Toll-Like Receptor 4 Contributes to Colitis Development but Not to Host Defense during *Citrobacter rodentium* Infection in Mice

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Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are noninvasive bacterial pathogens that infect their hosts' intestinal epithelium, causing severe diarrheal disease. These infections also cause intestinal inflammation, although the mechanisms underlying the inflammatory response, as well as its potential role in host defense, are unclear. Since these bacteria are gram-negative, Toll-like receptor 4 (TLR4), the innate receptor for bacterial lipopolysaccharide may contribute to the host response; however, the role of TLR4 in the gastrointestinal tract is poorly understood, and its impact has yet to be tested against this family of enteric bacterial pathogens. Since EPEC and EHEC are human specific, we infected mice with *Citrobacter rodentium*, a mouse-adapted attaching and effacing (A/E) bacterium that infects colonic epithelial cells, causing colitis and epithelial hyperplasia, using a similar array of virulence proteins as EPEC and EHEC. We demonstrated that *C. rodentium* activates TLR4 and rapidly induced NF- κ B nuclear translocation in host cells in a partially TLR4-dependent manner. Infection of TLR4-deficient mice revealed that TLR4-dependent responses mediate much of the inflammation and tissue pathology seen during infection, including the induction of the chemokines MIP-2 and MCP-1, as well as the recruitment of macrophages and neutrophils into the infected intestine. Surprisingly, spread of *C. rodentium* through the colon was delayed in TLR4-deficient mice, whereas the duration of the infection was unaffected, indicating that TLR4-mediated responses against this A/E pathogen are not host protective and are ultimately maladaptive to the host, contributing to both the morbidity and the pathology seen during infection.

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are among the most important bacterial causes of diarrheal disease worldwide (15, 35). EPEC infection leads to the deaths of hundreds of thousands of children each year in developing countries (35, 56), while EHEC, the causal agent of "hamburger disease," is a growing health threat in North America and Europe, causing sporadic diarrheal outbreaks and deaths (21, 29). Belonging to a family of related gram-negative pathogenic bacteria that includes the mouse pathogen *Citrobacter rodentium* (30), these bacteria are noninvasive, infecting their hosts by attaching to the surface of intestinal epithelial cells, effacing the epithelial microvilli and producing pedestal-like structures (3, 4, 12, 30). The formation of attaching and effacing (A/E) lesions correlates with the ability of these microbes to cause diarrheal disease (1, 14); thus, much work has been done to characterize the virulence mechanisms used by A/E bacteria. These studies have revealed a series of elegant strategies used by A/E bacteria to infect their hosts (16, 21, 56). Despite these advances, it is now becoming clear that the host also plays an important role in causing the tissue pathology and diarrheal disease seen during infection by these intestinal lumen-dwelling pathogens (10).

Recent reports indicate that EPEC and EHEC infections

induce significant intestinal inflammation, with fecal leukocytes more frequently seen in patients infected with EHEC than in those infected with other enteric pathogens, including *Salmonella* and *Shigella* spp. (28, 35, 56). Similarly, studies examining colonic biopsies from patients infected with A/E pathogens reveal inflammatory cell infiltration into the intestinal lamina propria and crypts of infected tissues (19, 27, 28). Although the mechanisms involved in triggering this inflammatory response are unclear, they likely require the activation of the host's innate immune system. Innate immunity in multicellular organisms involves a group of pattern recognition receptors that detect and respond to conserved molecules expressed by pathogenic microorganisms (17). Toll-like receptors (TLRs) are a family of these innate receptors (34, 51), the best described of which is TLR4, which in association with the LBP (lipopolysaccharide [LPS]-binding protein), CD14, and MD-2 proteins, specifically triggers an inflammatory response to LPS derived from gram-negative bacteria (34). We and others have demonstrated that TLR4-dependent responses play a critical role in host defense against *Salmonella enterica* serovar Typhimurium, a gram-negative bacterium that causes a systemic disease in mice. TLR4 expression limited the spread of this microbe through the host's spleen, liver, and other internal organs (36, 57) and, similarly, TLR4 has proven important in providing protection against a variety of other gram-negative bacterial pathogens, including uropathogenic *E. coli* (47), as well as *Bordetella* and *Brucella* spp. (8, 32).

In contrast to the actions of TLR4 during systemic bacterial infections, the role of TLR4 in recognizing and responding to gram-negative bacterial infections of the gastrointestinal (GI)

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tract has not been examined. In part this reflects the lack of suitable models, since most clinically important enteric bacterial pathogens, including EPEC and EHEC, do not cause a relevant gastroenteritis in laboratory animal species (30). As a result, it is unclear what aspects of innate immunity are activated during A/E bacterial infections of the GI tract or what role they play in intestinal host defense. In fact, although innate responses usually trigger an inflammatory response that is toxic to pathogens, inflammation can also disrupt mucosal integrity (45), weakening physical barriers that protect the intestine from enteric pathogens. Several *in vitro* and *in vivo* studies have indicated that innate inflammatory responses may actually aid bacterial pathogens such as *Shigella flexneri* colonize their host's intestines by disrupting the intestinal epithelium that normally prevents these microbes from invading their hosts (37, 44, 45). Since EPEC and EHEC utilize infectious strategies different from *Shigella*, the impact of innate immunity on intestinal host defense against A/E bacterial pathogens remains an unresolved, yet important issue.

To investigate the role of TLR4 in an A/E bacterial infection, we chose the *C. rodentium* mouse model. An excellent paradigm for EPEC and EHEC infections, *C. rodentium* infects the colonic epithelium of mice, triggering colitis and mucosal hyperplasia (3, 4, 12, 30). *C. rodentium* colonizes epithelial cells using a homologous array of virulence proteins to those expressed by EPEC and EHEC and was recently used to identify new translocated effectors shared by these pathogens (13). We and others have also begun to assess what roles T and B lymphocytes (7, 48, 55), as well as the cytokines interleukin-12 (IL-12), gamma interferon (49), and tumor necrosis factor alpha (23), play in host defense and the inflammatory response to *C. rodentium* infection. Thus far, however, innate receptors have not been assessed in this model. In the present study, we compared the susceptibility of wild-type (WT) mice and TLR4-deficient mice to *C. rodentium*, studying their inflammatory responses, as well as the disease symptoms and pathology they suffered during infection. We found that TLR4 deficiency significantly reduced tissue pathology and inflammatory cell recruitment into the infected gut and, surprisingly, the absence of TLR4 also delayed the spread of *C. rodentium* through the mouse colon, suggesting that the innate inflammatory response may facilitate the ability of *C. rodentium* to colonize the distal colon. These studies thus introduce the novel concept that TLR4-dependent innate responses in the GI tract are not host protective but instead may prove maladaptive during infection by A/E bacteria, contributing to the resulting disease and facilitating the ability of these pathogens to infect and spread through their hosts' GI tracts.

MATERIALS AND METHODS

Mice. Six- to eight-week-old wild-type (WT), TLR4-expressing mice (C57BL/10J and C3H/HeOuJ) were obtained from Jackson Laboratories (Bar Harbor, Maine). Several TLR4-deficient strains were used in the present study, including the C3H/HeJ and C57BL/10ScNJ strains that were also obtained from Jackson, while C57BL/10ScNcr mice were purchased from the National Cancer Institute (Frederick, MD). Mice were kept in sterilized, filter-topped cages; handled in tissue culture hoods; and fed autoclaved food and water under specific-pathogen-free conditions at our animal facilities. Sentinel animals were routinely tested for common pathogens. The protocols used were approved by the University of British Columbia's Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

Bacterial strains and infection of mice. Mice were orally inoculated with wild-type *C. rodentium* (formerly *C. freundii* biotype 4280), strain DBS100 (46). For inoculations, bacteria were grown overnight in Luria broth. Mice were infected by oral gavage using 0.1 ml of Luria broth containing ca. 2.5×10^8 CFU of *C. rodentium*. To minimize differences between infections, both WT and TLR4-deficient mouse strains were infected with the same bacterial preparation. Mice were euthanized at various time points postinfection (p.i.), and tissues were prepared for histological analysis, viable bacterial counts, or RNA isolation as described below.

Survival and body weight measurement. To assess the systemic effects of *C. rodentium* infection on the host, the survival of infected mice and changes in their body weight were measured over the course of infection. Mice were monitored throughout the infection, and any that showed extreme distress or became moribund were euthanized. Mice were also weighed just prior to infection and at specified intervals until day 24 p.i. The survival and body weight data presented are from a representative experiment ($n = 3$). Body weight data are presented as the mean percentage of the starting weight of 10 mice at each time point, whereas survival data are presented as the percentage of the initial 10 mice still surviving at each time point.

Tissue and stool collection and colon weight measurement. Over the course of the infection, mice were euthanized and, after careful dissection, the first 4 cm of each colon, beginning at the anal verge, was collected. Fecal pellets were removed before the colonic tissue was weighed. Tissues were then placed in 10% neutral buffered formalin (Fisher) for histological analysis. For viable bacterial count studies, the colons and pellets were collected in phosphate-buffered saline (PBS; pH 7.4) and kept on ice before being processed for viable bacterial counts. For studies on stool water content, the pellets were weighed immediately (wet weight) and then allowed to dry overnight in a 37°C dry oven. The next day the dried stool was weighed and, by subtracting the dry stool weight from the wet stool weight and dividing the result by the original wet stool weight, we determined the original water content of the stool.

Bacterial counts. Colonic tissues (plus any fecal stool pellets) or mesenteric lymph nodes (MLNs) were homogenized at low speed using a Kinematica tissue homogenizer (Brinkmann). Homogenates were then serially diluted and plated onto MacConkey agar plates. Bacterial colonies were enumerated the following day. *C. rodentium* colonies were easily distinguished from commensal flora by their size and appearance (pink center with white rim), as previously described (46). The validity of this approach was verified by PCR analysis for LEE genes.

Crypt heights and histopathological analysis. Full thickness colonic tissues were fixed in 10% neutral buffered formalin. Sections (3 μ m) were cut and stained with hematoxylin and eosin. Photomicrographs were taken using a Nikon Eclipse E400 microscope. Crypt heights were measured by micrometry by an observer blinded to the experimental condition, with 10 measurements taken in the distal colon for each mouse. Only well-oriented crypts were measured. Similarly, colon sections were assessed for quantitative analysis of intestinal inflammation by a blinded observer, using a previously described scoring system (5). In brief, the system assessed submucosal edema, polymorphonuclear infiltration into the lamina propria, goblet cell depletion, and epithelial integrity. The combined pathological score ranged from 0 to 13 arbitrary units and covers the following levels of inflammation: 0 = intestine intact with no sign of inflammation; 1 to 2 = minimal signs of inflammation; 3 to 4 = slight inflammation; 5 to 8 = moderate inflammation; and 9 to 13 = profound inflammation.

