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TLR Signaling Mediated by MyD88 Is Required for a Protective Innate Immune Response by Neutrophils to *Citrobacter rodentium*  

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Enteropathogenic *Escherichia coli*, enterohemorrhagic *E. coli*, and *Citrobacter rodentium* are classified as attaching and effacing pathogens based on their ability to adhere to intestinal epithelium via actin-filled membranous protrusions (pedestals). Infection of mice with *C. rodentium* causes breach of the colonic epithelial barrier, a vigorous Th1 inflammatory response, and colitis. Ultimately, an adaptive immune response leads to clearance of the bacteria. Whereas much is known about the adaptive response to *C. rodentium*, the role of the innate immune response remains unclear. In this study, we demonstrate for the first time that the TLR adaptor MyD88 is essential for survival and optimal immunity following infection. MyD88−/− mice suffer from bacteremia, gangrenous mucosal necrosis, severe colitis, and death following infection. Although an adaptive response occurs, MyD88-dependent signaling is necessary for efficient clearance of the pathogen. Based on reciprocal bone marrow transplants in conjunction with assessment of intestinal mucosal pathology, repair, and cytokine production, our findings suggest a model in which TLR signaling in hemopoietic and nonhemopoietic cells mediate three distinct processes: 1) induction of an epithelial repair response that maintains the protective barrier and limits access of bacteria to the lamina propria; 2) production of KC or other chemokines that attract neutrophils and thus facilitate killing of bacteria; and 3) efficient activation of an adaptive response that facilitates Ab-mediated clearance of the infection. Taken together, these experiments provide evidence for a protective role of innate immune signaling in infections caused by attaching and effacing pathogens. *The Journal of Immunology*, 2007, 179: 566–577.

Pathogenic strains of *Escherichia coli*, including enteropathogenic *E. coli* (EPEC)4 and enterohemorrhagic *E. coli* (EHEC), pose a significant public health risk especially in developing countries where they contaminate food and water supplies. EPEC causes infantile diarrhea (1), and the infection leads to dehydration and death in 25–70% of infected infants (2). EHEC infections cause hemorrhagic colitis and hemolytic-uremic syndrome, a potentially fatal disease (3, 4). EPEC, EHEC, and a closely related mouse pathogen *Citrobacter rodentium* are classified as attaching and effacing (A/E) pathogens based on their ability to adhere to intestinal epithelium, destroy microvilli, and induce actin filled membranous protrusions, “pedestals” at the site of attachment. Pedestal formation is associated with the formation of A/E lesions, breach of the epithelial barrier by the bacteria, and development of disease (5, 6).

Upon infection, A/E pathogens displace the commensal flora and cause intestinal inflammation characterized by crypt hyperplasia, goblet cell depletion, and damage to the epithelium (7). Additionally, infection with these pathogens induces infiltration of immune cells and edema within the lamina propria. An influx of neutrophils is associated with the formation of abundant crypt abscesses (8). Much of the pathology observed in response to A/E pathogens appears to result from a deleterious host response to the bacteria following breach of epithelial barrier; thus, administration of heat killed *C. rodentium* to mice with permeabilized colons has been shown to result in inflammatory disease nearly identical to that seen with live bacteria (8). Ultimately, an Ab response is necessary for clearance of the bacteria (9). Therefore, although host immune response to infection with *C. rodentium* results in a destructive colitis, it also serves to protect mice.

Although much is known about the etiology of A/E pathogen infections, the induction of the host immunity particularly as it relates to controlling the balance between protective and destructive responses is less well understood. To identify signaling cascades responsible for such immune responses to A/E pathogens, we considered the involvement of TLRs which are highly conserved type-I transmembrane proteins containing leucine rich repeats and a conserved Toll/IL-1R domain. TLRs recognize and respond to conserved motifs associated with microbes, which include proteins (e.g., flagellin), lipids (e.g., LPS), and nucleic acids.
(e.g., Cpg DNA). Signaling cascades initiated by engagement of TLRs with their ligands requires many adaptor proteins, including MyD88, Toll-IL-1R domain-containing adaptor protein/MyD88 adaptor-like (TIRAP/Mal), TIR domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). TLR signaling pathways ultimately lead to activation of transcription factors (e.g., NF-κB and IRF-3), which regulate production of cytokines and chemokines (e.g., IL-6, IFN-γ, TNF-α, IL-8, etc.). Most of these cytokines are strongly induced during an infection with A/E pathogens (8, 11).

Although most studies on TLR signaling have focused on cytokine responses to particular TLR ligands, several recent studies have described a role for MyD88-dependent signaling in detecting intact viruses (12), parasites (13), pathogenic bacteria (14, 15), and even commensal bacteria present in the intestine (16, 17). In these latter studies, dextran sodium sulfate (DSS) was used to induce epithelial injury and, as a consequence, acute colitis that resembles that seen in patients with inflammatory bowel disease. Notably, DSS-induced colitis was exacerbated in the absence of MyD88 and TLR4. Thus, TLR signaling appears to be required for innate immune responses to intestinal injury. However, how TLR signaling contributes to containment of pathogens within the intestinal tract is less clear (16, 17).

Several lines of evidence suggest that C. rodentium may provide an ideal system for evaluating how TLR signaling and the innate immune response contribute to intestinal inflammation and prevents dissemination of the bacteria of the colon. C. rodentium efficiently out-competes commensal strains in the intestine and can easily be cultured ex vivo. Recently, Khan et al. (18) have provided evidence that TLR4 signaling contributes to inflammation induced by C. rodentium. In this study, we show that MyD88-dependent signaling contributes to intestinal inflammation in response to C. rodentium and is required to protect the host from a disseminated infection. Moreover, we provide evidence that these effects are mediated by both nonhemopoietic and hemopoietic cells, and in particular neutrophils. Thus, although TLR signaling via MyD88 mediates a deleterious inflammatory response following infection, such signaling is also required for limiting the level of bacterial colonization throughout the body and for facilitating timely clearance. Experiments presented here may provide important information on means to mollify the destructive responses while facilitating protective ones.

Materials and Methods

Mouse strains and breeding

MyD88−/− mice on a C57BL/6 background (21) were the gift of D. Ungerhill (Institute for Systems Biology, Seattle, WA) and were originally generated in the lab of S. Akira (22). TIRAP−/− mice (C57BL/6-Tg(ACTB-EGFP)10sb/J). Animal care and use were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Emory University.

