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

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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 demonstrated that restoring intestinal innate immune tone by TLR5 stimulation 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 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-
bistance to \textit{C. difficile} infection. We found that flagellin protects mice from lethal \textit{C. difficile} infection. Flagellin inhibits \textit{C. difficile} growth in the intestine during the first 24 h following inoculation, prevents the development of intestinal pathology, and protects the intestinal epithelial barrier. This is the first report demonstrating that targeted TLR stimulation can protect against \textit{C. difficile} infection. These studies contribute to our understanding of resistance to \textit{C. difficile} and provide a basis for the potential development of therapeutics that enhance the safety of antibiotic administration.

\section*{MATERIALS AND METHODS}

\textbf{Mice and infection regimen.} C57BL/6 female mice (6 to 8 weeks of age) were purchased from The Jackson Laboratory. TLR5-deficient mice were provided by R. Flavell (Yale University, New Haven, CT), and the colony is maintained at Memorial Sloan-Kettering Cancer Center (MSKCC) by mating of TLR5$^{-/-} \times$ TLR5$^{-/-}$. Mice received antibiotic treatment prior to \textit{C. difficile} infection as follows. Antibiotics (kanamycin, 0.4 mg/ml; gentamicin, 0.035 mg/ml; colistin, 850 U/ml; metronidazole, 0.215 mg/ml; and vancomycin, 0.045 mg/ml) were administered in the drinking water on days −6 to −3. On day −3, mice were provided regular drinking water. On day −1, animals received a single dose of clindamycin (200 $\mu$g) intraperitoneally, and they were infected on day 0 with $10^5$ CFU \textit{C. difficile} strain VPI 10463 spores by gavage. Mice received flagellin (15 $\mu$g in phosphate-buffered saline (PBS)) or PBS intraperitoneally on the days indicated for each experiment. \textit{Salmonella enterica} serovar Typhimurium-derived flagellin was used for all experiments (InvivoGen). All mice were maintained in a specific-pathogen-free facility at Memorial Sloan-Kettering Cancer Center Animal Resource Center. Age- and gender-matched animals were used for all experiments. Experiments were approved under institutional guidelines (IACUC protocol 00-05-066).

\textbf{C. difficile culture.} \textit{C. difficile} strain VPI 10463 (ATCC 43255) was purchased from the American Type Culture Collection (ATCC) and cultured in BHIS media at 37°C in an anaerobic chamber (Coy Labs). After a single colony was obtained, bacteria were patched onto BHIS agar. Ten days later, bacteria were recovered from the plates and spores were obtained as previously described (28). Briefly, spores were released from the mother cell by multiple washes in ice-cold PBS. Pellets were resuspended in 20% HistoDenz (Sigma-Aldrich), layered over 50% HistoDenz, and centrifuged at 15,000 $\times$ g for 15 min. The HistoDenz gradient separates spores from vegetative bacteria (28). The absence of live vegetative bacteria was confirmed by microscopic examination and the inability to grow in the absence of taurocholate. After isolation of pelleted spores, they were washed five times in ice-cold PBS.

\textbf{Quantitative culture of \textit{C. difficile} spores and vegetative forms.} Stool pellets or intestinal contents from ileum, cecum, or colon were suspended in deoxygencated PBS. Ten-fold dilutions of the suspension were plated anaerobically on BHIS plates containing taurocholate, t-cycloserine, and cefoxitin for specific selection of \textit{C. difficile} and maximum recovery of both vegetative and spore forms of \textit{C. difficile}. The suspension was then wet heated at 60°C for 20 min to kill vegetative forms and then plated on BHIS plates containing taurocholate, t-cycloserine, and cefoxitin in order to quantify the number of \textit{C. difficile} spores in the intestinal contents or the stool samples. Plates were placed in a 37°C incubator within the anaerobic chamber overnight.

