with E. coli and grew them at 37 °C with shaking overnight. We placed aliquots of cultures onto LB agar plates and 6-mm Whatman discs loaded with the appropriate antibiotic onto the agar. We incubated plates overnight at 37 °C and measured the zone of bacterial growth inhibition. Antibiotic susceptibility profiling of the isolate was also performed by the Clinical Microbiology Laboratory at Kaiser Permanente. E. coli DH5α was used as a control.

16S rDNA quantitative PCR analyses. We gave mice regular drinking water or drinking water supplemented with AVNM for 7–10 d. We then harvested intestinal tissues, snap froze them in liquid nitrogen and stored them at −80 °C before analysis. We extracted DNA using the QiaGen Stool Kit per manufacturer’s instructions with a bead-beating step. Specifically, we put whole tissues into Lysing Matrix E tubes (MP Biomedicals) with 1.4 ml of buffer ASL continuously for the duration of the experiment. We extracted DNA using the QIAamp DNA Micro kit (Qiagen) and grew at 37 °C aerobically and anaerobically for 24 h. We counted bacterial colonies and then separated on the basis of colony appearance and performed colony 16S rDNA PCR and sequencing (see below).

E. coli O21:H+ was selected as a control.

16S rDNA sequencing. We amplified 16S rDNA sequences from the first PCR reaction using the primers 27F and 1492R and sequenced the amplicons on either a 3730xl (Applied Biosystems) or an Illumina MiSeq. The Illumina reads were demultiplexed and quality trimmed using Cutadapt47 and quality filtered using Trimmomatic48. Illumina reads were then aligned to the UCSC human reference assembly GRCh38 (hg38) using BWA49, and then the aligned reads were used to quantify gene expression with the program STAR50. Bacterial ribosomal RNA reads were classified using the Ribosomal Database Project classifier function51.

Accession numbers. The raw Illumina sequencing reads were deposited in the NCBI Sequence Read Archive under the following accession numbers: SRP111718 and SRP111719.
Motility assay. We inoculated single colonies of bacteria at a single point on soft motility LB agar (0.35%) and incubated overnight at 37 °C. As controls, we used S. Typhimurium strain LT2 and the flagellin mutant S. Typhimurium LT2 ΔfljB (a gift from A. Van der Velden and M. Starmbach).

Retroviral constructs and transductions. We produced retroviral constructs and executed transductions as previously described. Briefly, we generated retroviral particles by the transient transfection of Phoenix-Eco packaging cells with MSCV2.2-based retroviral vectors. We cultured bone marrow–derived cells (1 × 10⁶) for 48 h in a six-well Falcon brand non–tissue-culture–treated plate in medium containing macrophage colony-stimulating factor, and then we transduced cells with 2 ml of retrovirus-containing packaging cell supernatant. 24 h after the initial infection, we infected bone marrow–derived cells once again with 2 ml of retrovirus-containing packaging cell supernatant. We analyzed macrophages 4 d after transduction and analyzed for GFP expression on a Beckman Coulter FC-500 flow cytometer. We analyzed more than 25,000 cells per sample. We cloned the E. coli O21:H+ flaC gene using the primers flaC Xhol (5′-AAAACTGAGGCGCAGCATGGCAAGTCATTAATAC-3′) and flaC NotI (5′-TGGTTCCGGCGGCTTAAACCCTGCAAGA-3′).

LPS injections. We injected age-matched female wild-type and Il1b−/− mice intravenously with 5 mg per kg body weight LPS O55:5B (Sigma) and gave them food and water ad libitum. We monitored survival (time to moribund).

E. coli O21:H+ serotyping. Serotyping was done at the E. coli reference center, Pennsylvania State University.

Experiments with commercial vendor mice. For disease analyses, we kept Taconic and Jax mice in autoclaved isolator cages and gave them sterile-filtered water and food ad libitum. We inoculated single colonies of bacteria at a single point on soft motility LB agar (0.35%) and incubated overnight at 37 °C. As controls, we used S. Typhimurium strain LT2 and the flagellin mutant S. Typhimurium LT2 ΔfljB (a gift from A. Van der Velden and M. Starmbach).

