The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* infection

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Clostridium difficile infection (CDI) arises in the setting of antibiotic administration where disruption of the normal indigenous gut microbiota leads to susceptibility to *C. difficile* colonization and colitis. Using a murine model of CDI, we demonstrate that changes in the community structure of the indigenous gut microbiota are associated with the loss of colonization resistance against *C. difficile*. Several antibiotic regimens were tested in combination for the ability to overcome colonization resistance, including a five antibiotic cocktail consisting of kanamycin, gentamicin, colistin, metronidazole and vancomycin administered in drinking water for three days, a single intraperitoneal dose of clindamycin or 10 days of cefoperazone in drinking water. Following antibiotic treatment animals were challenged with 10^5 colony forming units of *C. difficile* strain VPI 10463 via oral gavage. Animals that received the antibiotic cocktail and clindamycin prior to *C. difficile* challenge followed one of two clinical courses, either becoming clinically ill and moribund within 2–4 days post challenge, or remaining clinically well. Animals that became clinically ill developed histologically severe colitis. These histopathologic findings were significantly less severe in animals that remained clinically well. Analysis of 16S rRNA gene sequences retrieved from gut tissue at necropsy demonstrated that Proteobacteria dominated the gut microbiota in clinically ill animals. In contrast, the gut microbial community of clinically well animals more closely resembled untreated animals, which were dominated by members of the Firmicutes. All animals that received cefoperazone treatment prior to *C. difficile* challenge were clinically ill and moribund by 2–5 days post challenge in a dose dependent manner. The gut communities in these animals were dominated by *C. difficile* suggesting that cefoperazone treatment resulted in a greater loss in colonization resistance. Thus, the severity of colitis that arises in this system reflects the interplay between the expansion of *C. difficile* in the gut community and the ecologic dynamics of the indigenous microbial community as it recovers from antibiotic perturbation. We demonstrate that altering the balance of these two opposing processes alters clinical outcome and thus may lead to novel preventative and therapeutic approaches for CDI.

Introduction

The gastrointestinal (GI) tract of mammals is inhabited by a complex microbial community that plays a crucial role in maintaining gut homeostasis.[1,2] The GI tract microbiota performs a number of beneficial metabolic functions[3] and also aids in the normal development of the mucosal epithelium and maturation of the mucosal immune system.[4-7] The indigenous microbiota protects the host from colonization by potentially pathogenic organisms, a function that is termed colonization resistance.[8] It has been hypothesized that following the successful colonization by a pathogen, the ultimate pathology depends on the interplay between the host, pathogen and the indigenous microbiota.[9] Thus, the resident microbiota can potentially modulate the outcomes of any pathogen/host interaction.

C. difficile is a Gram-positive, toxin-producing bacterium first described in 1935 as a commensal organism in the fecal microbiota of healthy newborn infants.[10] It is currently the most common cause of health care-associated diarrhea and colitis and is responsible for significant morbidity and increased healthcare cost.[11] *Clostridium difficile* infection (CDI) is associated with the use of broad-spectrum antibiotic therapy, increasing patient age and hospitalization.[12] In recent years, the appearance of an epidemic strain (BI/NAP1/027) with potentially increased virulence has prompted renewed interest in the pathogenesis and epidemiology of this bacterium.[11,12] Additionally, it appears that the overall incidence of *C. difficile* infection has been increasing.[13]

As *C. difficile* is not normally a significant component of the GI tract microbiota of adult humans, it is proposed that the indigenous gut microbiota is important in mediating colonization resistance against this pathogenic bacterium.[14,15] According to this hypothesis, disruption of the indigenous gut microbiota by the administration of antibiotics results in a decrease in colonization resistance. Furthermore, recurrent CDI appears to occur in the setting where the indigenous microbiota is sufficiently disturbed so that colonization resistance cannot be restored even after cessation of the inciting antibiotics and completion of specific treatment directed against *C. difficile*.[16] We have demonstrated that patients with recurrent *C. difficile* infection have decreased diversity of the indigenous gut microbiota which may reflect a corresponding defect in colonization resistance.[17]

A number of animal models have been developed to facilitate the study of *C. difficile* pathogenesis. The hamster model has been used extensively and it was in this host that Koch's postulates were fulfilled for *C. difficile* as the causative agent of antibiotic-associated colitis.[18] In this model colitis develops after exposure to clindamycin and subsequent *C. difficile* challenge. However, the resulting disease is severe and lethal within three days after initial infection. This does not represent the usual course and spectrum of CDI in humans, which can range from asymptomatic to severe colitis.[13] Furthermore, the limited availability of reagents to study host responses in hamsters has dampened the usefulness of this model. Gnotobiotic mice challenged with *C. difficile* also develop intestinal disease but this model precludes an examination of the role of indigenous microbiota in mediating colonization resistance.[19-21] Thus, the available animal models have limited studies of *C. difficile* pathogenesis.

It has been reported that treatment of mice with various antibiotics can render the animals susceptible to *C. difficile* colonization.[22] In some cases this can lead to the development of colitis.[23,24] In this present study, we utilized antibiotic-treated mice to demonstrate that altering the community structure of the indigenous gut microbiota is associated with both the loss of colonization resistance against *C. difficile* and differences in the severity of disease. Our results indicate that a better understanding of the role of the indigenous microbiota in CDI could lead to novel and improved mechanisms for prevention and treatment.

Results

Overcoming colonization resistance to *C. difficile*. To compare the ability of different antibiotic regimens to overcome colonization resistance against *C. difficile* six to eight week old C57BL/6 mice were treated with either an antibiotic cocktail (kanamycin, gentamicin, colistin, metronidazole and vancomycin), clindamycin or the combination of both prior to challenge with 1 x 10⁵ CFU of *C. difficile* (VPI 10463) via oral gavage (**Fig. 1A and B**). Control animals were challenged with *C. difficile* in the absence of any antibiotic pretreatment. Colonization was monitored by culture and *C. difficile*-specific PCR was performed on DNA isolated from stool pellets or from gut tissue harvested at necropsy, which occurred 2 to 4 days post-challenge. Animals were monitored daily for signs of clinical CDI including diarrhea,

weight loss and hunched posture. *C. difficile* was never recovered from animals that were challenged without antibiotic pretreatment. Animals that received the antibiotic cocktail without clindamycin prior to *C. difficile* challenge were also resistant to colonization. Of the 9 animals that received only a single dose of clindamycin prior to *C. difficile* challenge, 4 of them shed low amounts of the organism in their feces for the first 2 days following challenge, but the organism was no longer detectable in stool or tissue when the animals were euthanized four days following challenge. All 12 animals that received both the antibiotic cocktail and clindamycin prior to *C. difficile* challenge shed the organism in their feces throughout the experiment and *C. difficile* was found in tissue at the time of necropsy. These results indicate that the combination of the antibiotic cocktail and clindamycin is required to completely overcome colonization resistance against *C. difficile*.

Clinical disease in *C. difficile* infected mice. We monitored the development of disease in mice that received both the antibiotic cocktail and clindamycin prior to *C. difficile* challenge. Five of 12 animals that received both the antibiotic cocktail and clindamycin did not show overt clinical signs of CDI despite remaining colonized with *C. difficile*. The remaining seven mice exhibited signs of disease including diarrhea, hunched posture and significant (>20% from baseline) weight loss (**Fig. 2**). One animal was found dead at 2 days post challenge while 6 animals were moribund and euthanized 2 to 4 days post challenge.

A central feature of the pathogenicity of *C. difficile* is the production of two large glucosyltransferase toxins encoded by *tcdA* and *tcdB* that modify and inactivate the small GTPases Rho, Rac and Cdc42.[25] We measured the activity levels of *C. difficile* toxin in the gut using a tissue culture cytotoxin assay. High levels of *C. difficile* cytotoxin were detected in samples obtained from animals with severe clinical disease (**Fig. 3A**). Additionally, quantitative PCR analysis indicates that animals with severe clinical disease had significantly higher numbers of *C. difficile* in their gut at the time of necropsy compared to those animals that were clinically well (**Fig. 3B**). These results suggest that the animals that did not develop severe CDI in the multi-antibiotic treatment protocol had the ability to control the population size of colonizing *C. difficile* and to limit the production of toxin.