TLR4 activation. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in a 5% CO₂ incubator in Dulbecco modified Eagle medium containing 10% cosmic calf serum (HyClone, Logan, UT). The day prior to transfection, HEK293 cells were plated in a 96-well dish at 5×10^4 per well. Transient transfection was performed by using Polyfect reagent (QIAGEN, Chatsworth, CA). Cells were transfected with 150 ng of NF- κ B reporter (endothelial leukocyte molecule-1 [ELAM-1] firefly luciferase construct), 15 ng of thymidine kinase *Renilla* luciferase vector (pRL-TK; Promega, Madison, WI) to control for transfection efficiency, 20 ng of EF6 hTLR4, 100 ng of hMD-2, and 10 ng of hCD14, or a hTLR8 vector. The empty vector pEF6 was used as a control and to normalize the DNA concentrations for all transfections to 250 ng per well. At 20 h after transfection, the cells were stimulated with 10% supernatant from an overnight culture of *C. rodentium* in LB broth, 10 ng of Ultrapure LPS (*Salmonella enterica* serovar Minnesota R595; List Biological Laboratories, Campbell, CA)/ml, or LB alone. Cells were lysed after 4 h by using passive lysis buffer, and the luciferase activity was quantified by using the dual-luciferase reporter assay system (Promega). Firefly luciferase units were divided by *Renilla* units to normalize for transfection efficiency between wells.

Generation and infection of bone marrow-derived macrophages and CMT-93 epithelial cells: immunofluorescence studies. Bone marrow was isolated from the femurs of C57BL/10ScNJ (TLR4-deficient) or C57BL/10J (WT) mice, cultured

for 7 days in DMEM (HyClone) supplemented with 20% fetal bovine serum (Invitrogen)—2 mM L-glutamine—1 mM sodium pyruvate—30% L cell-conditioned medium at 37°C in 5% CO₂. The bone marrow-derived macrophages (BMDM) were seeded in 24-well plates (10⁵ cells per well). The BMDM, as well as CMT-93 murine colonic epithelial cells (10⁶ cells per well), were infected with 10 µl of *C. rodentium* grown overnight in LB. At 1 to 4 h p.i., cells were fixed in 2.5% paraformaldehyde at 37°C for 10 min, followed by extensive washing in PBS. Fixed cells were then permeabilized in PBS containing 10% (vol/vol) goat serum and 0.3% Triton X-100 for 30 min at room temperature. Primary rat antibodies to TLR4/MD2 (MTS510 kindly provided by Kensuke Miyake) and a mouse monoclonal directed to the p65 subunit of NF-κB (Santa Cruz Biotechnology), as well as Alexa-conjugated secondary antibodies (all at a 1:300 dilution) were applied in PBS—0.3% Triton X-100—5% goat serum for 1 h at room temperature. DAPI (4',6'-diamidino-2-phenylindole; Sigma) was used at 1 µg/ml to stain both host cell and bacterial DNA. Coverslips were mounted in Mowiol mounting medium (Aldrich) and viewed at 350, 488, and 594 nm with a Nikon microscope.

Immunofluorescence staining of colon tissues. Immunofluorescence staining of control and infected tissues was performed in a fashion similar to that described previously (14, 55). In brief, tissues were rinsed in ice-cold PBS, embedded in optimal cutting template compound (OCT; Sakura, Finetech), frozen with isopentane (Sigma) and liquid N₂, and stored at -70°C. Serial sections were cut at a thickness of 6 µm and fixed in ice-cold acetone for 10 min. Tissue sections were directly blocked with 1% bovine serum albumin, followed by the addition of polyclonal rabbit antisera (dilution 1:1,000) generated against LPS (*E. coli* Poly 8 [Biotec Laboratories, England]), rat antibodies to the macrophage marker F4/80 (Serotec; dilution 1:300), or rat antibodies to the neutrophil marker Gr1 (Serotec; dilution 1:100). After extensive washing with Tris-buffered saline, Alexa 488-conjugated goat anti-rabbit immunoglobulin G (IgG) antibodies or Alexa 568-conjugated goat anti-rat IgG antibodies (all at a dilution of 1:300) were added. Lastly, DAPI was used to stain the host cell DNA and visualized as described above.

RNA extraction, semiquantitative, and real time-PCR. Immediately after dissection, colonic tissues were transferred to TRIzol reagent (Gibco-BRL), frozen in liquid N₂, and stored at -70°C until required. RNA was purified according to the manufacturer's instructions. Total RNA was treated with DNase I (Ambion) to remove any contaminating DNA. DNase I was removed with DNase inactivation reagent (Ambion) according to the manufacturer's instructions. cDNA was synthesized with Superscript II reverse transcriptase (Gibco-BRL) using 3 µg of DNase-treated RNA. An aliquot (1/40) of the cDNA reaction was subject to PCR analysis according to the manufacturer's instructions for AmpliTaq Gold DNA polymerase (Perkin-Elmer). For TLR4 gene transcription, the oligonucleotide primers mTLR4+ (5'-GGG TCA AGG AAC AGA AGC AG-3') and mTLR4- (5'-AGC CTT CCT GGA TGA TGT TG-3') were used with cycling conditions of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for a total of 28 cycles. For semiquantitative chemokine analysis, the primers mMIP-2F (5'-TCC TCG GGC ACT CCA GAC-3') and mMIP-2R (5'-GCC TTG CCT TTG TTC AGT AT-3') or mMCP-1F (5'-CTT CCT CCA CCA CCA TGC AG-3') and mMCP-1R (5'-AAA ATG GAT CCA CAC CTT GC-3') were used with cycling conditions of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for a total of 35 cycles. As a control for a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with the oligonucleotides CR127 (5'-ACA TCA TCC CTG CAT CC-3') and CR128 (5'-GGA TGG AAA TTG TGA GG-3') with cycling conditions as follows: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for a total of 22 cycles. PCR products were separated by agarose gel electrophoresis.

Quantitative real-time PCR was performed by using the Bio-Rad Laboratories MJ mini Opticon Real-Time PCR System. For quantitative chemokine analysis, the primers MIP-2F (5'-GCC AAG GGT TGA CTT CA-3') and MIP-2R (5'-TGT CTG GGC GCA GTG) or MCP-1F (5'-TAC TCA TTA ACC AGC AAG AT-3') and MCP-1R (5'-TTG AGG TGG TGG TGG AA-3') were used, while PCR conditions were as previously described (20, 33). Expression of GAPDH was used as a reference, using the GAPDH primers mentioned above for semiquantitative analysis. PCR was conducted with IQ SYBR Green Supermix (Bio-Rad), using cycling conditions of 95°C for 5 min and 35 cycles of 95°C for 30 s, followed by 55°C for 30 s and 72°C for 30 s. To calculate the relative gene expression, the Pfaffl method was used, as outlined in the Gene Ex Macro software by Bio-Rad.

Data presentation and statistical analysis. All of the results are expressed as the mean value ± the standard error of the mean (SEM). The results presented are from one representative infection out of three. Statistical significance was calculated by using the nonparametric Mann-Whitney test. Multiple comparisons

FIG. 1. Lower infectious doses of *C. rodentium* reveal prolonged survival of TLR4-deficient compared to TLR4-expressing mice. TLR4-deficient C3H/HeJ mice (A) and TLR4-expressing C3H/HeOuJ mice (B) were inoculated with 2.5×10^8 (circles), 2.5×10^6 (squares), or 2.5×10^4 CFU (diamonds), and the survival of the C3H/HeJ mice (open markers) and the C3H/HeOuJ mice (filled markers) was monitored over the first 24 days of infection. In this experiment, the reduction in infectious doses dramatically prolonged the survival of the C3H/HeJ mice, whereas it had only a modest impact on the survival of the C3H/HeOuJ mice. Each datum point represents the percentage of surviving mice from an initial population of five mice. Survival data from one experiment representative of three is shown.

were performed by using the Neuman-Keuls multiple comparison test. A *P* value of <0.05 was considered significant.

RESULTS

Lower infectious doses reveal a maladaptive role for TLR4 during *C. rodentium* infection. In a previous study, it was noted that the TLR4-deficient mouse strain, C3H/HeJ, and the closely related, but TLR4-expressing C3H/HeOuJ mice were both highly susceptible to infection by *C. rodentium*, carrying bacterial burdens several logs higher than the less susceptible C57BL/6 and BALB/c mouse strains (54). Since both C3H/HeJ and C3H/HeOuJ mice ultimately succumbed to infection, we determined that TLR4 played no significant role in the host response to *C. rodentium* infection (52). However, considering the speed with which these mice succumbed, it was possible that the susceptibility of these strains and the large oral dose

FIG. 2. Immunolocalization of *C. rodentium* LPS in the infected colon. Immunofluorescence staining of an uninfected colon (A), as well as a *C. rodentium*-infected colon from C57BL/10J mice at day 10 p.i. (B and C). Panel A shows a low-magnification ($\times 40$) image, with the structures of the colonic mucosa and muscle layers highlighted by phalloidin staining of the actin cytoskeleton (red) and DAPI staining of host cell nuclei (blue). Note that there is no staining with the anti-*C. rodentium* LPS antisera (green). In panel B, a low-magnification image of an infected colon shows that *C. rodentium* LPS (green) is predominantly located in the lumen and on the colonic epithelial surface. Panel C shows a higher-magnification ($\times 1,000$) image, using the same staining as in panel B, demonstrating intact *C. rodentium* on the apical surface of colonic epithelial cells at the base of a colonic crypt. Note the spread of intact bacteria (arrows), and shed LPS across the epithelial barrier and into the underlying lamina propria.

