Critical Role for MyD88-Mediated Neutrophil Recruitment during *Clostridium difficile* Colitis

Irene Jarchum, Mingyu Liu, Chao Shi, Michele Equinda, and Eric G. Pamer

Immunology Program, Sloan-Kettering Institute, Infectious Diseases Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York, USA

Clostridium difficile can infect the large intestine and cause colitis when the normal intestinal microbiota is altered by antibiotic administration. Little is known about the innate immune signaling pathways that marshal inflammatory responses to *C. difficile* infection and whether protective and pathogenic inflammatory responses can be dissociated. Toll-like receptors predominantly signal via the MyD88 adaptor protein and are important mediators of innate immune signaling in the intestinal mucosa. Here, we demonstrate that MyD88-mediated signals trigger neutrophil and CCR2-dependent Ly6C^{hi} monocyte recruitment to the colonic lamina propria (cLP) during infection, which prevent dissemination of bystander bacteria to deeper tissues. Mortality is markedly increased in MyD88-deficient mice following *C. difficile* infection, as are parameters of mucosal tissue damage and inflammation. Antibody-mediated depletion of neutrophils markedly increases mortality, while attenuated recruitment of Ly6C^{hi} monocytes in CCR2-deficient mice does not alter the course of *C. difficile* infection. Expression of CXCL1, a neutrophil-recruiting chemokine, is impaired in the cLP of MyD88^{-/-} mice. Our studies suggest that MyD88-mediated signals promote neutrophil recruitment by inducing expression of CXCL1, thereby providing critical early defense against *C. difficile*-mediated colitis.

The incidence of *Clostridium difficile* infection is increasing worldwide (11). *C. difficile*, a Gram-positive rod, forms spores that are resistant to a variety of commonly used disinfectant agents (18). Orally acquired *C. difficile* spores can germinate and infect the large intestine following antibiotic-induced changes to the intestinal microbiota (18). Upon germination, *C. difficile* produces toxins A and B, which mediate the development of colitis (13). *C. difficile* colitis is the most common cause of diarrhea among hospitalized patients (18).

A single dose of clindamycin, an antibiotic that kills Gram-positive and obligate anaerobic bacteria, is sufficient to render mice susceptible to *C. difficile* infection and colitis (3). Challenge of antibiotic-treated mice with *C. difficile* spores results in diarrhea, weight loss, and mortality that peaks 2 to 4 days following inoculation (3, 9). Mice surviving the initial bout of colitis continue to shed *C. difficile* for many weeks but regain weight and resolve diarrhea beyond the fifth day of infection.

Antibiotics predispose to *C. difficile* infection by disrupting the composition and/or density of the microbiota. Clindamycin alters the intestine's microbial composition and diversity for up to 30 days (3, 14), and mice remain vulnerable to *C. difficile* infection for prolonged periods following antibiotic exposure. However, resolution of diarrhea and weight gain occur within 3 to 5 days, suggesting that innate immune defense mechanisms, and not restoration of the microbiota, mediate recovery from early, severe colitis.

Toll-like receptors (TLRs) respond to microbial molecules and drive the expression of antimicrobial proteins (16) and, in the setting of infection, trigger immune responses that lead to pathogen clearance (2, 15). The TLR adaptor molecule myeloid differentiation factor 88 (MyD88) is central to defense against intestinal pathogens (15). In mice infected with *Citrobacter rodentium*, MyD88 signaling promotes neutrophil recruitment to the colon, and MyD88-deficient mice succumb while wild-type mice recover (15). MyD88 deficiency also increases susceptibility to *C. difficile* infection (14, 19); however, the mechanism of MyD88-mediated

protection in wild-type mice remains undefined. Stimulation of TLR5 by exogenously administered flagellin reduces morbidity and mortality following *C. difficile* infection, indicating that enhanced activation of TLRs can be protective (9).