In vivo infections

C. rodentium (American Type Culture Collection, ATCC 31002) were prepared by overnight culturing (12–16 h) at 37°C in Luria-Bertani broth (BD Biosciences) without shaking. Cultures were harvested by centrifugation and resuspended in 20% sucrose distilled water. For infections of mice, food was withdrawn and drinking water was replaced with C. rodentium suspension overnight. Volume of suspension was measured before and after administration and the number of bacteria in the inoculum was calculated following retrospective plating. The average dosage was 4 × 108 CFU/mouse. Survival of infected mice and changes in body weight were monitored daily. Mice losing >15% of their original weight were euthanized. For immunization studies, drinking water was supplemented with a neomycin sulfate/polyoxymyxin B sulfate mixture (Sigma-Aldrich) 3 days after infection. Clearance of C. rodentium was assessed by measuring the number of colonies in fecal samples. Four weeks after initial infection, mice were reinfected with 4 × 109 CFU of C. rodentium.

Histology

For histology studies, colons, livers, and spleens were removed from uninfected or infected mice, fixed in 10% formalin, and embedded in paraffin. Sections (5 μm) were cut and stained with H&E by the Translational Research Lab at Emory University. Crypt height was measured by micrometry using a Zeiss 200M microscope, a ×20 NA1.4 lens and Slidebook software (Intelligent Imaging Innovations). Longitudinal sections, which displayed the entire lengths of crypts, were used for measurements. Two measurements were made on each image, and one image was taken per colon. The number of samples (n) refers to the number of colons measured rather than the number of measurements. Intestinal histopathology was assessed microscopically by one of us (C. A. Parkos). Samples were coded and the observations made in a blind fashion to avoid bias in the evaluation process.

TUNEL assay staining and scoring

To access level of apoptosis in colonic tissue, sections (5 μm) were cut from the same paraffin blocks described above and stained using an Apoptag Fluorescein In Situ Apoptosis Detection Kit (S1710) (Millipore) according to the manufacturer’s instructions. Briefly, the tissue was digested with TdT enzyme and stained with α-digoxigenin conjugated to fluorescein and 4',6-diamidino-2-phenylindole. The intensity of fluorescein staining was assessed from digital images using a Nikon Eclipse 80i microscope, a ×20 N.A. 0.75 objective and Spot software (Diagnostic Instruments). Longitudinal sections, which displayed the entire lengths of crypts, were used for measurements. Samples were coded and the observations made in a blind fashion to avoid bias in the evaluation process. Samples were given a score of 0, 1, 2, or 3, where a low score represented minimal TUNEL staining and a high score extensive staining.

 Colony counts of C. rodentium

To measure the CFU of C. rodentium, tissue samples of colon, liver, spleen, or mesenteric lymph node (MLN) weighing ~0.1–0.3 g were homogenized at low speed with a Tissuemizer (Fisher Scientific) in 1 ml of PBS. The lysate was plated on MacConkey agar plates at various dilutions, and C. rodentium colonies were recognized as pink with a white rim as described previously (25). Pink colonies were confirmed as C. rodentium by PCR with Translocated Intimin Receptor Toll-IL-1R-specific primers (25). Pink colonies were counted after 20 h of incubation at 37°C to determine the CFU per gram of tissue.

Passive immunization

To generate immune serum, C57BL/6 or MyD88−/− mice were infected with 4 × 108 CFU/mouse C. rodentium and rescued with antibiotic treatment as described above. Serum was harvested 3 or 4 wk postinfection (p.i.) from each strain. Samples from each strain were pooled and used to passively immunize naive μMT mice. To do this, naive μMT mice were infected with C. rodentium and injected daily i.p. on days 4–7 and 11–14 p.i. with 40 μl of serum from infected MyD88−/− or WT mice or serum from uninfected WT mice, as described previously (9).

ELISA analysis

For isotype analysis, Ab titers in sera were determined by ELISA. C. rodentium-specific IgG1, IgG2a, and IgG2b Abs were measured as described previously (25). Sandwich ELISA kits were used according to the specifications of the manufacturer to measure levels of IL-6 (BD Biosciences) and KC (BioSource International) in supernatant derived from colon or other tissues.

Bone marrow transplants

MyD88−/−→WT chimeric mice were generated essentially as described previously (26). Briefly, C57BL/6 recipient mice were irradiated in two sessions, separated by 3 h, for a total dosage of 11 Gy. Bone marrow from donor mice (3 × 106 cells in 200 μl of PBS) was injected into the tail vein the following day. Because MyD88−/− mice are more susceptible to infection by opportunistic pathogens, MyD88−/− recipients received a single dose of irradiation of 6 Gy. For 2 wk following the transplant, drinking water was replaced with water containing neomycin sulfate/polyoxymyxin B sulfate mixture (Sigma-Aldrich). To facilitate confirmation of reconstitution in sublethally irradiated animals, mice received WT marrow from...
C57BL/6 mice with GFP-labeled β-actin. Twelve weeks after reconstitution, blood samples were taken via tail or eye bleed. To confirm reconstitution, lymphocytes were isolated through centrifugation over Histopaque-1077 (Sigma-Aldrich) (27) from whole blood and were identified by GFP fluorescence. Based on flow cytometry measurements, donor marrow comprised 95.93% of the peripheral white blood cells.

**Myeloperoxidase (MPO) assays**

Tissue samples from colon weighing −0.2–0.3 g were homogenized in ice-cold potassium phosphate buffer (50 mM K2HPO4 and 50 mM KH2PO4 (pH 6.0)) containing hexadecyltrimethylammonium bromide (0.5% v/v, Sigma-Aldrich). The homogenates were then subjected to three freeze-thaw cycles, followed by sonication (Fisher Scientific) on ice for 10s (power level 5) before centrifugation at 14,000 rpm (Forma Scientific) for 15 min at 4°C. Aliquots of each supernatant or MPO standard (14 μl; Sigma-Aldrich) were added to 200 μl of substrate (0.167 mg of α-naphthol dissolved in 1 ml of 0.2 M H2O, 0.0005% H2O2, in potassium phosphate buffer), and the A450 was measured with a plate reader (Bio-Tek Instruments). Total protein levels were measured by the bicinchoninic acid protein assay (Bio-Rad). MPO activity was expressed as units per milligram of protein. One unit of enzyme activity was defined as the amount that consumes 1 μmol of H2O2/min.

