



Intestinal Inflammation and Altered Gut Microbiota Associated with Inflammatory Bowel Disease Render Mice Susceptible to *Clostridioides difficile* Colonization and Infection

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ABSTRACT *Clostridioides difficile* is a noteworthy pathogen in patients with inflammatory bowel disease (IBD). Patients with IBD who develop concurrent *C. difficile* infection (CDI) experience increased morbidity and mortality. IBD is associated with intestinal inflammation and alterations of the gut microbiota, both of which can diminish colonization resistance to *C. difficile*. Here, we describe the development of a mouse model to explore the role that IBD-induced changes of the gut microbiome play in susceptibility to *C. difficile*. *Helicobacter hepaticus*, a normal member of the mouse gut microbiota, triggers pathological inflammation in the distal intestine akin to human IBD in mice that lack intact interleukin 10 (IL-10) signaling. We demonstrate that mice with *H. hepaticus*-induced IBD were susceptible to *C. difficile* colonization in the absence of other perturbations, such as antibiotic treatment. Concomitant IBD and CDI were associated with significantly worse disease than observed in animals with colitis alone. Development of IBD resulted in a distinct intestinal microbiota community compared to that of non-IBD controls. Inflammation played a critical role in the susceptibility of animals with IBD to *C. difficile* colonization, as mice colonized with an isogenic mutant of *H. hepaticus* that triggers an attenuated intestinal inflammation maintained full colonization resistance. These studies with a novel mouse model of IBD and CDI emphasize the importance of host responses and alterations of the gut microbiota in susceptibility to *C. difficile* colonization and infection in the setting of IBD.

IMPORTANCE The incidence of *C. difficile* infection (CDI) has increased significantly among patients with IBD, independently of antibiotic use, yet the relationship between IBD and increased risk for CDI remains to be understood. Our study sought to describe and utilize an antibiotic-independent mouse model to specifically explore the relationship between the IBD-associated gut and susceptibility to *C. difficile* colonization and CDI development. We demonstrate that the development of IBD is sufficient to render mice susceptible to *C. difficile* colonization and results in significantly worse disease than IBD alone. Furthermore, this model requires IBD-induced inflammation to overcome colonization resistance to *C. difficile*. This model recapitulates human IBD and CDI comorbidity and will aid in developing new clinical approaches to predict, diagnose, and treat *C. difficile* infection in the IBD population.

KEYWORDS *Clostridium difficile*, *Helicobacter hepaticus*, animal models, gut inflammation, inflammatory bowel disease, intestinal colonization

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Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are chronic and progressive conditions characterized by inflammation of the digestive tract. The incidence of *Clostridioides difficile* infection (CDI) has significantly increased among hospitalized patients with IBD over the past 2 decades (1–3). *C. difficile* is a spore-forming bacterium that produces enterotoxins that damage the intestinal epithelium (4). *C. difficile* was initially described as a cause of antibiotic-associated diarrhea (5, 6). Normally, an intact intestinal microbiota provides resistance to *C. difficile* (7). However, antibiotic exposure can render otherwise healthy individuals susceptible to CDI due to disruption of the microbiota. Although antibiotic use is a well-known risk factor for CDI, other risk factors have been recognized, including immunosuppression and preexisting IBD (8, 9). The gut microbiota plays a critical role in the pathogenesis of IBD (10, 11), and CDI is associated with more severe intestinal microbiota disturbances among patients with IBD (12). In addition, underlying IBD lowers the long-term efficacy of fecal microbiota transplantation (FMT) to treat recurrent CDI, and this is associated with less robust engraftment of donor microbes (13). These results suggest that the pathophysiology of IBD influences gut microbiota composition and CDI outcomes.

The pathogenesis of IBD and CDI have long been studied in animal models (14, 15); however, a robust mouse model of comorbid IBD and CDI in the absence of antibiotic-induced perturbation of the microbiota has yet to be described. Colonization with enteric *Helicobacter* species, including *Helicobacter hepaticus*, has been shown to trigger colitis in genetically predisposed mice, such as those lacking the regulatory cytokine interleukin 10 (IL-10) (16–18). IL-10^{-/-} mice reared under *Helicobacter*-free, specific-pathogen-free (SPF) conditions develop colitis that resembles human IBD when colonized with *H. hepaticus* (16, 17, 19), and this intestinal inflammation is associated with alterations in gut microbiota community structures (20). Interestingly, the ability of *H. hepaticus* to trigger IBD in IL-10^{-/-} mice depends on the presence of an indigenous microbiota, as ex-germfree mice mono-colonized with *H. hepaticus* do not develop severe colitis (21). Most previously described mouse models of CDI require antibiotic administration to disrupt the intestinal microbiota and render animals susceptible to *C. difficile* colonization and disease (15, 22) and have revealed that colonization resistance and protection from CDI are mediated by the microbiota and (7, 23) host immune responses (24, 25).

In the present study, we sought to replicate the relationship between IBD and CDI in a mouse model. We wished to develop a system where we could evaluate the specific role of intestinal inflammation and gut microbiota in inducing susceptibility to *C. difficile* colonization and infection. We utilized a genetic model of murine IBD where intestinal inflammation is triggered by a normal member of the gut microbiota and show that the development of colitis is associated with a loss of colonization resistance to *C. difficile*.

RESULTS

Intestinal inflammation in IL-10^{-/-} mice colonized with *H. hepaticus* is associated with altered gut microbiota. Wild-type (WT) and IL-10^{-/-} C57BL/6 mice reared under specific-pathogen-free (SPF) conditions received *H. hepaticus* or sterile broth via oral gavage. Animals were monitored for the development of intestinal inflammation by measurement of the inflammatory marker lipocalin-2 in feces. We confirmed that wild-type mice did not develop signs of intestinal inflammation, regardless of *H. hepaticus* colonization status, whereas IL-10^{-/-} mice colonized with *H. hepaticus* did develop colitis. The level of lipocalin-2 was significantly increased in the feces of IL-10^{-/-} mice 7 days after *H. hepaticus* colonization, and this increase was sustained at 14 days post-colonization (Fig. 1A). Histological examination of colon sections harvested from IL-10^{-/-} mice colonized with *H. hepaticus* revealed pathology consistent with inflammatory bowel disease, including loss of goblet cells, inflammatory cell infiltration, and crypt elongation 14 days after *H. hepaticus* colonization, compared to that of WT mice of either genotype mock challenged with sterile tryptic soy broth (Fig. 1B).

