Toll-Like Receptor 5 Stimulation Protects Mice from Acute *Clostridium difficile* Colitis

Irene Jarchum,* Mingyu Liu, Lauren Lipuma, and Eric G. Pamer*

Immunology Program, Department of Medicine (Division of Infectious Disease), Memorial Sloan-Kettering Cancer Center, New York, New York

Received 9 November 2010/Returned for modification 28 December 2010/Accepted 5 January 2011

*Corresponding author. Mailing address: Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 9, New York, NY 10065. Phone for Eric G. Pamer: (646) 888-2367. Fax: (646) 444-0502. E-mail: pamere@mskcc.org. Phone for Irene Jarchum: (646) 888-2361. Fax: (646) 444-0502. E-mail: jarchumi@mskcc.org.

**Published ahead of print on 18 January 2011.**

*Clostridium difficile* is a spore-forming bacterium that infects the lower intestinal tract of humans and is the most common known cause of diarrhea among hospitalized patients. *Clostridium difficile* colitis is mediated by toxins and develops during or following antibiotic administration. We have used a murine model of *C. difficile* infection, which reproduces the major features of the human disease, to study the effect of innate immune activation on resistance to *C. difficile* infection. We found that administration of purified *Salmonella*-derived flagellin, a Toll-like receptor 5 (TLR5) agonist, protects mice from *C. difficile* colitis by delaying *C. difficile* growth and toxin production in the colon and cecum. TLR5 stimulation significantly improves pathological changes in the cecum and colon of *C. difficile*-infected mice and reduces epithelial cell loss. Flagellin treatment reduces epithelial apoptosis in the large intestine, thereby protecting the integrity of the intestinal epithelial barrier during *C. difficile* infection. We demonstrate that restoring intestinal innate immune tone by TLR signaling in antibiotic-treated mice ameliorates intestinal inflammation and prevents death from *C. difficile* colitis, potentially providing an approach to prevent *C. difficile*-induced pathology.

*Clostridium difficile* is a Gram-positive, spore-forming rod that is acquired by oral ingestion of spores and occurs most commonly in hospitals and long-term health care facilities (27). Perturbation of the normal intestinal microflora by antibiotics generally precedes the development of *C. difficile* colitis, which is mediated by toxins and is associated with a spectrum of illnesses that extend from mild to severe diarrhea and that can escalate to life-threatening toxic megacolon. *C. difficile* infection is the most common known cause of diarrhea among hospitalized patients, and its incidence, as well as the severity of *C. difficile* infection, has increased over the last decade (13). It is estimated that the cost of this disease exceeds $1.1 billion in the United States annually (16).

*C. difficile* produces two toxins, toxin A and toxin B, which are endocytosed via clathrin-coated vesicles (24). Both toxins are potential virulence factors (15) and, upon entering the cytoplasm, monoglucosylate Rho GTPases, thereby inactivating them. Subsequent cytoskeletal disruption results in loss of tight junctions and compromises the integrity of the intestinal mucosa (7, 23, 29, 34). It has been postulated that *C. difficile* toxins reach the lamina propria after disruption of the intestinal epithelial barrier and target immune cell populations such as monocytes, macrophages, and T cells, leading to the production of proinflammatory cytokines and macrophage and T cell death (20, 22, 27).

The mechanism by which antibiotics lead to markedly increased susceptibility to *C. difficile* infection is not clear. The intestinal commensal flora, which plays a critical role in maintenance of innate and adaptive immune homeostasis, is depleted by antibiotic treatment (10). Toll-like receptors (TLRs) recognize pathogen-derived molecular patterns and activate innate immune pathways. TLR signaling is decreased in mice that undergo antibiotic treatment (2, 14), rendering the host more susceptible to infection.

MyD88, an adaptor protein required for signaling through most TLRs, is required for resistance to intestinal infections (26). MyD88 signaling protects the intestinal epithelium from severe damage and from passage of bacteria across the epithelial cell barrier during infection with the mouse colonic pathogen *Citrobacter rodentium* (18). In a mouse model of *C. difficile* infection, MyD88-deficient mice are more susceptible to the development of colitis than wild-type mice, underscoring the critical role of TLR signaling in defense against *C. difficile* (17). However, the mechanisms contributing to resistance against *C. difficile* infection remain poorly elucidated, in part due to the fact that mouse models for the disease have only recently been reported (5, 17). The hamster model of *C. difficile* colitis, in which disease is acute and principally affects the cecum, has been used extensively to investigate *in vivo* pathogenesis (1). The use of mice for the study of *C. difficile* colitis has several advantages, including the availability of genetically altered animals, a broad range of reagents to study immune responses, and a colitis model that affects the colon and cecum (5).