**Manual neutrophil counts**

Crypts were observed in the slides stained with H&E as described above using a ×63 NA1.4 lens on a Zeiss 200M microscope. Neutrophils were identified by their distinctive nuclear morphology and counted in fifteen crypts per colon. The mean number of neutrophils per crypt was calculated for each colon observed. The number of samples (n) refers to the number of colons measured.

**Neutrophil killing assays**

*C. rodentium* bacteria were cultured overnight in Luria-Bertani broth and serially diluted in PBS. To isolate neutrophils, bone marrow from WT or MyD88−/− mice was removed, and RBC were lysed, 22–30% of cells were positive for GR-1 as measured by flow cytometry with an anti-GR-1 Ab conjugated to FITC (BD Biosciences). Bacteria (5 × 105) were added to 5 × 105 cells in 300 μl of RPMI 1640 medium without antibiotics. After 1, 2, or 3 h, the bacterium-cell culture was diluted 1/10 in water for 10 min at 4°C. Aliquots of each supernatant or MPO standard (14 μl; Sigma-Aldrich) were added to 200 μl of substrate (0.167 mg of α-naphthol dissolved in 1 ml of 0.2 M H2O, 0.0005% H2O2, in potassium phosphate buffer), and the A450 was measured with a plate reader (Bio-Tek Instruments). Total protein levels were measured by the bicinchoninic acid protein assay (Bio-Rad). MPO activity was expressed as units per milligram of protein. One unit of enzyme activity was defined as the amount that consumes 1 μmol of H2O2/min.

**α-GR1 Ab treatment**

C57BL/6 mice were rendered neutropenic by injection of 100 μg of α-GR1 Ab (eBiosource) in 300 μl of PBS on days 2 and 4 p.i. Peripheral blood samples were taken on days 1 and 5 p.i. to ensure that numbers of neutrophils were decreased. Mice were sacrificed on days 7 p.i. and various disease parameters measured.

**Statistical analysis**

For mortality curves, a two-sided Fisher’s exact test determined statistical significance of data. For crypt lengths in Fig. 3E, a Student’s t test assessed statistical significance. For all other experiments, level of statistical significance was determined by a Mann-Whitney rank-sum test. Results were considered significant if the p value was <0.01.

**Results**

**MyD88-dependent signaling is necessary to survive infection with C. rodentium**

To determine whether TLR signaling participates in host response to an AE pathogen in vivo, we infected mice deficient in individual TLRs or in adaptor proteins with *C. rodentium* and assessed survival. Upon infection, mice deficient in TLR4 (TLR4−/−) and their control strain, C57BL/6, all survived (data not shown) and appeared healthy four weeks later, the longest time assessed (data not shown). Thus, signaling through TLR4 alone is not required for a protective immune response to *C. rodentium*, a result similar to that reported by Khan et al. (18).

We considered that signaling from several TLRs might coordinate innate immune responses to an infection with *C. rodentium* in vivo. To test this possibility, we infected mice deficient in TIRAP (TIRAP−/−), an adaptor protein necessary for MyD88-dependent signaling from TLR2 and TLR4 complexes (23, 28), or MyD88 (MyD88−/−), an adaptor protein that mediates signaling from most TLRs (10). In accordance with our results with TLR4−/− mice, TIRAP−/− mice infected with *C. rodentium* survived at a rate comparable to that seen with the control background strain, B129P2F2J (data not shown). Infection of MyD88−/− mice with *C. rodentium* proved much more deleterious. All infected MyD88−/− mice died within 13 days (Fig. 1A); by contrast, all WT control mice (C57BL/6) survived. Taken together, these data suggest that MyD88-dependent signaling is required to protect mice from mortality following infection with *C. rodentium*.

**MyD88-dependent signaling protects mice from severe intestinal damage and facilitates repair of damaged epithelium in response to *C. rodentium***

To characterize the pathology associated with *C. rodentium* infection in MyD88−/− mice, colons were removed from WT and MyD88−/− strains and evaluated for both macroscopic and microscopic appearance, and crypt length. As seen in Fig. 1B, colons from MyD88−/− mice but not WT mice exhibited localized intra-colonic mural bleeding, which developed between 3 and 7 days p.i. Longitudinal sections of colonic tissue stained with H&E revealed that both WT and MyD88−/− mice suffer from edema and epithelial injury following infection with *C. rodentium* (Fig. 1, D and G). However, MyD88−/− colonic tissue additionally displayed pathological features consistent with gangrenous necrosis (Fig. 1G) characterized by foci of mucosal necrosis associated with large colonies of bacteria (Fig. 1G). Gangrenous mucosal damage was not evident in uninfected MyD88−/− mice (Fig. 1F) or in infected WT mice (Fig. 1D). Moreover, neither intramural colonic bleeding nor gangrenous mucosal necrosis was evident in TIRAP−/− mice. Together these data suggest that MyD88-dependent signaling, but not TIRAP-dependent signaling, occurring in response to activation of multiple TLRs provides protection from severe colonic pathology associated with unregulated bacterial growth.

Intestinal damage induced by DSS has been reported to induce a repair mechanism that replenishes epithelial cells and restores the integrity of the intestinal barrier in a TLR4 and MyD88-dependent manner (18, 29). In this process, stem cells present at the base of intestinal crypts differentiate into enterocytes, divide, and migrate along the crypt to the site of damage (30). Such hyperplasia, measurable as an increase in crypt length, is also a hallmark feature of AE pathogen infection (7) and is indicative of restoration of damaged intestinal epithelium. We observed that crypts of WT mice were nearly twice as long seven days following *C. rodentium* infection compared with those of uninfected mice (Fig. 1E). However, no increase in crypt length was evident following infection of MyD88−/− mice (Fig. 1E). Accordingly, histological examination of colons from infected MyD88−/− mice indicates sustained damage throughout the course of the infection. By contrast, upon infection of TIRAP−/− mice, hyperplasia was evident to the same degree as in the control strain, B129P2F2J (data not shown). Taken together, these data suggest that MyD88-dependent signaling is required for either sensing *C. rodentium* or for the repair response initiated upon damage to the epithelia.