We determined whether intestinal inflammation was associated with changes in the gut microbiota. *H. hepaticus* colonization in WT mice did not significantly impact

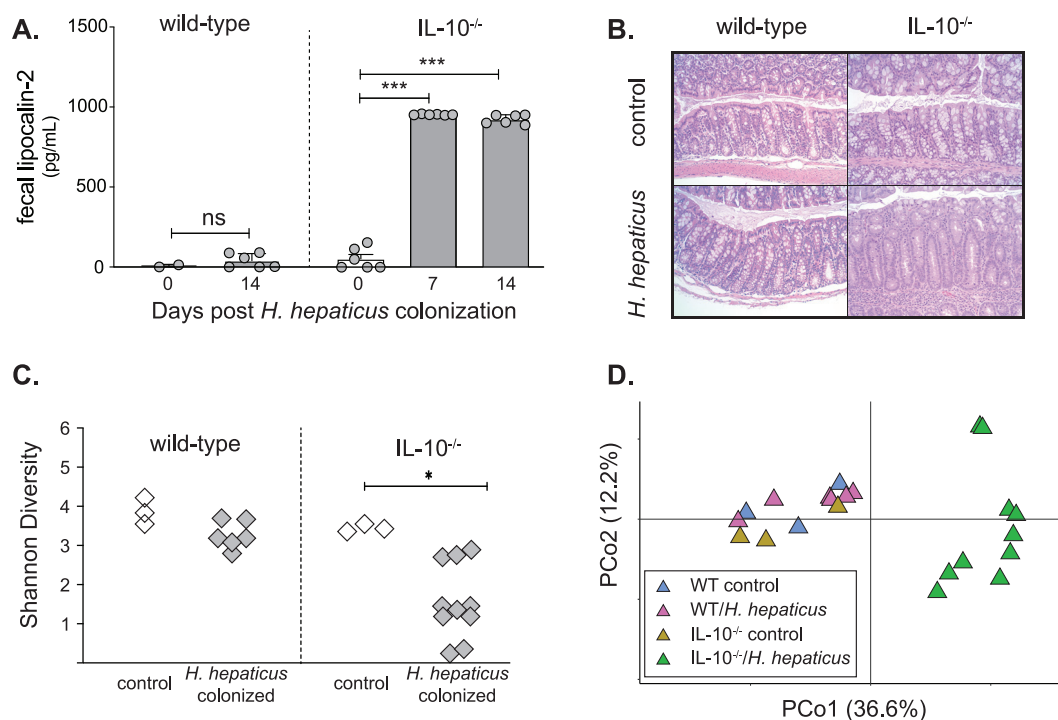


FIG 1 Intestinal inflammation is associated with altered intestinal microbiota in IL-10^{-/-} mice colonized with *H. hepaticus*. (A) Lipocalin-2 levels in feces from SPF wild-type mice or SPF IL-10^{-/-} mice were measured by ELISA at days 7 and 14 after colonization with *H. hepaticus* or mock challenged with sterile tryptic soy broth (control). ANOVA and the Tukey test were performed (***, $P < 0.001$). (B) Colonic histologic analysis in SPF wild-type mice or IL-10^{-/-} mice 14 days after colonization with *H. hepaticus* or mock colonization with sterile broth (control). No histopathologic changes are seen in wild-type animals regardless of *H. hepaticus* status or in control IL-10^{-/-} mice. The colons of IL-10^{-/-} mice colonized with *H. hepaticus* had notable epithelial hyperplasia with an acute mucosal and submucosal infiltrate. Representative hematoxylin and eosin images are shown ($\times 50$ original magnification). (C) Shannon diversity indexes of the microbial community in the lumens of the proximal colons of wild-type mice or IL-10^{-/-} mice 14 days after *H. hepaticus* colonization or control. Bacterial community diversity was lower in IL-10^{-/-} mice colonized with *H. hepaticus*, while no change was seen in wild-type animals following *H. hepaticus* colonization. A two-tailed unpaired *t* test was performed (*, $P < 0.05$). (D) Principal-coordinate (PCo) analysis plot of Bray-Curtis distances of bacterial communities in the luminal contents collected from the colons of wild-type mice or IL-10^{-/-} mice 14 days after *H. hepaticus* colonization or from control animals mock challenged with sterile broth.

the diversity of the colonic microbial community (Fig. 1C and D). However, intestinal inflammation in SPF IL-10-deficient animals induced by *H. hepaticus* colonization was associated with an altered colonic microbial community structure (Fig. 1D). Differences in microbial community structure were driven by treatment ($r^2 = 0.193$, $P = 0.0008$) and mouse genotype ($r^2 = 0.245$, $P = 0.004$) (Fig. 1D). Similar changes in the microbiota after the development of colitis were observed in both the cecum and proximal colon (Fig. S1). Taken together, these data demonstrate that the development of IBD in IL-10^{-/-} mice colonized with *H. hepaticus* is accompanied by alterations in the distal gut microbiota.

The development of IBD in IL-10^{-/-} animals results in susceptibility to *C. difficile* colonization. We explored whether mice with intestinal inflammation due to IBD are susceptible to CDI. SPF IL-10^{-/-} mice were colonized with *H. hepaticus* to trigger IBD prior to challenge with spores of *C. difficile* strain VPI 10463 (Fig. 2A). As a control for the development of CDI in IL-10^{-/-} animals, we utilized our standard CDI model with antibiotic pretreatment, administering the broad-spectrum antibiotic cefoperazone for 10 days, followed by *C. difficile* spore challenge (22). Both groups of animals were monitored for *C. difficile* colonization and signs of clinical disease. As noted above, intestinal inflammation triggered by *H. hepaticus* colonization was associated with a fecal microbiota structurally distinct from that of noncolonized controls. Furthermore, animals treated with cefoperazone had a fecal microbiota at the time of *C. difficile* challenge that was different from those of the other two experimental groups (Fig. 2B).