Horizontal transfer experiments. We obtained 5-week-old C57BL/6 male mice from Jackson Laboratories (Sacramento, CA) and kept them single-housed in isolator cages or co-housed in isolator cages with age- and sex-matched C57BL/6 mice bred in our colony. We then treated mice with AVNM ad libitum for 7 d, after which we harvested and homogenized intestinal tissues and plated them on LB agar plates supplemented with AVNM to determine the levels of AVNM-resistant bacteria colonization. To analyze disease severity (temperature, body weight and time to moribund), we gave mice drinking water supplemented with AVNM plus 5% DSS after the initial 7-d course of AVNM treatment. We compared disease severity to single-housed controls.

Oral infection of commercially purchased mice. We obtained 5-week-old C57BL/6 female mice from Jackson Laboratory (Sacramento, CA), kept them in isolator cages and treated them with AVNM ad libitum for 7 d. On 4, 5 and 6 d, we gavaged mice with 200 µl of an overnight culture of E. coli O21:H+, washed the bacteria and resuspended them to 2.5 × 10⁶ in sterile PBS. We gavaged control mice with 200 µl of sterile PBS. On 7 d, we either dissected mice for CFU analysis or gave them drinking water supplemented with AVNM+5% DSS and monitored temperature and weight.

Whole-genome sequencing of E. coli O21:H+. Library preparation, Illumina HiSeq 2000 SE50 sequencing and mapping were done by the Tufts Core Facility (TUCF) at Tufts University. For library preparation, we grew E. coli overnight in LB medium shaking at 37 °C and isolated genomic DNA using the Qiagen DNeasy kit and submitted DNA to TUCF for library preparation and sequencing. Reads were aligned to eleven publicly available E. coli whole-genome sequences (E. coli HS CP008002, E. coli E24377 ATEC NC_0098801, E. coli 53638 EIEC AAAB0000000, E. coli 042 EAEC FN54766, E. coli 0127:H6 EPEC NC_011601, E. coli IA139 ExPEC NC_011750, E. coli 858 ExPEC NC_011742, E. coli O157:H7 EHEC NC_002695, E. coli APEC O1 CP000468, E. coli UT89 EXPEC CP000243, E. coli CFT073 ExPEC AE014075) using the CLC Genomics workbench software. Annotation of the resulting consensus sequences was done using RAST (rapid annotations using subsystem technology) (http://rast.nmpdr.org/).

Phylogenetic analysis of flaC. We performed analysis using Phylogeny.fr (http://www.phylogeny.fr/) one-click mode and the publicly available E. coli flaC gene sequences (E. coli strain B2F1 DQ459008.1, E. coli strain U111–44 AY250004.1, E. coli O111:H– str. 11128 AP010960.1, E. coli O113:H21 DQ862122.1, E. coli ABU 83972 CP001671.1, E. coli VTH–15 GQ423574.1, E. coli CFT073 AE014075.1, E. coli 042 FN54766. E. coli O55:H7 str. CB961 CP001846.1, E. coli IHE304 CP001969.1, E. coli O157:H7 str. Sakai NC_002695, E. coli HS CP000802, E. coli IA139 NC_011750, E. coli UT89 CP000243, E. coli S88 NC_011742, E. coli UMN026 C194261.3, E. coli SM5–3 NC_010498, E. coli O127:H6 str. E2348 NC_011601, E. coli APEC O1 NC_008563) and Legionella pneumophila flaA gene sequence as the outgroup NC_006368.1.

IL-1R-specific antibody experiments. We gave colony-born wild-type mice drinking water supplemented with AVNM for 7 d. We then gave mice drinking water supplemented with AVNM and 5% DSS to induce gut injury. At 1, 24 and 48 h after DSS administration, we administered 100 µg (100 µl total volume) of IL-1R-specific antibody (Amen) intraperitoneally or control IgG antibody (Sigma M8642 resuspended to 1 µg/µl in sterile PBS) to mice and monitored survival (time to moribund) over the course of the disease.

Statistics. We analyzed Kaplan-Meier plots using log-rank analysis. All other data are expressed as mean ± s.d. and were analyzed using a Student’s t test or Mann-Whitney test. We considered a P value less than 0.05 to be significant.