We also explored the effect of MyD88-dependent signaling on epithelial apoptosis. TLR4−/− mice show increased apoptosis after intestinal injury with DSS, which can be reversed with exogenous PGE2 (31). Furthermore, mutant *Salmonella* strains that fail to activate TLR5 signaling induce extensive apoptosis, which exacerbates disease (32). These studies raise the possibility that MyD88-dependent signaling protects from *C. rodentium* through blocking epithelial apoptosis and preserving the integrity of the epithelial...
FIGURE 1. MyD88-dependent signaling is necessary for survival and prevention of gangrenous mucosal necrosis following infection with C. rodentium. A, Survival curves of 12- to 16-wk-old MyD88^−/− mice (n = 12, □) and their C57BL/6 controls (WT; n = 12, ◀) infected with 4 × 10^8 CFU of C. rodentium. MyD88^−/− mice succumbed within 12 days, while age-matched controls survived. B, Colon sections from MyD88^−/− (upper) and WT (lower) mice 7 days p.i. Magnification, ×400. C, H&E staining of colonic tissue from uninfected WT mice. D, H&E staining of colonic tissue from WT mice 7 days p.i. Magnification, ×400. Colonic tissues exhibit pathology characterized by 1) loss of goblet cells, 2) edema, and 3) epithelial injury. E, Crypt lengths of colonic tissue from uninfected WT or MyD88^−/− mice, or from infected WT mice or MyD88^−/− mice 3 or 7 days p.i. *p < 0.001. The number of animals ranged from 5–12 for each bar. F, H&E staining of uninfected colonic tissue from MyD88^−/− mice. Magnification, ×400. G, H&E staining of MyD88^−/− colonic tissue 7 days p.i. Colonic tissue exhibited gangrenous mucosal necrosis characterized by 1) visible bacterial colonies (both with (inset 1′) and without (inset 1″) neutrophil infiltration), 2) mucosal injury, 3) neutrophil infiltration (+, 4) edema, 5) apoptosis (†), 6) intramural bleeding, and 7) epithelial injury. Neutrophils (+) can be more readily distinguished at higher magnification in the inset. Magnification, ×400 (inset 3 × 800, inset 1′ and 1″ × 1260).

Barrier. To test this, we assessed apoptosis using TUNEL staining of infected colon sections at 3 and 7 days p.i. Quantitation of these data is shown in Fig. 2G. Although some apoptosis was evident in areas of gangrenous mucosal necrosis, overall we observed no significant increase in apoptosis in colonic tissue from MyD88^−/− animals compared with WT animals at 3 or 7 days p.i. (compare Fig. 2, A with B, C with D, and E with F). Taken together, these data suggest that MyD88-dependent signaling does not contribute to apoptosis in response to C. rodentium infection, and the exacerbated pathology seen in infected MyD88^−/− mice is likely indicative of increased necrosis, not apoptosis.

Levels of C. rodentium colonization correlate with intramural colonic bleeding

Damage to the colon caused by A/E pathogens can facilitate transit of bacteria across the epithelial barrier and dissemination throughout the body (7). In accordance with this idea, colonic sections with the highest degree of damage, evidenced by isolated intramural colonic bleeding, also contained the highest numbers of C. rodentium (Fig. 3A). Intramural colonic bleeding in MyD88^−/− mice appeared to progress distally over time. Such a progression was correlated with the colonization pattern of C. rodentium, which began in the cecal patches, but within days was found in the medial and eventually the distal colon (33). These data suggest that bleeding and high levels of colonization in MyD88^−/− colons may facilitate dissemination of C. rodentium throughout the body.

MyD88^−/− mice fail to control the level of C. rodentium colonization

To test whether colonic damage facilitates bacteremia, we next assessed the extent to which C. rodentium disseminates to peripheral organs in MyD88^−/− mice. Dissemination of bacteria from the colon to the MLNs was evident one day after infection (data not shown), and to the liver and spleen by 3 days p.i. (Fig. 3B). The level of C. rodentium colonization in various tissues increased until day 7 p.i. (Fig. 3C), after which time levels began to decrease.
...but are impaired in their ability to class-switch an affective adaptive immune response to parasites, bacteria, and viruses (12–15). To assess the efficacy of the adaptive immune response of MyD88<sup>-/-</sup> mice following infection with C. rodentium, we determined whether the Ab produced was sufficient to protect MyD88<sup>-/-</sup> mice from mortality. To do this, MyD88<sup>-/-</sup> mice were infected with C. rodentium and then treated three days later with antibiotics for a period of 4 wk. Ninety-five percent of mice treated with antibiotics survived (22 of 23 mice; data not shown). The mice were then reinfected with C. rodentium and their survival assessed. As seen in Fig. 4A, 73% of the reinfected MyD88<sup>-/-</sup> mice survived the second infection although clearance of C. rodentium in reinfected survivors occurred at least 2 wk later than in WT mice (data not shown). Interestingly, low levels of C. rodentium were still present in colonic tissue of 27.7% of MyD88<sup>-/-</sup> mice after 4 wk of continuous antibiotic treatment, and colons of these mice showed evidence of pathology (data not shown). Thus, the percentage of mice that succumbed following reinfection was the same as that in which colonization and pathology was evident following antibiotic treatment. Notably, MyD88<sup>-/-</sup> mice produced decreased levels of IL-6 (Fig. 4B), a cytokine required for the efficient induction of an adaptive response at the site of infection.

An adaptive Ab response is sufficient to protect MyD88<sup>-/-</sup> mice from mortality

Previous studies have shown that MyD88<sup>-/-</sup> mice mount an effective adaptive immune response to parasites, bacteria, and viruses (12–15), but are impaired in their ability to class-switch autantibodies in a systemic lupus erythematosus model (35). To determine whether the Ab produced was sufficient to protect MyD88<sup>-/-</sup> mice from mortality, we transferred serum from immunized MyD88<sup>-/-</sup> animals into μMT mice, which lack B cells (37), and are extremely susceptible to infection from this bacteria (34, 38). Notably, the level of C. rodentium colonization in μMT mice is lower than that seen in MyD88<sup>-/-</sup> mice 7 days p.i. (data not shown). Furthermore, examination of the colons of μMT mice indicated no evidence of the intramural colonic bleeding or severe colitis seen in MyD88<sup>-/-</sup> mice. As shown in Fig. 4D, serum from immunized MyD88<sup>-/-</sup> mice conferred survival on 40% of μMT mice infected by C. rodentium. The rate of survival was similar to that seen in μMT mice that received serum from immunized WT mice. Together, these data indicate that MyD88<sup>-/-</sup> mice can mount a protective C. rodentium-specific Ab response. The increased mortality of immunized MyD88<sup>-/-</sup> mice compared with WT mice (Fig. 4A) suggest a deficiency in another protective process in these mice, such as wound repair or neutrophil response.