Our laboratory demonstrated that exogenous administration of TLR ligands restricts intestinal colonization with vancomycin-resistant *Enterococcus* (VRE) (2, 14). Both oral lipopolysaccharide (LPS) and systemic flagellin (TLR4 and TLR5 agonists, respectively) dramatically decrease VRE colonization upon administration to antibiotic-treated mice. Administration of flagellin, in contrast to LPS, is less inflammatory and is not associated with sepsis and severe organ pathology (6, 33). TLR5 stimulation by truncated flagellin has also been shown to protect mice from the harmful effects of radiation on the intestine (4, 33).

Here we investigated whether TLR5 ligation enhances re-
FIG. 1. Flagellin administration protects mice from *C. difficile*-induced death. Mice (*n* = 10 per group) received a cocktail of antibiotics in the drinking water on days −6 to −3 and were then switched to regular water. On day −1, mice received a single dose of clindamycin (200 μg) intraperitoneally, and they were infected with 10³ CFU of *C. difficile* spores (strain VPI 10463) the following day. Mice received three doses of flagellin (15 μg) or PBS intraperitoneally on days −1, 0, and 1 and were followed for survival. The data shown in panel A are representative of those from several independent experiments, which are tabulated in panel B.

**RESULTS**

Flagellin protects against *C. difficile* colitis. To determine if flagellin can protect mice from development of *C. difficile* colitis and death, we challenged mice with *C. difficile* after pretreatment with an antibiotic regimen previously described to confer susceptibility to *C. difficile* infection (5) and described in detail in Materials and Methods. Briefly, mice received antibiotics in their drinking water from day −6 to day −3 and on day −1 received a single dose of clindamycin. Mice were infected with 10³ CFU *C. difficile* spores by gavage on day 0, and on days −1, 0, and 1 they were treated with PBS or flagellin (15 μg per dose) intraperitoneally. Consistent with published reports of this mouse model (5), PBS-treated mice died within the first 5 days following infection. However, as shown in Fig. 1A, flagel-
lin administration markedly protected mice from death, with 10% mortality in mice that received flagellin, compared to 70% mortality in PBS-treated mice (P = 0.0043). In Fig. 1B, five independent experiments are tabulated, which showed highly reproducible results, similar to those in Fig. 1A. Flagellin administration substantially protects antibiotic-treated mice from *C. difficile* infection.

**TLR5 expression is required for flagellin-mediated protection.** To demonstrate that flagellin-mediated protection is dependent on signaling through TLR5, we tested whether flagellin treatment can protect TLR5−/− mice from *C. difficile* colitis. To equilibrate the intestinal flora, TLR5−/− and C57BL/6 mice were cohoused for at least 2 weeks, which, based on our experience and that of others, allows transfer of bacterial species, including those that adhere tightly to the intestinal epithelium, between the animals (8, 11). Mice received antibiotics in the drinking water on days −3 to −1, followed by a single dose of clindamycin on day 0. Mice were infected with *C. difficile* spores on day 0 and received three doses of flagellin or PBS on days −1, 0, and 1. As shown in Fig. 2A, C57BL/6 mice that received flagellin were protected from *C. difficile* colitis. In contrast, TLR5−/− mice treated with flagellin succumbed to disease (Fig. 2B). C. difficile-infected C57BL/6 mice were protected from severe weight loss following treatment with flagellin (Fig. 2C). Flagellin-treated mice lost less than 10% of their weight, while mice that received PBS underwent approximately 20% weight loss after *C. difficile* infection. However, flagellin treatment did not protect TLR5-deficient mice from extreme weight loss (Fig. 2D). These results demonstrate that flagellin-mediated protection from *C. difficile* colitis requires TLR5 expression in the infected host.

**Lower burden of *C. difficile* early in infection.** We next investigated whether flagellin mediates protection by decreasing *C. difficile* growth in the intestine. We pretreated mice with antibiotics and challenged them with 10^3 CFU *C. difficile*. Mice received two doses of flagellin or PBS on days −1 and 0, and we sacrificed animals on day 2 postinfection. TLR5 stimulation in *C. difficile*-infected mice with flagellin lowered the density of *C. difficile* in the cecum and colon by a factor of approximately 10,000 (Fig. 3A). Further, while *C. difficile* toxin was present in high concentrations in mice that were not treated with flagellin, cytotoxicity was undetectable in the intestinal contents of flagellin-treated mice (Fig. 3B).