(2.5×10^8 CFU) initially used had obscured any potential role for TLR4. To test this hypothesis, we infected both strains of susceptible mice with the original dose, as well as lower doses, of *C. rodentium* (2.5×10^6 CFU and 2.5×10^4 CFU). Although the C3H/HeJ and C3H/HeOuJ strains succumbed at similar times (7.5 ± 1.2 days and 8.1 ± 1.9 days p.i., respectively) after the high infectious dose, we observed a distinct delay in the time of death of the TLR4-deficient C3H/HeJ mice (see Fig. 1 for representative infections) compared to their WT C3H/HeOuJ controls at the lower doses. The delay was ca. 3 days ($12.6 \text{ days} \pm 2.5$ versus 9.7 ± 2.1 days p.i.) at the 2.5×10^6 CFU dose and was even more pronounced at the lower dose of 2.5×10^4 CFU, with a significant delay of almost 6 days (17.3 ± 3.3 days versus 10.8 ± 2.6 days p.i. [$P < 0.05$]). These data suggested that mice expressing a functional TLR4 gene might suffer a more severe disease during *C. rodentium* infection than TLR4-deficient mice. Unfortunately, because of the low infectious doses required to observe this effect in C3H/HeJ mice, the resulting variability of disease onset made the C3H/HeJ mouse less than ideal for exploring this newly hypothesized role for TLR4 during *C. rodentium* infection. Therefore, these studies were continued with TLR4-deficient C57BL/10ScNJ mice (39) and their WT (C57BL/10J) TLR4-expressing mice that are on a genetic background that is relatively resistant to *C. rodentium* infection.

***C. rodentium* LPS is found in both the colonic lumen and lamina propria of infected mice.** As gram-negative bacteria, both EHEC and EPEC produce LPS, with the O157 serotype being the most prevalent EHEC isotype associated with human disease. *C. rodentium* are also gram-negative bacteria, and the structure of their LPS was recently described in detail (31). As previously demonstrated, antisera generated against the O152 LPS serotype recognize *C. rodentium* LPS (55). In the present

study, these antisera were used to examine the location of *C. rodentium* LPS within the colons of infected C57BL/10J mice, to determine which host cells were exposed to the LPS during infection. No LPS staining was seen in uninfected colonic tissues, confirming the specificity of the antisera (Fig. 2A), but when an infected colon was assessed (Fig. 2B), the vast majority of *C. rodentium* were found in the colonic lumen or adherent to the colonic epithelium. Similarly, at a higher magnification (Fig. 2C), *C. rodentium* organisms were observed primarily on the apical surface of colonic epithelial cells. Although not all epithelial cells were directly infected, it was apparent that most epithelial cells in the infected colon were exposed to the LPS shed by this pathogen. In addition to the epithelium-adherent population, a small number of *C. rodentium* and shed LPS were clearly seen spreading out of infected crypts and into the underlying lamina propria. Since *C. rodentium* is not a classically invasive bacterium (30), this bacterial translocation probably reflects a disruption of the colonic epithelial barrier. Based on these observations, it appears that both colonic epithelial cells and professional inflammatory cells in the colonic lamina propria would be exposed to *C. rodentium* LPS during an infection by this pathogen.

TLR4 mRNA is expressed in naive and *C. rodentium*-infected mouse colons. We next assessed whether the TLR4 gene is expressed in the mouse colon, and whether its expression was altered during infection by *C. rodentium*. Colonic tissues from the TLR4-deficient C57BL/10ScNJ strain (39) and WT (C57BL/10J) mice were collected both prior to and during *C. rodentium* infection, and mRNA were isolated. As shown in Fig. 3A, we found that TLR4 mRNA was expressed in the colons of uninfected WT mice (lane 4), and similar mRNA levels were observed at day 6 p.i. (lane 5) and at day 10 p.i. (lane 6). We also identified a comparable pattern of TLR4 expression in the ceca

FIG. 3. TLR4 is expressed in the mouse colon and *C. rodentium* supernatants activate TLR4. (A) Colonic tissues taken from wild-type (TLR4⁺) and TLR4-deficient (TLR4⁻) mice were assessed for the expression of TLR4 mRNA under uninfected conditions and at days 6 and 10 of *C. rodentium* infection. reverse transcription-PCR for TLR4 showed expression under control conditions (lane 4), with only slight increases in TLR4 expression at days 6 (lane 5) and 10 p.i. (lane 6). In contrast, colonic tissues from TLR4 deficient mice lacked any TLR4 gene expression under uninfected or infected conditions (lanes 1 to 3), in keeping with their spontaneous loss of the TLR4 gene. GAPDH was used as the housekeeping gene control. (B) Luria broth (LB) (□) and the supernatants from *C. rodentium* cultures grown overnight in LB (■) were used to stimulate HEK 293 cells transiently transfected with an NF-κB reporter construct along with either an empty vector, vectors encoding TLR4 (as well as MD-2 and CD14), or a vector encoding TLR8. The NF-κB activation (as assessed by firefly luciferase units) resulting from this stimulation was normalized for transfection efficiency and compared to the response with the empty vector. The asterisk indicates that the level of NF-κB activation by the *C. rodentium* supernatant is significantly greater (*, $P < 0.01$) in cells transfected with the TLR4 vector than the activation caused by LB alone, whereas the supernatant caused no significant NF-κB activation of cells transfected with the TLR8 vector.

of WT mice (not shown). As expected, no TLR4 mRNA expression was detected in tissues taken from TLR4-deficient mice under either uninfected (lane 1) or infected conditions (lanes 2 and 3). These results clearly demonstrate that the TLR4 gene is expressed in the colons of WT mice both prior to and during *C. rodentium* infection.

Exposure to *C. rodentium* supernatant activates cells through TLR4. We next assessed whether mammalian TLR4 recognizes and responds to the LPS produced by *C. rodentium*. HEK293 cells were transfected with the ELAM-luciferase reporter to measure NF-κB activity and cotransfected with TLR4, MD-2, and CD14. We assessed NF-κB activity by measuring luminescence of transfected cells after exposure to either purified LPS or to the supernatant taken from overnight cultures of *C.*

rodentium. Ultrapure *Salmonella enterica* serovar Minnesota LPS (10 ng/ml) activated NF-κB in TLR4-transfected cells by two- to fourfold compared to unstimulated cells or cells transfected with empty vector. Similarly, *C. rodentium* supernatant activated the TLR4 reporter by 13- to 14-fold (see Fig. 3B) relative to TLR4 transfectants treated with an equivalent volume of LB, confirming that *C. rodentium* can activate cells through TLR4. This activation was specific, since *C. rodentium* supernatant did not activate NF-κB in HEK293 cells transfected with TLR8, whereas stimulation with the TLR8 agonist R848 led to a five- to eightfold increase in NF-κB-dependent luciferase activity. No significant NF-κB activity was measured in cells transfected with empty vector and stimulated under the same conditions.

Exposure to *C. rodentium* causes NF-κB nuclear translocation: partial TLR4 dependence. Once we had confirmed that *C. rodentium* activated TLR4, we assessed the impact of TLR4 on the inflammatory response of host cells. A/E pathogens most closely interact with intestinal epithelial cells; however, several studies have demonstrated that colonic epithelial cells are normally hyporesponsive to LPS (17, 22, 25, 38). Although we did find that the CMT-93 murine colonic epithelial cell line expresses TLR4 mRNA (not shown), we were unable to immunofluorescently detect TLR4 protein expression by these cells (Fig. 4A and B) using MTS510, an antibody that recognizes the complex formed by TLR4 and its coreceptor MD-2 (2). Moreover, we were unable to detect overt NF-κB activation in the CMT93 cells following *S. enterica* serovar Minnesota LPS stimulation (not shown), confirming the hyporesponsiveness of these epithelial cells to LPS. In contrast, professional phagocytes such as macrophages and dendritic cells are known to express functional levels of this innate receptor and respond to purified LPS (42). Since previous immunostaining studies of uninfected mouse colons had identified numerous resident macrophages, but few resident dendritic cells (C. Ma and B. A. Vallance, unpublished data), we examined TLR4 protein expression and its role in the inflammatory response to *C. rodentium* in macrophages. For these studies, viable *C. rodentium* from overnight cultures were added to macrophages derived from the bone marrow of WT mice or from TLR4-deficient mice. After 1 to 4 h of exposure, cells were fixed and processed for immunofluorescence. The localization of TLR4/MD-2 was determined by using the MTS510 antibody (2). As assessed by immunofluorescence staining, *C. rodentium* were phagocytosed by both types of macrophages, while bacterial counts revealed similar numbers of *C. rodentium* were phagocytosed by the two macrophage populations (not shown). TLR4/MD-2 staining (red) was readily seen in WT-derived macrophages (Fig. 4C) but not in macrophages derived from TLR4-deficient mice (Fig. 4D). Much of the TLR4/MD-2 staining appeared to localize to the perinuclear region, presumably within the Golgi apparatus, as previously described (34). The specificity of the TLR4 antibody was confirmed when control slides receiving irrelevant isotype-matched antibodies, or the secondary antibody alone, showed no staining (not shown).

We next tested the ability of *C. rodentium* to trigger an inflammatory response in BMDM, as assessed by the nuclear translocation of the proinflammatory transcription factor NF-κB. Macrophages were exposed to *C. rodentium* for the times described above; the cells were then fixed and immunostained

FIG. 4. Immunostaining of TLR4/MD-2 and NF- κ B in epithelial cells and macrophages exposed to *C. rodentium*. (A and B) Immunofluorescence staining demonstrating the lack of TLR4/MD-2 immunoreactivity (red) in uninfected murine CMT93 colonic epithelial cells (A), as well as in CMT93 cells exposed to *C. rodentium* (B). The cell nuclei are DAPI stained (blue). (C) In contrast, to the lack of TLR4/MD-2 staining in colonic epithelial cells, there is considerable expression of TLR4/MD-2 in WT BMDM exposed to *C. rodentium*. Note the immunolocalization of TLR4/MD-2 (in red, see arrow) within the cytoplasm, and the internalized bacteria (an arrowhead indicates the DAPI-stained bacteria in blue). (D) In contrast, macrophages derived from TLR4-deficient mice showed no evidence of immunoreactive TLR4, despite the presence of phagocytosed *C. rodentium* (arrowhead). (E) Localization of NF- κ B was predominantly cytoplasmic in naive BMDM derived from WT mice, with little if any staining in the nucleus (arrow). (F) A similar cytoplasmic localization of NF- κ B was seen in naive BMDM derived from TLR4-deficient mice (see arrow for nucleus). (G) Within 2 h of exposure to *C. rodentium*, there was a dramatic recruitment of NF- κ B to the nucleus (arrow) of WT derived cells. (H) Under the same conditions, TLR4-deficient BMDM showed modest but significantly attenuated recruitment of NF- κ B. All images were taken at $\times 800$ magnification.

for NF- κ B. As shown in Fig. 4E and F, uninfected WT and TLR4-deficient macrophages both showed punctate NF- κ B staining throughout the cytoplasm, with little nuclear staining. Within 2 h of exposure to *C. rodentium*, there was a dramatic recruitment of NF- κ B to the nucleus of WT macrophages (Fig.

