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The microbiota and immune response during *Clostridium difficile* infection

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Abstract

Clostridium difficile is a gram-positive, spore forming anaerobe that infects the gut when the normal microbiota has been disrupted. *C. difficile* infection (CDI) in the United States is the most common cause of hospital acquired infection, and the leading cause of death due to gastroenteritis. Patients suffering from CDI have varying symptoms which range from mild diarrhea to pseudomembranous colitis and death. The involvement of the immune response to influence disease severity is just beginning to be investigated. There is evidence that the immune response can facilitate either protective or pathogenic phenotypes, suggesting it plays a multifaceted role during CDI. In addition to the immune response, the microbiota is pivotal in dictating the pathogenesis to CDI. A healthy microbiota effectively inhibits infection by restricting the ability of *C. difficile* to expand in the colon. Thus, understanding which immune mediators and components of the microbiota play beneficial roles during CDI will be important to future therapeutic developments. This review outlines how the microbiota can modulate specific immune mediators, such as IL-23 and others, to influence disease outcome.

1. Introduction

Clostridium difficile is a spore forming, Gram-positive, toxin-producing anaerobe that infects the gut when the natural flora has been disrupted, primarily through use of antibiotics. It is currently the leading cause of nosocomial infection in the United States, surpassing methicillin resistant *Staphylococcus aureus* (MRSA) [1–3]. The Centers for Disease Control and Prevention (CDC) lists *C. difficile* as one of three urgent threats in the United States and it is estimated to cause approximately 453,000 infections per year with 29,300 related deaths [4]. Moreover, a 30-day mortality rate has been observed in up to 15%

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of *C. difficile* patients [5]. Disease can range from asymptomatic colonization to mild diarrhea, pseudomembranous colitis, and life threatening toxic megacolon. Treatment for *Clostridium difficile* infection (CDI) costs the US health care system an estimated \$4.8 billion annually in acute health settings alone, with an additional substantial burden seen in long-term care facilities [6]. Despite therapy, recurrent disease is seen in 10–35% of patients after initial infection and secondary relapses are observed in 35–65% of patients after primary reoccurrence [7–9]. Risk factors include antibiotic exposure, acute or long term care facility exposure, advanced age, comorbidities such as inflammatory bowel disease, and use of proton pump inhibitors [6,10]. The prevalence of *C. difficile* cases have been increasing annually in both health care and community settings and hypervirulent strains of *C. difficile*, most notably BI/NAP1/Ribotype 027 strains, are also becoming more common [11]. Additionally, the ability of *C. difficile* spores to survive in harsh conditions including resistance to alcohol-based cleaners contributes to disease transmissibility. In the past ten years, there has been a five-fold rise in disease incidence in the North American population, emphasizing the need for better treatment and management strategies [12,13].

2. Pathogenesis of CDI

Disruption of the host's endogenous microbiota, a state called dysbiosis, provides an ideal environment for CDI to occur. Several components of a healthy microbiota contribute to preventing host susceptibility to infection, outlining the importance of commensal bacteria to combat *C. difficile*. Bacterial spores are transmitted through the fecal-oral route and germinate into vegetative cells in the intestine of susceptible hosts. These cells infiltrate the mucus layer surrounding the epithelial cell layer and adhere to its surface [14]. Once adhered, the bacteria produce toxins that mediate a robust inflammatory response. Toxin A (TcdA) and toxin B (TcdB) are the primary virulence factors of *C. difficile* and are released during the late log phase and stationary phase of vegetative growth [15]. TcdA and TcdB are able to glucosylate and inactivate Rho and Ras family small GTPases causing disruption of the actin cytoskeleton, cell rounding, inhibition of cell division and cell death [16,17]. This process is especially harmful to the integrity of the epithelial barrier. The breakdown of the epithelium causes permeability of the barrier and allows for both pathogenic and commensal bacteria to translocate into the lamina propria. Collectively, these actions induce the release of proinflammatory mediators from epithelial and immune cells in the lamina propria that subsequently recruit additional immune effector cells to the site of infection [16,18–20]. Neutrophils are the hallmark cell subset recruited to the intestinal barrier during infection and are found in pseudomembranous lesions during severe disease. However, the role of the immune response during infection remains incompletely understood as there is evidence to support both protective and pathogenic roles during CDI. The dual role of the immune response coupled with the knowledge that a healthy microbiota prevents infection demonstrates the importance of both commensal bacteria and the host inflammatory response during CDI.