Inflammatory cell infiltration of the colonic mucosa is a hallmark of *C. difficile* infection in mice and humans (4). It has been suggested that massive neutrophil infiltration may exacerbate colitis and delay recovery (10). A recent study, however, demonstrated that the innate immune receptor nucleotide-binding oligomerization domain-1 (NOD-1) contributes to neutrophil recruitment during *C. difficile* colitis (8) and NOD-1 deficiency increases mortality following *C. difficile* infection. Thus, neutrophil recruitment to the colonic lamina propria (cLP) may play an important role in the defense against *C. difficile* colitis (8).

In this study, we investigated the role of MyD88 signaling during *C. difficile* colitis. We find that MyD88 is critical for defense against early *C. difficile* infection and that recruitment of inflammatory monocytes and neutrophils to the cLP is markedly attenuated in mice lacking MyD88. Specific depletion of neutrophils with Ly6G-specific monoclonal antibodies increased susceptibility to *C. difficile* infection, while diminished inflammatory monocyte recruitment in CCR2-deficient mice did not alter the course of infection. These findings reveal an important role for neutrophils during the early stages of murine *C. difficile* colitis, contributing to a better understanding of the immune factors that play a role in defense against *C. difficile* colitis.

Received 30 April 2012 Returned for modification 22 May 2012

Accepted 1 June 2012

Published ahead of print 11 June 2012

Editor: A. J. Bäuml

Address correspondence to Eric G. Pamer, pamere@mskcc.org.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.00448-12

FIG 1 Increased mortality of MyD88-deficient mice during *C. difficile* colitis. (A) MyD88^{-/-} and C57BL/6 (B6) mice without prior antibiotic treatment were challenged with a high dose of *C. difficile* VPI10463 spores (10^5) and followed for weight loss. Results are representative of two independent experiments (both with $n = 4$ or 5/group). (B to D) Mice received clindamycin on day -1 and were infected with *C. difficile* VPI10463 spores on day 0. Mice ($n = 10$ /group) were followed for weight loss (B) and survival (C). The log-rank (Mantel-Cox) test was used for statistical analysis of survival. Results are representative of 2 independent experiments. (D) *C. difficile* burden in the cecum was assessed 2 days postchallenge in MyD88^{-/-} and wild-type mice. Results are pooled from two independent experiments (B to E). (E) Mice received a single dose of clindamycin and were challenged with 10^4 spores of *C. difficile* strain CD196.

MATERIALS AND METHODS

Mice and infection. C57BL/6 female mice were purchased from The Jackson Laboratory. MyD88^{-/-} mice (1) were obtained from Shizuo Akira (Osaka, Japan) and were bred to C57BL/6J mice in our laboratory for more than 10 generations prior to their use in this study. CCR2.GFP mice (22) were generated and maintained in the Memorial Sloan-Kettering Cancer Center vivarium. For *C. difficile* infections, mice received a single dose of clindamycin (200 μ g per dose) by intraperitoneal injection 1 day prior to challenge with 10^3 CFU of *C. difficile* spores (strain VPI 10463). CD196 (Fig. 1E) was administered at 10^4 CFU/mouse. *C. difficile* spores were obtained as previously described (9) and administered by gavage. To equilibrate the microbiota, MyD88^{-/-} and CCR2^{-/-} mice were co-housed with C57BL/6 mice for 2 to 4 weeks (at a ratio of 1:1) prior to antibiotic administration. All mice were maintained in a specific-pathogen-free facility at the Memorial Sloan-Kettering Cancer Center Animal Resource Center. Age- and gender-matched animals were used for all experiments. Experiments were approved by institutional guidelines.

Quantitative culture of *C. difficile*. For quantitation of *C. difficile*, cecal contents were suspended in deoxygenated phosphate-buffered saline (PBS), and 10-fold dilutions of the suspension were plated anaerobically on beef heart infusion supplement (BHIS) plates containing taurocholate, D-cycloserine, and cefoxitin for specific selection of *C. difficile*. Plates were placed in a 37°C incubator within the anaerobic chamber (Coy labs) overnight. Bacterial burden in the mesenteric lymph nodes (MLN) was determined by mashing the lymph nodes between frosted glass slides and plating onto Columbia 5% sheep blood agar (Gibco), followed by anaerobic incubation at 37°C for 48 h.