Histopathologic changes were seen in both the cecal and colonic tissue of all animals that received the antibiotic cocktail, clindamycin and *C. difficile*. Pathologic changes consisted of neutrophilic inflammation in the mucosa and submucosa with varying degrees of submucosal edema (**Fig. 4**). Of note, animals that were infected and clinically ill had significantly more severe colonic inflammation and particularly, submucosal edema than animals that were infected but remained clinically well (**Fig. 4E and F**). In the most severely affected animals, there were areas of erosion and in rare cases, ulceration. Occasionally, severely affected animals had luminal exudates comprised of degenerate neutrophils, hemorrhage and sloughed epithelium embedded in a fibrinous matrix suggestive of pseudomembranes (**Fig. 4D**). Untreated animals or those that received antibiotics without *C. difficile* challenge had no histological alterations.
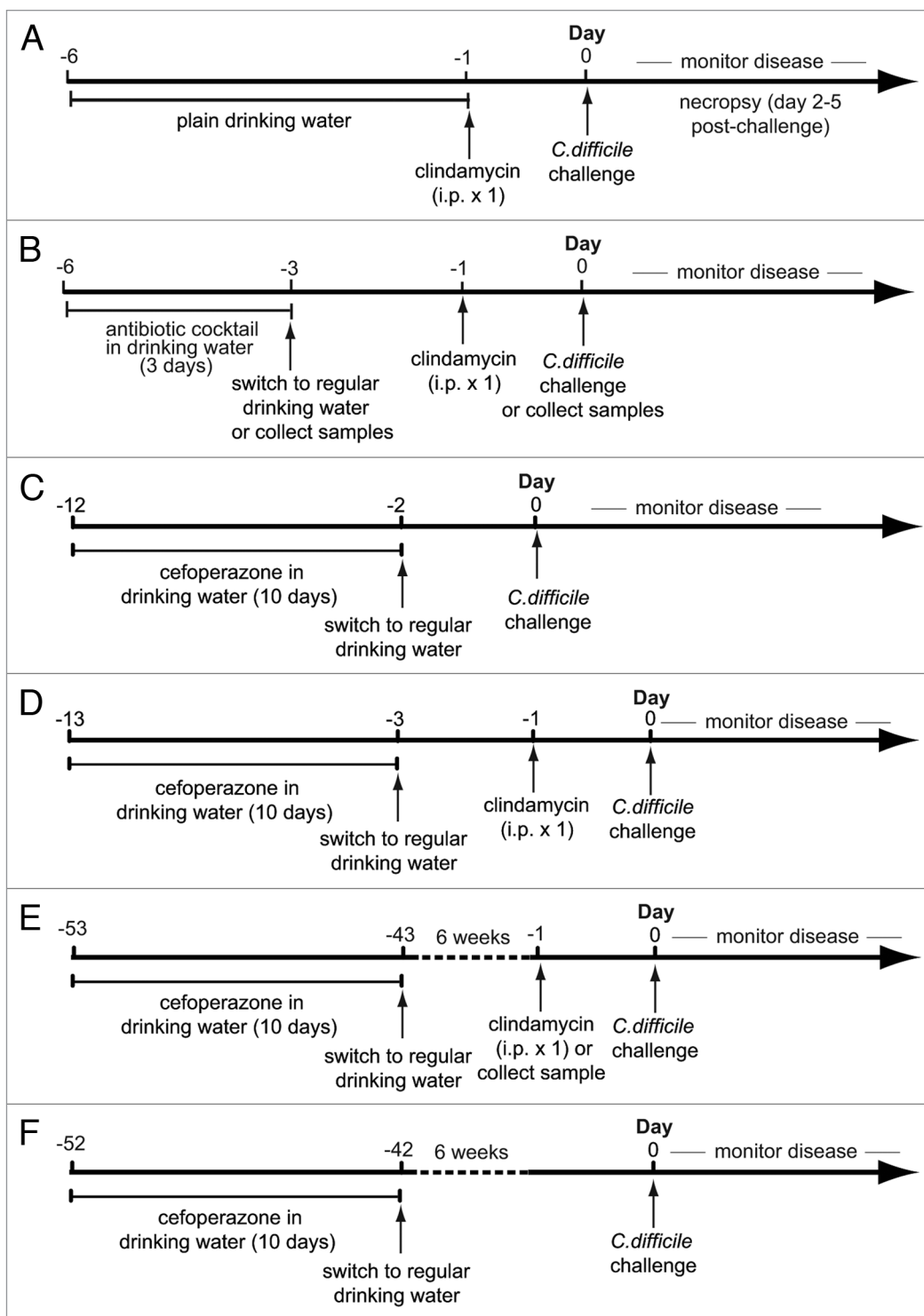
Figure 1. Experimental designs for alteration of microbiota and *C. difficile* infection. Wild-type mice were treated with: (A) a single intraperitoneal dose of clindamycin and challenged with 10[5] CFU of *C. difficile* (VPI 10463). (B) a 5 antibiotic cocktail in drinking water for 3 days; a 5 antibiotic cocktail in drinking water for 3 days followed by a 2-day period without the drug and a single dose of clindamycin; or a 5 antibiotic cocktail in drinking water for 3 days followed by a 2-day period without the drug and a single dose of clindamycin followed by challenge with 10[5] CFU of *C. difficile* one day later. (C) 10 days cefoperazone treatment followed by 2 days off drug with or without *C. difficile* challenge. (D) 10 days cefoperazone treatment followed by 2 days off drug, a single dose of clindamycin followed by one day recovery with or without *C. difficile* challenge. (E) 10 days cefoperazone treatment followed by 6 weeks off drug, a single dose of clindamycin followed by one day recovery prior to *C. difficile* challenge. (F) 10 days cefoperazone treatment followed by 6 weeks off drug then *C. difficile* challenge.
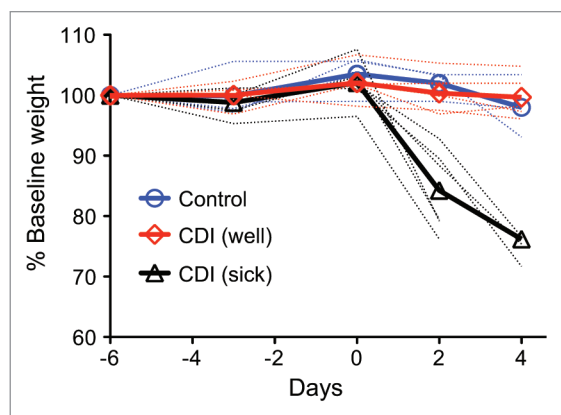
Figure 2. Weight loss in *C. difficile* infected mice. Weight loss curves for untreated (control) animals (n = 5) and animals treated with the 5 antibiotic cocktail for 3 days (days -6 to -3) then given a single dose of clindamycin after 2 days off antibiotics followed by challenge with *C. difficile* one day later (n = 11). 6 of 11 mice lost >20% body weight (sick) while one animal died 1–2 days post challenge (not shown). The remaining 5 animals did not lose significant body weight (well) when compared to control animals. Weight loss percentage is based on the starting weight at day -6 with thick lines showing the average for animals in each clinical group and dotted lines showing the data for each individual. CDI-*C. difficile* infection.

Shifts in microbial ecology associated with antibiotic treatment and *C. difficile* infection. We have previously shown that antibiotic administration can decrease the overall mass of bacteria within the gut.[26] The administration of the 5 antibiotic cocktail significantly decreased the overall bacterial population by 20-fold when measured immediately after the treatment period (data not shown). However, a single administration of clindamycin did not change the total microbial population size when measured 24 hours after the dose was given. Furthermore, following

administration of both the antibiotic cocktail and clindamycin, at the time corresponding to *C. difficile* challenge, the overall bacterial population size was similar to untreated animals. Therefore, the loss of colonization resistance against *C. difficile* following antibiotic administration was not directly related to changes in the overall density of the gut microbiota.