**Flagellin prevents intestinal damage during *C. difficile* infection.** We next investigated whether TLR5 stimulation protects intestinal tissues from damage during *C. difficile* infection. Mice were pretreated with antibiotics and infected with 10^3 CFU *C. difficile*. On days −1, 0, and 1, mice received flagellin or PBS, and they were sacrificed on day 2 postinfection. Histologic analysis of colon (Fig. 4A and B) and cecum (Fig. 4A...
and C) from these mice revealed that TLR5 stimulation protects tissues of the lower intestine from damage. As shown in the representative images in Fig. 4A, at 2 days postinfection, inflammatory cell infiltration (mostly neutrophils), edema, and epithelial cell loss in the colon and cecum are evident in mice that received PBS. However, intestinal tissues and epithelial cells in flagellin-treated mice maintain structural integrity. Interestingly, we found that epithelial cell loss in the colon is

FIG. 3. TLR5 stimulation results in decreased CFU at 1 day postinfection. Mice were infected on day 0 with $10^5$ CFU C. difficile spores following antibiotic treatment. Mice received two doses of PBS or flagellin on days −1 and 0 and were sacrificed at 24 h postinfection. (A) Vegetative C. difficile CFU (spores are generally undetectable at this time point) in cecum and colon were quantified. (B) Cytotoxicity in cecal and fecal contents was quantified using a cell-based assay. Results in panel A are representative of at least two independent experiments. For panel B, results from three independent experiments were pooled. ND, not detectable.

FIG. 4. TLR5 stimulation prevents tissue damage in the lower intestine. Three groups of mice were treated with antibiotics and either kept uninfected or infected with $10^3$ CFU C. difficile spores. Infected mice were treated with PBS or flagellin on days −1, 0, and 1. On day 2, mice were sacrificed and their colons and ceca were isolated and fixed. Histology sections stained with hematoxylin and eosin were scored for edema, inflammatory cell infiltration, epithelial cell loss, and goblet cell loss. Scale bars represent 200 μm for large images and 50 μm for insets. The colons (A and B) and ceca (A and C) of flagellin-treated mice are largely protected from tissue damage. Particularly, epithelial cell loss is greatly reduced in mice that received PBS. However, intestinal tissues and epithelial cells in flagellin-treated mice maintain structural integrity. Interestingly, we found that epithelial cell loss in the colon is

Each symbol represents one mouse.
significantly reduced in mice that received TLR5 stimulation (Fig. 4A and D), suggesting that flagellin administration protects intestinal epithelial cells from apoptosis and/or stimulates their replenishment.

**Integrity of the intestinal epithelial barrier is maintained in flagellin-treated mice.** We performed experiments to determine the effect of TLR5 stimulation on the intestinal epithelial barrier in *C. difficile*-infected mice. Antibiotic-treated mice were infected with *C. difficile* and received three doses of flagellin or PBS on days −1, 0, and 1. Mice were sacrificed on day 2, and cecal tissue was analyzed by immunohistochemistry for detection of apoptosis by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (Fig. 5A). In contrast to the case for untreated mice, fewer epithelial cells undergo apoptosis in *C. difficile*-infected mice that received flagellin, suggesting that the integrity of the intestinal barrier may be maintained in these mice. Indeed, *C. difficile*-infected mice treated with flagellin have decreased permeability of the intestinal barrier to FITC-labeled dextran compared to PBS-treated mice. As shown in Fig. 5B, FITC-dextran was found in the sera of mice that received PBS. In contrast, FITC-dextran was detectable in flagellin-treated mice to the same extent as in uninfected mice. These experiments demonstrate that TLR5 stimulation maintains the integrity of the epithelial barrier in the intestine.

**DISCUSSION**

In this study, we demonstrate for the first time that flagellin-mediated TLR5 stimulation protects mice from death during *C. difficile* colitis. We find that at 24 h after infection of antibiotic-treated mice with *C. difficile*, the density of *C. difficile* in mice that received flagellin is about 10,000 times lower than that in PBS-treated mice. Our experiments established that flagellin maintains the structural integrity of the epithelial layer of the large intestine during *C. difficile* infection. Striking edema and epithelial cell loss in PBS-treated mice contrasts with largely normal mucosal architecture of colonic and cecal tissues of flagellin-treated mice. Further, apoptosis is decreased in the large intestine and the epithelial intestinal barrier is protected in flagellin-treated mice infected with *C. difficile*.

Previous studies show that TLR5 stimulation limits the harmful effects of radiation by protecting the intestinal epithelial barrier (4). Administration of a truncated version of *Salmonella*-derived flagellin that retains the ability to stimulate NF-κB activation but has reduced toxicity and immunogenicity protects mice from a lethal dose of irradiation. Interestingly, that study also found that flagellin has an antiapoptotic effect on intestinal cells of irradiated mice and induces proliferation of crypt cells. Flagellin treatment has been shown to reduce inflammation and neutrophil infiltration in the dextran sulfate sodium (DSS)-colitis model (33). Recent studies show that mucosal administration of flagellin can protect against lung infection in murine models of acute pneumonia, underscoring the effectiveness of this TLR ligand in eliciting productive innate immune responses during infection (21, 36).