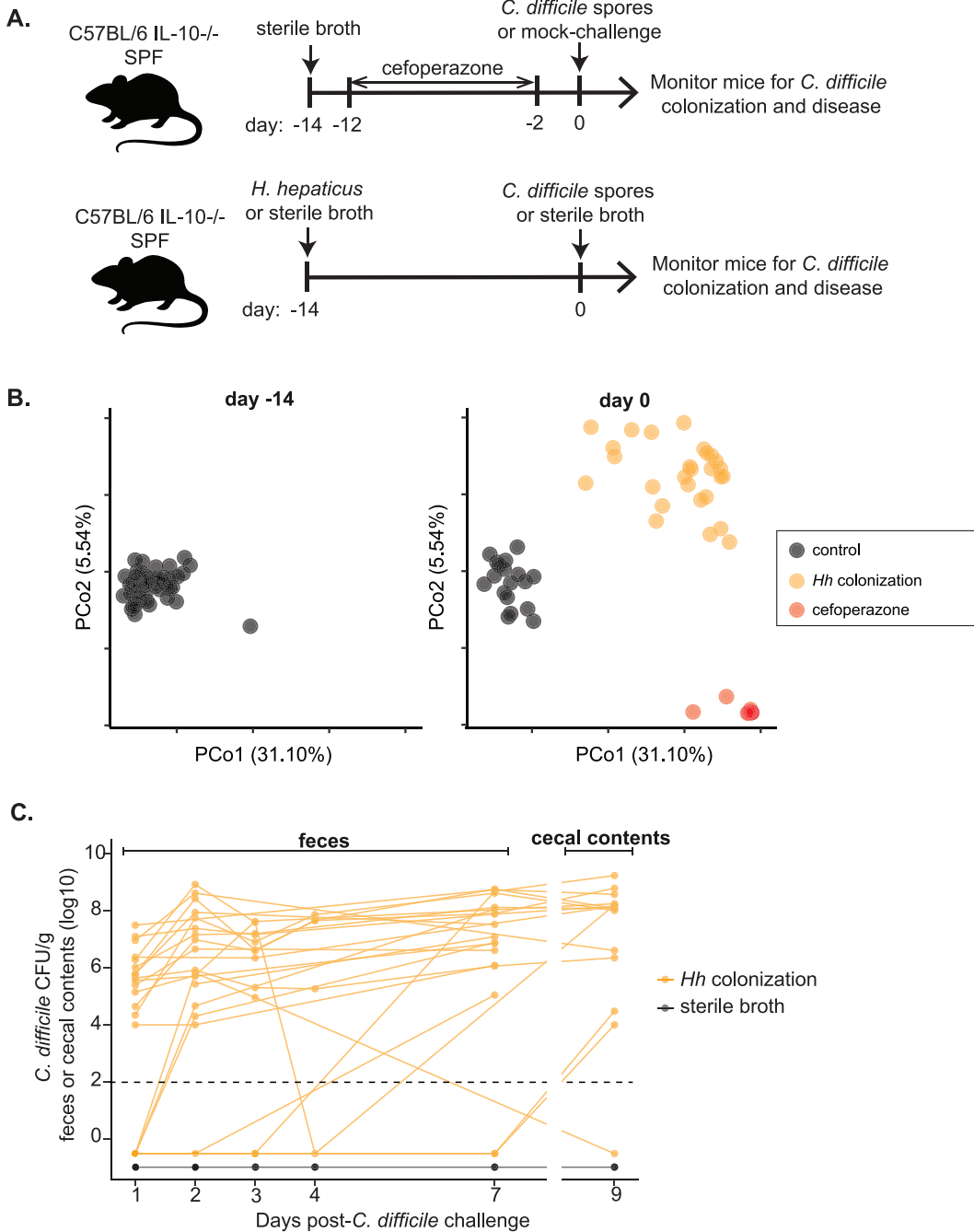


FIG 2 The development of colitis in IL-10^{-/-} animals following colonization with *H. hepaticus* results in a loss of colonization resistance against *C. difficile*. (A) Experimental timeline for models of *C. difficile* infection in IL-10^{-/-} mice. Animals were either treated with the antibiotic cefoperazone to alter their gut microbiota or infected with *H. hepaticus* to trigger colitis. (B) Principal-coordinate plots of Bray-Curtis distances of bacterial communities in feces collected at baseline (day -14, prior to any experimental treatment) and on the day of *C. difficile* spore challenge (day 0). Animals that received sterile broth had no change in bacterial community structure at day 0 compared to baseline, while cefoperazone treatment and the development of colitis after *H. hepaticus* colonization caused marked, but differing, alterations in the microbiota. (C) Colonization dynamics in IL-10^{-/-} mice following *C. difficile* spore challenge. IL-10^{-/-} mice that had developed colitis after *H. hepaticus* colonization shed variable amounts of *C. difficile* in their feces after challenge. Lines show the colonization trajectory of individual mice. All colitic IL-10^{-/-} mice had *C. difficile* detectable in their feces at some point in the 7 days after challenge, and 8 of 9 mice had *C. difficile* isolated from cecal contents at the time of necropsy 9 days after spore challenge. No *C. difficile* was ever recovered from the feces or cecal contents of noncolitic (i.e., noncolonized with *H. hepaticus*) mice at any point after challenge. The dotted line indicates the limit of detection for *C. difficile* quantification (10² CFU). Data represent results from 2 independent experiments. A two-tailed unpaired *t* test was performed (*P* < 0.01).

IL-10^{-/-} mice challenged with *C. difficile* spores in the absence of *H. hepaticus*-triggered colitis or antibiotic pretreatment were resistant to *C. difficile* colonization (Fig. 2C). As we have observed previously in wild-type animals, cefoperazone-treated IL-10^{-/-} mice had high levels of *C. difficile* colonization 1 day after the spore challenge (Fig. S2). Interestingly, 68% of IL-10^{-/-} animals with *H. hepaticus*-triggered colitis shed *C. difficile* in their feces 1 day after challenge (Fig. 2C). At 7 days after spore challenge, 89% of mice with IBD shed *C. difficile* (Fig. 2C). As opposed to what we had observed in antibiotic-treated mice, there was temporal variability in the shedding of *C. difficile* in the feces of mice with IBD. All mice with IBD had a detectable level of *C. difficile* colonization at some point during the course of monitoring post-spore challenge, but variation in the levels was seen over the 9 days of the experiment (Fig. 2C).