Since the loss of colonization resistance against *C. difficile* was not associated with an overall decrease in bacterial density we analyzed 16S rRNA gene sequences retrieved from gut tissue at the time of necropsy to examine the specific changes in the community structure of the gut microbiota that resulted from antibiotic treatment and *C. difficile* infection. In control mice that never received antibiotics, the gut microbial community was dominated by members of the phylum Firmicutes with lower numbers of Bacteroidetes. The administration of either the antibiotic cocktail, clindamycin or both resulted in a significant change in the structure of the gut microbial community (**Fig. 5A and B**). The administration of the antibiotic cocktail resulted in a shift in the community structure to one dominated by bacteria from the family Lactobacillaceae. Clindamycin treatment alone shifted the community composition to a dominance of Proteobacteria belonging to the family Enterobacteriaceae (**Fig. 5A**). When clindamycin was administered following treatment with the antibiotic cocktail, there was again a predominance of Proteobacteria in the gut community (**Fig. 5B**).

Interestingly, subsequent changes in the gut microbial community structure following *C. difficile* challenge followed two distinct courses. Animals that developed severe clinical disease harbored a gut microbial community at the time of necropsy that remained dominated by Proteobacteria (**Fig. 5B**). Animals that remained clinically well and had significantly less severe histologic colitis at the time of necropsy possessed a gut microbiota that appeared to be returning towards the baseline state. Members of the Firmicutes again became significant members of the community and Proteobacteria were no longer dominant. 16S rRNA gene sequences corresponding to *C. difficile* were detected in the gut communities of both clinically well and sick animals but in agreement with the quantitative PCR results, *C. difficile* sequences composed 8.5% (±7.7) of those recovered in clinically ill mice, but only 1.1% (±1.5) of the sequences in well mice. Thus, mice that were clinically well harbored an indigenous microbial community that was more similar to the baseline state seen in untreated controls than those with severe clinical CDI.
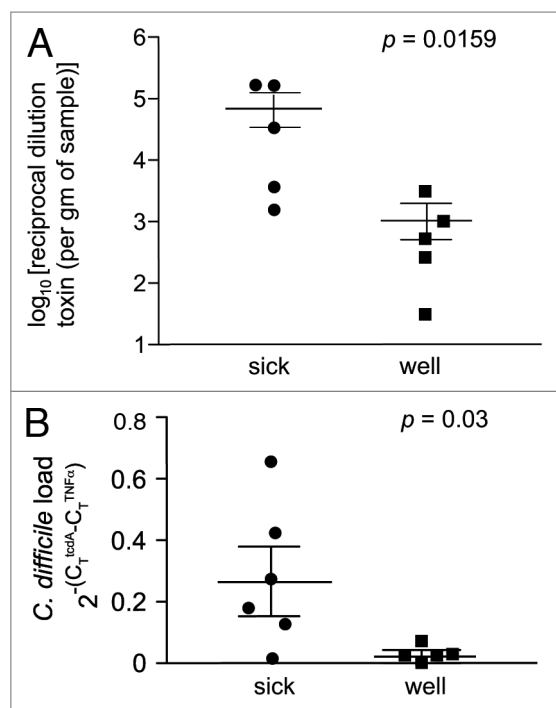