MyD88-dependent signaling in both hemopoietic and nonhemopoietic cells is necessary to survive C. rodentium infection

To determine whether MyD88-dependent signaling in hemopoietic cells, in nonhemopoietic cells or in both was necessary for protection from C. rodentium, we performed reciprocal bone marrow transplants. Thus, bone marrow from MyD88<sup>-/-</sup> mice was transferred into lethally irradiated WT mice, creating MyD88<sup>-/-</sup>→WT chimeric mice, to determine the necessity of MyD88-dependent signaling in hemopoietic cells. Likewise, we created WT→MyD88<sup>-/-</sup> chimeric mice, to determine the necessity of MyD88-dependent signaling in nonhemopoietic cells. As controls, we created WT→WT and MyD88<sup>-/-</sup>→MyD88<sup>-/-</sup> chimeric mice. Reconstitution was confirmed after twelve weeks by flow cytometry.
Upon infection with \textit{C. rodentium}, 100\% of MyD88\(^{−/−}\) mice died within 10 days at a rate similar to that seen in MyD88\(^{−/−}\) or MyD88\(^{−/−}\)→MyD88\(^{−/−}\) mice (Fig. 5A). By contrast, nearly all control WT→WT mice survived (Fig. 5A). Therefore, MyD88-dependent signaling from hemopoietic cells is required for survival in response to infection with \textit{C. rodentium}. Notably, bone marrow from WT mice provided only marginal protection to MyD88\(^{−/−}\) mice infected with \textit{C. rodentium} as only 77.8\% of WT→MyD88\(^{−/−}\) chimeric mice died compared with 100\% of MyD88\(^{−/−}\) or MyD88\(^{−/−}\)→MyD88\(^{−/−}\) mice (Fig. 5A). Moreover, the WT→MyD88\(^{−/−}\) mice that survived cleared the infection with kinetics comparable to those seen with WT and WT→WT mice (data not shown). Taken together, these data suggest that both hemopoietic and nonhemopoietic cells are required for survival upon infection with \textit{C. rodentium}.

Pathological examination of infected MyD88\(^{−/−}\)→WT mice indicated that colons displayed phenotypes characteristic of those induced by \textit{C. rodentium} in MyD88\(^{−/−}\) mice. Thus, colons from

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** MyD88\(^{−/−}\) mice have higher levels of colonization of \textit{C. rodentium}, which correlates with intramural colonic bleeding. A, MyD88\(^{−/−}\) colons were harvested 8–12 days p.i. and cut into four sections: cecum (n = 10), proximal (n = 10), medial (n = 10), and distal (n = 10). Bleeding was apparent in the distal section of each colon. Colonization was determined for each section as described in Materials and Methods. * Statistical significance in comparison with other colonic sections, p < 0.001.

B. Level of colonization in the distal colon of WT mice 7 days p.i. (n = 14). B and C, Various tissues were harvested aseptically from MyD88\(^{−/−}\) and WT mice 3 days (B) or 7 days p.i. (C) \textit{C. rodentium} CFU were counted as described in Materials and Methods. The number of mice ranged from 9 to 20 for each bar. * Statistical significance in comparison to WT samples, p < 0.001.

**FIGURE 4.** The Ab response in MyD88\(^{−/−}\) mice is protective, but not optimal (A) Survival curves of MyD88\(^{−/−}\) mice (n = 12, empty gray boxes), WT mice (n = 12, ◦), immunized MyD88\(^{−/−}\) mice (n = 11, filled gray circles), and immunized WT mice (n = 10, ▲). Immunized mice were infected with \textit{C. rodentium}, and treated with antibiotics for 4 wk, and then reinfected with a dose lethal to naive MyD88\(^{−/−}\) mice (4 × 10\(^{8}\) CFU). The survival of immunized MyD88\(^{−/−}\) mice is significantly higher than naive MyD88\(^{−/−}\) mice, p < 0.001. B, Colon supernatants from MyD88\(^{−/−}\) (n = 10) and WT (n = 10) were assessed for IL-6 levels via ELISA 7 days p.i. The level of IL-6 is significantly higher in WT mice than in MyD88\(^{−/−}\) mice, p < 0.01. C, Immunized MyD88\(^{−/−}\) mice generate higher titers of Abs to \textit{C. rodentium} compared with WT mice, likely reflecting the greater bacterial loads in these animals. D, Survival curves of WT (n = 12, ◦), μMT mice (n = 4, gray asterisks), μMT mice that received WT immune serum (n = 3, empty gray triangles), and μMT mice that received MyD88\(^{−/−}\) immune serum (n = 5, empty gray circles). Note that serum from immunized WT or MyD88\(^{−/−}\) mice is partially protective.
MyD88−/−WT mice showed evidence of isolated intramural colonic bleeding (compare Figs. 5B and 1B), a phenotype never observed in WT→WT mice (Fig. 5B), or in WT mice (Fig. 1B). Colons of uninfected MyD88−/−WT mice likewise showed no evidence of bleeding or inflammation (Fig. 5C). Additionally, histological evaluation of longitudinal sections of colons in MyD88−/−→WT mice revealed mucosal and epithelial injury, including gangrenous mucosal necrosis similar to those observed in MyD88−/− mice (Fig. 1F). Such findings were not evident in WT→WT mice (Fig. 5D). These results indicate that MyD88-dependent signaling on hemopoietic cells is necessary to prevent localized intramural colonic bleeding and severe intestinal pathology.

