Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis

Masayuki Fukata, Kathrin S. Michelsen, Rajaraman Eri, Lisa S. Thomas, Bing Hu, Katie Lukasek, Cynthia C. Nast, Juan Lechago, Ruliang Xu, Yoshikazu Naiki, Antoine Soliman, Moshe Arditi, and Maria T. Abreu. 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, 2005; doi:10.1152/ajpgi.00328.2004.—Inflammatory bowel disease (IBD) arises from a dysregulated mucosal immune response to commensal bacteria. Several lines of evidence support this model of perturbed host-microbial interactions. First, patients with IBD have immunologic reactivity to commensal flora or their products (8, 11, 28). Fecal diversion is effective in reducing intestinal inflammation (25, 45, 46), and treatment with antibiotics has a modest effect on improving disease activity (40). The first gene to be associated with Crohn’s disease (23, 37), CARD15/NOD2, encodes an intracellular pattern-recognition receptor (PRR) that recognizes bacteria-derived muramyl dipeptide (17, 24). Crohn’s disease-associated polymorphisms in the CARD15/NOD2 gene result in a protein that is impaired in its ability to activate NF-κB in response to peptidoglycan (7, 17, 24). These data collectively suggest that a defect in the innate immune response to commensal or pathogenic bacteria may culminate in chronic intestinal inflammation.

Thus far research has focused on the role of the adaptive immune response in the development of colitis. It is likely, however, that the innate immune response may be equally important, especially in the early phases of colitis. In mammalian cells, LPS and other bacterial products are recognized by a class of PRRs known as Toll-like receptors (TLRs) (6). TLR4 is required for recognition of LPS present in the cell wall of gram-negative bacteria. The interaction of TLR4, and its co-receptors CD14 and MD-2, with LPS results in the recruitment of the adapter molecule myeloid differentiation marker 88 (MyD88), phosphorylation of the IL-1 receptor-associated kinase, and recruitment of the adapter molecule TNF receptor-associated factor (TRAF)6 (30, 62). Recruitment of TRAF6 leads to the phosphorylation of IκB and release of NF-κB. An alternative MyD88-independent pathway through the adapter molecule Trif results in delayed NF-κB activation and interferon regulatory factor activation (15, 61). We (1, 2, 31) and others (34, 52) previously demonstrated that intestinal epithelial cells are hyporesponsive to the TLR4 ligand LPS and TLR2 ligands such as peptidoglycan and phenol-soluble modulin because of low expression of TLR4.

Given the requirement for bacteria in the initiation of IBD, we hypothesized that TLR signaling is an important link between luminal bacteria and intestinal inflammation. To test this hypothesis, we used the dextran sodium sulfate (DSS) model of murine colitis and asked whether TLR4 deficiency

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amelinates development of colitis. In this model, oral adminis-
istration of DSS-containing water results in acute colitis char-
acterized by epithelial injury and an acute inflammatory infil-
trate. Surprisingly, TLR4-deficient (TLR4−/−) mice develop
more bleeding compared with wild-type mice after DSS ex-
sposure. Histological examination of the colon revealed that
TLR4−/− mice have no neutrophilic infiltrate, whereas con-
trol mice have a dense neutrophilic infiltrate. An increase in
gram-negative bacterial translocation to mesenteric lymph
nodes (MLN) was seen in TLR4−/− mice more frequently
than wild-type littermates given DSS. Similar results were seen
in MyD88−/− mice. The reason underlying the decrease in
neutrophil recruitment is diminished chemokine production by
lamina propria macrophages and a diminished chemotactic
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 MATERIALS AND METHODS

Reagents. DSS, with a molecular weight of 36,000–50,000, was
obtained from ICN Biomedicals (Aurora, OH).

Animals. TLR4−/− mice and MyD88−/− mice were kindly
provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) and
backcrossed to C57BL/6 mice obtained from Jackson Laboratory
(Jackson Laboratory, Bar Harbor, ME). Control mice were derived
from the wild-type littermates derived from the backcrosses and
matched by gender. All animal experiments were approved by the
Cedars-Sinai Medical Center Institutional Animal Care and Use
Committee. Five- to eight-week-old mice were given 2.5% DSS in
their drinking water for 5 days. Animals were killed at the end of 5
days. For recovery studies, DSS was administered for the first 5 or 7
days, as indicated, and then DSS was removed from the drinking
water and animals were killed 7 days after cessation of DSS treatment.