Histology and immunofluorescence. Mice were euthanized, the cecum and colon were obtained from naïve or infected mice, and the bulk of the content was gently removed. For histology, tissues were fixed in 4% paraformaldehyde overnight, washed, and dehydrated in ethanol prior to

paraffin embedding. Sections were cut and stained for hematoxylin and eosin. The histological score was determined by examination by a single blinded histopathologist. The severity of tissue damage was quantified in each section, taking into account the following parameters: epithelial cell loss, cellular infiltration, and edema. A score of 0 to 3, denoting increasingly severe abnormality, was assigned for each of these parameters (10). For immunofluorescence studies, tissues were fixed for 6 h in fresh 4% paraformaldehyde and incubated in 30% sucrose overnight prior to snap-freezing and sectioning. Slides were blocked with undiluted donkey serum and stained with 15 μ g/ml 1A8 antibody, followed by Texas Red-conjugated anti-rat IgG. Images were obtained in an inverted Leica microscope.

Flow cytometry and antibodies. To characterize infiltration of the cLP by neutrophils and monocytes during *C. difficile* infection, cLP leukocytes were isolated using a modification of a previously published protocol (26). Briefly, the colon was placed on ice immediately after isolation, opened longitudinally, and cut into 1- to 2-cm sections. The tissue was washed in cold PBS and placed in 30 mM EDTA in Hank's balanced salt solution (HBSS) at 37°C for 30 min with gentle shaking. Tissues were then cut into small fragments with a razor blade and placed in 0.5 mg/ml collagenase IV at 37°C for 30 min with gentle shaking, and cells were recovered and placed in cold Dulbecco's modified Eagle medium containing 10% fetal calf serum. Tissue fragments were incubated a second time in collagenase for 15 min, and cells were pooled with those recovered earlier. Leukocytes were separated by Percoll gradient. For flow cytometry, inflammatory monocytes were defined as CD45⁺Ly6G^{hi}CD11b⁺ and neutrophils as CD45⁺Ly6G^{hi}CD11b⁺ (see Fig. 3A). For antibody depletion studies, RB6-8C5 and 1A8 were purified from hybridoma cultures in our laboratory. Three doses of antibody (0.3 mg/ml per dose) were administered intraperitoneally every other day beginning on the day prior to *C. difficile* challenge.

FIG 2 Intestinal damage in MyD88^{-/-} and C57BL/6 (B6) mice during *C. difficile* colitis. Mice were challenged with *C. difficile* 1 day following clindamycin administration. Two days postinfection, the cecum and colon were removed, fixed, and stained with hematoxylin and eosin. (A) Representative images of cecum and colon from infected mice. (B) Blind scoring of three parameters of intestinal damage (epithelial cell loss, edema, and cellular infiltration) revealed a similar overall degree of tissue inflammation and destruction in the cecum and colon of MyD88^{-/-} mice. (C) Histological scores for each of the parameters graded in panel B are shown. Scale bars represent 200 μ m for large images and 50 μ m for insets. Arrows point to areas of cellular infiltration.

Real-time PCR. To determine expression levels of CXCL1 in colon, tissue was obtained and homogenized in a bead beater. RNA was isolated by TRIzol separation (Invitrogen). Primers for CXCL1 and GAPDH were obtained from Qiagen and used in SYBR green real-time PCR.

RESULTS

MyD88^{-/-} mice rapidly succumb to *C. difficile* infection. To determine the susceptibility of MyD88^{-/-} mice in our colony to *C. difficile* infection, we first cohoused MyD88^{-/-} and C57BL/6 mice for at least 2 weeks to equilibrate the microbiota. To determine whether impaired innate immune signaling renders mice susceptible to *C. difficile* colitis even without prior antibiotic treatment, we challenged untreated wild-type and MyD88^{-/-} mice with a high dose of *C. difficile* spores (10^5). In the absence of prior antibiotic treatment, MyD88^{-/-} mice are resistant to *C. difficile* colitis (Fig. 1A). Next, we treated wild-type and MyD88^{-/-} mice with clindamycin prior to *C. difficile* challenge (10^3 CFU) and