The clinical course of IL-10^{-/-} animals that were challenged with *C. difficile* after pretreatment with cefoperazone followed what we had reported for wild-type animals (22, 26). At the time of necropsy, animals had gross colitis. Histopathologic analysis revealed severe colitis with edema, epithelial damage, and a marked neutrophilic infiltrate (Fig. S2). Animals with concurrent IBD and CDI lost significantly more weight than mice with IBD alone at day 7 and day 9 after *C. difficile* spore challenge (Fig. 3A). Overall, IL-10^{-/-} animals with *H. hepaticus*-triggered colitis and infected with *C. difficile* had higher clinical disease scores than mice with IBD alone (Fig. 3B). These results suggest that IBD superimposed with CDI results in more severe clinical disease than IBD alone.

Nine days after challenge with *C. difficile* spores, the IL-10^{-/-} animals were euthanized and tissue was collected for histopathologic analysis. The ceca and colons of mice were examined by a veterinary pathologist and scored for edema, inflammatory infiltrate, and epithelial damage (22). While clinical disease was greater in animals with comorbid CDI and IBD, the histopathology scores were not different between mice with IBD alone and mice with comorbid CDI (Fig. 3C). As the pathogenesis of *C. difficile* infection is due to the production of the toxins TcdA and TcdB (27), we measured the production of toxin in the ceca of animals at the time of necropsy and quantified the *C. difficile* burden in the cecal contents of animals with IBD and superimposed CDI. As a group, the level of *C. difficile* colonization in animals with CDI and IBD was not significantly different from that of animals that were rendered susceptible by antibiotic administration (Fig. 3D). Despite these similar levels of colonization, mice with CDI following cefoperazone treatment had significantly more active *C. difficile* toxin in their cecal contents than mice with comorbid IBD and CDI (Fig. 3E). Several mice with IBD did not have detectable *C. difficile* toxin activity, despite being colonized with *C. difficile*.

Inflammation following *H. hepaticus* colonization is necessary to render IL-10-deficient mice susceptible to *C. difficile* colonization. Our data demonstrate an association between *H. hepaticus* colonization, the development of gut inflammation, and microbiota changes with susceptibility to *C. difficile* infection. In an effort to disentangle these potential factors leading to the loss of colonization resistance against *C. difficile*, we utilized an isogenic mutant of *H. hepaticus* that is unable to produce cytolethal distending toxin (CDT), a bacterial genotoxin known to modulate immune responses (28). We previously demonstrated that IL-10^{-/-} mice colonized with an *H. hepaticus* strain deficient in CDT production (*HhCDT*⁻) develop significantly attenuated colitis despite colonization at the same levels as for wild-type *H. hepaticus* (*HhCDT*⁺) (29, 30). We used this isogenic mutant to further explore the contribution of *H. hepaticus* colonization and the development of colitis to *C. difficile* susceptibility in IL-10^{-/-} animals. IL-10-deficient mice were colonized with either the wild-type strain (*HhCDT*⁺) or the *HhCDT*⁻ mutant and compared to controls that received sterile broth via oral gavage. Fourteen days later, all three groups of mice were challenged with *C. difficile* strain VPI 10463 spores and monitored for *C. difficile* colonization and disease (Fig. 4A). Mice colonized with wild-type *H. hepaticus* had significantly higher levels of fecal lipocalin-2 (Fig. 4B) than those colonized with the CDT-deficient mutant and a significantly higher degree of histopathologic intestinal inflammation (Fig. S3).