Figure 3. Increased cytotoxin activity and *C. difficile* load are associated with increased CDI severity. (A) Vero cell tissue culture was used to determine the log$_{10}$ reciprocal cytotoxin dilution per gram of sample. Each point represents individual animals that were either sick (n = 5) or well (n = 5) after treatment with antibiotics/clindamycin and exposure to *C. difficile*. Sick animals had increased levels of cytotoxin production compared to animals with less clinical disease. (B) Quantitative PCR was performed on cecal DNA from animals treated with antibiotics/clindamycin and exposed to *C. difficile*. Values represent the relative abundance of the *tcdA* gene normalized to the single copy mouse tumor necrosis factorα (TNFα) gene. Sick animals (n = 6) had increased levels of *C. difficile* compared to well animals (n = 5) with less clinical disease.
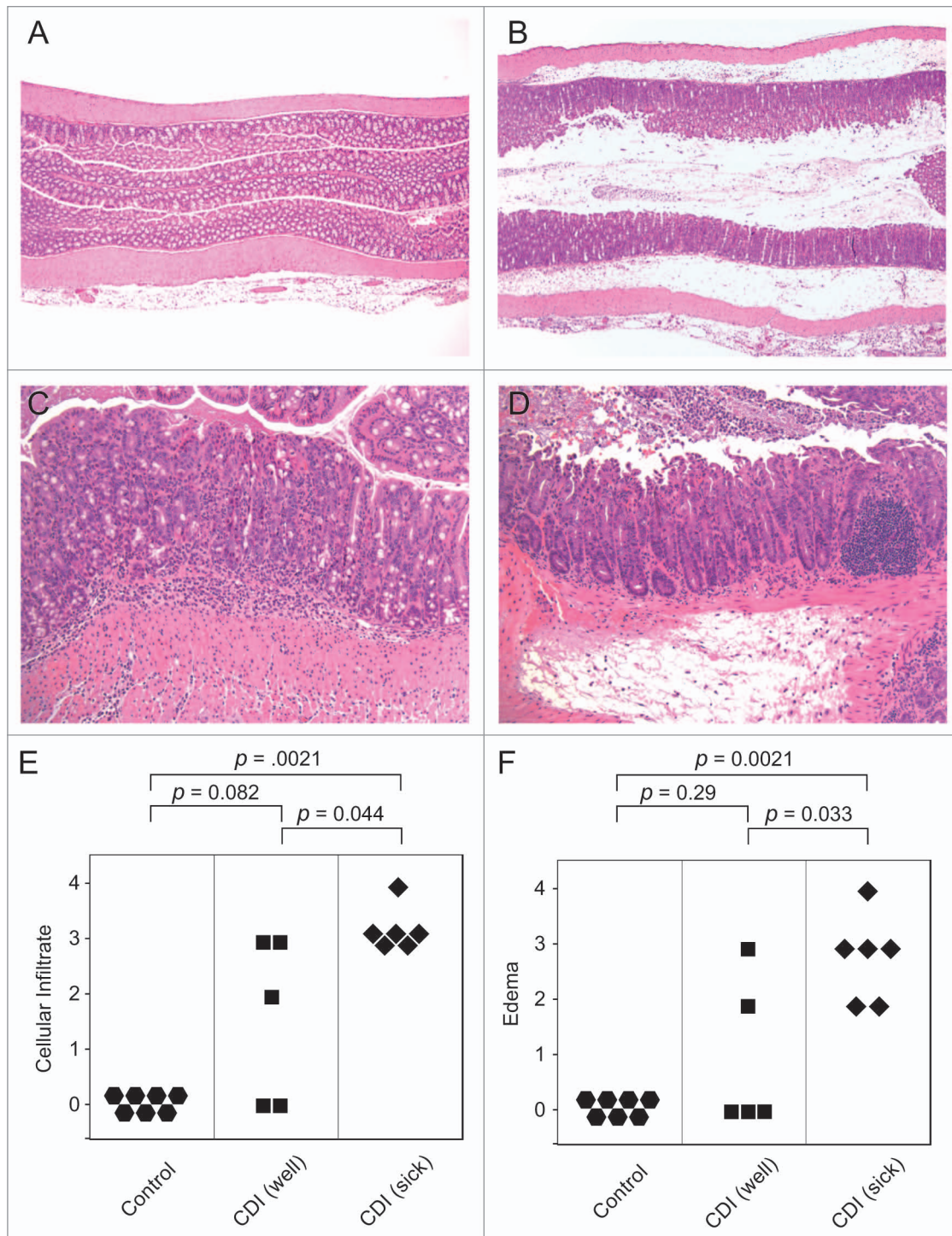
Figure 4. Animals with clinically severe CDI have increased colonic histopathology. (A) Proximal colon of an untreated (control) mouse. HE. Original magnification x40. (B) Colon of a sick antibiotic treated mouse infected with *C. difficile* showing severe submucosal edema. HE. Original magnification x40. (C) Increased magnification of colon from a well antibiotic treated mouse infected with *C. difficile* showing moderate neutrophilic mucosal and submucosal inflammation but lacking significant submucosal edema. HE. Original magnification x200. (D) Sick *C. difficile* infected mouse showing marked submucosal edema in addition to neutrophilic inflammation. There is also a pseudomembrane on the luminal surface consisting of degenerate neutrophils, sloughed epithelial cells and hemorrhage within a fibrinous matrix. HE. Original magnification x200. (E) Categorical cellular infiltration scores of untreated (control) animals, *C. difficile* infected well animals and *C. difficile* infected sick animals. (F) Categorical edema scores of untreated (control) animals, *C. difficile* infected well animals and *C. difficile* infected sick animals. Comparisons between groups were performed using the non-parametric Krustal Wallis test.
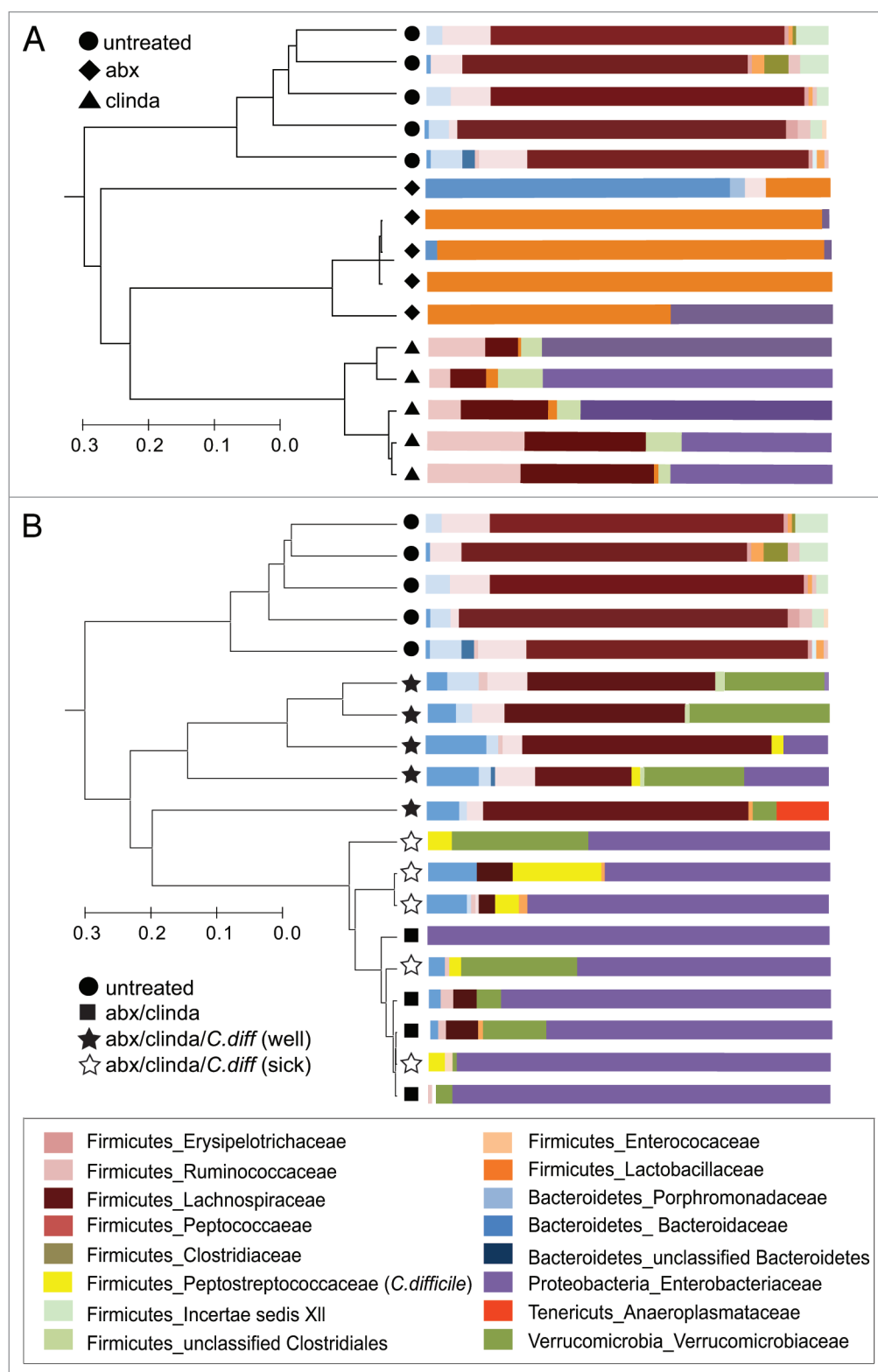
Figure 5. For figure legend, see page 151.

Cefoperazone treatment renders mice susceptible to colonization and colitis following *C. difficile* challenge. We previously demonstrated that administration of the beta-lactam antibiotic cefoperazone had significant and long-lasting effects on the indigenous gut microbiota.[26] Even after a six-week recovery period following a 10-day course of cefoperazone, the gut microbiota exhibited altered community structure and diminished diversity. The 5 antibiotic cocktail did not have such long-lasting

effects as gut microbial composition returned to baseline within four weeks of discontinuing the drug (data not shown).

To determine if the greater disturbance of the gut microbiota associated with cefoperazone administration differentially altered the course of experimental *C. difficile* infection, six to eight week old C57BL/6 mice were treated with cefoperazone prior to oral challenge with *C. difficile*. One group of mice received cefoperazone in drinking water for 10 days followed by a 2-day period on plain water (**Fig. 1C**). Another group received the same cefoperazone treatment followed by a single dose of clindamycin after 2 days (**Fig. 1D**). Two final groups received 10 days of cefoperazone followed by a 6-week period without the drug with or without a single dose of clindamycin (**Fig. 1E and F**). All four groups of animals then received a challenge of 1 x 10^5 CFU of *C. difficile* via oral gavage. All animals in the 2 groups of mice that were challenged 2–3 days after cefoperazone treatment (with or without clindamycin) were moribund by 2 days post challenge while one animal died between 1–2 days post challenge. Similarly, all animals treated with cefoperazone followed by a 6-week recovery period that received a single dose of clindamycin and *C. difficile* challenge also exhibited signs of CDI and were moribund by 4 days post challenge. However, animals that were challenged with *C. difficile* after the 6-week recovery period without a dose of clindamycin were not colonized (data not shown).

16S rRNA-encoding gene analysis on tissue collected from cefoperazone-treated animals indicated that a 10-day treatment with this antibiotic resulted in a bacterial gut community dominated by bacteria in the family Pseudomonadaceae (**Fig. 6A**). Following a 2-day period without cefoperazone and a single dose of clindamycin, the gut microbiota became dominated by members of the Lactobacillaceae (**Fig. 6A**). After infection with *C. difficile* the communities of all animals pretreated with cefoperazone with or without clindamycin became dominated by *C. difficile* (**Fig. 6B**) with between 48–92% (69 ± 15%) of the 16S rRNA-encoding gene sequences retrieved corresponding to this organism. *C. difficile* 16S rRNA-encoding gene sequences were detected in 3 of the 4 animals infected 6 weeks after cefoperazone treatment followed by a dose of clindamycin and *C. difficile* challenge (12.8 ± 10%).