4G), and this was maintained until at least 4 h postexposure (not shown). In contrast, NF- κ B translocation to the nuclei of macrophages derived from TLR4-deficient mice was attenuated at 2 h (Fig. 4H) compared to WT-derived cells. Although the extent of nuclear translocation did increase over time (not shown), it always remained less than that seen in WT macrophages. These results demonstrate that exposure to *C. rodentium* triggers NF- κ B nuclear translocation in macrophages in a partially TLR4-dependent manner.

Early inflammatory cell recruitment during *C. rodentium* infection is TLR4 dependent. We next assessed the impact of TLR4 expression on the host inflammatory response to *C. rodentium* infection. In a recent study by Wiles et al. (58), it was demonstrated that after oral inoculation, *C. rodentium* initially colonize the cecum and, after bacterial growth at that site, the infection progresses down the length of the colon. Therefore, we examined cecal tissues at day 6 p.i., the earliest time point when we identified the cecum to be consistently and heavily colonized. Infected tissues were immunostained and then compared to control tissues for the presence of macrophages and neutrophils by using antibodies recognizing F4/80 and Gr1 as the respective markers. Under control conditions, occasional resident F4/80-positive macrophages were identified at the base of cecal crypts and in subepithelial locations in both WT and TLR4-deficient mice (Fig. 5A and C). Similarly, we saw few other inflammatory cells, such as Gr1-positive neutrophils, in control cecal tissues (Fig. 5E and G).

By day 6 p.i., the ceca of both WT mice and TLR4-deficient mice were heavily colonized [$(2.6 \pm 0.5) \times 10^8$ CFU versus $(1.9 \pm 0.3) \times 10^8$ CFU, respectively] by *C. rodentium*. In contrast to the sporadic network of resident macrophages seen in the mucosa of uninfected mice, immunostaining revealed a dramatic infiltration of F4/80-positive macrophages into the mucosa and submucosa of cecal tissues taken from infected WT mice (Fig. 5B). Similarly, whereas neutrophils were only rarely seen in uninfected tissues, infection of WT mice was associated with the recruitment of numerous Gr1-positive neutrophils into the cecal mucosa and submucosa (Fig. 5F). Compared to this dramatic inflammatory response, only minimal inflammatory changes were identified in the cecal tissues of infected TLR4-deficient mice. The small population of macrophages found in infected tissues (Fig. 5D) was similar to that seen under control conditions and, similarly, only a few scattered neutrophils were identified in the tissues of infected TLR4-deficient mice (Fig. 5H). It should be noted that by day 10 p.i., we did observe some neutrophils and macrophages within the cecal mucosa of TLR4-deficient mice; however, the number of cells was still dramatically less than that observed in tissues taken from infected WT mice (not shown).

TLR4-dependent changes in chemokine gene expression in the infected cecum. To explore the basis for the impaired inflammatory cell recruitment seen in the TLR4-deficient mice, we examined the gene expression of the neutrophil chemoattractant MIP-2 (11), as well as the monocyte/macrophage chemokine MCP-1 (50) through both semiquantitative PCR (Fig. 6A) and quantitative real-time PCR (Fig. 6B). We chose these two chemokines as representative of the chemokine response to *C. rodentium* infection, since we had previously determined their expression to be upregulated in colonic tissues during infection in WT mice (C. Ma and B. A. Vallance, unpub-

FIG. 5. Macrophage and neutrophil recruitment during *C. rodentium* infection is TLR4 dependent. Cecal tissues from uninfected WT (A and E) and TLR4-deficient (C and G) mice, as well as day 6 post-*C. rodentium*-infected WT (B and F) and TLR4-deficient (D and H) mice, were stained for the presence of F4/80-positive macrophages (A to D) and Gr1-positive neutrophils (E to H). Inflammatory cells are shown in red, and DAPI-stained host cell nuclei are in blue. A resident network of macrophages is seen in both uninfected WT (A) and TLR4-deficient (C) mice, as shown in panel B, numerous infiltrating F4/80-positive macrophages were seen in the cecal submucosa and mucosa of *C. rodentium*-infected WT mice. (D) However, no infiltrating macrophages were observed in the TLR4-deficient mice. The few F4/80-positive macrophages detected in the mucosa of infected TLR4-deficient mice (B) were similar in location and number to the network of resident macrophages seen in uninfected mice (C). Few if any neutrophils were seen in uninfected WT (E) and TLR4-deficient (G) mice, whereas large numbers of infiltrating Gr1-positive neutrophils were also detected in the cecal tissues of infected WT mice (F). (H) This recruitment was also significantly attenuated in the TLR4-deficient mice. All images were taken at $\times 200$ magnification and are representative of 10 sections assessed for each group.

lished data). As shown in Fig. 6, MCP-1 was expressed at similarly low levels in cecal tissues taken from uninfected WT (lane 1) and TLR4-deficient (lane 3) mice, whereas MIP-2 gene expression was very low in WT mice, and even lower in TLR4-deficient mice. Infection led to a significant (88-fold) increase in MIP-2 expression in WT mice (lane 2), whereas only a twofold increase was seen in tissues taken from infected TLR4-deficient mice (Fig. 6A, lane 4, and B). Similarly, MCP-1 expression was upregulated by sixfold in tissues from infected WT mice, but no change in expression was detected in TLR4-deficient mice. Based on these findings, a lack of chemokine induction after infection may be the basis for the impaired recruitment of inflammatory cells into the ceca of infected TLR4-deficient mice. Corresponding to the observed infiltration of more inflammatory cells at later time points in infected TLR4-deficient mice, we also detected a modest increase in these chemokines at later time points during the infection (not shown).

Infection induced weight loss and mortality are attenuated in TLR4-deficient mice. In our previous collaborative studies assessing the role of TLR4 in a mouse model of serovar Typhimurium infection (57), TLR4 expression was found to be critical in controlling the severity and ultimately the lethality of the infection. Using a similar approach with *C. rodentium*, we

monitored changes in body weight, as well as the number of infected mice that required euthanization, as measures of disease severity. Similar to our previous studies infecting other immunocompetent mouse strains with *C. rodentium*, the TLR4-expressing WT strain suffered a moderate mortality rate ranging from 20 to 30%, which occurred between days 10 and 14 p.i. (Fig. 7A). The mortality occurred at the same time as the maximal weight loss (Fig. 7B), when the mice had lost ca. 15% of their body weight. In contrast, no TLR4-deficient mice died or required euthanization after *C. rodentium* infection (Fig. 7A). Moreover, weight loss in the TLR4-deficient C57BL/10ScNJ mice was minimal and significantly less ($P < 0.05$) than that seen in the WT mice at days 2, 10, and 14 p.i. (Fig. 7B). Thus, mortality and weight loss during *C. rodentium* infection were significantly attenuated in this TLR4-deficient mouse strain.

***C. rodentium* colonization and spread through the colon are delayed in TLR4-deficient mice.** We next studied whether TLR4 expression modulated *C. rodentium* infection dynamics, including the spread of the infection from the cecum to the distal colon. We continued these studies with our original WT and TLR4-deficient mice (on a C57BL/10 genetic background), since these mice survived *C. rodentium* infection, whereas infec-

FIG. 6. Increased chemokine expression during *C. rodentium* infection is TLR4 dependent. (A) Cecal tissues from WT (TLR4+) and TLR4-deficient (TLR4-) mice were assessed for the expression of the chemokine genes, MIP-2 and MCP-1. Tissues were collected from uninfected mice and at day 6 p.i. (A) Both mouse strains expressed detectable but low mRNA levels of the chemokine MCP-1 but no expression of MIP-2 (lanes 1 and 3) under uninfected conditions. By day 6 p.i., the expression of both chemokine genes (lane 2) strongly increased in WT mice but not in TLR4-deficient cecal tissues (lane 4). GAPDH was used as the housekeeping gene control. (B) The same samples (from uninfected and day 6 p.i. WT and TLR4 knockout mice) were subjected to real-time PCR analysis, and the expression of MCP-1 (■) and MIP-2 (□) were quantified relative to the mRNA levels in uninfected WT samples. The asterisks indicate that the expression levels of both chemokines were significantly increased in WT day 6 p.i. tissues over that of uninfected control tissues (*, $P < 0.01$). In contrast, the modest increase in the expression of these genes in infected TLR4 knockout mice did not reach significance.

tion almost always proved lethal to the C3H/HeJ and C3H/HeOuJ mice. Orally inoculated *C. rodentium* (dose given was 2.5×10^8 CFU) heavily colonized the ceca of both WT and TLR4-deficient mice by day 6 p.i., and similar numbers of *C. rodentium* were identified in the cecum at day 10 p.i. (Fig. 8A). In WT mice the cecal phase of the infection was accompanied by the rapid spread of *C. rodentium* to the distal colon, resulting in a mean bacterial load of $(5.1 \pm 0.7) \times 10^8$ CFU by day 6 p.i. in the colons of WT mice (Fig. 8B). In contrast, *C. rodentium* spread to the colon more slowly in the TLR4-deficient mice, with $\sim 10^3$ -fold fewer bacteria recovered from the distal colon at day 6 p.i. [$(7.3 \pm 1.2) \times 10^5$ CFU; $P < 0.001$].

FIG. 7. Infection induced weight loss and mortality are attenuated in TLR4-deficient mice. (A) Mice were inoculated with 2.5×10^8 CFU, and the survival of WT (●) and TLR4 deficient mice (○) was monitored over the first 24 days of infection. In this experiment, 30% of the WT mice in this particular experiment required euthanasia between days 10 and 14 p.i.; however, no TLR4-deficient mice required euthanasia over the 24 days. Each datum point represents the percentage of surviving mice from an initial population of ten mice. Survival data from one experiment representative of three is shown. (B) The body weights of WT (●) and TLR4-deficient mice (○) were followed over the first 24 days of a *C. rodentium* infection. Each datum point represents average weight data pooled from ten mice and is expressed as the percentage of the initial body weight. Although both mouse strains lost weight during the course of infection, the body weight loss was dramatically greater for WT mice. The asterisks indicate that the body weight of the WT mice was significantly less than that found for TLR4-deficient mice at days 2, 10, and 14 p.i. (*, $P < 0.05$).