Immunofluorescent and immunohistochemical studies. Colonic tis-
sues from the proximal and distal portion of the colon were freshly
isolated, and each was divided into two pieces. One piece was frozen
in optimum cutting temperature (OCT) compound with dry ice
(Sakura Finetek, Torrance, CA). The other was embedded in paraffin
wax after fixation with 10% neutral buffered formalin. First, paraffin
sections (4 μm thick) were stained with anti-CD68 to detect macroph-
ages. After paraffin was removed, sections were incubated with
0.1% trypsin (Sigma) dissolved in 0.05M Tris buffer pH 7.6 for 15 min
at 37°C. Subsequently, sections were blocked in 5% skim milk for 1 h
and then incubated with the rat anti-CD68 antibody (MCA1957S;
Serotec) overnight at 4°C. Sections were washed and incubated with
tetramethylrhodamine isothiocyanate-conjugated rabbit anti-rat IgG
as a secondary antibody for 1 h at room temperature. After being
washed with PBS, the sections were mounted in nonfluorescent
glycerol in 0.05 M Tris-HCl.

Double immunofluorescent staining of CD68 and macrophage
inflammatory protein (MIP)-2 was performed with frozen OCT com-
pound-embedded sections. Acetone-fixed cryosections (8 μm thick)
were stained with anti-CD68 as described above. Sections were then
incubated with the goat anti-MIP-2 antibody (R&D Systems) over-
night at 4°C, followed by reincubation with 5% skim milk. After
washing with PBS, sections were incubated with FITC-conjugated
rabbit anti-goat IgG F(ab’)2 (ICN Biomedicals) for 1 h at room
temperature. The working dilutions of the primary and secondary
antibodies for the CD68 staining were 1:20 and 1:150, respectively.
For the MIP-2 staining, the working dilutions of the primary and
secondary antibodies were both 1:100. The specificity of immuno-
activity was confirmed with appropriate isotype-matched controls.

The number of immunoreactive CD68-positive cells was counted in
three different high-power fields of the lamina propria and submu-
cosa. The average number of CD68-positive cells was then calculated
and compared between TLR4−/− or MyD88-knockout mice and their
respective wild-type controls. Next, the number of MIP-2-positive
cells/CD68-positive cells infiltrating the lamina propria and submu-
cosa was quantified with double-stained slides. Slides were viewed on
an Olympus BX51 immunofluorescence microscope, and photographs
were taken with a digital camera and the Magnalite 2.0 software
program.

The number of proliferating cells was detected by immunoperox-
idase staining for the thymidine analog bromodeoxyuridine (BrdU).
One and a half hours before death, mice (n = 10; 4 TLR4−/− mice
and 6 wild-type littermates from a minimum of 2 separate experi-
ments) were injected intraperitoneally with 5-bromo-2′-deoxyuridine
(Sigma) at a concentration of 100 mg/kg. Sections (4 μm) of paraffin-
embedded colonic tissue were deparaffinized and heated by micro-
wave for 5 min. Endogenous peroxidases were blocked with 3% H2O2
in methanol for 15 min. Sections were incubated with 2 N HCl for 1 h,
washed in PBS, and then incubated in 0.1% pepsin for 15 min at 37°C.
Nonspecific binding was blocked with 5% skim milk for 30 min.
Subsequently, sections were incubated with rat anti-BrdU (1:40;
Novus Biologicals) overnight at 4°C. The secondary antibody used
was biotinylated goat anti-rat IgG (1:150; Novus Biologicals) fol-
lowed by 1-h incubation of streptavidin-horseradish peroxidase (HRP
(Santa Cruz Biotechnology). The number of BrdU-positive cells per cel-
lar-orientated crypt were calculated in every three crypts for each
colon segment at high magnification under light microscopy. The
results are shown as means ± SE and compared between TLR4−/−
mice and wild-type littermates.

For bacterial translocation studies, colonic sections (n = 10; 4
TLR4−/− mice and 6 wild-type littermates from a minimum of 2
separate experiments) were stained with anti-Pseudomonas fluores-
cens MAb (1:10; Biogenesis). Paraffin-embedded colonic sections
were deparaffinized and blocked for both endogenous peroxidases
and nonspecific binding as described above. After overnight incubation
with anti-P. fluorescens MAb, HRP-conjugated protein A (1:1,000;
Upstate) was used as a substitute for a secondary antibody. Primary
antibody was omitted for negative control. HRP was then visualized
by incubation with 3,3′-diaminobenzidine, and sections were counter-
stained with 1% methyl green.