Given the apparent increased susceptibility to *C. difficile* infection after cefoperazone treatment, we determined if decreasing the challenge dose would alter the severity of the resultant CDI. Groups of 9 to 10 animals were treated with a 10-day course of cefoperazone followed by a 2-day recovery period without the drug, and were then challenged with varying doses of *C. difficile* ranging from 2 x 10^2 CFU to 2 x 10^5 CFU. The animals were monitored for weight loss and clinical signs of severe disease, and euthanized when the appropriate clinical endpoints were reached. There was a strict dose dependence on the rate at which clinical endpoints were reached (**Fig. 7**). The majority of animals receiving the highest dose of *C. difficile* became moribund within 2 days post challenge while all of the animals receiving the lowest dose of 10^2 CFU remained clinically well until 5 days post challenge at which time all became ill and required euthanasia.

Discussion

The term "colonization resistance" was coined to refer to the ability of an established gut microbial community to resist invasion by additional microbes.[27-29] Although this initially applied to pathogenic microbes, the concept was derived from concepts of community robustness derived from studies of classical ecologic systems (for example grasslands and lakes) and thus could be applied to any invading microbe.[30] Current hypotheses suggest that the normal indigenous microbiota is not permissive for the establishment of colonization by *C. difficile*.[15] In rare cases where normal individuals are colonized by *C. difficile* without overt clinical disease, it is further hypothesized that the normal indigenous microbiota can at least limit the production of toxin, perhaps by directly interfering with toxin production or limiting the population size of *C. difficile* and preventing significant amounts of toxin from accumulating in the gut.[15,31] Accordingly, disruption of the indigenous microbiota by antibiotics leads to a loss of colonization resistance, making the gut vulnerable to colonization by exogenous *C. difficile* spores or, in previously colonized patients, expansion and toxin production. In support of this concept, Wilson and colleagues provided evidence for the ability of the normal gut microbiota to inhibit *C. difficile* by demonstrating that administration of normal cecal homogenates would decrease the number of viable *C. difficile* and prevent colitis in antibiotic-challenged hamsters.[15,32]

Using a murine model of *C. difficile* infection involving pretreatment of mice with antibiotics to overcome colonization resistance, we found that administration of 10^5 CFU of *C. difficile* to animals treated with a cocktail of five antibiotics and clindamycin results in uniform colonization and a mortality rate of about 60%. The initial description of this model noted that by increasing the challenge dose of *C. difficile*, mortality would increase in direct relationship to the dose of organism.[23] In the current study, we also found that a more significant disruption of the microbiota, using the antibiotic cefoperazone could also result in uniform mortality in animals that were challenged with a dose
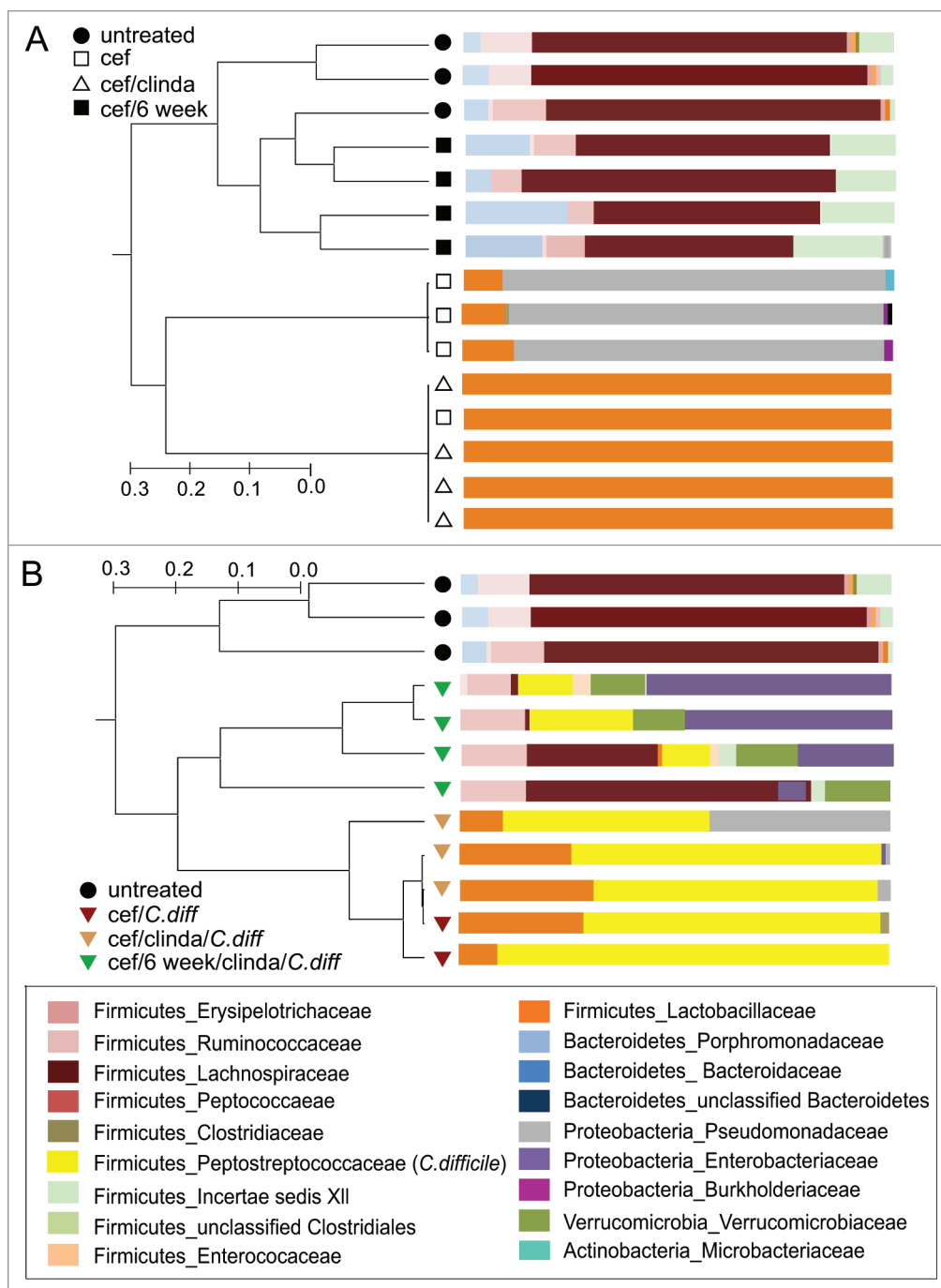
Figure 6. Shifts in microbial community structure and composition associated with cefoperazone administration and *C. difficile* infection. The composition of the gut microbiota was determined by 16S rRNA clone library construction. (A) The microbial communities in animals that were treated with cefoperazone with or without clindamycin were altered from that seen in untreated controls. Six weeks after cefoperazone treatment, the communities returned to a community structure that resembled untreated controls. (B) Animals that were challenged with *C. difficile* within three days after stopping cefoperazone treatment (with or without clindamycin treatment) had very high relative levels of *C. difficile* in their microbial communities. Animals that were challenged with *C. difficile* 6 weeks after antibiotic treatment was stopped followed by a single dose of clindamycin had less *C. difficile* present. The relative abundance of *C. difficile* appeared to directly correlate with the amount of Proteobacteria and inversely with the abundance of Lachnospiraceae. Dendrograms were constructed using Morisita-Horn similarities based on >97% sequence similarity while taxonomic assignments were made using the Ribosomal Database Project Classifier. cef-cefoperazone, clinda-clindamycin, *C.diff-C. difficile.*

of *C. difficile* that was lethal to only about half the animals that were treated with the five antibiotic cocktail and clindamycin.