At day 10 p.i., the bacterial load in the colons of WT mice averaged $(6.4 \pm 1.1) \times 10^8$. At the same time point, the bacterial burden carried by TLR4-deficient mice had increased, but only to $(5.8 \pm 2.3) \times 10^7$, still 10-fold lower than that found in WT mice ($P < 0.01$). By day 14 p.i., *C. rodentium* numbers had reached a similar plateau in the two mouse strains, with WT and TLR4-deficient mice carrying $(5.7 \pm 1.1) \times 10^8$ and $(3.2 \pm 0.6) \times 10^8$ CFU, respectively. After day 14, the infection began to clear from the colons of WT mice, with the population of *C. rodentium* declining at days 18 and 21 p.i., until by day 24 p.i., when only a few thousand *C. rodentium* remained. Bacterial clearance from the TLR4-deficient mice was slightly delayed, not

FIG. 8. *C. rodentium* spread to the colon is delayed in TLR4-deficient mice. WT and TLR4-deficient mice were inoculated with 2.5×10^8 CFU, and the number of *C. rodentium* CFU recovered from the ceca of individual infected WT mice (●) and TLR4-deficient mice (○) are presented on days 6 and 10 p.i. (A), while panel B indicates the number of *C. rodentium* CFU recovered from the colons of these mouse strains on days 6, 10, 14, 18, and 21 p.i.. The mean CFU values are indicated by the horizontal solid lines for WT mice and horizontal dashed lines for TLR4-deficient mice. Asterisks denote the recovery of significantly more bacteria (mean value) from the colons of WT mice compared to TLR4-deficient mice at days 6 and 10 p.i. and significantly more bacteria recovered from the TLR4-deficient mice at day 18 p.i. (*, $P < 0.01$). Each group contained five mice, and the data shown are from one experiment representative of three.

beginning until after day 18 p.i., but by day 21 p.i. the *C. rodentium* numbers were reduced to a level similar to that found in WT mice, indicating that TLR4 expression was not essential for the clearance of infection. These time course studies were repeated in three independent infections, and a consistent delay in *C. rodentium* colonization of the distal colon was always observed in TLR4-deficient mice compared to WT mice. Moreover, clearance of the infection always occurred at a similar time in the two mouse strains.

Considering that TLR4 frequently limits bacterial growth during systemic gram-negative bacterial infections, the discovery that *C. rodentium* colonization of the distal GI tract was delayed by 4 to 8 days in the TLR4-deficient mice was a surprising result. To evaluate whether this was a common feature in murine hosts lacking TLR4 function, we assessed the time course of *C. rodentium* infection in the other two spontaneously TLR4-defi-

cient mouse strains: the C57BL/10ScNcr strain and the C3H/HeJ mouse strain. Using the same oral dose (2.5×10^8 CFU), *C. rodentium* colonization of the C57BL/10ScNcr mice also showed a significant delay, carrying 10- to 100-fold fewer bacteria than their WT controls at day 6 p.i., and 2- to 5-fold fewer bacteria at day 10 p.i. (not shown). Because the TLR4-deficient C3H/HeJ strain is highly susceptible to infection, we used a smaller infectious dose of *C. rodentium* in order to visualize any potential differences in colonization rates. After an oral dose of 10^4 CFU, heavy colonization of the WT C3H/HeJ strain occurred at day 7 p.i.. With the same dose, heavy colonization of the C3H/HeJ strain was delayed and did not occur until 4 to 6 days later (not shown). These results confirm that the loss of TLR4 function in the host delays *C. rodentium* colonization and spread to the murine colon.

***C. rodentium* dissemination to the MLNs is TLR4 independent.** Although *C. rodentium* are primarily found in the colonic lumen of their hosts, some bacteria do translocate across the intestinal epithelial barrier, reaching the lamina propria (Fig. 2C), and the MLNs of infected mice (54). Since we and others have shown that serovar Typhimurium and other invasive gram-negative bacteria rapidly proliferate and spread through the internal organs of TLR4-deficient mice, we examined whether TLR4 expression also impacted on *C. rodentium* translocation and systemic survival in their murine hosts. The *C. rodentium* populations found in the MLN of WT (C57BL/10J) and TLR4-deficient (C57BL/10ScNJ) mice were enumerated during the peak phase of the infection, days 10 to 14 p.i., when both strains carried large *C. rodentium* burdens in their colons. As shown in Table 1, the numbers of *C. rodentium* recovered from the MLN of infected WT mice were $5.9 \pm 1.7 \times 10^6$ on day 10 p.i., with the number falling to $4.5 \pm 1.5 \times 10^5$ CFU on day 14 p.i. Similar, but consistently lower numbers [$(8.4 \pm 3.3) \times 10^5$ and $(1.1 \pm 0.6) \times 10^5$ CFU] were recovered from the TLR4-deficient mice. Interestingly, the MLN numbers in both mouse strains correlated well with the load of *C. rodentium* found in their colons, reflecting ca. 1.6% of the colonic *C. rodentium* population at day 10 p.i. and 0.1% at day 14 p.i.. These results indicate that the loss of TLR4 expression did not increase *C. rodentium* translocation to, or growth in, the MLNs but instead that the *C. rodentium* population in the MLN was directly related to the load of this pathogen in the colon.

Infection-induced colitis and colonic pathology are attenuated in TLR4-deficient mice. A number of previous studies have characterized *C. rodentium* infection in mice as causing significant intestinal inflammation, as well as severe colonic pathology characterized by heavier colon weights, increased crypt heights, and the disruption of normal colonic architecture, including a depletion of goblet cells (30). To determine the role of TLR4 in these pathological events, we weighed the colons from infected C57BL/10ScNJ mice over the full course of the infection and took tissues for histological analysis to assess crypt heights and the degree of colitis. The extent of pathology was determined by using a previously described scoring system designed for serovar Typhimurium-induced colitis (24) that assesses submucosal edema, polymorphonuclear infiltration into the lamina propria, goblet cell depletion, and epithelial integrity.

TABLE 1. *C. rodentium* dissemination to the MLN is TLR4 independent^a

Mouse group	Mean $\pm$ SD			
	Day 10 p.i.		Day 14 p.i.	
	CFU in MLN	% colonic CFU	CFU in MLN	% colonic CFU
WT	$(5.9 \pm 1.7) \times 10^{6*}$	(1.52 ± 0.35)	$(4.5 \pm 1.5) \times 10^5$	0.09 ± 0.04
TLR4 deficient mice	$(8.4 \pm 3.3) \times 10^5$	(1.78 ± 0.51)	$(1.1 \pm 0.6) \times 10^5$	0.06 ± 0.03

^a WT and TLR4-deficient mice were inoculated with 2.5×10^8 CFU. *C. rodentium* CFU recovered from the MLNs and colons of WT and TLR4-deficient mice were enumerated at days 10 and 14 p.i. As shown, the number of bacteria recovered from the MLNs of WT mice at day 10 p.i. was significantly greater (*, $P < 0.05$) than in the MLNs of TLR4-deficient mice. However, when the MLN populations are considered as a percentage of the colonic *C. rodentium* population recovered from the same mouse, no difference in the relative populations was found. At day 14 p.i., no significant differences between the mouse strains were identified with regard to the MLN populations or in the percentage of *C. rodentium* found in the MLNs, indicating TLR4 independence. Each group contained five mice, and the data shown are from one representative experiment out of three.

(i) **Colon weights.** After *C. rodentium* infection, the colons of infected WT mice rapidly increased in weight, as shown in Fig. 9A, demonstrating a significant increase ($P < 0.05$) over the weight of uninfected colons from days 10 to 21 p.i. Colon weights increased from a mean of 105 ± 8 mg in uninfected mice to 118 ± 12 mg at day 6 p.i. and 185 ± 16 mg at day 10 p.i. The colon weights continued to rise to 235 ± 18 mg at day 14 p.i. but peaked at day 18 p.i., reaching weights of 275 ± 8 mg, after which they fell to 220 ± 15 mg at day 21 p.i. Although the TLR4-deficient mice also exhibited a time-dependent increase over control values, their increases were only significant over their uninfected values between days 14 and 21 p.i. As a result of this attenuated response, the mean colon weights of the TLR4-deficient mice were significantly lower than that seen in WT mice at all time points during infection.

(ii) **Crypt heights.** A similar but not identical trend was observed when the mean crypt heights were determined for WT and TLR4-deficient mice over the same course of infection (see Fig. 9B). We found that the crypt heights increased rapidly and dramatically in WT mice, increasing from 160 ± 8 μ m under uninfected conditions to 251 ± 7 μ m at day 6 p.i. and then to 272 ± 26 μ m at day 10 p.i. Thereafter, crypt heights reached a plateau, remaining at heights between 328 and 342 μ m between days 14 and 21 p.i. Thus, the crypt heights in WT mice were significantly increased ($P < 0.05$) over control values at all of the time points studied. The rapid increase in crypt heights was significantly attenuated ($P < 0.01$) in TLR4-deficient mice at day 6 and 10 p.i., with crypt heights showing little change from control levels (155 ± 7 μ m) to day 10 levels (158 ± 9 μ m). However, by day 14 p.i., a significant increase in crypt heights had occurred, reaching 276 ± 16 μ m and incrementally increasing thereafter to 296 ± 11 μ m and 300 ± 9 μ m at day 18 and 21 p.i., respectively. While the trend toward lower crypt heights in the TLR4-deficient mice was observed throughout the infection, the differences with WT values were no longer significant after day 10 p.i.

(iii) **Colitis/inflammation score.** Although the TLR4-dependent attenuation of the initial inflammatory response in the cecum was dramatic, since the infection progressed into the distal colon, inflammation, and tissue damage were observed in both WT and TLR4-deficient mice, but to a lesser degree in the TLR4-deficient mice. As shown in Fig. 9C, colitis in WT mice was evident by day 6 p.i. (score, 2.4 ± 0.4 U), with the score primarily due to polymorphonuclear infiltration and epithelial sloughing. By days 10 to 14 p.i., neutrophil infiltration was less prominent in WT mice, but submucosal edema, epi-

thelial damage, and goblet cell depletion (Fig. 9D) were prominent aspects of the colonic pathology, increasing the inflammatory score to over 6.0 U. The colitis score was maintained in WT mice at day 18 p.i. (score, 6.3 ± 0.5 U), but by day 21 p.i., as the infection began to clear, the inflammation score in the WT mice decreased to 1.8 ± 0.2 U. In contrast, the inflammatory response seen in the TLR4-deficient mice was reduced and more transient, showing little inflammation except at day 14 p.i. As a result, the inflammation scores in the TLR4-deficient mice were significantly attenuated compared to WT mice (Fig. 8E) at all time points studied, except days 14 and 21 p.i. Even on day 14 p.i., when the inflammation peaked in the TLR4-deficient mice, the score was primarily due to epithelial disruption and polymorphonuclear cell infiltration, while other pathological features such as edema and the degree of goblet cell depletion remained attenuated compared to WT mice.