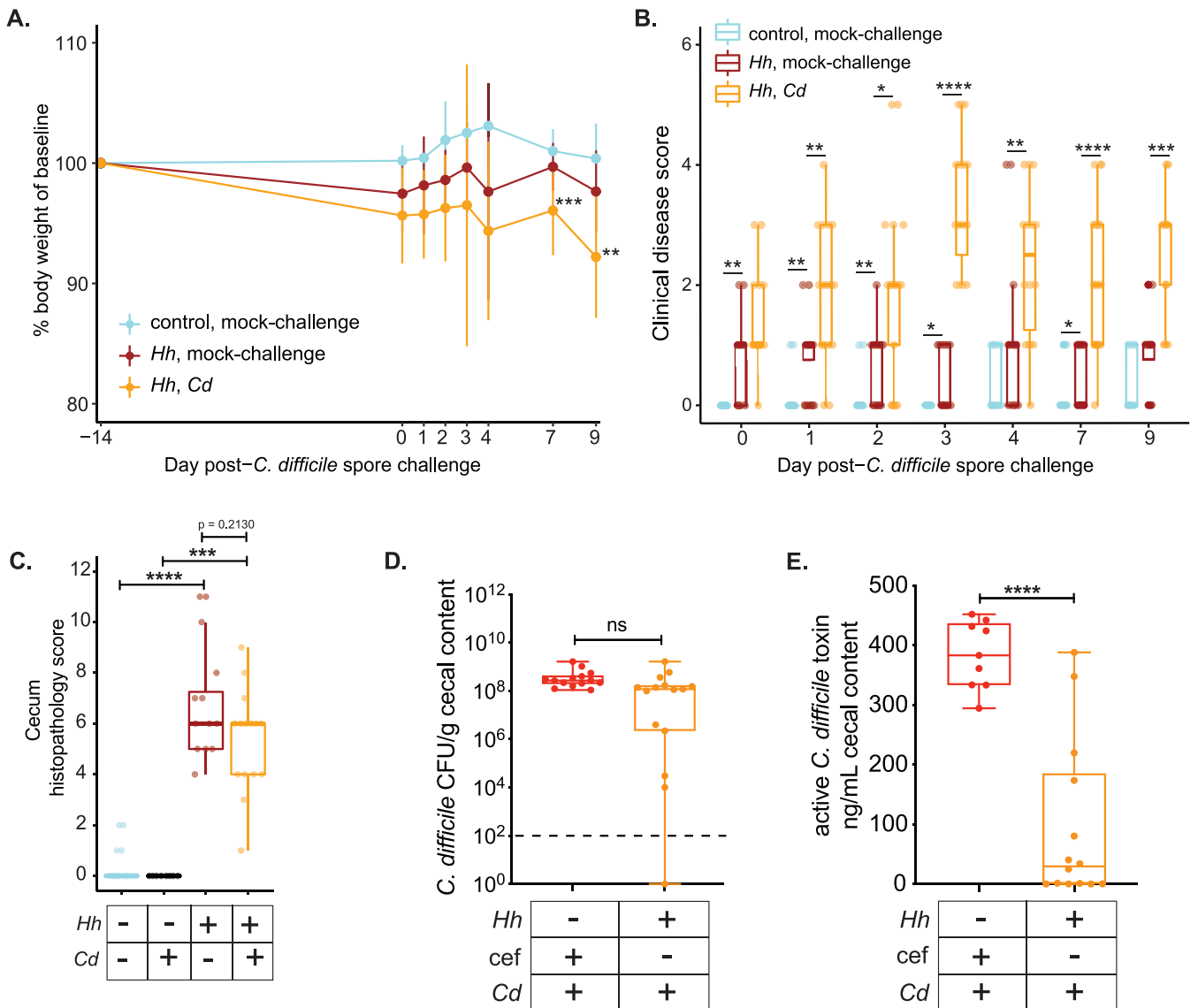


FIG 3 Clinical disease, histopathology, *C. difficile* colonization, and *C. difficile* toxin production in IL-10^{-/-} mice with and without colitis following *C. difficile* spore challenge. IL-10^{-/-} mice were colonized with *H. hepaticus* (*Hh*) or mock colonized with sterile broth (control). Fourteen days later, both groups of animals received either *C. difficile* spores (*Cd*) or sterile saline via oral gavage (mock-challenge). (A) Mice that had colitis triggered by *H. hepaticus* colonization and were subsequently challenged with *C. difficile* spores (*Hh*, *Cd*) had increased weight loss compared to the weights of animals that developed *H. hepaticus*-triggered colitis alone (*Hh*, mock-challenge) or IL-10^{-/-} animals that received neither *H. hepaticus* nor *C. difficile* (control, mock-challenge). (B) Clinical disease scores. IL-10^{-/-} animals with *H. hepaticus*-triggered colitis alone (*Hh*, mock-challenge) had higher clinical scores than control animals without colitis (control, mock-challenge). Clinical disease severity was even greater in animals with *H. hepaticus*-triggered colitis and superimposed *C. difficile* infection (*Hh*, *Cd*). (C) Cecal histopathology (composite score of edema, epithelial damage, and inflammation) was observed in IL-10^{-/-} animals colonized with *H. hepaticus*. Superimposed *C. difficile* colonization in colitic animals did not result in worse histopathology than in mice with colitis alone. (D) *C. difficile* colonization levels (CFU per gram of cecal contents) in colitic animals challenged with *C. difficile* spores were not different from those of animals in which colonization resistance was abrogated by administration of the antibiotic cefoperazone (cef). (E) Despite similar cecal burdens of *C. difficile* in cefoperazone-treated animals and colitic animals challenged with *C. difficile* spores, greater *C. difficile* toxin activity was seen in the ceca of animals that had been treated with cefoperazone. Data represent results from 2 to 3 independent experiments and were analyzed by the *t* test (D and E), ANOVA with the *post hoc* Tukey test (A), or the Kruskal-Wallis test (B and C) (*, *P* > 0.05; **, *P* > 0.01; ***, *P* > 0.001; ****, *P* > 0.0001; ns, not statistically significant).

Colonization of IL-10^{-/-} mice with either *Hh*CDT⁺ or *Hh*CDT⁻ was associated with lower microbial diversity in the cecum than in mice that received sterile broth (Fig. 4C). Similarly, examination of the cecal microbial community structure demonstrated that animals colonized with either strain of *H. hepaticus* were distinct from animals that received sterile broth (Fig. 4D; Fig. S4). The colonization treatment conditions explained 40.7% of the variation in microbial community structure (*P* = 0.0002) (Fig. 4D). Colonization with either *Hh*CDT⁺ or *Hh*CDT⁻ was associated with an expansion of *Enterobacteriaceae* and *Lactobacillaceae* and a loss in *Lachnospiraceae* (Fig. S1).