found that MyD88 deficiency markedly increases mortality (Fig. 1C). However, the burdens of *C. difficile* in the ceca of MyD88^{-/-} and wild-type mice were similar (Fig. 1D). Although *C. difficile* strain VPI 10463 has been widely studied and is commonly used in mouse models of *C. difficile* colitis (4, 9, 17, 21), we wanted to confirm the importance of MyD88 in defense against the disease by using another toxin-producing strain of *C. difficile*, CD196 (25). As shown in Fig. 1E, C57BL/6 mice infected with CD196 do not succumb to *C. difficile* colitis; however, 50% of MyD88^{-/-} mice die of the disease by day 5 postinfection. These results demonstrate that MyD88 signaling makes a major contribution to defense against *C. difficile* colitis. Interestingly, while MyD88^{-/-} mice have higher mortality following *C. difficile* challenge, weight loss is less pronounced than in C57BL/6 mice (Fig. 1B).

To investigate the possible mechanism by which MyD88 defi-

FIG 3 MyD88 signaling is required for neutrophil and monocyte recruitment to the cLP during *C. difficile* colitis. MyD88^{-/-} and wild-type C57BL/6 (B6) mice received a single dose of clindamycin on day -1 and were challenged with *C. difficile* the following day. Two days postinfection, mice were sacrificed, and the percentage of neutrophils (A and B) and monocytes (A and C) in the cLP was assessed by flow cytometry. The gating strategy is shown in panel A, and results are shown as percentages of total cells in the cLP. Results are pooled from two independent experiments. Student's *t* test (Mann-Whitney) was used for statistical analysis.

ciency results in increased mortality following *C. difficile* infection, we performed histological analyses of the lower intestine (Fig. 2). The histology of the colonic and cecal mucosa of infected mice was evaluated for tissue damage by scoring for edema, inflammatory cell infiltration, and epithelial destruction. Although the aggregate scores for these parameters were similar in wild-type and in MyD88^{-/-} mice infected with *C. difficile* (Fig. 2A and B), we noticed that the cecum and colon of MyD88^{-/-} mice had fewer inflammatory cells infiltrating the lamina propria, and quantification of this parameter revealed a marked difference in cellular infiltration in the colon of MyD88^{-/-} and wild-type mice (Fig. 2A and C).

MyD88 signaling is required for recruitment of inflammatory cells during *C. difficile* infection. Our histologic observations suggested that MyD88 signaling may be involved in recruitment of inflammatory cells to the cLP. To further address this, we treated MyD88^{-/-} and C57BL/6 mice with a 200- μ g dose of clindamycin on day -1 and challenged them with 10^3 CFU of *C. difficile* VPI 10463 spores the following day. Two days later, we assessed cellular infiltration in the cLP by flow cytometry. As shown in Fig. 3A to C, there is marked infiltration of the cLP by neutrophils and Ly6C^{hi} monocytes in *C. difficile*-infected mice. In contrast, recruitment of neutrophils and monocytes to the cLP was reduced in MyD88^{-/-} mice. These results indicate that MyD88 signaling is required for monocyte and neutrophil recruitment to the cLP during *C. difficile* colitis.

Monocyte and neutrophil recruitment to the cLP during *C. difficile* colitis. We next investigated the kinetics of neutrophil and monocyte recruitment to the colon during *C. difficile* colitis. Mice were infected with *C. difficile* following clindamycin treatment, and cellular populations infiltrating the cLP were characterized by flow cytometry 2, 4, 7, and 10 days later. As shown in Fig. 4A, the neutrophil population markedly increases 4 days following infection, reaching peak frequencies of 40 to 50% of all leukocytes in the cLP. Monocytes reach the highest levels on day 2, constituting up to 10% of leukocytes in the cLP (Fig. 4B). By day 10, monocyte and neutrophil frequencies are restored to preinfection levels (Fig. 4A and B).