Infection-induced diarrhea is less severe in TLR4-deficient mice. Both EPEC and EHEC induce profuse watery diarrhea in infected human hosts (29, 35); however, *C. rodentium* infection of mice causes a less severe form of intestinal dysfunction, resulting in soft and watery stools (30). At the most severe stage of *C. rodentium* infection, the colons of infected mice contain no formed stool but rather a mixture of fecal material, mucus, and fluids. Since diarrhea is such an important component of A/E bacterial infections, we studied what role TLR4 played in these pathophysiological changes using a scoring system assessing the water content of the stool. The stools from uninfected mice were well formed and relatively dry (water content 41%), but by day 6 p.i. the *C. rodentium* infection had already altered the stool found in WT mice, with fewer stools present and the average water content increasing from control values to 82% (Fig. 10). By days 10 and 14 p.i., this response had worsened (water content of 85 to 90%), with most animals carrying only unformed watery fecal material. Although TLR4-deficient mice also developed watery stool during infection, this response was significantly attenuated at day 6 and 10 p.i., with stool water content determined to average 56 and 72%, respectively. At day 14 p.i., the water content in the stool from TLR4-deficient mice reached more than 75% and was no longer significantly different from WT mice. After day 14 p.i., the stool water content in both mouse strains began to return to control levels, falling at day 18 p.i. to 55% in the WT mice and to 45% at 21 p.i. A similar trend was seen in the TLR4-deficient mice and, although the water content was consistently

FIG. 9. Infection-induced colitis and pathology are attenuated in TLR4-deficient mice. WT and TLR4-deficient mice were inoculated with 2.5×10^8 CFU and assessed for colitis and intestinal pathology for 21 days. (A) Both WT (■) and TLR4-deficient mice (□) developed significant increases in colon weights during the course of *C. rodentium* infection ($P < 0.05$). The WT tissues showed a significant increase over uninfected controls from days 10 to 21 p.i., whereas the TLR4-deficient mice showed significant increases from days 14 to 21 p.i. However, at all time points between days 6 to 21 p.i. inclusive, the mean colon weights (in milligrams) were significantly greater in the WT mice than in the TLR4-deficient mice (indicated by asterisks). Error bars represent standard errors, and each group contained five mice. *, $P < 0.05$. (B) WT mice (■) show significantly increased colonic crypt heights compared to uninfected controls at all time points tested during the course of *C. rodentium* infection, whereas TLR4-deficient mice (□) only showed significant increases from days 14 to 21 p.i. When the two strains are compared, the mean crypt heights (in microns) in the colons of WT mice were significantly greater than those from TLR4-deficient

higher in the stool from WT mice, the difference between strains was not significant at either day 18 or 21 p.i.

DISCUSSION

There is a growing recognition that the immune response triggered during enteric bacterial infections not only protects the host (10) but also inadvertently causes much of the associated disease (43, 55). Therefore, our laboratory and others have begun to explore how the hosts' immune system recognizes and combats A/E bacterial infections in the GI tract (48, 49, 53). However, our knowledge of how the innate immune system responds to these enteric pathogens is limited. Among the key issues that remain unresolved are the identity of the innate receptors that are expressed and function in the GI tract and whether these receptors respond to the presence of A/E bacteria. Moreover, we have yet to distinguish whether the innate responses that are induced actually protect the host or, alternatively, merely cause inflammation and tissue damage without providing significant protection against infection. We began our investigations by testing the role of the innate LPS receptor, TLR4, in the host response against the model pathogen, *C. rodentium*. Surprisingly, despite the prominent health threat posed by A/E bacteria, this is the first study to specifically assess the role of an innate receptor in the host response to these pathogens.

Our initial examination of infected mice found the vast majority of *C. rodentium* residing on the luminal surface of the gut epithelium. However, these microbes also translocated out of infected colonic crypts and into the lamina propria, exposing not just the epithelium but resident macrophages and other professional immune cells to *C. rodentium* LPS during infection. The basis for this translocation is unclear, but EPEC infection has been repeatedly shown to disrupt intestinal epithelial barrier function in tissue culture cells (26). Similarly, we recently found that *C. rodentium* infection causes increased permeability in gut segments (unpublished data); therefore, the passage of *C. rodentium* and its products across the epithelium likely reflects passive movement across a weakened intestinal barrier rather than an active invasive process.

Our examination of TLR4 expression found TLR4 mRNA to be constitutively expressed in the mouse colon, with little change in expression during the course of *C. rodentium* infec-

mice at days 6 and 10 p.i. (*, $P < 0.01$). (C) Both WT (■) and TLR4 deficient mice (□) show evidence of inflammation and tissue damage in the distal colon during *C. rodentium* infection, but the response was increased and more prolonged in the WT mice. Inflammation scores of WT tissues were significantly greater than those from TLR4-deficient mice at days 6, 10, and 18 p.i. (*, $P < 0.05$). For each panel, error bars represent standard errors. Each group contained five mice, and the data shown are from one representative experiment out of three. (D and E) Low-magnification ($\times 40$) histological images of colons taken from WT (D) and TLR4-deficient (E) mice at day 10 p.i. Significant inflammation, crypt hyperplasia, superficial epithelial damage, and goblet cell depletion are all evident in the tissues from WT mice. In contrast, only a limited inflammatory infiltrate is seen in the TLR4-deficient mouse tissues, with few signs of mucosal hyperplasia or other overt signs of pathology. The epithelium appears relatively undamaged and numerous goblet cells are evident in the TLR4-deficient tissue.

FIG. 9—Continued.

tion. While immunostaining for TLR4/MD-2 showed diffuse staining in a limited number of cells within the colonic mucosa (not shown), we were unable to clearly identify which cell types expressed these proteins. However, most previous studies on this subject suggest that resident macrophages and other inflammatory cells are the primary source of TLR4/MD-2 expression in the colon (25), with colonic epithelial cells expressing little if any TLR4 protein, in keeping with their relative nonresponsiveness to LPS (17, 40). Our results support these studies, since although we did detect TLR4 mRNA in the CMT-93 murine colonic epithelial cell line, we were unable to detect immunoreactive TLR4/MD-2 in these cells, whereas it was readily detectable in murine BMDM. Future studies will be needed to examine whether the colonic epithelium of a *C. rodentium*-infected mouse becomes more responsive to LPS

during the course of infection, potentially in response to the inflammatory mediators released during infection. Interestingly, while previous studies have shown that EPEC possesses strong antiphagocytic abilities (9), *C. rodentium* were rapidly phagocytosed by BMDM. This may reflect differences between EPEC and *C. rodentium* in their pathogenic strategies or, alternatively, it may simply reflect that the infectivity of *C. rodentium* as well as the secretion of their type III effectors is attenuated in tissue culture medium compared to EPEC (14).

Using nuclear translocation of NF- κ B as a measure of inflammatory activation (38), we found that exposure to *C. rodentium* caused a rapid activation of NF- κ B in BMDM in a partially TLR4-dependent manner. The limited TLR4-independent response likely reflects the activation of other innate receptors in the BMDM, since A/E bacteria are sources of a number of other proinflammatory molecules aside from LPS, such as flagellin (6) and bacterial DNA. Since NF- κ B activation is a central player in inflammation, our *in vitro* assays led us to test what role TLR4 plays in the inflammatory response during *C. rodentium* infection in mice.

C. rodentium infection *in vivo* triggers the recruitment of large numbers of macrophages and neutrophils to the infected intestine (30), and these inflammatory cells undoubtedly contribute to the tissue damage and disease suffered during infection. Prior to infection, we found few neutrophils in the cecal mucosa; however, immunostaining did reveal a broad network of resident macrophages within these tissues in both mouse strains. A similar network of macrophages has been previously identified in other regions of the mammalian GI tract (18) and may function to provide early recognition of translocating intestinal pathogens. We observed the rapid and dramatic recruitment of macrophages, as well as neutrophils, into the cecal mucosa and submucosa of WT mice during *C. rodentium* infection; however, few of these inflammatory cells were in direct proximity to the infected epithelium and the luminal *C. rodentium* population. Despite similarly large numbers of *C. rodentium* recovered from the cecal tissues of TLR4 deficient mice, we found few signs of macrophage or neutrophil recruitment in these mice, demonstrating a clear TLR4 dependence for this early inflammatory response. Similarly, we found a clear TLR4 dependence in the induction of MIP-2 and MCP-1 mRNA

FIG. 10. Infection-induced diarrhea is less severe in TLR4-deficient mice. The percentage of water in the colonic stool contents of WT mice infected by *C. rodentium* increased in a time-dependent manner, with the water content significantly elevated over uninfected control levels at days 6, 10, and 14 p.i. (all $P < 0.01$). While the percent water content of the stool also increased in TLR4-deficient mice, it was only significantly elevated over control levels at day 10 and 14 p.i. ($P < 0.05$). When the two strains are directly compared, the water content in the stool of WT mice was significantly greater than in TLR4-deficient mice at days 6 and 10 p.i. (*, $P < 0.05$). Each group contained five mice, and the data shown are from one representative experiment out of three.

expression within the infected ceca. These two chemokines potently recruit neutrophils and macrophages, respectively (11, 50). These findings are similar to an earlier characterization of the role of TLR4 during hepatic serovar Typhimurium infection in mice (57). In that study, serovar Typhimurium infection led to the upregulated expression of MIP-2 and other chemokines in the infected livers of WT mice. In contrast, these chemokine responses were attenuated in serovar Typhimurium-infected TLR4-deficient mice. Furthermore, the impaired inflammatory response ultimately proved fatal to TLR4 deficient mice as the serovar Typhimurium infection spread through their internal organs, leading to sepsis (36, 57).

Our present findings indicate that TLR4 plays a more complex role during *C. rodentium* infection, with infected WT mice suffering more severe morbidity and greater mortality rates than TLR4-deficient mice. While these results contrast with an earlier study that found no difference in susceptibility to *C. rodentium* infection between TLR4 deficient C3H/HeJ mice and their TLR4 expressing controls (54), we hypothesized that the heightened susceptibility of the C3H mice as well as the large oral bacterial inocula given in that earlier study masked any role for TLR4. In the present study, gavaging these mice with lower infectious doses revealed a previously unrecognized delay in disease onset in the C3H/HeJ mice compared to their WT controls, confirming that TLR4 expression worsens the disease outcome in this infection model.