Although the combination of the antibiotic cocktail and clindamycin was able to overcome colonization resistance, it is important that neither alone had the same effect. Therefore, loss of colonization resistance is not simply associated with creating an overall "depauperate" community but is dependent on the specific changes to the community structure as well. Administration of the antibiotic cocktail alone significantly decreased the overall biomass of the community, but this decrease in bacterial community size alone did not lead to a loss of colonization resistance. Furthermore, after administration of the antibiotic cocktail and clindamycin, at the time of successful challenge with *C. difficile*, the overall bacterial population had recovered, but the community structure was markedly altered from baseline. This further supports the idea that the specific changes brought on by antibiotic administration determine susceptibility to *C. difficile* colonization. This is consistent with the clinical observation that the risk of subsequent CDI differs with different antibiotics[33] and in vitro and animal studies that also differentiate antibiotics on the basis of their ability to overcome colonization resistance against *C. difficile*.[31,34,35]

Taken together these results suggest that this murine infection model accurately represents competition between two

opposing processes that are thought to be at the center of the pathogenesis of CDI (**Fig. 8**). On one hand there is the expansion of the population of *C. difficile* once it has colonized an altered/susceptible microbial community and the subsequent production of toxin. On the other hand, there is the tendency of stable microbial communities to return towards their baseline state following a perturbation, in this case, antibiotic administration.[26,30,36,37] According to this model, the observation of 50% mortality in animals treated with the antibiotic cocktail and clindamycin and then challenged with 10^5 CFU of *C. difficile* reflects a point at which the two processes are in close balance. In this case, if the pathogen can grow and produce toxin more rapidly than the recovery of the indigenous microbiota clinically severe disease would result. Alternately, if the microbiota recovers prior to sufficient expansion of *C. difficile* there could be control of the infection. This balance can be shifted in favor of *C. difficile* colonization and severe disease either by administering a larger challenge dose of *C. difficile* or causing a greater perturbation in the microbial community structure by administering cefoperazone. Alternately, administration of a smaller inoculum of *C. difficile* results in less disease.

Figure 7. Dose dependence of disease in cefoperazone-treated mice infected with *C. difficile*. Kaplan-Meier survival plot for mice infected with different doses of *C. difficile* VPI 10463 (2 x 10^2, 2 x 10^3, 2 x 10^4, 2 x 10^5 CFU) after 10 days of cefoperazone pretreatment and 2-day recovery. (n = 9 for 10^2, 10^3 groups and n = 10 for 10^4, 10^5 groups). Survival curves are significantly different (p = 0.0123 by the Log-rank (Mantel-Cox) Test) and there is a significant trend (p = 0.001).

In terms of the antibiotic cocktail with clindamycin, decreasing the inoculum prevented the development of clinical disease, but in the setting of cefoperazone, this merely delayed the onset of disease. This further supports the idea that cefoperazone administration results in a greater disturbance of the indigenous gut microbiota. However, if the gut microbial community is allowed to recover sufficiently from cefoperazone treatment (6 weeks), colonization resistance is restored and *C. difficile* did not colonize without further perturbation of the indigenous microbiota.

The mechanisms by which the indigenous microbiota can resist colonization and limit disease are not clear. Although direct competition between organisms within the gastrointestinal tract is possible,[38] it has been recently demonstrated that changes in the gut microbial community can indirectly affect colonization resistance via differential host responses. For example, decreasing the overall bacterial community through the administration of antibiotics can result in decreased host production of the antimicrobial peptide RegIIIγ.[39] RegIIIγ binds the surface-exposed peptidoglycan layer of Gram-positive organisms with high affinity in a calcium-independent manner.[40] Expression of RegIIIγ appears to be driven by the indigenous gut microbiota through host sensing of microbial-associated molecular patterns, primarily from Gram-negative organisms. RegIIIγ has been shown to be important 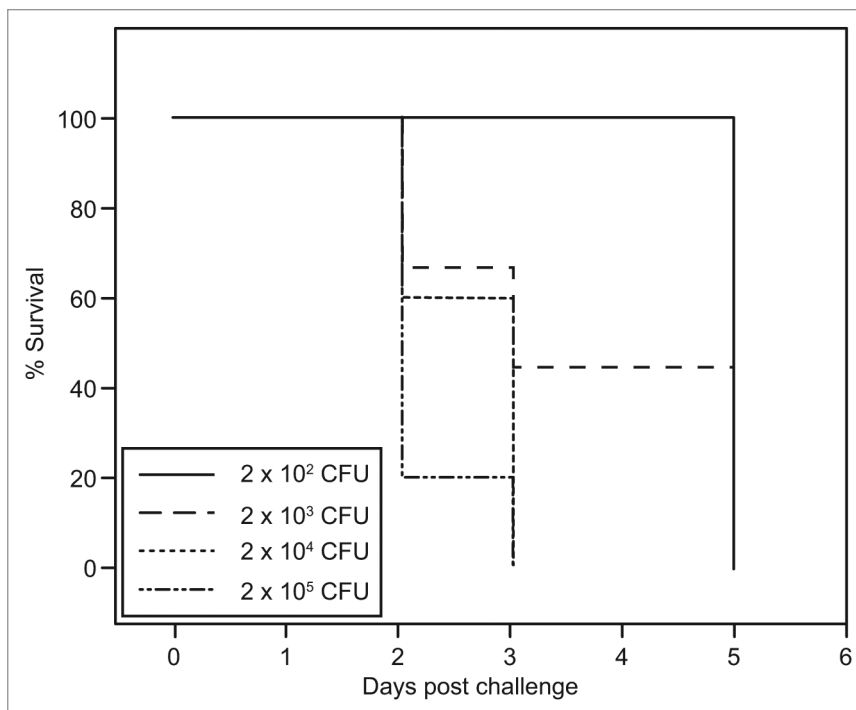in mediating colonization resistance to vancomycin resistant Enterococcus.[41] However, in our animals we did not find a direct relationship between changes in RegIIIγ expression following antibiotic treatment and colonization resistance to *C. difficile* (data not shown).

The development of specific host immune responses against *C. difficile* appears to have an important role in determining the severity of CDI, including the development of recurrent disease.[42] This observation underscores the exploration of *C. difficile* vaccines as a novel treatment/prevention modality.[43-46] Our current model is characterized by the acute development of disease and employs naïve mice, and thus *C. difficile*-specific adaptive responses are not thought to play a role. However, since this model can be manipulated such that the disease is not uniformly fatal (unlike the hamster model of disease), it remains to be determined if this model will be useful for studying adaptive immunity in CDI. There are recent reports that this is a useful model for studying the role of innate immune responses in CDI.[47,24] It is important to note that the role of immune responses in CDI is likely not independent of that of the indigenous microbes. It is clear that the gut microbiota has a key role in modulating the development of mucosal immune responses.[6,7] Therefore, changes in the gut microbiota, can influence the response to pathogens by altering the nature of host immunity.[48,49]