MyD88-dependent signaling on either hemopoietic or nonhemopoietic cells is sufficient to facilitate repair of intestinal epithelia in response to C. rodentium infection

In contrast to results from MyD88−/− mice (Fig. 1E), hyperplasia was evident in colonic tissue from MyD88−/−→WT mice infected with C. rodentium (Fig. 6A). Thus, crypt length was significantly longer than that observed in uninfected MyD88−/−→WT colonic tissue (Fig. 6A), but not significantly different from that found in infected WT→WT mice (data not shown). As expected, crypt lengths from infected WT→WT mice were significantly longer than those found in infected MyD88−/−→MyD88−/− mice (data not shown). These data indicate that MyD88-dependent signaling on nonhemopoietic cells is sufficient for the development of hyperplasia in response to C. rodentium. Histological examination of colons from WT→MyD88−/− mice also showed evidence of hyperplasia (Fig. 6A), suggesting that hemopoietic cells also contribute to the development of hyperplasia. Taken together, these data suggest that MyD88-dependent signaling in either hemopoietic or nonhemopoietic cells is necessary to facilitate repair of colonic epithelium. However, crypt lengths were significantly longer in WT→MyD88−/− mice compared with those seen in MyD88−/−→WT mice, indicating that MyD88 signaling in hemopoietic cells may contribute in a more significant way to the repair process than nonhemopoietic cells. Nevertheless, signaling in both hemopoietic and nonhemopoietic cells is required to protect mice from severe colitis and death upon infection with C. rodentium. A similar requirement for MyD88-dependent signaling in multiple cell types has been described in a murine pulmonary inflammation model (26).
FIGURE 6. MyD88-dependent signaling is required in both hemopoietic and nonhemopoietic to prevent bacteremia (A) Crypt lengths of uninfected or infected colonic tissue from WT→MyD88−/− mice (days 6–12 p.i.; n = 9, red bars with blue diagonal stripes) and MyD88−/−→WT mice (days 5–9 p.i.; n = 6, blue bars with red diagonal stripes). *, Statistical significance in comparison to uninfected samples, p < 0.001. **, Statistical significance between WT→MyD88−/− and MyD88−/−→WT samples, p < 0.001. B and C. Level of colonization by C. rodentium in organs. B, Various organs were harvested from infected WT→WT mice (n = 4, blue bars with blue horizontal stripes) and MyD88−/−→MyD88−/− mice (n = 3, red bars with red horizontal stripes). Data shown with MyD88−/− (red bars) and WT (blue bars) mice is from Fig. 3C. C. Various organs were harvested from WT→MyD88−/− mice (n = 6, red bars with blue diagonal stripes) and MyD88−/−→WT mice (n = 9, blue bars with red diagonal stripes). For comparison, colonization of WT→WT mice (blue bars with blue horizontal stripes), and MyD88−/−→MyD88−/− (red bars with red horizontal stripes) are shown. CFU were counted as described in Materials and Methods.

Control of C. rodentium colonization requires MyD88-dependent signaling in hemopoietic and nonhemopoietic cells

To further characterize pathology in the chimeric mice, organs were harvested from the mice and bacterial colony counts were measured. In each organ, the degree of colonization of the WT→WT mice was similar to that seen in WT (Fig. 6B). Likewise; the degree of colonization in organs of MyD88−/−→MyD88−/− mice was similar to that seen in MyD88−/− mice (Fig. 6B). The level of colonization seen in organs removed from both MyD88−/−→WT and WT→MyD88−/− mice more closely matched that found in MyD88−/− mice and was higher than the levels seen in organs from WT organs or WT→WT mice, especially in peripheral organs (Fig. 6C). Taken together, these data suggest that MyD88-dependent signaling on both hemopoietic and nonhemopoietic cells is necessary to control levels of C. rodentium colonization.

A role for neutrophils in the protective response to C. rodentium infection

Bone marrow transplants indicated that MyD88-dependent signaling on hemopoietic and nonhemopoietic cells is necessary for protection. We next set out to characterize more precisely which hemopoietic cell types were required. The observation that MyD88−/− mice cannot control C. rodentium colonization (Fig. 3C) suggested a role for neutrophils, which kill bacteria. To investigate the role of neutrophils, we measured the levels of MPO activity in colons of WT and MyD88−/− mice. Because MPO is expressed predominantly in neutrophils and to a lesser extent in macrophages (39), it enabled us to selectively distinguish the role of neutrophils. Levels of MPO in colons of WT mice increased by 13.2-fold at 3 days p.i., and remained at a high level at 7 days p.i. The increase in MPO levels in the medial and distal colon mirrored the increased levels of colonization seen in these regions as the infection progressed (Figs. 3C and 7A). By contrast, MPO levels in colons of MyD88−/− mice measured 3 days p.i. significantly lower (2.7-fold) than those seen in WT mice (Fig. 7A). MPO levels in the colons of MyD88−/− mice did increase by 7 days p.i. (Fig. 7A), although to a lesser extent than that seen in WT mice, despite the fact that colonization was two orders of magnitude higher (Fig. 3C). Counts of neutrophils in colonic tissues corroborated results of the MPO assays (Fig. 7B). Taken together, these data suggest that recruitment of neutrophils to the colon of MyD88−/− mice was delayed at a time when bacterial numbers at this site were rapidly expanding.

To determine whether there was an intrinsic defect in neutrophils from MyD88−/− mice, we assessed the capacity of neutrophils to migrate and to kill bacteria in vitro. As shown in Fig. 7C, MyD88−/− neutrophils migrated in vitro in response to the chemokine CXCL1/KC with the same efficacy as WT neutrophils, confirming previous observations (16). In addition, bactericidal activity of neutrophils isolated from both MyD88−/− and WT mice was indistinguishable (Fig. 7D). The addition of serum from convalescent WT or MyD88−/− mice to the killing assay did not influence the efficiency or rate of killing by neutrophils (data not shown). We were unable to measure MPO levels in peripheral organs of WT and MyD88−/− mice due to limitations in sensitivity of the assay. Thus, neutrophil levels in infected liver samples were below the level of detection, and levels of splenic neutrophils were high in uninfected samples and we could not distinguish an increase upon infection. Taken together, these data indicate that neutrophils from MyD88−/− mice did not display an intrinsic functional defect, although we cannot rule out the possibility that MyD88-dependent signaling regulates specific neutrophil functions in vivo.