Bacterial cultures. MLN and blood were removed under sterile
conditions. MLN were prepared with a sterile grinder and then platted
onto sheep’s blood agar, MacConkey agar, chocolate agar, and thio-
glycollate broth. Blood was plated directly onto the medium. Cultures
were incubated at 35°C and examined at 24-h intervals for 3 days.
Any bacterial growth in the broth or on the agar plates was quantitated
and identified with standard microbiological methods. All gram-
negative bacilli were identified with the GNS card on the Vitek 2
Identification and Susceptibility System (bioMerieux, St. Louis, MO).
Only identifications with 99% probability were accepted.

Assessment of disease activity. Body weight was assessed at base-
line and every day for the duration of the experiment. Weight change
was calculated as percent change in weight compared with baseline.
Fecal blood was tested with Serocult cards (Propper Manufacturing,
Long Island City, NY) and graded as follows: 0 = no blood, 1 = trace
blood, 2 = positive, and 4 = gross blood. Assessment of inflammation
was performed by a pathologist masked to the mouse genotype and
treatment. The cecum was removed, and the remainder of the colon
was divided into proximal and distal halves. Tissue was fixed in 10%
buffered formalin, paraffin embedded, sectioned, and stained with
hematoxylin and eosin. Histological score was a combined score of
acute inflammatory cell infiltrate (0–4), chronic inflammatory cell
infiltrate (0–3), and crypt damage (0–4) (5, 9, 49). Specifically,
the crypt damage was scored in the following manner. A score of 0 was
given to an intact crypt, 1 = loss of the basal one-third of crypt, 2 =
loss of basal two-thirds of crypt, 3 = entire loss of crypt, and 4 = loss
of crypt and surface epithelium (9). Two slides from each section of

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the colon were assessed per mouse, and at least three areas on each slide were examined because the injury from DSS is patchy.

Bone marrow-derived macrophages. Bone marrow cells were flushed from femurs and tibias of mice with complete medium (DMEM, 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin and streptomycin) and washed three times with complete medium. Cells were cultured for 3 days in complete medium supplemented with murine granulocyte macrophage colony-stimulating factor (GM-CSF; Biosource International, Camarillo, CA). On day 3, adherent cells were fed with fresh GM-CSF-containing medium. Adherent cells were fed with complete DMEM on day 6 and used in experiments on day 9.

Bone marrow-derived macrophages were then harvested and seeded in 12-well plates at a concentration of 2 × 10⁵ cells/well in the absence of antibiotics. On the following day, 2 × 10⁵ P. fluorescens, which were obtained from the MLN culture from TLR4−/− mice, were added per well and cocultured for 6 h at 37°C. Supernatants were harvested and assessed for MIP-2 release with a murine MIP-2 ELISA kit according to the manufacturer’s instructions (R&D Systems).

Neutrophil chemotaxis assays. Polymorphonuclear neutrophils (PMN) were isolated from peripheral blood of TLR4−/− or wild-type mice by using 3% dextran in HBSS followed by density-gradient centrifugation with Ficoll-Paque. The remaining erythrocytes were removed by quick hypotonic lysis. After cell viability was checked by trypan blue, PMN were suspended in DMEM-1% endotoxin-free BSA-10 mM HEPES and placed in the upper insert of a 24-well Transwell chamber (5-μm pore size, 6.5-mm diameter; Costar Corning) at a concentration of 1 × 10⁶/100 μl. The same medium containing recombinant MIP-2 (600 μl; R&D Systems) was placed in the bottom well at a final concentration of 10 ng/ml. Alternatively, the supernatant (harvested as above) from bone marrow-derived macrophages from TLR4−/− or wild-type mice stimulated with P. fluorescens was also applied to the bottom well. After 45-min incubation at 37°C in 5% CO₂, the cells in the bottom well were harvested by adding 50 μl of 70 mM EDTA and counted with a hemocytometer.

Statistical analysis. Student’s t-tests, Fisher’s exact test, and standard deviations were performed with the statistics package within Microsoft Excel. Mann-Whitney U-test and standard error were calculated with StatView, Kaplan-Meier and log rank tests were used for comparisons of mortality. P values were considered significant when <0.05.