Another mechanism by which altered gut microbial communities could affect a pathogen is by changing the overall
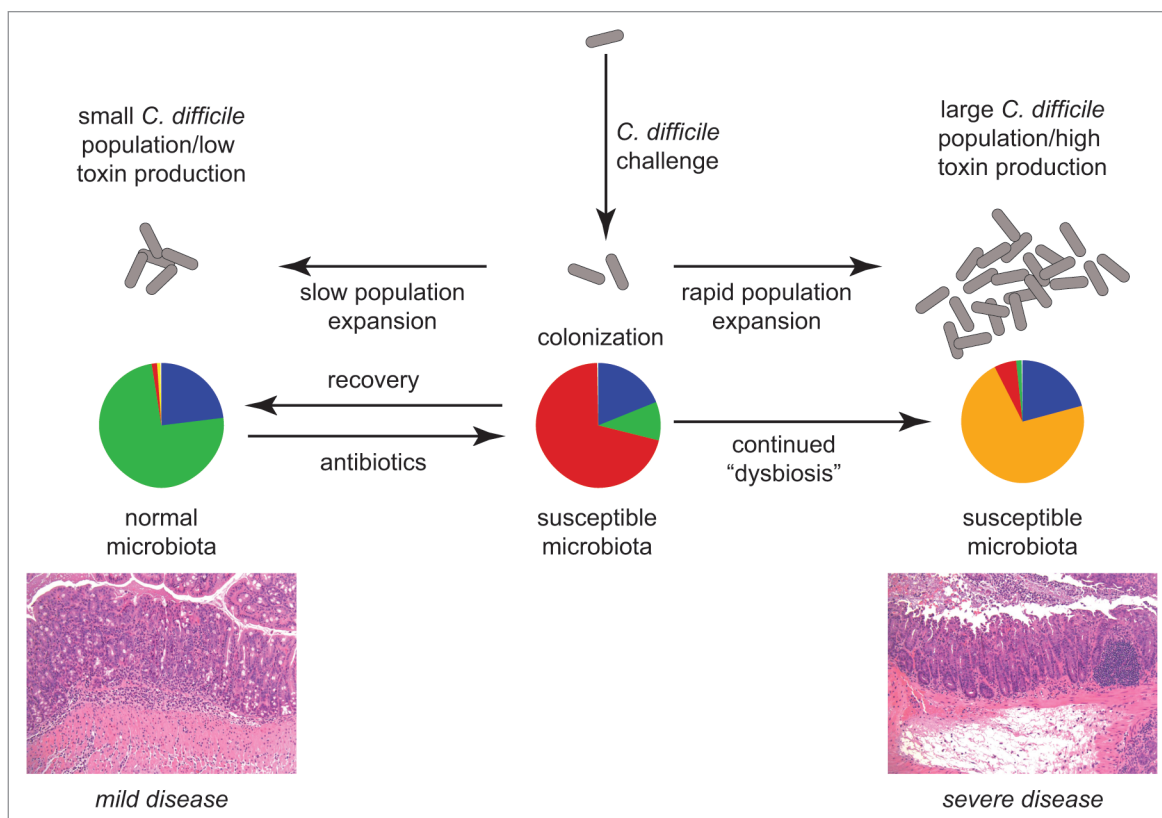
Figure 8. Model of the interaction between dynamics of the gut microbiota and *C. difficile* in antibiotic-treated mice. Antibiotic administration alters the community structure of the indigenous gut microbiota to a state that is susceptible to colonization with *C. difficile*. Subsequent clinical outcome is determined by the balance between the recovery of the gut microbiota following withdrawal of antibiotics and the expansion of the population of *C. difficile* and toxin production.

chemical environment of the gut. Changes in the community structure of the gut microbiota can dramatically alter the concentrations of microbial metabolites.[3,50] When comparing the gut microbial communities found in our animals that were clinically well versus those that were succumbing to CDI, the most obvious differences were the dominance of Proteobacteria in the ill animals and the return of Firmicutes, specifically members of the family Lachnospiraceae in well animals. These latter organisms are notable in that many are able to ferment complex carbohydrates to short-chain fatty acids (SCFA), which have an important role in maintaining intestinal homeostasis.[51-53] With regards to *C. difficile*, SCFA are able to inhibit the growth of the organism and decrease toxin production in vitro.[54]

In summary, our results demonstrate that the community structure (not the absolute level) of the indigenous gut microbiota plays a crucial role in shaping the outcome of *C. difficile* infection. The use of tractable murine models of disease should provide insight into the role that the indigenous gut microbiota plays in defense against pathogenic microbes. It remains to be seen which of the possible interactions between the host, indigenous microbiota and pathogen are important in determining the clinical outcome of infection. However, further study could lead to novel methods for the treatment and prevention of this increasing clinically important infection.

Experimental Procedures

Ethics statement. All animal protocols used during the conduct of these experiments were reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, Ann Arbor (protocol number 10212). The protocol was reviewed following guidelines for the care and use of laboratory animals set by the Office of Laboratory Animal Welfare, United States Department of Health and Human Services.

Animals and housing. The infection studies were performed with wild type C57BL/6 mice from a breeding colony established using animals purchased from Jackson Laboratories. Mice were housed with autoclaved food, bedding and water. Cage changes were performed in a laminar flow hood. Animals experienced a cycle of 12 h of light and 12 h of darkness.

***C. difficile* growth conditions.** The reference strain of *C. difficile*, strain VP1 10463 (ATCC 43255) was obtained and cultured on brain heart infusion agar containing 5% cysteine. An anaerobic environment was maintained at all times using an anaerobic chamber (Coy Industries). An incubation temperature of 37°C was used for growth. *C. difficile* suspensions for animal challenge were prepared by inoculating a single colony of *C. difficile* from a culture plate into brain heart infusion (BHI) broth, containing 5% cysteine, and allowing for overnight growth. Cells were

harvested by centrifugation (5,000 rpm for 15 min) and washed three times with pre-reduced PBS, pH 7.4. Bacterial enumeration was done to ensure that the correct dose of *C. difficile* vegetative cells was reached at the time of challenge.

Antibiotic administration and infection with *C. difficile.* Mice were divided into treatment groups consisting of 5 to 8 animals that were six to eight weeks old. An antibiotic mixture of 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) was prepared in sterile drinking water.[23] Antibiotics were purchased from Sigma-Aldrich (cat# K1377, G1914, C4461, M1547, V2002, C5269 and C4292). The antibiotic cocktail was administered for 3 days then the animals were switched to regular autoclaved drinking water for 2 days. All mice in each experiment were housed under the same conditions and were fed standard autoclaved chow. A single dose of clindamycin (10 mg/kg) was administered intraperitoneally one day before *C. difficile* challenge. Cefoperazone (0.5 mg/ml) was prepared in sterile drinking water and administered for 10 days. The cefoperazone drinking water was replaced with a fresh supply every 48 hours for the duration of cefoperazone administration. Animals were then switched to regular autoclaved drinking water for 2 days. A single dose of clindamycin (10 mg/kg) was administered intraperitoneally one day before *C. difficile* challenge. Some animals were allowed to recover for 6 weeks after cefoperazone treatment then a single dose of clindamycin was administered prior to *C. difficile* infection. Animals were infected by oral gavage with 1×10^5 CFU of *C. difficile* strain VPI 10463. Animals were monitored daily for signs of disease such as diarrhea, hunched posture and weight loss.

Necropsy and histological procedures. Mice were euthanized by CO_2 asphyxiation. The tip of the cecum of each mouse was removed, halved and rinsed in phosphate-buffered saline to remove luminal contents. Approximately 5 mm of proximal colon and terminal ileal tissue and luminal contents were collected from each animal. All samples were snap frozen in liquid nitrogen and stored at -80°C. The remaining cecum, colon and ileal tissue were placed intact into histology tissue cassettes and stored in 10% buffered formalin for 24 hours then transferred to 70% ethyl alcohol.[56] Tissue cassettes were then processed, paraffin embedded and then sectioned. Haematoxylin and eosin stained slides were prepared for histologic examination (McClinchey Histology Lab Inc.).

Histopathologic examination. Histological slides were coded, randomized and scored in a blinded manner by one of the authors (ILB) who is a board-certified veterinary pathologist. A scoring system was adapted from a previously published method.[23,57] Edema, cellular infiltration and epithelial damage in each tissue (colon, cecum, ileum) were scored from 0–4 according to the following defined criteria: Edema scores-(0): no edema; (1) mild edema with minimal (<2x) multifocal submucosal expansion; (2) moderate edema with moderate (2–3x) multifocal sub-mucosal expansion; (3) severe edema with severe (>3x) multifocal sub-mucosal expansion; and (4) same as score 3 with diffuse sub-mucosal expansion. Cellular infiltration

scores were graded as follows: (0) no inflammation; (1) minimal multifocal neutrophilic inflammation; (2) moderate multifocal neutrophilic inflammation (greater submucosal involvement); (3) severe multifocal to coalescing neutrophilic inflammation (greater submucosal ± mural involvment); and (4) same as score 3 with abscesses or extensive mural involvement. Epithelial damage was scored as follows: (0) no epithelial changes; (1) minimal multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); (2) moderate multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); (3) severe multifocal epithelial damage (same as above) ± pseudomembrane (intraluminal neutrophils, sloughed epithelium in a fibrinous matrix); and (4) same as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of epithelium).