To determine the localization of infiltrating monocytes in the cLP during *C. difficile* infection, we infected CCR2.GFP mice (22). We identified inflammatory monocytes in the cLP by green fluorescent protein (GFP) fluorescence and infiltrating neutrophils by staining with a Ly6G-specific monoclonal antibody. Immunohistologic analyses of the colon and cecum are shown in Fig. 4C. Monocytes and neutrophils can be readily identified in the cLP, and their localization is distinct. While both cell types are found in areas of edema, particularly in the cecum, monocytes are distributed throughout the lamina propria and do not enter the intestinal lumen. Neutrophils, in contrast, are closely associated with the intestinal epithelium, which, as shown in Fig. 2, is discontinuous secondary to toxin-mediated damage.

Gr-1⁺ cells are critical for protection against *C. difficile* colitis. Given the apparent role of MyD88 signaling for inflammatory cell recruitment to the cLP during *C. difficile* infection, we hypothesized that depletion of inflammatory cells would recapitulate the effects of MyD88 deficiency. We depleted Gr-1⁺ cells by administering RB6-8C5 antibody every other day, beginning on the day prior to *C. difficile* challenge. RB6-8C5-treated mice had markedly higher mortality following *C. difficile* infection, with approximately 80% mortality within 3 days of infection (Fig. 5A). Increased mortality, however, was not associated with increased growth or expansion of *C. difficile* in the large intestine on days 1 or 2 postinfection (Fig. 5B). To determine if RB6-8C5 depletion renders mice susceptible to systemic dissemination of commensal bacteria from the gut, we quantified viable bacteria in mesenteric lymph nodes 2 days following infection. RB6-8C5 treatment resulted in higher bacterial counts (Fig. 5C), suggesting that Gr-1⁺ cells play a role in containing luminal bacteria and preventing systemic bacterial dissemination during *C. difficile* colitis.

Neutrophil recruitment to the cLP is required for defense against *C. difficile*. Because Gr-1⁺ cells include both Ly6G⁺ neutrophils and Ly6C⁺ monocytes (7) and both are depleted by administration of RB6-8C5 antibody (Fig. 5D and E), we aimed to determine which of these cell populations promotes survival following *C. difficile* infection. In a strategy similar to that used for depletion of Gr-1⁺ cells, we used 1A8 (anti-Ly6G) antibody to

FIG 4 Monocyte and neutrophil infiltration of the cLP in *C. difficile*-infected mice. C57BL/6 mice were infected with 10^3 CFU *C. difficile* following a single dose of clindamycin the day before. Leukocytes were isolated from the cLP and analyzed by flow cytometry to determine neutrophil (A) and monocyte (B) infiltration during the course of infection. Results are expressed as the percentage of CD45⁺ cells in the cLP and are pooled from two independent experiments. (C) CCR2.GFP mice were challenged with *C. difficile* following clindamycin administration and sacrificed 3 days later. Frozen sections of cecum and colon were stained with 1A8 antibody (red, anti-Ly6G) to identify neutrophils. Hoechst (blue) was used for nuclear staining. L, lumen; E, edema; M, muscle layer. White arrows point to neutrophils within the luminal content. Scale bars represent 50 μ m. Images are representative of 3 independent experiments with 2 to 5 mice each.

FIG 5 Depletion of Gr1⁺ cells increases mortality during *C. difficile* colitis. Mice received a single dose of clindamycin on day -1, followed by infection with *C. difficile* VPI10463 the next day. Mice received three doses of RB6-8C5 antibody (anti-Gr1, 0.3 mg/dose) or PBS every other day starting on day -1. (A) Mice ($n = 10$ /group) were followed for survival. The log-rank (Mantel-Cox) statistical test was used. (B) The burden of *C. difficile* in the cecum of animals treated with RB6-8C5 or PBS was quantified on days 1 and 2. In panels A and B, results are pooled from at least two independent experiments. (C) Mice received two doses of RB6-8C5 or PBS on days -1 and 1. On day 2 postinfection, bacterial counts in the mesenteric lymph nodes were determined. (D and E) Mice were infected with *C. difficile* 1 day following clindamycin and either RB6-8C5 or PBS administration. On day 1 postinfection, the percentages of neutrophils and monocytes were assessed in the cLP of mice treated with RB6-8C5 or PBS and found to be significantly reduced.