RESULTS

TLR4 and MyD88 are required for neutrophil recruitment in DSS-induced colitis. Colitis induced by DSS is characterized by epithelial damage and a neutrophilic infiltrate (9). We examined the cecum, proximal colon, and distal colon for degree of histological damage with respect to crypt epithelial damage, acute inflammatory cell infiltrate, and chronic inflammatory cell infiltrate (Fig. 1A). Although control mice and TLR4−/− mice had similar degrees of DSS-induced crypt epithelial damage (Fig. 1, A and C), TLR4−/− mice had significantly less acute inflammatory cell infiltrate (Fig. 1). Specifically, TLR4−/− mice have significantly fewer neutrophils in the lamina propria and submucosa compared with control mice. Peripheral blood neutrophils are normal in number in TLR4−/− mice (3, 4). Similar results were seen with MyD88−/− mice, suggesting that TLR4 signaling to MyD88 is required for neutrophil recruitment to the damaged mucosa.

To address the time point of maximal injury, DSS treatment was continued for a total of 7 days followed by 7 days without DSS, i.e., recovery. The degree of epithelial damage was most severe between days 8 and 11 and was minimal by day 14 (Fig. 2A). A spectrum of injury from frank ulceration (grade 3–4) to minimal damage (grade 1) was seen in all animals. The longitudinal extent of injury per tissue section was also similar. These results were evaluated by two independent gastrointestinal pathologists masked to mouse genotype and treatment protocol. Despite similarities in the degree and extent of epithelial damage, TLR4−/− and MyD88−/− mice had decreased acute inflammatory infiltrate, Rakoff-Nahoum et al. (42) recently showed increased epithelial injury in TLR4−/− and MyD88−/− mice in the setting of reduced epithelial proliferation. Consistent with their data, we find a decrease in epithelial proliferation in TLR4−/− and MyD88−/− mice after DSS injury (Fig. 2, B and C). These changes in proliferative rate, however, do not seem to underlie the degree of acute epithelial damage in response to DSS, which is similar for all animals.

TLR4−/− and MyD88−/− mice have more severe bleeding compared with control mice. Given the absence of an acute inflammatory infiltrate, we hypothesized that TLR4−/− mice and MyD88−/− mice would have similar or reduced signs of colitis compared with control mice. Interestingly, the amount of rectal bleeding was significantly greater and occurred earlier in TLR4−/− mice and MyD88−/− mice compared with control mice (Fig. 3A). These differences became significant by day 2. All TLR4−/− mice had gross blood in the stool by day 4 of DSS compared with only 25% of the control mice (Fig. 3C). For MyD88−/− mice, all mice had gross blood by day 3 compared with only one of eight wild-type littermate controls. One MyD88−/− mouse who was having severe bleeding succumbed on day 3. By day 5, all mice had gross blood in the stool. These observations were mirrored in significantly lower hemoglobin in TLR4−/− and MyD88−/− mice compared with wild-type mice (TLR4−/− = 5.6 g/dl compared with wild-type = 11.2 g/dl; MyD88−/− = 3.4 g/dl compared with wild-type = 8.3 g/dl). Differences in weight between TLR4−/− mice or MyD88−/− mice and control mice were not significant (Fig. 3B). When DSS is continued for a total of 7 days followed by 7 days of recovery, all mice lose weight between days 8 and 11 but MyD88−/− mice have increased mortality at days 8–10 (mortality MyD88−/− 100% vs. wild type 25% or TLR4−/− 20%; P = 0.03 for MyD88−/− compared with wild type). At the time of death, the average hemoglobin in MyD88−/− mice was 5.2 g/dl, suggesting that blood loss contributed to mortality. These data suggest that TLR4−/− and MyD88−/− mice have greater clinical signs of colitis compared with control mice. The increased mortality seen in MyD88−/− mice compared with TLR4−/− mice suggests that the MyD88 pathway may play a broader protective role in colonic injury.

TLR4-deficient mice have increased bacterial translocation compared with control mice. We reasoned that the impairment in neutrophil recruitment to the lamina propria of DSS-treated TLR4−/− and MyD88−/− mice might result in diminished bacterial clearance from the lamina propria and increased bacterial translocation. The first observation we made was that MLN from TLR4−/− mice appeared larger than those from wild-type mice. Indeed, the average weight of MLN from TLR4−/− was more than twice the weight of MLN from wild-type mice (TLR4−/− mice = 39.9 (SD 12.4) mg and wild-type mice = 16.7 (SD 10.8) mg; P = 0.005). No differences were seen in splenic weight after DSS treatment (TLR4−/− mice = 93.0 (SD 20.8) mg and wild-type mice =
96.4 (SD 16.8) mg; \( P = 0.4 \), suggesting an intestine-specific event. MLN weights were similar in all mice before DSS (data not shown). Cultures of MLN revealed the presence of gram-negative enteric bacteria, especially *Escherichia coli* and *P. fluorescens*, more frequently in TLR4\(^{-/-}\)/MyD88\(^{-/-}\) mice compared with wild-type mice (Table 1). These species of bacteria were also cultured from the stool of these mice, suggesting that they originated from the intestinal lumen. Finally, direct staining of intestinal tissue with an antibody that recognizes *P. fluorescens* demonstrated the presence of gram-negative rods deep in the epithelial layer in TLR4\(^{-/-}\)/MyD88\(^{-/-}\) mice but only on the apical surface in the mucus layer of wild-type mice.