3. The role of the microbiota during *Clostridium difficile* infection

Antibiotic exposure remains the leading risk factor of disease, stressing the beneficial role of the microbiota in host protection [21]. Disruption of a 'healthy' microbiota or the reduction

of its diversity is directly linked to host susceptibility to CDI. The microbiota of patients in the hospital are commonly in a dysbiotic state due to increased incidence of antibiotic treatment, modulation of diet, and other treatments such as chemotherapy. Dysbiosis coupled with enhanced exposure to *C. difficile* spores in the hospitals may explain why the majority of CDI cases are associated with health care facilities. The loss of disease resistance associated with alterations of the endogenous flora is an important initial step in the pathogenesis of CDI (Figure 1). The necessity of antibiotic pretreatment to render mice susceptible to CDI has since been established in mouse models [22]. The microbiota has been shown to protect against infection through a process called colonization resistance, which involves commensal microbes outcompeting the pathogen for space and nutrients in the intestine [23]. Wilson and colleagues originally described colonization resistance by demonstrating that the transfer of cecal contents from an untreated hamster to a vancomycin-treated hamster effectively prevented susceptibility to CDI [24]. It was later shown that bacteria with similar nutrient and spatial demands are capable of excluding *C. difficile*. In fact, a series of experiments demonstrated that pre-infection with a non-toxigenic strain of *C. difficile* was capable of successfully protecting hamsters from subsequent infection with a toxigenic *C. difficile* strain [25]. Although, the immune response to non-toxigenic *C. difficile* was not examined as a potential mechanism of disease prevention, the authors conclude that similar niche requirements utilized by non-toxigenic *C. difficile* results in protection from CDI [24]. Furthermore, recent studies have identified that alterations in the microbiota in response to antibiotic treatment induced spikes in succinate and sialic acids which are then exploited by *C. difficile* to facilitate its expansion in the gut [26,27]. Together, this data supports a role for the microbiota to outcompete *C. difficile* resulting in inhibition of infection.

In addition to colonization resistance through competition for space and nutrients, the microbiota has also been observed to regulate primary and secondary bile salts to inhibit *C. difficile* outgrowth. The primary bile salt taurocholate was identified as an *in vivo* germinant of *C. difficile* spores into vegetative cells that cause disease [28]. It was later shown that derivatives of cholate activate spore germination when combined with glycine, whereas derivatives of chenodeoxycholate suppress germination, supporting a role for bile salts in regulating the outgrowth of *C. difficile* [29,30]. Interestingly, antibiotic treatment and subsequent changes in the endogenous microbiota lead to increased taurocholate in the cecum and reduced levels of the inhibitory secondary bile salt deoxycholate, which is toxic to vegetative cells [31]. Thus, antibiotic treatment supports the outgrowth of *C. difficile* by inducing bile salts that enhance germination and reducing bile salts that suppress the expansion of vegetative cells. In fact, transfer of bacteria from the cecal contents of antibiotic treated mice supported expansion of *C. difficile in vivo*, while transfer of contents from untreated mice increased host resistance to infection [32]. Buffie et al. associated protection observed in mice receiving cecal contents from untreated mice with the presence of enhanced secondary bile acids [32]. The elevation of secondary bile acids in protected mice could be achieved by transferring a cocktail of four specific bacteria, with a primary role for bacterium *Clostridium scindens* [32]. This study supports the hypothesis that specific components of the microbiota have the ability to protect against CDI.

In addition to its well-defined role in preventing host susceptibility, there is emerging evidence for the ability of the microbiota to resolve active CDI. Fecal microbiota therapy (FMT) involves the transfer of 'healthy' donor stool to *C. difficile* infected patients with the goal of restoring bacterial diversity in the colon and expelling *C. difficile*. Studies in mice are now beginning to explore the ability of a transferred microbiota to clear CDI from mice and prevent relapsing disease. In fact, the transfer of six phylogenetically diverse intestinal bacteria was sufficient to clear CDI in these mice [33]. Moreover, Tvede and Rask-Madsen successfully performed FMT in six CDI patients in 1989, although the treatment has not received mainstream attention until recent years [34]. A recent study from the New England Journal of Medicine observed an approximate 90% cure rate in relapsing CDI patients [35], indicating that the microbiota has a beneficial role to play at both the resistance and resolution stages of disease. Furthermore, restoration of microbiota through FMT has shown more promise in preventing disease reoccurrence than vancomycin treatment [35]. This may be due to the ability of vancomycin to target beneficial members of the microbiota in addition to *C. difficile* and prevent the recovery of a healthy microbiota and patient recovery.