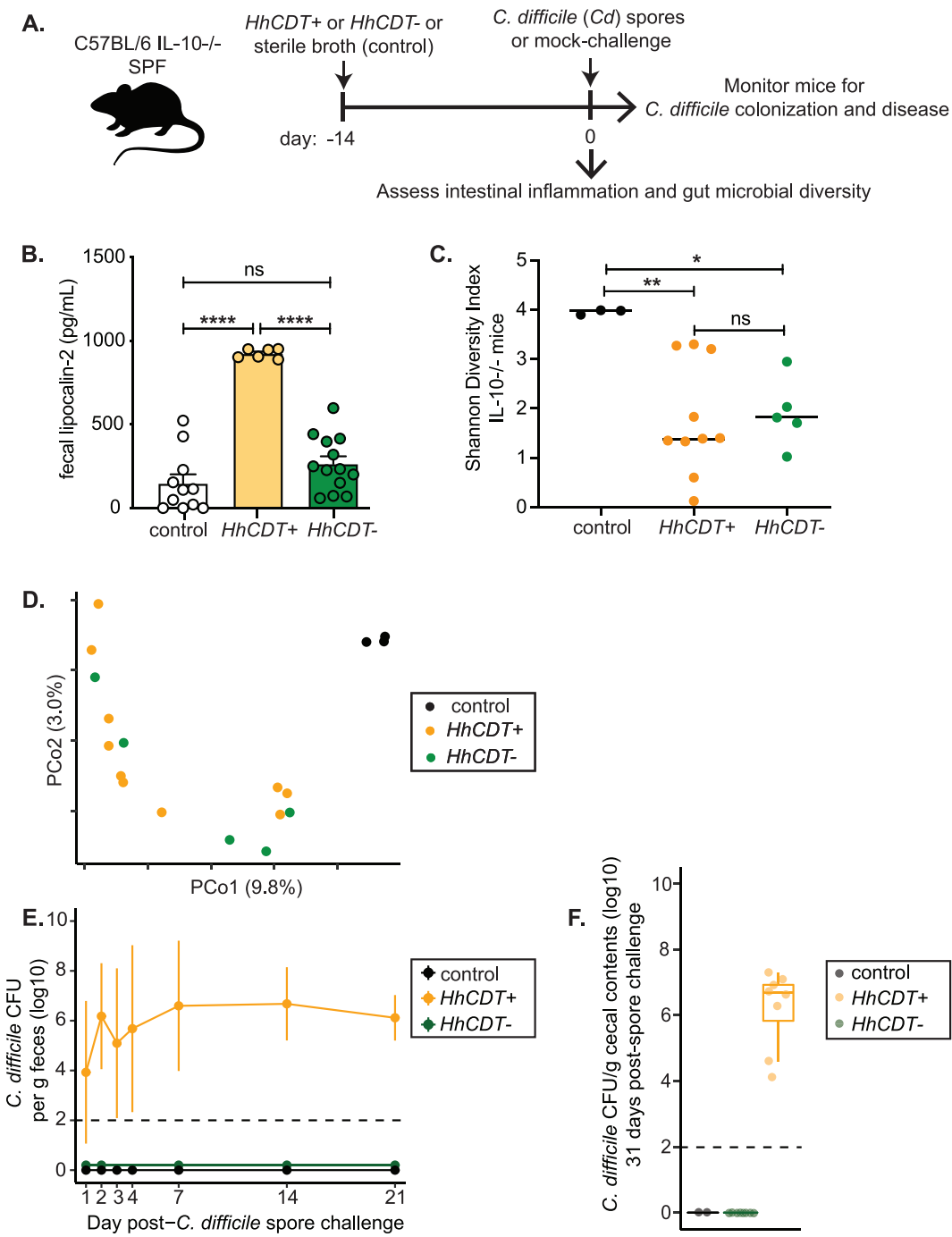


FIG 4 Inflammation in the setting of *H. hepaticus* colonization of IL-10^{-/-} mice is required to overcome colonization resistance to *C. difficile*. (A) Experimental design. IL-10^{-/-} animals were colonized with either wild-type *H. hepaticus* (*HhCDT*⁺) or an isogenic mutant of *H. hepaticus* that no longer produced the immunoregulatory genotoxin cytolethal distending toxin (*HhCDT*⁻). Mice colonized with either of the *H. hepaticus* strains were challenged with spores from *C. difficile* (Cd) strain VPI 10463 and monitored for up to 31 days for *C. difficile* colonization and clinical severity of disease. (B) Lipocalin-2 levels in feces from IL-10^{-/-} mice measured by ELISA at day 14 postcolonization with wild-type *H. hepaticus* (*HhCDT*⁺) or CDT-deficient *H. hepaticus* (*HhCDT*⁻). Compared to animals that received only sterile tryptic soy broth (control), animals colonized with wild-type *H. hepaticus* developed colitis, whereas animals colonized with the CDT-deficient isogenic *H. hepaticus* mutant did not develop significant intestinal inflammation. (C) Shannon diversity indexes plotted with the group mean. (D) Principal-coordinate analysis plot of Bray-Curtis distances of bacterial communities in the luminal contents collected from the ceca of IL-10^{-/-} mice 14 days after colonization with either *HhCDT*⁺ or *HhCDT*⁻ or after receiving sterile broth (control). Similar changes to the microbiota are seen in animals that were colonized with either strain of *H. hepaticus*. (E) Shedding of *C. difficile* in feces collected from mice over time. (F) Cecal contents at day 31 following *C. difficile* spore challenge were plated anaerobically on selective agar plates to quantify *C. difficile* burdens (plotted as means ± standard deviations). Only animals that were colonized with wild-type *H. hepaticus* were colonized with *C. difficile*.

Despite similar changes to the gut microbiota in animals colonized with *HhCDT*⁺ and *HhCDT*⁻, challenge with *C. difficile* spores demonstrated differential effects of these two *H. hepaticus* strains on colonization resistance. *C. difficile* was not detectable in the feces of IL-10^{-/-} mice colonized with *HhCDT*⁻ at any time point following *C. difficile* spore challenge (Fig. 4E). At the time of necropsy, 31 days after challenge with *C. difficile* spores, we confirmed this finding in cecal contents and found no detectable *C. difficile* colonization in mice colonized with *HhCDT*⁻, while animals initially colonized with *HhCDT*⁺ had high levels of *C. difficile* in their cecal contents (Fig. 4F). These data suggest that changes in the microbiota following colonization of IL-10^{-/-} animals with *H. hepaticus* is not sufficient to lower colonization resistance against *C. difficile* in the absence of a threshold degree of inflammation.

DISCUSSION

The clinical intersection between inflammatory bowel disease and *C. difficile* infection has long been recognized (2). Patients with underlying IBD have an increased incidence of CDI, and this is associated with a worse clinical course (31). Despite our recognition of this relationship between the two conditions, the mechanisms that underlie this confluence between IBD and CDI are not well defined. The indigenous microbiota is one obvious link between these diseases, as alterations in the gut microbiota are thought to play a key role in the pathogenesis of IBD (10, 32). Additionally, an altered microbiota structure and function underly the loss of colonization resistance to *C. difficile* (33). However, in patients with IBD, the presence of an altered microbiota can be due to a number of factors. It may be related to changes in the microbiota that predispose to IBD, the treatment of IBD with antibiotics or biologics, or the effect of chronic inflammation on the host and the indigenous bacteria. Determining which of these lead to the susceptibility to CDI seen in patients with IBD would be important to help guide rational prevention and treatment strategies.

Clinically, the poor outcomes seen in patients with IBD who have CDI may reflect the direct effect that infection with toxin-producing *C. difficile* has on the altered epithelium/immune system present in patients with IBD. While this is a straightforward hypothesis, it is interesting to note that a recent retrospective study suggests that patients with more severe IBD are more prone to CDI, and thus, the observed relationship may simply reflect the baseline severity of patients with IBD rather than the *C. difficile* infection increasing the severity of disease (34). Disentangling the complex relationship between IBD and CDI is challenging due to the lack of appropriate animal models to model this interaction. Recently, reports have investigated how gut inflammation induced by dextran sodium sulfate (DSS) administration in mice influenced subsequent *C. difficile* infection. Zhou et al. demonstrated that mice with concurrent DSS-induced colitis had a worse clinical outcome when infected with *C. difficile* (35). Saleh et al. subsequently demonstrated more severe clinical disease due to CDI even when the animals were challenged with *C. difficile* 3 weeks after cessation of DSS treatment, at a time when the acute colitis due to DSS administration had resolved (36). In both of these studies, susceptibility to *C. difficile* colonization and increased disease manifestations required the administration of antibiotics before challenge with spores of *C. difficile*. Zhou et al. did note that about 40% of animals that received DSS without antibiotic treatment could be colonized by *C. difficile* without antibiotic administration, but in this case, worsened histopathologic colitis was not observed in animals that harbored *C. difficile* (35). We previously showed that DSS administration alters the fecal microbiota prior to development of severe histopathologic disease (37), and it is possible that this alteration of the microbiota leads to a loss of colonization resistance.