Fig. 1. Histology in Toll-like receptor-4-null (TLR4\(^{-/-}\)) and myeloid differentiation marker 88-null (MyD88\(^{-/-}\)) mice after dextran sodium sulfate (DSS)-induced colitis. A: histology was assessed with respect to acute inflammatory infiltrate, chronic inflammatory infiltrate, and crypt epithelial damage on a scale of 0–3 in the cecum, proximal colon, and distal colon of TLR4\(^{-/-}\) mice \((n = 6)\), MyD88\(^{-/-}\) mice \((n = 5)\), and wild-type littermate control mice \((n = 9)\) as indicated. Standard error is shown. \(* P < 0.05\). B: polymorphonuclear neutrophil (PMN) counts were performed by a pathologist masked to mouse treatment or genotype. PMN per high-power field (HPF) were counted in the cecum, proximal colon, and distal colon of TLR4\(^{-/-}\) mice \((n = 6)\), MyD88\(^{-/-}\) mice \((n = 5)\), and wild-type littermate controls \((n = 9)\). TLR4\(^{-/-}\) and MyD88\(^{-/-}\) mice have significantly fewer PMN compared with wild-type littermate controls \((n = 9)\). Standard error is shown. \(* P < 0.05\). C: photomicrographs of representative areas from TLR4\(^{-/-}\), MyD88\(^{-/-}\), and wild-type littermate controls as indicated. Arrows highlight PMN infiltrate (original magnifications are \( \times 200 \) at left, \( \times 400 \) at right).
mice (Fig. 4). These data suggest that TLR4 is required to limit bacterial translocation after intestinal epithelial injury.

**TLR4-deficient macrophages have defective MIP-2 production in response to Pseudomonas stimulation.** Neutrophils are recruited to the site of bacterial invasion through the local expression of chemokines that results in neutrophil chemotaxis (39). To understand the mechanism of decreased neutrophil recruitment in TLR4−/− and MyD88−/− mice, we first asked whether macrophage numbers were decreased and whether expression of the neutrophil chemoattractant MIP-2 was reduced. Our findings demonstrate that CD68-positive macrophages are only modestly but significantly reduced in TLR4−/− and MyD88−/− mice compared with their littermate controls (Fig. 5, A and C). The more dramatic difference,
However, was in the expression of MIP-2. Whereas nearly all the CD68-positive cells in DSS-treated wild-type mice expressed MIP-2, <20% of CD68-positive cells from TLR4−/− or MyD88−/− mice expressed MIP-2 (Fig. 5, B and D). These data suggest that diminished expression of chemokines in the intestinal mucosa contributes to the paucity of neutrophils recruited to the site of intestinal epithelial injury.

In addition to decreased expression of MIP-2, we hypothesized that the decrease in neutrophil infiltrate was due to a defect in the neutrophil’s response to a chemotactic stimulus. To test this hypothesis, we added peripheral blood neutrophils from TLR4−/− or wild-type mice to the top well of a Transwell plate. The bottom well contained supernatant from bone marrow-derived macrophages from TLR4−/− or wild-type mice stimulated with P. fluorescens or medium with recombinant MIP-2 (Fig. 5E). Wild-type neutrophils had the highest level of chemotaxis in response to recombinant MIP-2 or supernatant from wild-type macrophages cocultured with P. fluorescens isolated from MLN cultures of TLR4−/− mice. Chemotaxis was diminished, however, in the presence of supernatant from TLR4−/− macrophages cocultured with P. fluorescens. These data suggest that indeed TLR4−/− macrophages do not provide the full complement of chemokines in response to a bacterial challenge. Neutrophils derived from TLR4−/− mice had significantly diminished chemotaxis in response to supernatant from either wild-type or TLR4−/− macrophages cocultured with P. fluorescens. In contrast, they were not impaired with respect to their response to recombinant MIP-2. These data suggest that, at physiological local concentrations of chemo-