While *C. difficile* has a gene encoding flagellin (30), the main component of flagella, our experiments have not revealed higher susceptibility to *C. difficile* infection in TLR5-deficient mice. Therefore, we speculate that *C. difficile*- or microbiota-derived flagellin does not play a major role in eliciting a protective immune response upon infection. TLR5 is expressed on intestinal cells of irradiated mice and induces proliferation of crypt cells. Flagellin treatment has been shown to reduce inflammation and neutrophil infiltration in the dextran sulfate sodium (DSS)-colitis model (33). Recent studies show that mucosal administration of flagellin can protect against lung infection in murine models of acute pneumonia, underscoring the effectiveness of this TLR ligand in eliciting productive innate immune responses during infection (21, 36).

While *C. difficile* has a gene encoding flagellin (30), the main component of flagella, our experiments have not revealed higher susceptibility to *C. difficile* infection in TLR5-deficient mice. Therefore, we speculate that *C. difficile*- or microbiota-derived flagellin does not play a major role in eliciting a protective immune response upon infection. TLR5 is expressed on the basolateral surface of intestinal epithelial cells (9), on endothelial cells of the intestine (19), and on a subset of lamina propria dendritic cells (31). Therefore, *C. difficile*-derived flagellin would not signal through TLR5 until the intestinal epithelial barrier has been destroyed by the action of the toxins. At this time, however, the toxins themselves elicit a rapid and robust recruitment of immune cells and cytokine production (20, 22). It is likely that exogenous administration of flagellin, as we have done in our study, protects mice from *C. difficile* infection by triggering TLR5 signaling prior to disruption of the intestinal epithelial layer.

The mechanism by which flagellin prevents the accumulation of *C. difficile* in the large intestine is unclear. At least two scenarios are possible: first, flagellin may directly or indirectly

**FIG. 5. Flagellin treatment protects against damage to the intestinal epithelial barrier.** C57BL/6 mice received antibiotics in the drinking water on days −6 to −3 and a single dose of clindamycin on day −1. This was followed by infection by gavage with 10⁶ CFU *C. difficile* spores and flagellin or PBS treatment on days −1, 0, and 1. (A) Mice were sacrificed at 2 days postinfection, and the cecum was obtained and stained for the detection of cell death with TUNEL. TUNEL-positive cells were quantified blindly. (B) At 2 days postinfection, mice were fasted from food and drink for 4 h and were administered 15 μg FITC-dextran by gavage. Four hours later, mice were sacrificed and bled by cardiac puncture, and the presence of FITC-dextran in serum was assessed. For panels A and B, results from two independent experiments were pooled.
inhibit C. difficile germination, or second, it may prevent C. difficile proliferation. Work from our laboratory has demonstrated that TLR stimulation in mice infected with vancomycin-resistant Enterococcus (VRE) results in decreased coloni- zation with VRE. The antimicrobial peptide RegIIIγ, which is upregulated by LPS and flagellin administration, can kill VRE and other Gram-positive bacteria (2, 3, 14). Flagellin administration during C. difficile infection also leads to the upregu-lation of RegIIIγ expression in the large intestine (our unpublished results), and thus it is possible that RegIIIγ-mediated killing decreases the density of C. difficile within the first day following infection of antibiotic-treated mice. TLR5 stimulation results in production of the inflammatory cytokine interleukin-22 (IL-22) (14, 32), which is required for flagellin-mediated restriction of colonization by VRE (14). IL-22 is also crucial for resistance to the murine intestinal pathogen Citrobacter rodentium (37). IL-22 production in the gut activates the transcription factor STAT3 in epithelial cells, and specific depletion of STAT3 expression in intestinal epithe-lium leads to increased apoptosis and decreased proliferation of intestinal epithelial cells (25). Further, IL-22 increases expression of RegIIIγ (14, 25). It is not known, however, whether IL-22 is required for TLR5-mediated protection against C. difficile infection.

A recent study indicates that MyD88-deficient mice have in-creased susceptibility to C. difficile infection (17), consistent with the notion that TLR signaling induced by the intestinal microbi-ota maintains homeostatic innate immune defenses, thereby con-ferring resistance to C. difficile colitis. It was unclear, however, whether specific TLR signaling is sufficient to prevent death follow-ing C. difficile infection of antibiotic-treated mice. In the pres-ent study we demonstrate for the first time that flagellin-mediated stimulation of TLR5 protects against C. difficile colitis. In hospi-talized patients in whom antibiotic administration is unavoidable, TLR5 engagement by exogenous ligand administration may be a successful approach to ameliorate C. difficile infection.

ACKNOWLEDGMENTS

This work was supported by grants R01 AI042135 and R37 AI039031 to E.G.P.

REFERENCES