The observation that neutrophils from MyD88−/− mice did not display an intrinsic defect led us to hypothesize that chemokines derived from hemopoietic or nonhemopoietic cells or both might facilitate migration of neutrophils to the colon following infection. In this regard, CXC chemokines such as KC are released by epithelial cells and have been shown to participate in the recruitment of neutrophils following infection by a variety of pathogens (40). As shown in Fig. 7E, levels of KC were ~3 orders of magnitude higher in colons of WT mice at day 3 p.i. compared with MyD88−/− mice. By 7 days p.i., KC levels in WT and MyD88
animals were nearly equivalent (data not shown). Taken together, these data suggest that production of KC or other chemokines required for proper recruitment of neutrophils, was significantly delayed in colons of MyD88−/− mice in response to C. rodentium, thus mirroring the changes in MPO levels in these mice. To determine whether neutrophils are required for innate immune protection from C. rodentium, we next set out to deplete neutrophils in WT mice by injecting the neutralizing mAb GR-1. To do this anti-GR-1 Ab was injected on days 2 and 4 following infection with C. rodentium. Using this protocol, the number of neutrophils was reduced to undetectable levels (data not shown). Injection of GR-1 mAb resulted in 22% mortality by day 7 p.i. compared with 0% for PBS-treated WT mice (n = 9). Significance at the p < 0.01 level is indicated by an asterisk (*). Colonization data from MyD88−/− mice (□) is from Fig. 3C.

Discussion

A/E pathogens, including EPEC, EHEC, and C. rodentium, cause disease characterized by intestinal inflammation and diarrhea. Previous reports suggest that inflammatory responses occur following a breach of the intestinal barrier and are mediated by innate and adaptive immune mechanisms (34, 38, 41). Whereas the adaptive responses to A/E pathogens have been well characterized (9, 34, 38), the role of innate immune responses is less well understood. We initiated this study to determine whether reducing innate immune recognition of the bacteria could mollify the colitis caused by C. rodentium. However, data presented here suggest that innate immune signals through the TLR adaptor protein MyD88 protect the host from bacteremia and severe pathology in the colon including gangrenous mucosal necrosis that is associated with uncontrolled growth of the pathogen. Thus, although an innate immune response associated with inflammation appears deleterious and maladaptive, it also affords significant protection as the adaptive response is generated.

Innate and adaptive immune responses control bacterial dissemination

Khan et al. (18) provide evidence that TLR4-dependent responses mediate inflammation and tissue pathology during C. rodentium infection with C. rodentium. How-

FIGURE 7. Neutrophil recruitment to the colons of MyD88−/− mice is delayed, leading to increased bacterial colonization in the liver and spleen. A, Neutrophil recruitment in colons was quantified by MPO assay for MyD88−/− (n = 6–12) and WT mice (n = 7–12). *, Statistical significance in comparison to uninfected MyD88−/− samples, p < 0.01. **, Statistical significance in comparison to uninfected C57BL/6 samples, p < 0.001. B, Manual neutrophil counts in crypts from WT (n = 6–7) and MyD88−/− (n = 5–6) colons. *, Statistical significance in comparison to uninfected samples, p < 0.01. C, Neutrophils derived from MyD88−/− (n = 4) and WT (n = 4) mice are able to respond the chemoattractant fNLP with equal efficacy. D, C. rodentium (5 × 10^5) incubated with neutrophils (5 × 10^5) from WT or MyD88−/− mice were killed with equal efficacy within 1 h. E, Colon supernatants from MyD88−/− (n = 12) or WT (n = 12) mice 3 days p.i. were assessed for KC levels by ELISA. The difference between these groups was significant at p < 0.01. F, Treatment of WT mice with α-GR1 Abs rendered mice neutropenic. Significantly higher levels of C. rodentium colonization were evident in liver and spleen in α-GR1-treated WT mice compared with PBS-treated WT mice (n = 9). Significance at the p < 0.01 level is indicated by an asterisk (*). Colonization data from MyD88−/− mice (□) is from Fig. 3C.
infection. Our data with MyD88-deficient mice extend the results of Khan et al. (18) and point to MyD88 as a key mediator not only of inflammation, but also of protection from bacteremia and severe colonic pathology. The cell types involved include both hemopoietic cells including neutrophils, and nonhemopoietic cells, most notably the epithelium (Figs. 5–7).

Control of bacterial dissemination to peripheral organs following C. rodentium infection in the colon appears to be mediated by both innate and adaptive immune responses. For example, infection of mice lacking B cells, T cells, or both have greater pathogen loads in colonic and peripheral tissues (9, 34). However, adaptive responses do not alone appear necessary for survival. A significant proportion of T and B cell-deficient mice survive infection with C. rodentium (9, 34, 42), although the infection appears more severe than in WT mice. Moreover, mast cell-deficient mice suffer bacteremia and death after infection with C. rodentium, although the adaptive response appears unaffected (25). Finally, we provide evidence here that MyD88−/− mice have greater bacterial loads both in the colon and in peripheral tissues (Fig. 3C), which strongly correlates with decreased neutrophil infiltration into the colon (Fig. 7F). These data suggest a prominent role for the innate immune system early in the infection, although they do not preclude a requirement for Ab-mediated responses later on.

Innate immune mechanisms that provide protection by killing bacteria or limiting dissemination are not limited to A/E pathogens. In this regard, previous studies of MyD88−/− mice infected with lymphocytic choriomeningitis virus (12), Borrelia burgdorferi (13), Brucella abortus (14), or Salmonella typhimurium (15) have also demonstrated that MyD88-dependent signaling is required to limit severity of disease, dissemination, and pathogen numbers. For example, following inoculation of MyD88−/− mice, spread of B. abortus to peripheral organs occurs with similar kinetics to that seen with C. rodentium (Ref. 14; Fig. 3B). Moreover, MyD88−/− mice display delayed clearance of B. burgdorferi and B. abortus infections (13, 14), and strains of lymphocytic choriomeningitis virus, which are cleared in WT mice, cause chronic disease in MyD88−/− mice (12), indicating a suboptimal adaptive response in the absence of MyD88-dependent signaling.

Our results with immunized mice provide novel information on the cross-talk between the innate and adaptive responses. Two aspects of the experiments with antibiotic treated MyD88−/− mice were unexpected, and may provide important information on the role of innate immune cells in bacterial clearance. First, continuous administration of an antibiotic mixture was not sufficient to clear the pathogen in all MyD88−/− mice, although all WT mice cleared the infection. It is possible that high bacterial loads might account for such a result although at the time of antibiotic administration bacterial loads in WT and MyD88−/− mice were equivalent (Fig. 3B). However, we cannot rule out that significantly more bacteria were present in the MyD88−/− mice at the time the antibiotics took effect. It is also possible that bacteria were in stationary phase and thus resistant to bactericidal effects of the antibiotics, thereby facilitating development of resistant strains (43). In this regard, preliminary experiments suggest that C. rodentium strains recovered after long-term antibiotic treatment were relatively resistant to the administered antibiotics (S. L. Lebeis, unpublished observations), demonstrating that the antibiotic treatment in MyD88−/− mice provides selective pressure on the bacteria. These results may also suggest that an innate immune response is required for effective bacterial clearance in conjunction with an antibiotic, or for suppressing the development of resistant bacteria.