Table 1. Mesenteric lymph nodes: microbiological results

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<th>TLR4−/−</th>
<th>MyD88−/−</th>
<th>Wild-Type Control</th>
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<tr>
<td>Rate of positive culture in MLN, %</td>
<td>100 (4/4)</td>
<td>100 (4/4)</td>
<td>10^4 (1/10)</td>
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<tr>
<td>Species of bacteria cultured in MLN</td>
<td>Pseudomonas fluorescens, Escherichia coli</td>
<td>E. coli</td>
<td>E. coli</td>
</tr>
<tr>
<td>Amount of bacteria cultured in 24 h, CFU/mg</td>
<td>&gt;10^6</td>
<td>7.7 × 10^4 ± 2.5 × 10^4</td>
<td>&lt;10^4</td>
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MLN, mesenteric lymph nodes; CFU, colony-forming units; TLR, Toll-like receptor; MyD88, myeloid differentiation marker 88. Mean ± SE amount of bacteria is shown for MyD88−/−. *P = 0.02 compared with TLR4−/−, MyD88−/− (Fisher’s exact test); †P < 0.001 compared with TLR4−/−, MyD88−/− (t-test).
advantage of the presence of bacteria as well as protect against DSS-induced colitis (19, 43). Thus these historical data support the use of the DSS model to query the role of the innate immune response in the development of acute colitis.

Controversy exists with respect to the role of TLR4 in the development of murine colitis. Older studies had found that C3H/HeJ mice with a spontaneous mutation in the TLR4 gene have a response to DSS similar to that of their congenic, LPS-sensitive controls (50). Others found, however, that LPS-sensitive mice have worse colitis compared with LPS-resistant mice (29). A recent study used C3H-Tlr4+/− and TLR2−/− null mutants to examine the role of TLR4 and TLR2 in the development of acute colitis. This work elegantly demonstrated that introduction of enteric bacteria into germ-free mice activates a complex and reproducible genetic program by intestinal epithelial cells characterized by the expression of nutrient- and transport-related genes. Below the layer of lining epithelial cells lie immune cells consisting of lamina propria macrophages, B cells, T cells, and dendritic cells. All these cell types may be important in the response to injury and prevention of bacterial translocation when natural or unnatural breaks occur in the epithelial lining.

In this study, we examined the role of TLR4 and its downstream adapter molecule MyD88 in an animal model of acute colitis. We chose the DSS model of colitis for several reasons. We wished to examine the role of TLR4 signaling in the early stages of colitis when the innate immune response may be more relevant. This model does not depend on the adaptive immune system, given that immunodeficient mice are susceptible to DSS injury (10). Antibiotics can ameliorate the severity of DSS-induced colitis (19, 43). Thus these historical data support the use of the DSS model to query the role of the innate immune response in the development of acute colitis.

Finally, to directly test the ability of macrophages from TLR4−/− mice to generate MIP-2, we cultured bone marrow-derived macrophages from TLR4−/− mice or wild-type littermates in the presence of P. fluorescens as described above and measured MIP-2 production (Fig. 5F). Wild-type macrophages produced significantly more MIP-2 than TLR4−/− macrophages. These data together demonstrate that the defect in neutrophil recruitment to the lamina propria of DSS-treated TLR4-deficient mice is largely due to diminished chemokine production by TLR4-deficient macrophages, especially MIP-2.

**DISCUSSION**

The intestinal immune system has developed under the dual pressure of protecting the host from pathogenic infections and coexisting with the myriad commensal organisms in the lumen. These same commensal bacteria elicit a potent proinflammatory response across other mucosal surfaces such as the lung and bladder. Because of its coexistence with bacteria, the intestine has developed a variety of mechanisms that both take advantage of the presence of bacteria as well as protect against their possible invasion. The work of Hooper et al. (21, 22) has elegantly demonstrated that introduction of enteric bacteria into germ-free mice activates a complex and reproducible genetic program by intestinal epithelial cells characterized by the expression of nutrient- and transport-related genes. Below the layer of lining epithelial cells lie immune cells consisting of lamina propria macrophages, B cells, T cells, and dendritic cells. All these cell types may be important in the response to injury and prevention of bacterial translocation when natural or unnatural breaks occur in the epithelial lining.