In the current study, we describe a novel murine system that can be used to study the intersection between IBD and CDI in a model that does not require antibiotic administration to render animals susceptible to *C. difficile* colonization. Furthermore, the model of IBD that we employ is characterized by the development of gut inflammation in a susceptible host that exhibits a dysregulated immune response to the

normal gut microbiota. In our SPF colony of IL-10^{-/-} mice, colonization with *H. hepaticus* rapidly triggers the development of typhlocolitis (20, 29), while no colitis is seen in wild-type animals carrying *H. hepaticus*. *H. hepaticus* and other enteric *Helicobacter* species are found as naturally occurring members of the gut microbiota of wild rodents (38–40). Recent studies have demonstrated that inbred laboratory mice carrying microbiota derived from wild mice, which includes *Helicobacter* species, exhibited immune responses that more closely reflected that seen in humans (41, 42). Therefore, the model described here recapitulates the complex relationships between the host, the indigenous microbiota, and the pathogen seen in patients with IBD who are at risk for the development of *C. difficile* infection.

Our results demonstrate that loss of colonization resistance against *C. difficile* is not due solely to the changes in microbiota structure that follow colonization with *H. hepaticus*. While we demonstrate that *H. hepaticus* colonization of IL-10^{-/-} mice results in an altered cecal and colonic microbiota, the development of inflammation is a critical requirement to overcome colonization resistance. Similar changes in the microbiota of the distal gastrointestinal tract were observed in IL-10^{-/-} animals colonized with either wild-type *H. hepaticus* or an isogenic *H. hepaticus* mutant that does not express cytolethal distending toxin. However, only animals infected with wild-type *H. hepaticus*, which developed much more severe baseline intestinal inflammation, were susceptible to colonization with *C. difficile*. This indicates that in this system, changes in the microbiota itself do not lead to susceptibility to *C. difficile*. Only when these changes in the microbiota are accompanied by the development of intestinal inflammation is a luminal environment created that is permissible for the establishment of *C. difficile* colonization.

The relationship between inflammation, microbiota alterations, and pathogen susceptibility is a theme that has emerged as an important factor in the pathogenesis of a number of gastrointestinal bacterial infections (43). *Salmonella* has been shown to utilize the alternative electron acceptors present in the lumen of the inflamed intestine to provide a growth advantage over the indigenous microbiota (44). Furthermore, intestinal inflammation limits the availability of micronutrients, including transition metals, such as iron and zinc (45). Some bacterial pathogens have evolved systems to deal with this aspect of so-called “nutritional immunity.” Indeed, the levels of zinc within the gastrointestinal tract have been shown to be a key factor in protection against *C. difficile* (46).

C. difficile has been shown to be metabolically flexible, allowing it to occupy multiple nutrient niches within the gastrointestinal tract (47). One metabolic feature that has been shown to favor the growth of *C. difficile* is the presence of luminal amino acids, such as proline, which can be used by the pathogen in energy-generating Stickland fermentation reactions (48). Competition for proline is a critical determinant for the success of *C. difficile* versus other gut microbes (49). Furthermore, amino acid availability has been shown to be increased in patients with diarrhea, including inflammatory diarrhea, affording a permissive environment for *C. difficile* (50). The triggering of gut inflammation by the activity of *C. difficile* toxins was recently shown to specifically alter the intestinal environment in a manner that favors the growth and persistence of *C. difficile* (51). These findings are in concordance with our current finding that the development of inflammation in a murine model of IBD leads to susceptibility to *C. difficile* colonization.

The system described here will permit detailed studies of the complex interplay between the indigenous microbiota, host inflammatory responses, and pathogen that underlies the clinical relationship observed between *C. difficile* infection and inflammatory bowel disease. This model (i) will permit mechanistic studies of the interaction between altered host responses and gut microbes that leads to a breakdown in host-microbe homeostasis and (ii) also will serve as a test bed for novel strategies to prevent and treat *C. difficile* infection in patients with IBD.

MATERIALS AND METHODS

Mice. Male and female C57BL/6 wild-type or IL-10-deficient mice were maintained under specific-pathogen-free (SPF), *Helicobacter*-free conditions. Mice were at least 8 weeks of age at the start of experiments. All mice were from a breeding colony at the University of Michigan that was originally derived from the Jackson Laboratories in 2002. Euthanasia was carried out via CO₂ inhalation at the conclusion of the experiment. Animal studies were approved by the University of Michigan's Committee on the Care and Use of Animals, and animal husbandry was performed in an AAALAC-accredited facility.

Bacterial strains and growth conditions. *H. hepaticus* strain 3B1 (ATCC 51488) was obtained from the American Type Culture Collection (Manassas, VA). The isogenic mutant 3B1::Tn20 has a transposon inserted near the start of *cdtA* and no longer produces cytolethal distending toxin (CDT) (29). Wild-type *H. hepaticus* 3B1 and 3B1::Tn20 were grown on tryptic soy agar (TSA) supplemented with 5% sheep blood at 37°C for 3 to 4 days in a microaerobic chamber (1 to 2% oxygen; Coy Laboratories). The isogenic mutant 3B1::Tn20 is chloramphenicol resistant and was grown on medium supplemented with 20 µg/ml chloramphenicol (Sigma, St. Louis, MO). Spores of *C. difficile* reference strain VPI 10463 (ATCC 43255) were prepared and used as previously described by Theriot et al. (22). Spores were enumerated by plating them on prereduced taurocholate cycloserine cefoxitin fructose agar (TCCFA), prepared as previously described (26).

Infection studies. *H. hepaticus* suspensions for animal inoculation were prepared by harvesting organisms from culture plates into Trypticase soy broth (TSB). Mice were challenged with 10⁸ CFU of *H. hepaticus* by oral gavage. *H. hepaticus* colonization status was confirmed by PCR of the *cdtA* gene (52) on fecal DNA extracted using a DNeasy UltraClean microbial kit (Qiagen) by following the manufacturer's instructions. TCCFA plates with fecal or cecal samples or spore inoculum were incubated in an anaerobic chamber (Coy Industries) at 37°C for 18 h prior to colony enumeration.