deplete neutrophils and thus prevent their recruitment to the cLP. Administration of 1A8 antibody resulted in approximately 3-fold reduction in neutrophils (Fig. 6B), which was sufficient to markedly increase mortality during *C. difficile* colitis (Fig. 6A). However, this finding does not exclude a potentially important role for monocytes in defense. The role of CCR2 in monocyte recruitment from the bone marrow is well established (23). We corroborated that CCR2^{-/-} mice have normal neutrophil but impaired monocyte recruitment to the cLP during *C. difficile* infection (Fig. 6D). We treated these mice with clindamycin and challenged them with *C. difficile* spores. As shown in Fig. 6C, impaired monocyte recruitment to the cLP does not render CCR2^{-/-} mice more susceptible to severe *C. difficile* colitis. These results demonstrate that neutrophils are key players in defense against *C. difficile*, such that a 60% reduction in neutrophil recruitment renders mice highly susceptible to the disease. However, attenuation of monocyte recruitment did not have an effect on survival during *C. difficile* colitis.

MyD88-mediated signals promote neutrophil recruitment to the colon. Our findings suggest that a mechanism contributing to the susceptibility of MyD88^{-/-} mice to *C. difficile* colitis may involve impaired neutrophil recruitment. We next wanted to determine whether MyD88-mediated signals are required for the expression of CXCL1, a potent neutrophil-recruiting chemokine,

in the colon. As shown in Fig. 7A, there is an approximately 1,000-fold reduction in CXCL1 expression in the colon of MyD88^{-/-} mice during *C. difficile* infection. We corroborated that in these mice there was impaired neutrophil recruitment, and similar to the results shown in Fig. 3, there was a striking reduction in neutrophil infiltration into the cLP (Fig. 7B). Our studies suggest that MyD88-dependent expression of CXCL1 in the colon is required for neutrophil recruitment to the cLP during *C. difficile* infection.

DISCUSSION

In this study, we investigated the role of MyD88-mediated signaling in *C. difficile* colitis. We find that mortality following *C. difficile* infection is markedly increased in antibiotic-treated MyD88^{-/-} mice. While our demonstration of increased susceptibility in MyD88^{-/-} mice is consistent with two previous studies (14, 19), we extend previous work by demonstrating that MyD88-mediated signals are essential for recruitment of neutrophils and monocytes to the cLP during *C. difficile* infection. We have used specific depletion strategies to demonstrate that neutrophils provide a barrier in the aftermath of intestinal epithelial destruction during *C. difficile* infection, thereby preventing systemic dissemination of intestinal bacteria.

MyD88-mediated signaling is essential for intestinal homeostasis and defense against a range of infections. Our experiments

FIG 6 Neutrophil deficiency increases mortality during *C. difficile* colitis. (A and B) Mice received clindamycin on day -1 and were challenged with *C. difficile* spores the following day. Three doses of 1A8 antibody (anti-Ly6G, 0.3 mg/dose) or PBS were administered every other day starting on day -1. (A) Mice ($n = 8$ /group) were followed for survival. (B and D) Mice were sacrificed on day 2 postinfection, and neutrophil and monocyte infiltration was assessed by flow cytometry. CCR2^{-/-} mice are not more susceptible to *C. difficile* colitis than their wild-type C57BL/6 counterparts (B6) (C). Results are representative of two independent experiments. Log-rank and Student's *t* test were used for statistical analysis.

show that induction of the chemokine CXCL1 (KC) in the colon during *C. difficile* infection is dependent on MyD88 signaling. CXCR2 is a chemokine receptor that binds CXCL1 and is expressed by neutrophils. Upon stimulation by CXCL1, CXCR2-

mediated signals counter constitutive retention signals in the bone marrow that are mediated by CXCR4, thereby enabling neutrophils to traffic out of the bone marrow and to circulate in the bloodstream. CXCR2 is also believed to enable neutrophils to respond to CXCL1 in peripheral tissues, enabling neutrophil infiltration into sites of infection (20). Interestingly, the innate immune receptor NOD-1 also plays a role in CXCL1 expression, as demonstrated by low serum levels of the chemokine in NOD-1-deficient mice that were infected with *C. difficile* intraperitoneally (8).