4. The dual role of the immune response during *Clostridium difficile* infection

The role of the immune response during *C. difficile* infection remains controversial as there is evidence to support protective and pathogenic phenotypes during disease. In animal models of *C. difficile* infection, the absence of an intact immune response is disadvantageous to the host. The importance of neutrophils is supported by evidence that mice infected with *C. difficile* that lack the capability to recruit neutrophils to the gut suffered from enhanced mortality compared to controls. [36,37]. TLR4^{-/-} and MyD88^{-/-} mice experienced enhanced morbidity, likely through the observed decrease in MyD88-dependent neutrophil recruitment to the gut [36–39]. Likewise, leptin signaling, a hormone that is associated with innate and adaptive immune regulation, enhances *C. difficile* clearance and is associated with increased inflammatory mediators during CDI in mice [40]. In addition, innate lymphoid cells (ILCs), primarily group 1 ILCs, have been recognized as a critical cell type involved in resolving infection by producing IFN- γ , a type 1 proinflammatory cytokine, to reduce CDI-associated mortality [41,42]. The activation of IL-22, a type 17 proinflammatory cytokine, has also been shown to enhance survival during CDI mice. IL-22 functioned to enhance complement-mediated bacterial phagocytosis, which targeted pathobionts to limit the detrimental systemic translocation of these bacteria [43]. The culmination of these data supports the hypothesis that an intact inflammatory response is beneficial to combat bacterial burden and resolve the infection.

While the recruitment of neutrophils appears to be essential to combat the pathogen and resolve infection, it has been postulated that these cells may also exacerbate disease severity through off-target influences that lead to tissue damage and the formation of pseudomembranous colitis. In support of this hypothesis, leukocytosis, an indicator of systemic inflammatory response, correlates to increased disease severity and a negative prognosis in hospitalized patients with diarrhea [44]. Additionally, recent studies have indicated that increases observed in inflammatory markers are a more accurate way to

predict poor patient outcome than increased *C. difficile* bacterial burden. For instance, a slower recovery rate in adults and children was associated with higher levels of the intestinal mediators CXCL5 and IL-8, which both function to recruit neutrophils to the gut. Interestingly, the burden of *C. difficile* bacteria in the gut did not correlate with delayed patient recovery [45,46]. Furthermore, a polymorphism resulting in increased expression of the IL-8 gene was associated with enhanced recurrence of CDI, highlighting the potential role for overabundant inflammation as a mechanism of severe infection. [47]. Evidence that induction of certain immune mediators may enhance disease severity was directly demonstrated in a mouse model of CDI investigating the role of IL-23, a cytokine with potent neutrophil recruiting capabilities. In this study, mice were significantly protected from CDI-associated mortality and morbidity in two distinct models which lacked IL-23 signaling [48]. Moreover, toxin B has been demonstrated to activate an ASC containing inflammasome and *in vitro* studies observed an inflammasome-dependent increase in IL-23p19 mRNA and protein expression from cells stimulated by two individual strains of *C. difficile* [49,50]. Together, these studies indicate that *C. difficile* induced IL-23 signaling may tip the immune balance towards a robust proinflammatory environment that results in enhanced intestinal damage and pathology during CDI. In addition to data derived from human and mouse CDI studies, the negative role of the immune response is most evident in mouse models of intoxication with purified *C. difficile* virulence factors TcdA and TcdB. Using these models researchers can specifically investigate the influence of *C. difficile* toxins on host tissue *in vivo*. Intoxication with TcdA in IFN γ -deficient mice displayed dampened inflammation and less severe pathology compared to control mice [51]. Likewise, TcdA/B intoxication in mice treated with an IL-1R antagonist had reduced inflammation and exhibited a similar protective phenotype [52]. Together, there is evidence to support a deleterious role for certain immune mediators in both models of intoxication and infection.