DNA extraction. Total DNA from fecal and tissue samples was extracted using the MagNA Pure DNA isolation protocol (Roche, cat# 03730964001). Samples were placed in an Ultra Clean fecal bead tube (MoBio) to which 500 μl of MagNA Pure bacterial lysis buffer (Roche) was added. Samples were bead beaten for 1 min with a mini bead beater (Biospec), digested with proteinase K, incubated at 65°C, bead beaten for 1 min and then was heat inactivated at 95°C. Samples were placed in the MagNA Pure (Roche) and the MagNA Pure nucleic isolation kit protocol for bacterial DNA was followed as recommended by the manufacturer.

16S ribosomal rRNA-encoding gene clone libraries. The community structure of infected and uninfected mice was analyzed by the construction of 16S rRNA clone libraries.[58,59] PCR targeting bacterial 16S rRNA genes using primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') [60] was performed on each extracted DNA sample. PCR was performed using Illustra Pure Taq Ready-To-Go PCR beads (GE Healthcare, cat# 27955901). Reaction mixtures were set up with 100 ng of template DNA, 20 pmol of each primer, and water to a total volume of 25 μl. The reaction mixtures were subjected to amplification in a DNA thermal cycler (Eppendorf Mastercycler gradient) with the following cycling conditions: initial denaturation at 94°C for 2 min followed by 20 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec and extension at 72°C for 1.5 min. A final extension at 72°C for 10 min was performed. Control amplifications with sterile water were included in each amplification reaction and never gave visible amplicons. Amplicons were purified using a kit Illustra MicroSpin Column (GE Healthcare, cat# 27514001) according to the recommendations of the manufacturer. The purified PCR products were ligated into a plasmid vector (pCR 2.1; Invitrogen).

DNA sequencing and analysis. Plasmid purification and DNA sequence determination of 96 randomly selected clones from each library were performed by the DNA Sequencing Core facilities at the University of Michigan. Each clone was sequenced with a single primer (8F) that typically yielded 750 bases of readable sequence. Sequences with numerous ambiguous base calls or with fewer than 350 total bases were excluded from further analysis.

Sequences were analyzed for the formation of chimeras using the Chimera Check program from the Ribosomal Database Project.[60] Potential chimeric sequences were excluded from additional analysis. Sequences were also aligned to a phylogenetically diverse collection of 16S rRNA gene sequences using the RDP Classifier.[60]

Partial 16S rRNA sequences were initially analyzed using mothur[61] to calculate pair wise Morisita-Horn distances which was exported to Mega4 [62] software package and then UPGMA analysis was used to create dendrograms.

Quantitative PCR. Quantitative PCRs were used to assay the quantity of rRNA operons in the DNA samples relative to a single-copy host gene (mouse tumor necrosis factor alpha [TNFα]) as detailed in Antonopoulos et al.[27] Assays used the LightCycler 480 Probes Master reaction mixture (Roche, cat# 04707494001) at 1x concentration and appropriate primer-probe sets to increase the specificity of the signals detected from the sample DNA (100 ng). For detection of the bacterial signal, 100 nmol of each of the forward and reverse primers and the flourescent probe were included in the reaction mixtures. Sequences for the forward primer (5'-TCC TAC GGG AGG CAG CAG T-3'), the reverse primer (5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3') and the probe (5'-[6-carboxyfluorescein]-CGT ATT ACC GCG GCT GCT GGC AC-[6-carboxytetramethylrhodamine]-3') were based on the work of Nadkarni et al. Signals were detected with a LightCycler 480 instrument (Roche). Detection of the host signal used 200 nmol of the forward (TNFα_mu_se; 5'-GGC TTT CCG AAT TCA CTG GAG-3') and reverse (TNFα_mu_as; 5'-CCC CGG CCT TCC AAA TAA A-3') primers and 100 nmol of the probe (TNFα_mu_probe; 5'-Cy5-ATG TCC ATT CCT GAG TTC TGC AAA GGG A-Iowa Black RQ-3') adapted from Nitsche et al. Relative bacterial loads were compared via the C_T method by normalizing the 16S rRNA gene signal to the host signal.[65]

Monitoring *C. difficile* colonization. The colonization status of *C. difficile* infected animals was monitored using a *C. difficile* toxin multiplex qPCR assay of fecal pellets collected at various time points pre and post challenge from mice in each group. For the *C. difficile* Toxin Multiplex qPCR (LightCycler 480) 8 and 10 pmol/μl for *tcdA* and *tcdB* primers, respectively, were prepared from 200 pmol/μl original stocks. Primer and probe sets are as follows: tcdA_F: 5'-GGT AAT AAT TCA AAA GCG GCT, tcd_R: 5'-AGC ATC CGT ATT AGC AGG TG, tcdA_probe_FAM: 5'-6FAM-AGC CTA ATA CAG CTA TGG GTG CGA A-BHQ1, tcdB_F: 5'-GAA AGT CCA AGT TTA CGC TCA AT, tcdB_R: 5'-GCT GCA CCT AAA CTT ACA CCA, tcdB_probe_Hex: 5'-Hex-ACA GAT GCA GCC AAA GTT GTT GAA TT-BHQ1 (James Versalovic, personal communication,

manuscript in preparation). For each 20 μl reaction, 4 μl template, 10 pmol *tcdA* primers, 12.5 pmol *tcdB* primer, 1.6 pmol *tcdA* probe and 2 pmol *tcdB* probe were used. The following cycling conditions were used for the qPCR run: Activation-95°C for 15 min, 95°C for 15 sec, Cycling (X45)-60°C for 20 sec, 72°C for 10 sec, Hold-37°C for 30 sec. Values were normalized to mouse TNFalpha gene content and the mean fold change of *tcdA tcdB* gene content were calculated using the $2^{-\Delta Ct}$ method.[66]

***C. difficile* cytotoxin assay.** The assay was performed in 96-well flat-bottom microtiter plates (Corning) and was adapted from Corthier et al. Green African monkey kidney epithelial cells (Vero) (provided by M. Imperiale, University of Michigan) were grown to confluency in DMEM (GIBCO Laboratories, cat# 11965) containing 10% heat inactivated fetal bovine serum (GIBCO Laboratories, cat# 16140) and 1% penicillin streptomycin solution (GIBCO Laboratories, cat# 15140). The cells were trypsinized using 0.25% trypsin (GIBCO Laboratories, cat# 25200) and washed with 1 volume of DMEM medium. Cells were diluted in DMEM medium and approximately 1×10^5 cells were distributed per well and incubated at 37°C with 5% CO_2 for 18–24 hours. Samples of luminal contents or intestinal tissue were weighed and 500 μl of 1x PBS was added. Intestinal tissue was homogenized using a Medimachine (Becton Dickenson). Samples were vortexed then spun at 13,000 rpm for 5 minutes and then the supernatant was filtered through a 0.2 μm membrane. Each sample was titrated in two-fold dilutions within the wells to a maximum dilution of 2^{-12} and each well had a corresponding control to which both antitoxin (TechLabs, cat# T5000) and sample were added. After an overnight incubation at 37°C, plates were fixed with 10% buffered formalin for 2 hours then stained with geimsa (50 μl per well) for 15 minutes followed by a wash with 1x PBS. Wells with approximately 100% round cells were easily recognized under 200x magnification. The cytotoxic titer was defined as the reciprocal of the highest dilution that rounds 100% of Vero cells per gram of sample. Vero cells with purified *C. difficile* toxin and antitoxin (TechLabs, cat# T5000) were used as controls.

Statistical analysis. Statistical analyses were performed using Prism 5 for Mac OS X GraphPad Software. t tests were used for treatment group comparisons, except for categorical histology scores, where the nonparametric Krustal Wallis test was used. Statistical significance was set at a p value of < 0.05.

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