During review of the present article, Rakoff-Nahoum et al. (42) described similar findings with a DSS model of colitis in TLR4−/−, TLR2−/−, and MyD88−/− mice. Namely, they found increased bleeding and mortality in MyD88−/− mice with intermediate degrees in TLR4−/− and TLR2−/− mice. They attribute the increased bleeding and mortality to increased epithelial damage due to decreased epithelial proliferation. Colitis in response to DSS is classically characterized by epithelial damage and the presence of an acute and chronic inflammatory infiltrate (9). Indeed, DSS induces apoptosis of intestinal epithelial cells (58) and has been shown to decrease proliferation as measured by BrdU incorporation (54). Prior studies demonstrated that administration of LPS protects against radiation-induced damage in the intestine (44). In our study, we graded epithelial damage according to the method described by Cooper et al. (9), in which the degree of crypt and surface epithelial loss is rated on a scale from 0 to 4 (see MATERIALS AND METHODS). With this scoring system and two independent gastrointestinal pathologists at two institutions, there was no apparent difference between crypt epithelial damage (Figs. 1, A and C, and 2A) when the patchy nature of the damage caused by DSS is taken into account. It should be noted that we used a higher concentration of DSS, 2.5% (vs.
2%), and a different scoring system than Rakoff-Nahoum et al., which may result in differences in the outcome. Like Rakoff-Nahoum et al., we found that epithelial proliferation was reduced in TLR4−/− mice (Fig. 2, B and C). These data suggest that changes in proliferation rate are unlikely to explain the severity of epithelial injury in the acute setting but may result in delayed recovery in knockout mice. In addition, the amount of colonic blood loss is likely to be multifactorial, including mucosal injury, hemostatic defects, and possibly erythropoietic defects.
Rakoff-Nahoum et al. (42) tested the hypothesis that mortality in DSS-treated MyD88−/− mice was due to bacteremia by culturing spleens and treating mice with broad-spectrum antibiotics. Bacteremia was not found, and antibiotics did not reduce mortality in their study. We also did not find systemic bacteremia but found the presence of bacteria in MLN, which may be relevant in the pathogenesis of early IBD. In our study we found a striking paucity of neutrophils recruited to the site of epithelial damage. Similar results have been seen in TLR4−/− mice in models of bacterial pneumonia or cystitis (3, 47). In the Rakoff-Nahoum et al. study, the results are reported as “infiltrating leukocyte score” and not broken down by acute (PMN) vs. chronic (lymphocytes), which may account for the differences in our findings.

Among the novel observations in our study is the presence of gram-negative bacteria in the MLN of DSS-treated TLR4−/− and MyD88−/− mice. We believe that the absence of a neutrophilic infiltrate is an important contributor to the escape of bacteria from the mucosa. MIP-2 was previously shown to recruit neutrophils to the intestinal mucosa (38). MIP-2 expression by monocytes and macrophages is regulated through TLR signaling (13, 59). Decreased expression of MIP-2 by intestinal macrophages and P. fluorescens-stimulated macrophages in our model provides one mechanistic explanation for the observed decrease in neutrophil recruitment to the colon. Previous studies showed that neutrophils from TLR4-deficient mice are poorly responsive to MIP-2-induced chemotaxis (13). In our system, the decrease in chemotaxis of TLR4−/− neutrophils in response to supernatants from wild-type macrophages demonstrates that chemotaxis in response to local, physiological concentrations of MIP-2 and other chemokines is reduced. Furthermore, we used a relevant stimulus for macrophage activation, namely, the very bacteria, P. fluorescens, recovered from cultures of MLN in our TLR4−/− mice. Finally, Ogawa et al. (36) demonstrated that human intestinal microvascular endothelial cells express TLR4 and respond to LPS with increased expression of adhesion molecules, e.g., ICAM-1 and VCAM-1. Thus several mechanisms may be at play in both immune and nonimmune cells to explain the phenotype of decreased neutrophil recruitment.

Neutrophils play several roles at the site of epithelial injury. They are essential for clearance of bacteria through phagocytosis and lysis of bacteria with potent bactericidal proteins and reactive oxygen species (14, 27). Macrophage engulfment of apoptotic neutrophils is required for wound healing (20, 32, 53). Neutrophils have also been demonstrated to express hepatocyte growth factor, which can aid in tissue regeneration (18). Our observation of perturbed mucosal barrier function in TLR4−/− and MyD88−/− mice may be directly or indirectly related to defective neutrophil recruitment. Another contributor to the decrease in proliferative response of the epithelium to damage may be more directly related to TLR4. In addition to recognition of LPS, TLR4 recognizes heat shock proteins, which may be released by dead or dying intestinal epithelial cells (56). Thus, in addition to impaired TLR4 signaling in response to luminal pathogen-associated molecular patterns in our model, there may also be impaired signaling in response to necrotic debris caused by DSS and delayed epithelial healing.