Understanding how A/E bacterial pathogens colonize their hosts is obviously of great importance in combating these infections, and a recent study found that after oral inoculation of mice, *C. rodentium* initially colonizes the epithelium that overlies lymphoid tissue in the cecum (known as the cecal patch) (58). From that site, the bacteria replicate within the cecum and then pass from that site to colonize the distal colon of infected mice. The mechanisms that underlie the spread from the cecum to the distal colon are unclear. Our data indicate that *C. rodentium* was able to colonize the ceca of both WT and TLR4-deficient mice to roughly equal levels but the spread to and colonization of the distal colon by *C. rodentium* was significantly delayed in TLR4 deficient mice, while TLR4 ultimately had little impact on pathogen clearance from the gut. Similarly, TLR4 expression did not directly affect the population of *C. rodentium* found in the MLN during infection; therefore, we conclude that TLR4 does not contribute to host defense against *C. rodentium* in the GI tract or its associated lymphoid tissues or to bacterial clearance from infected mice.

To explore possible reasons for the delayed colonization of the colons in the TLR4 deficient mice, we assessed pathological factors characteristic of *C. rodentium* infection, including increased colon weights and crypt heights, as well as intestinal inflammation (30) and water content in the stools of infected mice. Although *C. rodentium* does not cause overt diarrhea in infected mice, infected mice do exhibit watery stool. There was evidence that compensatory inflammatory pathways were activated in the absence of TLR4; however, all of the pathological features that we assessed were attenuated and/or delayed in TLR4-deficient mice, suggesting that TLR4-mediated inflammatory changes may facilitate the ability of *C. rodentium* to colonize the distal colon. Even after bacterial burdens had reached similar levels in the two mouse strains, the degree of inflammation and pathology was still significantly greater and

longer lasting in the WT mice, indicating that differences in bacterial burdens are unable to explain the dramatic TLR4 dependence of the inflammation and tissue damage seen in this model.

Our results with *C. rodentium*-induced colitis agree with the contention that TLR4 contributes to intestinal inflammation and pathology, as proposed in two recent studies testing the role of TLR4 in the dextran sodium sulfate (DSS)-induced colitis model (22, 41). In both studies, TLR4-deficient mice suffered less inflammation than WT mice, with Fukata et al. linking this attenuated response to reduced MIP-2 production by TLR4-deficient macrophages. Curiously, despite the reduced inflammation, both groups found TLR4-deficient mice suffered greater body weight loss, intestinal bleeding, and mortality than WT mice during DSS-induced colitis. This heightened susceptibility was linked to impaired epithelial proliferation and increased commensal bacterial translocation to the MLN (22). Epithelial cell hyperplasia was also impaired in our *C. rodentium*-infected TLR4-deficient mice; in contrast, we saw less morbidity and mortality in these mice, and no significant increase in bacterial translocation. This difference in pathological outcomes may reflect the nature of the injurious agent and the severity of the resulting colitis. DSS is a sulfated polysaccharide known to be directly toxic to colonic epithelium, resulting in rapid and significant epithelial cell injury and apoptosis irrespective of the inflammatory response (22). In contrast, the colitis that develops during *C. rodentium* infection occurs more slowly, causes only limited apoptosis (54), and appears to be predominantly mediated by the host immune response (10, 55). We therefore hypothesize that differences in the mode and degree of epithelial damage differentiate the impact of TLR4 deficiency in these two models of colitis.

Overall, our results were unexpected, since in most previously published reports, the loss of TLR4 signaling leads to increased susceptibility to gram-negative bacterial infections. We hypothesize that the lack of a beneficial effect of TLR4 against *C. rodentium* infection may reflect the environmental niche occupied by this pathogen and the cell types it colonizes. In previous studies, TLR4 expression has been shown to protect mice against infection by *Salmonella* and uropathogenic *E. coli*, as well as other gram-negative bacterial pathogens (47, 57). In these models, salmonellae preferentially infect macrophages, whereas uropathogenic *E. coli* infects bladder epithelial cells. Both cell types express functional TLR4 (47, 57), and several studies have shown that infection of these cells by gram-negative bacteria induces a rapid inflammatory response. In contrast, there is little evidence that under basal conditions, the colonocytes preferentially infected by *C. rodentium* express TLR4 or its required cofactors at the protein level. While *C. rodentium* can induce an inflammatory response in macrophages, very few of the *C. rodentium* found in the gut lumen would directly contact these cells in vivo. Instead intestinal macrophages might be activated by translocating bacteria or by LPS, passively entering the colonic mucosa. The resulting innate response, as we have shown with TLR4-deficient mice, contributes to both the tissue damage and the disease symptoms caused by this infection; however, *C. rodentium* and perhaps other luminal pathogens may be sufficiently distant from these cells to be protected from their responses. Our findings therefore may prove relevant to the wide variety of gram-

negative bacterial pathogens that preferentially infect the intestinal epithelium. Interestingly, our results bear some similarity to work showing that broadly innate immune-impaired MyD88-deficient mice suffer less severe gastroenteritis than WT mice after oral infection with serovar Typhimurium (24).

While it was surprising that TLR4 deficient mice would be less susceptible to infection than WT mice, the ability of bacterial pathogens to exploit inflammation in order to colonize the colon is not unprecedented. *Shigella* spp. are known to trigger an inflammatory response in order to invade and spread through the host's colonic epithelium (37, 44, 45). Although A/E pathogens do not invade the epithelium like *Shigella* organisms do, several of the translocated virulence proteins used by A/E pathogens are homologous to *Shigella* effector proteins (52), suggesting these pathogens may share common virulence strategies. While more studies are needed to determine whether *C. rodentium* is truly exploiting the TLR4-induced host response, there are many reasons why colonization of the colon might be facilitated by a strong innate host response. As we demonstrated, TLR4 responses aid in the depletion of goblet cells, potentially weakening luminal protection against infection. Also, the aggravated diarrheal response seen in infected WT mice, along with the sloughing of colonized enterocytes, would increase fecal shedding and transmission of the organism down the GI tract and to other potential hosts. Similarly, the rapid epithelial cell proliferation and mucosal thickening seen during *C. rodentium* infection would provide a rapidly renewing supply of uninfected cells, as well as a larger and potentially less protected epithelial surface area for bacterial colonization. Another possibility is that TLR4-dependent responses may alter the population of commensal microflora found in the GI tract, providing *C. rodentium* with a selective advantage in colonizing this tissue.

In summary, our studies indicate that TLR4 expression mediates much of the colitis and tissue pathology seen during *C. rodentium* infection; however, in contrast to the host protective role observed in other types of gram-negative infections, TLR4 expression demonstrated no obvious benefit to the host during infection by this A/E bacterium. The putative maladaptive role played by TLR4 in this model may reflect the unique environment of the colon and the luminal niche occupied by A/E bacteria. While translocating *C. rodentium* and shed LPS appear to activate TLR4-dependent inflammation, the vast majority of these bacteria reside within the colonic lumen, a location that is relatively safe from the resulting inflammatory response. It should be noted, however, that other, more broadly acting, host immune factors do contribute to host defense in this model, since mice deficient in downstream inflammatory mediators such as IL-12, gamma interferon (49), and NF- κ B (B. A. Vallance, unpublished data) suffer increased susceptibility to *C. rodentium* infection. Therefore, although our findings indicate that TLR4 activation is not effective in promoting host defense against *C. rodentium*, other inflammatory pathways may prove beneficial, presumably differing from TLR4-dependent responses in their timing, location, or the cell types involved. Although more studies are needed to establish how inflammation may facilitate *C. rodentium* colonization of the host gut, it is clear that the role of innate immunity in the lower GI tract cannot simply be extrapolated from tissue culture or systemic infection studies. Considering the importance of this site, and the vast number of pathogens

that target the GI tract, additional studies examining the actions of the innate immune system in relevant enteric infection models should be a high priority for the field, to aid in the rational design of vaccines and therapies.

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REFERENCES

1. Abe, A., U. Heczko, R. G. Hegele, and B. B. Finlay. 1998. Two enteropathogenic *Escherichia coli* type III secreted proteins, EspA and EspB, are virulence factors. *J. Exp. Med.* **188**:1–10.
2. Akashi, S., R. Shimazu, H. Ogata, Y. Nagai, K. Takeda, M. Kimoto, and K. Miyake. 2000. Cutting edge: cell surface expression and lipopolysaccharide signaling via the Toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. *J. Immunol.* **164**:3471–3475.
3. Barthold, S. W., G. L. Coleman, P. N. Bhatt, G. W. Osbaldiston, and A. M. Jonas. 1976. The etiology of transmissible murine colonic hyperplasia. *Lab. Anim. Sci.* **26**:889–894.
4. Barthold, S. W., G. W. Osbaldiston, and A. M. Jonas. 1977. Dietary, bacterial, and host genetic interactions in the pathogenesis of transmissible murine colonic hyperplasia. *Lab. Anim. Sci.* **27**:938–945.
5. Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W. D. Hardt. 2003. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect. Immun.* **71**:2839–2858.
6. Berin, M. C., A. Darfeuille-Michaud, L. J. Egan, Y. Miyamoto, and M. F. Kagnoff. 2002. Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF- κ B and MAP kinase pathways and the upregulated expression of interleukin 8. *Cell Microbiol.* **4**:635–648.
7. Bry, L., and M. B. Brenner. 2004. Critical role of T cell-dependent serum antibody, but not the gut-associated lymphoid tissue, for surviving acute mucosal infection with *Citrobacter rodentium*, an attaching and effacing pathogen. *J. Immunol.* **172**:433–441.
8. Campos, M. A., G. M. Rosinha, I. C. Almeida, X. S. Salgueiro, B. W. Jarvis, G. A. Splitter, N. Qureshi, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira. 2004. Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. *Infect. Immun.* **72**:176–186.
9. Celli, J., M. Olivier, and B. B. Finlay. 2001. Enteropathogenic *Escherichia coli* mediates antiphagocytosis through the inhibition of PI 3-kinase-dependent pathways. *EMBO J.* **20**:1245–1258.
10. Clare, S., A. Huett, and G. Dougan. 2002. Host/pathogen interactions at mucosal surfaces: immune consequences. *Res. Microbiol.* **153**:455–459.
11. Daly, C., and B. J. Rollins. 2003. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation* **10**:247–257.
12. Deng, W., Y. Li, B. A. Vallance, and B. B. Finlay. 2001. Locus of enterocyte effacement from *Citrobacter rodentium*: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. *Infect. Immun.* **69**:6323–6335.
13. Deng, W., J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vazquez, J. Barba, J. A. Ibarra, P. O'Donnell, P. Metalnikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B. B. Finlay. 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc. Natl. Acad. Sci. USA* **101**:3597–3602.
14. Deng, W., B. A. Vallance, Y. Li, J. L. Puente, and B. B. Finlay. 2003. *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. *Mol. Microbiol.* **48**:95–115.
15. Donnenberg, M. S., B. B. Finlay, and J. B. Kaper. 1997. Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends Microbiol.* **5**:109–114.
16. Donnenberg, M. S., and T. S. Whittam. 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *J. Clin. Investig.* **107**:539–548.