Using CCR2^{-/-} mice, we demonstrated that a 90% reduction in monocyte recruitment to the cLP does not affect mortality during *C. difficile* colitis. Monocytes mediate clearance of many infectious pathogens, including intestinal pathogens such as *C. rodentium* (12) and *Toxoplasma gondii* (6). During *C. rodentium* infection, monocytes promote Th1 differentiation of responding T cells and enhance T cell-dependent IgG production, which contribute to recovery from infection (12). However, the role of monocytes in protection against these intestinal infections appears to be crucial at later stages of infection. Neutrophil-depleted mice infected with *C. difficile* succumb to colitis 2 to 4 days following infection, and it is possible, therefore, that monocytes are essential for defense against later stages of *C. difficile* infection. While impaired monocyte recruitment in CCR2^{-/-} mice does not result in higher susceptibility to *C. difficile* colitis up to 2 weeks

FIG 7 MyD88 deficiency results in impaired neutrophil recruitment from the bone marrow during *C. difficile* infection. (A) Mice were sacrificed 2 days postinfection, and the level of expression of the neutrophil-recruiting chemokine CXCL1 (KC) was assessed by quantitative PCR of colonic tissue. (B) The percentage of neutrophils in cLP was assessed by flow cytometry in the same mice as for panel A. Results are pooled from two independent experiments. AU, arbitrary units; B6, C57BL/6 mice.

postinoculation, it remains possible that CCR2-independent trafficking of Ly6Chi monocytes may contribute to immune defense against *C. difficile* infection.

Neutrophils provide early innate immune defense in a broad range of infections. In the gut, neutrophils are required for protection against the murine intestinal pathogen *C. rodentium* (15). In *C. difficile* infection, a role for neutrophils in protection has been suggested by RB6-8C5 depletion (8). However, the Gr-1 epitope targeted by RB6-8C5 is also highly expressed on monocytes, which are also depleted with RB6-8C5 treatment (5). Our experiments using the Ly6G-specific 1A8 antibody therefore demonstrate more specifically that neutrophils are critical for defense against *C. difficile* infection. Neutrophils, however, do not appear to mediate defense by decreasing the density of intestinal infection with *C. difficile* or reducing the concentration of toxin in the intestinal lumen. The burden of *C. difficile* in the cecum is not increased in neutrophil-depleted mice, and *C. difficile* is not disseminating systemically (our unpublished observations). However, dissemination of commensal intestinal bacteria to mesenteric lymph nodes is increased in *C. difficile*-infected mice that have undergone neutrophil depletion, suggesting a role for neutrophils in preventing systemic dissemination of intestinal bacteria, consistent with the findings of Hasegawa et al. (8).

We demonstrate that neutrophils aggregate near the intestinal lumen in areas where *C. difficile* infection has damaged the intestinal epithelial barrier. Neutrophils appear to create a barrier to prevent bacterial dissemination into underlying tissues and the bloodstream. Our immunohistologic studies demonstrate that neutrophils invade the cecal and colonic lumen while Ly6Chi monocytes remain within the cLP. Thus, while MyD88-mediated signals are required for the recruitment of neutrophils and monocytes to the cLP, once they enter the cLP these two cell populations presumably respond to different cues for further localization. This may result from distinct chemokine receptor expression by neutrophils and monocytes, or it may result from distinct responsiveness to adhesion molecules. For example, inflammatory monocytes recruited to hepatic foci of *Listeria monocytogenes* infection localize in a peripheral ring upon binding ICAM1, while neutrophils directly invade the site of bacterial infection (24). It is possible, therefore, that Ly6Chi monocytes bind adhesion molecules and become lodged in the lamina propria while neutrophils traffic toward bacterial populations in the intestinal lumen. Further studies will be required to identify the signals that explain the distinct trafficking of neutrophils and inflammatory monocytes during *C. difficile* infection. Defining the relative contributions of neutrophils and monocytes in defense against *C. difficile* infection and in pathogenesis has important potential clinical implications, particularly in immunocompromised patient populations that are at particularly high risk of infection.