At first blush the finding that a decrease in the acute inflammatory response bodes a worse outcome may seem counterintuitive; however, there are important parallels between our findings and human IBD. Patients with IBD are known to have an inappropriate adaptive immune response to the microflora (11). These responses can be assessed clinically through serological markers that measure antibody responses to commensal bacterial or yeast products (33). Antibodies against oligomannans of Saccharomyces cerevisiae (ASCA) are associated with Crohn’s disease, and their levels correlate with aggressive small bowel disease (33, 57). A recent study has found that low levels of mannan-binding lectin, a pathogen recognition receptor, is associated with ASCA positivity in patients with Crohn’s disease, suggesting that a defect in clearing yeast may play a role in Crohn’s disease pathogenesis (48). In our model, TLR4−/− and MyD88−/− mice frequently had P. fluorescens isolated from MLN. Studies from the laboratory of Jonathan Braun (51, 60) have demonstrated that P. fluorescens DNA can be isolated from the lamina propria mononuclear cells of patients with Crohn’s disease and that patients with Crohn’s disease frequently have antibodies against a pseudomonal protein sequence, I2. The first gene to be identified as a disease-susceptibility gene in Crohn’s disease is CARD15/NOD2, an intracellular PRR (23, 37). Crohn’s disease-associated polymorphisms in the CARD15/NOD2 gene result in a protein that is impaired in its ability to activate NF-κB in response to peptidoglycan (7, 17, 24). The exact mechanism by which an impaired response to bacteria can culminate in chronic intestinal inflammation is unclear, although a recent study describes that CARD15/NOD2 may normally dampen TLR2-mediated signals (35). These data demonstrate that patients with IBD, especially Crohn’s disease,

Fig. 5. Macrophage infiltrate and macrophage inflammatory protein (MIP)-2 expression is decreased in TLR4−/− and MyD88−/− compared with wild-type littermate controls. A: CD68 staining of intestinal macrophages in TLR4−/− and MyD88−/− compared with wild-type littermate controls (n = 4, 2 each). Arrows indicate positive cells. C: CD68-positive cells per HPF were counted in proximal colon and distal colon in mice. Significantly fewer CD68-positive cells were seen in TLR4−/− (n = 4) or MyD88−/− (n = 4) compared with wild-type littermate controls (n = 8, 4 each). Standard error bars are shown. *P < 0.05. D: % of CD68-positive cells that also express MIP-2 is significantly reduced in TLR4−/− and MyD88−/− mice compared with littermate controls. MIP-2-positive cells and CD68-positive cells per HPF were counted in proximal colon and distal colon in mice. Standard deviation is shown. *P < 0.05. E: TLR4-deficient neutrophils have impaired chemotaxis compared with wild-type neutrophils. Bone marrow-derived macrophages (Md) were generated from TLR4−/− or wild-type mice as described in MATERIALS AND METHODS. Macrophages were then cultured in the presence of P. fluorescens derived from mesenteric lymph nodes. The supernatant derived from these macrophage cultures was used in a neutrophil chemotaxis assay. TLR4−/− or wild-type neutrophils were added to the upper well of a Transwell, and supernatant from either TLR4−/− or wild-type macrophages was added to bottom well. As a positive control we used recombinant (r) MIP-2 (10 ng/ml), and as a negative control we used medium alone. The number of cells migrating to the bottom well was counted 6 h after addition of the supernatants. Neutrophils from TLR4−/− mice have a reduced capacity to migrate in response to supernatant from wild-type, bacterially stimulated macrophages (2nd set of bars). In addition, supernatant derived from TLR4−/− macrophages has a diminished capacity to chemotact wild-type and TLR4−/− neutrophils (3rd set of bars) *P < 0.05. F: MIP-2 production is decreased from TLR4−/− macrophages. MIP-2 levels were measured in the supernatants of macrophages from TLR4−/− (n = 2) or wild-type mice (n = 2) cultured in the presence of P. fluorescens. A high-sensitivity ELISA for MIP-2 was performed. Data are expressed as averages with standard deviation. MIP-2 production is significantly reduced in TLR4−/− macrophages. *P < 0.05.
may develop inappropriate adaptive immune responses to commensal organisms and that the primary defect may lie in a failure of the innate immune response.

On the basis of our data, we propose that TLR4 signaling to MyD88 in the intestine is important for recruitment of neutrophils, control of bacterial translocation, and triggering repair of the intestinal epithelium. This defect in mounting an appropriate acute response to a breach in the epithelial barrier may provide the groundwork for chronic adaptive immune responses to luminal bacteria. Future studies will examine the relative contributions of immune vs. nonimmune TLR4-expressing cells in this response.

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