17. Eckmann, L. 2004. Innate immunity and mucosal bacterial interactions in the intestine. *Curr. Opin. Gastroenterol.* **20**:82–88.
18. Eskandari, M. K., J. C. Kalf, T. R. Billiar, K. K. Lee, and A. J. Bauer. 1997. Lipopolysaccharide activates the muscularis macrophage network and suppresses circular smooth muscle activity. *Am. J. Physiol.* **273**:G727–G734.
19. Fagundes Neto, U., V. Ferreira, F. R. Patricio, V. L. Mostaco, and L. R. Trabulsi. 1989. Protracted diarrhea: the importance of the enteropathogenic *Escherichia coli* (EPEC) strains and *Salmonella* in its genesis. *J. Pediatr. Gastroenterol. Nutr.* **8**:207–211.
20. Fine, J. S., A. Rojas-Triana, J. V. Jackson, L. W. Engstrom, G. S. Deno, D. J. Lundell, and L. A. Bober. 2003. Impairment of leukocyte trafficking in a murine pleuritis model by IL-4 and IL-10. *Inflammation*. **27**:161–174.
21. Frankel, G., A. D. Phillips, I. Rosenshine, G. Dougan, J. B. Kaper, and S. Knutson. 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol. Microbiol.* **30**:911–921.
22. Fukata, M., K. S. Michelsen, R. Eri, L. S. Thomas, B. Hu, K. Lukasek, C. C. Nast, J. Lechago, R. Xu, Y. Naiki, A. Soliman, M. Arditi, and M. T. Abreu. 2005. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **288**:G1055–G1065.
23. Goncalves, N. S., M. Ghaem-Maghami, G. Monteleone, G. Frankel, G. Dougan, D. J. Lewis, C. P. Simmons, and T. T. MacDonald. 2001. Critical role for tumor necrosis factor alpha in controlling the number of luminal pathogenic bacteria and immunopathology in infectious colitis. *Infect. Immun.* **69**:6651–6659.
24. Hapfelmeier, S., B. Stecher, M. Barthel, M. Kremer, A. J. Muller, M. Heikenwalder, T. Stallmach, M. Hensel, K. Pfeffer, S. Akira, and W. D. Hardt. 2005. The *Salmonella* pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow *Salmonella* serovar Typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms. *J. Immunol.* **174**:1675–1685.
25. Hausmann, M., S. Kiessling, S. Mestermann, G. Webb, T. Spottl, T. Andus, J. Scholmerich, H. Herfarth, K. Ray, W. Falk, and G. Rogler. 2002. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. *Gastroenterology* **122**:1987–2000.
26. Hecht, G. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. VII. Enteropathogenic *Escherichia coli*: physiological alterations from an extracellular position. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**:G1–G7.
27. Hill, S. M., A. D. Phillips, and J. A. Walker-Smith. 1991. Enteropathogenic *Escherichia coli* and life threatening chronic diarrhoea. *Gut* **32**:154–158.
28. Kallas, M. R., F. R. Patricio, and U. Fagundes Neto. 1995. Morphometrics of the small intestine in children with diarrhea due to classical enteropathogenic *Escherichia coli* and to environmental asymptomatic enteropathy. *Rev. Assoc. Med. Bras.* **41**:162–166.
29. Kaper, J. B. 1998. Enterohemorrhagic *Escherichia coli*. *Curr. Opin. Microbiol.* **1**:103–108.
30. Luperchio, S. A., and D. B. Schauer. 2001. Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. *Microbes Infect.* **3**:333–340.
31. MacLean, L. L., and M. B. Perry. 2001. Structural studies on the O-polysaccharide of the lipopolysaccharide produced by *Citrobacter rodentium* (ATCC 51459). *Eur. J. Biochem.* **268**:5740–5746.
32. Mann, P. B., M. J. Kennett, and E. T. Harvill. 2004. Toll-like receptor 4 is critical to innate host defense in a murine model of bordetellosis. *J. Infect. Dis.* **189**:833–836.
33. Marriott, I., D. L. Gray, D. M. Rati, V. G. Fowler, Jr., M. E. Stryjewski, L. S. Levin, M. C. Hudson, and K. L. Bost. 2005. Osteoblasts produce monocyte chemoattractant protein-1 in a murine model of *Staphylococcus aureus* osteomyelitis and infected human bone tissue. *Bone* **37**:504–512.
34. Miyake, K. 2004. Endotoxin recognition molecules, Toll-like receptor 4-MD-2. *Semin. Immunol.* **16**:11–16.
35. Nataro, J., and J. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**:142–201.
36. O'Brien, A. D., E. S. Metcalf, and D. L. Rosenstreich. 1982. Defect in macrophage effector function confers *Salmonella typhimurium* susceptibility on C3H/HeJ mice. *Cell. Immunol.* **67**:325–333.
37. Perdomo, O. J., J. M. Cavaillon, M. Huerre, H. Ohayon, P. Gounon, and P. J. Sansonetti. 1994. Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. *J. Exp. Med.* **180**:1307–1319.
38. Philpott, D. J., S. Yamaoka, A. Israel, and P. J. Sansonetti. 2000. Invasive *Shigella flexneri* activates NF- κ B through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells. *J. Immunol.* **165**:903–914.
39. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**:2085–2088.
40. Pugin, J., C. C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc. Natl. Acad. Sci. USA* **90**:2744–2748.
41. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. *Cell* **118**:229–241.
42. Rescigno, M., M. Urbano, M. Rimoldi, B. Valzasina, G. Rotta, F. Granucci, and P. Ricciardi-Castagnoli. 2002. Toll-like receptor 4 is not required for the full maturation of dendritic cells or for the degradation of gram-negative bacteria. *Eur. J. Immunol.* **32**:2800–2806.
43. Ritchie, J. M., C. M. Thorpe, A. B. Rogers, and M. K. Waldor. 2003. Critical roles for *stx2*, *eae*, and *tir* in enterohemorrhagic *Escherichia coli*-induced diarrhea and intestinal inflammation in infant rabbits. *Infect. Immun.* **71**:7129–7139.
44. Sansonetti, P. J. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. III. Shigellosis: from symptoms to molecular pathogenesis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280**:G319–G323.
45. Sansonetti, P. J. 2001. Rupture, invasion and inflammatory destruction of the intestinal barrier by *Shigella*, making sense of prokaryote-eukaryote cross-talks. *FEMS Microbiol. Rev.* **25**:3–14.
46. Schauer, D. B., and S. Falkow. 1993. The *eae* gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. *Infect. Immun.* **61**:4654–4661.
47. Schilling, J. D., S. M. Martin, C. S. Hung, R. G. Lorenz, and S. J. Hultgren. 2003. Toll-like receptor 4 on stromal and hematopoietic cells mediates innate resistance to uropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **100**:4203–4208.
48. Simmons, C. P., S. Clare, M. Ghaem-Maghami, T. K. Uren, J. Rankin, A. Huett, R. Goldin, D. J. Lewis, T. T. MacDonald, R. A. Strugnell, G. Frankel, and G. Dougan. 2003. Central role for B lymphocytes and CD4⁺ T cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. *Infect. Immun.* **71**:5077–5086.
49. Simmons, C. P., N. S. Goncalves, M. Ghaem-Maghami, M. Bajaj-Elliott, S. Clare, B. Neves, G. Frankel, G. Dougan, and T. T. MacDonald. 2002. Impaired resistance and enhanced pathology during infection with a noninvasive, attaching-effacing enteric bacterial pathogen, *Citrobacter rodentium*, in mice lacking IL-12 or IFN- γ . *J. Immunol.* **168**:1804–1812.
50. Standiford, T. J., S. L. Kunkel, and R. M. Strieter. 1997. Role of chemokines in antibacterial host defense. *Methods Enzymol.* **288**:220–241.
51. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* **17**:1–14.
52. Tomson, F. L., V. K. Viswanathan, K. J. Kanack, R. P. Kanteti, K. V. Straub, M. Menet, J. B. Kaper, and G. Hecht. 2005. Enteropathogenic *Escherichia coli* EspG disrupts microtubules and in conjunction with Orf3 enhances perturbation of the tight junction barrier. *Mol. Microbiol.* **56**:447–464.
53. Vallance, B. A., W. Deng, M. De Grado, C. Chan, K. Jacobson, and B. B. Finlay. 2002. Modulation of inducible nitric oxide synthase expression by the attaching and effacing bacterial pathogen *Citrobacter rodentium* in infected mice. *Infect. Immun.* **70**:6424–6435.
54. Vallance, B. A., W. Deng, K. Jacobson, and B. B. Finlay. 2003. Host susceptibility to the attaching and effacing bacterial pathogen *Citrobacter rodentium*. *Infect. Immun.* **71**:3443–3453.
55. Vallance, B. A., W. Deng, L. A. Knodler, and B. B. Finlay. 2002. Mice lacking T and B lymphocytes develop transient colitis and crypt hyperplasia yet suffer impaired bacterial clearance during *Citrobacter rodentium* infection. *Infect. Immun.* **70**:2070–2081.
56. Vallance, B. A., and B. B. Finlay. 2000. Exploitation of host cells by enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97**:8799–8806.
57. Vazquez-Torres, A., B. A. Vallance, M. A. Bergman, B. B. Finlay, B. T. Cookson, J. Jones-Carson, and F. C. Fang. 2004. Toll-like receptor 4 dependence of innate and adaptive immunity to *Salmonella*: importance of the Kupffer cell network. *J. Immunol.* **172**:6202–6208.
58. Wiles, S., S. Clare, J. Harker, A. Huett, D. Young, G. Dougan, and G. Frankel. 2004. Organ specificity, colonization and clearance dynamics in vivo following oral challenges with the murine pathogen *Citrobacter rodentium*. *Cell Microbiol.* **6**:963–972.