ACKNOWLEDGMENTS

This work was supported by grants R01AI042135 and R37AI039031 to E.G.P. and a Career Development Award from the Northeast Biodefense Center to I.J.

We declare no conflict of interest.

REFERENCES

1. Adachi O, et al. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9:143–150.
2. Brandl K, Plitas G, Schnabl B, Dematteo RP, Pamer EG. 2007. MyD88-

- mediated signals induce the bactericidal lectin RegIII[gamma] and protect mice against intestinal *Listeria monocytogenes* infection. *J. Exp. Med.* 204:1891–1900.
3. Buffie CG, et al. 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infect. Immun.* 80:62–73.
4. Chen X, et al. 2008. A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology* 135:1984–1992.
5. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J. Leukoc. Biol.* 83:64–70.
6. Dunay IR, et al. 2008. Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. *Immunity* 29:306–317.
7. Fleming TJ, O'Huigin C, Malek TR. 1993. Characterization of two novel Ly-6 genes. Protein sequence and potential structural similarity to alpha-bungarotoxin and other neurotoxins. *J. Immunol.* 150:5379–5390.
8. Hasegawa M, et al. 2011. Nucleotide-binding oligomerization domain 1 mediates recognition of *Clostridium difficile* and induces neutrophil recruitment and protection against the pathogen. *J. Immunol.* 186:4872–4880.
9. Jarchum I, Liu M, Lipuma L, Pamer EG. 2011. Toll-like receptor-5 stimulation protects mice from acute *Clostridium difficile* colitis. *Infect. Immun.* 79:1498–1503.
10. Kelly CP, et al. 1994. Neutrophil recruitment in *Clostridium difficile* toxin A enteritis in the rabbit. *J. Clin. Invest.* 93:1257–1265.
11. Kelly CP, LaMont JT. 2008. *Clostridium difficile*—more difficult than ever. *N. Engl. J. Med.* 359:1932–1940.
12. Kim YG, et al. 2011. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. *Immunity* 34:769–780.
13. Kuehne SA, et al. 2010. The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* 467:711–713.
14. Lawley TD, et al. 2009. Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect. Immun.* 77:3661–3669.
15. Lebeis SL, Bommarius B, Parkos CA, Sherman MA, Kalman D. 2007. TLR signaling mediated by MyD88 is required for a protective innate immune response by neutrophils to *Citrobacter rodentium*. *J. Immunol.* 179:566–577.
16. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229–241.
17. Reeves AE, et al. 2011. The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* infection. *Gut Microbes* 2:145–158.
18. Rupnik M, Wilcox MH, Gerding DN. 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat. Rev. Microbiol.* 7:526–536.
19. Ryan A, et al. 2011. A role for TLR4 in *Clostridium difficile* infection and the recognition of surface layer proteins. *PLoS Pathog.* 7:e1002076. doi: 10.1371/journal.ppat.1002076.
20. Sadik CD, Kim ND, Luster AD. 2011. Neutrophils cascading their way to inflammation. *Trends Immunol.* 32:452–460.
21. Savidge TC, et al. 2011. Host S-nitrosylation inhibits clostridial small molecule-activated glucosylating toxins. *Nat. Med.* 17:1136–1141.
22. Serbina NV, Hohl TM, Cherny M, Pamer EG. 2009. Selective expansion of the monocytic lineage directed by bacterial infection. *J. Immunol.* 183:1900–1910.
23. Serbina NV, Pamer EG. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat. Immunol.* 7:311–317.
24. Shi C, et al. 2010. Monocyte trafficking to hepatic sites of bacterial infection is chemokine independent and directed by focal intercellular adhesion molecule-1 expression. *J. Immunol.* 184:6266–6274.
25. Stabler RA, et al. 2010. In-depth genetic analysis of *Clostridium difficile* PCR-ribotype 027 strains reveals high genome fluidity including point mutations and inversions. *Gut Microbes* 1:269–276.
26. Weigmann B, et al. 2007. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nat. Protoc.* 2:2307–2311.