\textbf{C. difficile toxin A and toxin B determination.} The presence of \textit{C. difficile} toxins was determined using a cell-based cytotoxicity assay. Human embryonic lung fibroblast WI-38 cells (ATCC) were plated at 10$^4$ cells/well in a 96-well plate overnight for formation of a monolayer. Intestinal or fecal contents were resuspended in sterile PBS and spun at 10,000 $\times$ g for 10 min. Ten-fold dilutions of the supernatant were incubated with either PBS or antitoxin antisera (Techlab, Blacksburg, VA) for 20 min at room temperature. These were then added to WI-38 cells for overnight incubation, after which the effect of intestinal content supernatants on cell rounding was assessed. The presence of \textit{C. difficile} toxins A and B was confirmed by neutralization by antitoxin antisera. Data are expressed as the log$_{10}$ reciprocal value of the first dilution at which cell rounding was not observed.

\textbf{Histologic analysis of cecum and colon.} Cecum and colon tissues were removed from mice, fixed in biopsys specimen-embedding cassettes using freshly made ice-cold 4% paraformaldehyde or Carnoy's fixative, and incubated overnight at 4°C. After fixation, the samples were washed twice with PBS and dehydrated prior to processing for embedding and sectioning. Tissues were stained with hematoxylin and eosin. The severity of enteritis was quantified using a grading system (12) that includes evaluation (on a scale of 0 to 3 for each parameter) of edema of the mucosa, inflammatory cell infiltration, epithelial cell loss, and goblet cell loss. Histologic analysis and scoring of these parameters was performed blindly by a single pathologist.

\textbf{In vivo permeability assay.} To assess the integrity of the intestinal epithelial barrier, mice were fasted for 4 h and were administered 15 mg fluorescein isothiocyanate (FITC)-dextran (average molecular weight, 5,000 to 5,000; Sigma-Aldrich) by gavage. Four hours later, mice were sacrificed by CO$_2$ inhalation and bled by cardiac puncture. The presence of FITC-dextran was assessed in serum 4 h later (excitation, 485 nm; emission, 530 nm) (35).

\textbf{Statistical analysis.} Student’s $t$ test was used throughout the study, except for survival curves, for which the log rank (Mantel-Cox) test was used.

\section*{RESULTS}

\textbf{Flagellin protects against \textit{C. difficile} colitis.} To determine if flagellin can protect mice from development of \textit{C. difficile} colitis and death, we challenged mice with \textit{C. difficile} after pre-treatment with an antibiotic regimen previously described to confer susceptibility to \textit{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 they received a single dose of clindamycin. Mice were infected with $10^3$ CFU \textit{C. difficile} spores by gavage on day 0, and on days −1, 0, and 1 they were treated with PBS or flagellin (15 $\mu$g 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, flagell-
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 −6 to −3, followed by a single dose of clindamycin prior to infection with 10^3 CFU of C. difficile spores on day 0. (A) C57BL/6 mice received three doses of PBS or flagellin on days −1, 0, and 1 and were followed for survival. (B) TLR5-deficient mice that underwent the antibiotic regimen and received three doses of flagellin were not protected from C. difficile colitis. (C and D) Similar results were found when flagellin- and PBS-treated C57BL/6 (C) and TLR5−/− (D) were followed for weight loss. C57BL/6 mice that received PBS lost approximately 20% of their body weight, while mice that received flagellin were greatly protected from weight loss (P = 0.0146) (C). However, TLR5−/− mice that received flagellin (n = 9) were not protected from weight loss compared to mice that received PBS (n = 9) (D).

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 1 (24 h following infection and the last dose of flagellin) and obtained samples of intestinal contents from cecum and colon. 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 $10^7$ 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
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 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
inhibit C. difficile germination, or second, it may prevent C. difficile proliferation. Work from our laboratory has demonstrated that TLR signaling induced by the intestinal microbiota maintains homeostatic innate immune defenses, thereby conferring resistance to C. difficile colitis. It was unclear, however, whether specific TLR signaling is sufficient to prevent death following C. difficile infection.

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

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