For our previously published model of *C. difficile* infection following antibiotic administration, mice received 0.5 mg/ml cefoperazone (MP Pharmaceuticals) in sterile distilled drinking water (Gibco) *ad libitum*. The antibiotic-supplemented water was provided for 10 days, followed by 2 days of drinking water without antibiotics (22). Animals were then challenged by oral gavage with 10³ to 10⁴ CFU of *C. difficile* spores suspended in 50 µl of distilled water (Gibco) or mock challenged with water. To develop a model of *C. difficile* challenge after the development of colitis, 2 weeks after colonization with *H. hepaticus* or mock colonization with sterile TSB, animals were challenged by oral gavage with 10³ to 10⁴ CFU of *C. difficile* spores suspended in 50 µl of distilled water (Gibco) or mock challenged with water. Over the course of each experiment, mice were regularly weighed, and feces were collected for quantitative culture. Fresh feces were collected from each mouse into a preweighed sterile tube. Immediately following collection, the tubes were reweighed to determine fecal weight and passed into an anaerobic chamber (Coy Laboratories). Each sample was then diluted 10% (wt/vol) with prereduced sterile phosphate-buffered saline (PBS) and serially diluted onto prereduced TCCFA plates. The plates were incubated anaerobically at 37°C, and *C. difficile* colonies were enumerated after 18 to 24 h of incubation.

Clinical disease severity, necropsy, and histopathologic scoring. Mice were monitored daily for clinical signs of disease. Disease scores were averaged based on scoring of the following features for signs of disease: weight loss, activity, posture, coat, diarrhea, and eyes/nose. A 4-point scale was assigned to score each feature, and the sum of these scores determined the clinical disease severity score (53). At the termination of each experiment, animals were euthanized by CO₂ inhalation. The cecum and colon were harvested and fixed in formalin. Sections were stained with hematoxylin and eosin and scored by a veterinary pathologist (Ingrid L. Bergin) in a blind manner. Histopathologic damage was scored using epithelial destruction, immune cell infiltration, and edema on a 4-point scale for each category, and the sum of these scores determined the histological score (22, 26, 54).

Quantitative detection of *C. difficile* toxin in cecal contents. The levels of functional *C. difficile* toxin were measured using a real-time cellular analysis (RTCA) assay (55). The RTCA assay was used to detect changes in cell-induced electrical impedance in cultured colorectal cell monolayers in response to cecal contents collected from mice with CDI to determine concentrations of active toxin. Cecal contents collected from mice at the time of euthanasia were weighed and diluted 1:1,000 (wt/vol) with sterile PBS. After homogenization, particulate matter was allowed to settle in the original collection tubes prior to transference of supernatant aliquots to fresh tubes. Cecal content supernatants were then filtered through a sterile 0.22-µm 96-well filter plate, and plates were centrifuged at 5,000 × g for 10 min at room temperature. HT-29 cells, a human colorectal adenocarcinoma cell line with epithelial morphology (ATCC HTB-38), were seeded in electrode-lined 96-well plates (E-Plate View 96; ACEA Biosciences) in Dulbecco's modified Eagle medium (DMEM) and allowed to grow to a confluent monolayer overnight prior to loading of the processed cecal content supernatant. Samples were run in triplicate. Prior to addition of the cecal content supernatant samples to the plates containing HT29 monolayers, an aliquot of each sample, also run in triplicate, was incubated in parallel with antitoxin specific for *C. difficile* toxins A and B (*C. difficile* toxin/antitoxin kit T5000; TechLab, Blacksburg, VA) for 40 min at room temperature as a specificity control for the presence of *C. difficile* toxin A and B in samples. Active *C. difficile* toxin has cytotoxic effects on HT29 cells, which results in a dose-dependent and time-dependent decrease in cell impedance (CI). A standard curve was generated using wells that received purified *C. difficile* toxin A (List Biological Labs). CI data following incubation with cecal contents from mice with CDI were acquired and analyzed using the xCELLigence RTCA system and software (ACEA Biosciences, San Diego, CA). A normalized CI was calculated for each sample by normalizing the CI to the last CI measured at the time point prior to the addition of cecal content to the well.

Fecal lipocalin-2 quantification. Fresh feces were collected from individual mice and stored at -80°C until processed and assayed by enzyme-linked immunosorbent assay (ELISA). Fecal pellets were

weighed and homogenized in PBS with 0.1% Tween 20. The fecal suspension was centrifuged, and lipocalin-2 levels in the supernatant were quantified using the mouse lipocalin-2/NGAL DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

DNA extraction and 16S rRNA gene sequencing. Cecal and colon luminal contents were separately collected from mice with IBD and without IBD at the time point immediately preceding *C. difficile* spore challenge. The University of Michigan Microbiome Core extracted total DNA from cecal and colon contents and prepped DNA libraries as previously described (56). The V4 region of the 16S rRNA gene was amplified from each sample using the dual-indexing sequencing strategy as described previously (57). Sequencing was done on the Illumina MiSeq platform using the MiSeq reagent kit V2 (MS-102-2003) to sequence the amplicons (500 total cycles), with modifications found in the Schloss SOP (https://github.com/SchlossLab/MiSeq_WetLab_SOP). The V4 region of the mock community (ZymoBIOMICS Microbial Community DNA Standard; Zymo Research) was also sequenced to supervise sequencing error. Data were analyzed using mothur (v 1.42.3) (58).

Statistics. Statistical analysis for continuous variables was performed using the unpaired Student *t* test or one-way analysis of variance (ANOVA), and Tukey's *post hoc* test was performed using R. A *P* value less than 0.05 was considered statistically significant. Categorical and ordinal variables were analyzed using nonparametric tests as indicated.

Data availability. Code and processing information are available in GitHub repository at the following URL: https://github.com/AbernathyClose/AbernathyClose_IbdCdi_mBio_2020.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 2.1 MB.

FIG S2, EPS file, 2.8 MB.

FIG S3, EPS file, 2.7 MB.

FIG S4, EPS file, 1.6 MB.

FIG S5, EPS file, 2.1 MB.

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