A second unexpected observation was the development of a high-titer Ab response despite undetectable levels of IL-6 in the colon (Fig. 4, B and C). The presence of IL-6 has been long been considered crucial for development of Ab responses (36). However, our results showing protection of μMT mice that had received serum from immunized MyD88−/− mice suggest that IL-6 is not necessarily required to generate protective Ab (Fig. 4, B and D). Of note, the immunization protocol was not completely protective in MyD88−/− mice: only 73% of immunized MyD88−/− mice survived a challenge infection, and those mice that do survive clear the infection with delayed kinetics (Fig. 4A). Thus, it remains possible that either IL-6 is required for optimal Ab responses or MyD88-dependent responses (such as phagocytosis of opsinized bacteria) are needed in conjunction with Ab to effectively clear the bacteria. Experiments to extend these observations are currently in progress.

Repairing the breach

A/E pathogens gain access to the basolateral surface by causing breakdown of the epithelial barrier using virulence factors such as EspF that are secreted into epithelial cells by a type III secretion system (44). Several lines of evidence indicate that epithelial hyperplasia constitutes a proliferation of crypt stem cells that repairs the breach in response to DSS, and that macrophages facilitate the repair process in a MyD88-dependent manner (29). Accordingly, infected MyD88−/− mice display substantial damage to the intestinal epithelium but little evidence of hyperplasia (Fig. 1, E and F). However, the degree of hyperplasia evident in MyD88−/−→WT mice was comparable to that seen in WT mice. These results suggest that MyD88-dependent signaling on nonhemopoietic cells is sufficient for epithelial repair following infection with C. rodentium. Moreover, hyperplasia was evident in WT mice by day 3, the same time at which neutrophils are recruited. Thus, protection may in part be mediated by wound repair of epithelial barrier breach that contains the infection within the colon and prevent dissemination to distal sites.

Repair in the intestine also involves protection from apoptosis. TLR4 and MyD88 are responsible for up-regulation of Cox-2 expression, which mediates antiapoptotic signals (31). As a result, TLR4−/− and MyD88−/− mice have increased apoptosis, reduced proliferation, and increased epithelial injury following DSS exposure (16, 31). Moreover, mutant Salmonella strains that do not stimulate TLR5 signaling induce greater epithelial damage due to the lack of MyD88-dependent protection from apoptosis (32). In contrast, we do not detect an increase in apoptosis in the MyD88−/− mice following infection with C. rodentium, and infected TLR4−/− mice actually have milder pathology than WT (18). Taken together, these observations suggest that DSS colitis, Salmonella infection, and A/E bacterial infection each stimulate distinct protective responses in the host with different etiology. Thus, in A/E pathogen infections, MyD88 signaling facilitates clearance by inducing recruitment of neutrophils, which controls bacterial load, and repair of the epithelial barrier. In the absence of MyD88, necrosis rather than apoptosis exacerbates the infection, causing additional damage to the epithelial barrier.

A role for neutrophils in protection

Several lines of evidence suggest that neutrophils and nonhemopoietic cells are together required for protection from C. rodentium...
infection. First, depletion of neutrophils in WT mice results in increased dissemination of bacteria and mortality (Fig. 7F). Second, neutrophil accumulation in colonic tissues is delayed in the absence of MyD88-dependent signaling (Fig. 7, A and B). This delay does not appear to be a result of an intrinsic defect in neutrophil function because MyD88<sup>−/−</sup> neutrophils retain the capacity to migrate and to kill bacteria in vitro (Fig. 7, C and D). Notably, the delay occurs at a time when bacteria are beginning to disseminate and numbers are rapidly expanding (Fig. 3B). Our data suggest that failure to restrict bacterial growth early in the infection results in loads that may be beyond the capabilities of innate or adaptive defenses to control. Although not addressed here, macrophages can also kill <i>C. rodentium</i> (S. L. Lebeis, unpublished observations), so they may act with neutrophils to restrict bacterial loads. The role of macrophages in recovery of <i>C. rodentium</i> infection is the subject of current studies.

The migration of neutrophils throughout the body is orchestrated by several chemokines, including those of the CXC class (45). It is possible that several chemokines orchestrate the recruitment of neutrophils in response to <i>C. rodentium</i>. We found at least one of these, KC, is produced in a MyD88-dependent manner (Fig. 7E). Our results also demonstrate that KC is significantly reduced at day 3 p.i. compared with WT mice, though it is present at levels equivalent to those in WT animals by day 7, thereby mirroring the delay in neutrophil recruitment (Fig. 7, A and B).

**A model of innate immune responses during infection with C. rodentium**

Data presented here suggest that MyD88-dependent signaling by both hemopoietic and nonhemopoietic cells is required to protect the host from disseminated bacterial infection (Fig. 8). According to this view and our results, we propose the following model: <i>C. rodentium</i> causes attaching and effacing lesions on intestinal epithelia, which leads to disruption of the epithelial barrier and simultaneous activation of innate immune signaling initiated by TLRs. Production of chemokines (e.g., KC) in a MyD88-dependent manner, likely by nonhemopoietic cells (e.g., epithelium), leads to recruitment of neutrophils, which contain the infection by killing bacteria. Concurrently, recruitment of macrophages facilitates repairs the damage to the epithelial barrier in a MyD88-dependent fashion (29). Despite these defenses, some bacteria manage to disseminate to MLNs and subsequently to liver and spleen. Ultimately, an adaptive response clears the infection. MyD88-dependent signaling contributes to the quality of the Ab response and facilitates clearance in a timely fashion. In the absence of MyD88-dependent signaling, neutrophil recruitment is delayed resulting in expansion of bacterial numbers in the lamina propria, and transit of additional bacteria from the lumen due to a failure of macrophages to repair the epithelial barrier. Bacterial dissemination continues unabated, and overwhelms the animal before an effective adaptive response can be mounted. In summary, our results provide a crucial role for MyD88 in host responses that confer protection from infection.

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**Disclosures**

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