The role of the immune response during CDI is likely multifaceted, as there is data to support that inflammation can be both protective and pathogenic (Figure 2). Thus, maintaining a balanced inflammatory response to combat infection while limiting off-target tissue damage is likely beneficial during CDI. The varying roles for inflammation in modulating disease outcome supports the notion that the severity of disease suffered by the patient hinges on intensity or the type of immune response elicited.

5. The Microbiota and Immune Responses

Crosstalk between the microbiota and the intestinal epithelium plays a role in shaping the mucosal immune response. This observation is supported by evidence that germ-free mice have under-developed innate and adaptive immune responses [53]. Therefore, alterations in the composition of the microbiota, due to antibiotic or environmental changes, can influence how immune cells react to insults and could contribute to host severity during CDI. It is well understood that both the immune response and the microbiota can influence the pathogenesis of CDI. Therefore, understanding how the microbiota can influence the immune response is important to consider when discussing this infection.

Influences of the microbiota on mucosal immune responses can be attributed to distinct microbial signals recognized by cells within the intestine. Many of these microbial signals,

such as flagellin and prokaryotic DNA, are recognized by Pattern Recognition Receptors (PRRs) on epithelial and immune cells [54]. These signals can alter immune responses not only by influencing the repertoire of immune cells present in the lamina propria, but also by affecting the functionality of cells. Thus, a reduction in microbial abundance or diversity can lead to an unbalanced immune response in the intestine that may be harmful during CDI. This is supported by evidence that signals from the microbiota can induce a proportion of regulatory T cells (Tregs) in colonic tissue [55]. The method by which a 'healthy' microbiota induces Tregs may be through the production of short-chain fatty acids (SCFA), which are dependent on microbiota fermentation of dietary components and have been demonstrated to promote the induction of Treg cells [56]. As a result, induction of Tregs by the microbiota was associated with the reduction in Type 17 T cells (Th17) [57]. Activation of type 17 responses culminates in granulopoiesis and recruitment of neutrophils to the site of infection [58]. Additionally, intestinal microbes have been suggested to play a regulatory role in the intestine by controlling the mucus barrier and gene expression of *muc2*, a major component of mucin. The induction of intestinal mucus is not only capable of reducing inflammation by creating a physical barrier between the lumen and the epithelium, but also actively skews mucosal dendritic cells to function in a regulatory capacity [59,60]. On the other hand, commensal organisms such as segmented filamentous bacteria (SFB) have been demonstrated to induce a strong Th17 response, including IL-23 and IL-17A expression and suggest that the composition and presence of the microbiota plays an important role in maintaining homeostasis between proinflammatory and regulatory responses in the colon [61]. Therefore, the disruption of Treg/Th17 homeostasis with antibiotic treatment may play a pivotal role in disease outcome

Antibiotic treatment has also been demonstrated to create a proinflammatory environment by enhancing the translocation of native commensal bacteria out of the intestine [62]. In this study antibiotic treatment was associated with enhanced CXCL1, IFN γ , and IL-17 protein in the mesenteric lymph nodes increased intestinal inflammation by histological analysis [62]. Additionally, antibiotic-induced translocation of commensals led to worsened DSS-induced colitis [62]. In a second study, high fat diets in mice were demonstrated to facilitate commensal translocation to the blood and adipose tissues and enhanced proinflammatory cytokine TNF- α . Translocation events and resulting inflammation were reversed with probiotic *Bifidobacterium animalis* treatment, indicating that certain species of the microbiota are capable of reducing bacterial translocation [63]. This is interesting, as it has been observed that IL-22 induced complement reduces CDI-associated morbidity by limiting the translocation of commensals [43]. Therefore, in addition to establishing host susceptibility by providing a beneficial niche for *C. difficile* prior to infection, antibiotic treatment may also be pathogenic to the host throughout CDI by supporting bacterial translocation and a subsequent unbalanced immune response.

Immunoglobulin A (IgA) is the dominant immunoglobulin at mucosal sites and plays an important role in maintaining intestinal barrier integrity and protecting host tissue from pathogenic and commensal microbes [64]. The microbiota supports the induction of plasma cells and IgA responses as evidenced by decreased levels in germ-free mice [65]. Interestingly, reductions in B cells, plasma cells, and IgA responses have been observed in CDI patients and the lowest levels were found in the most severe CDI patients with

pseudomembranous colitis [66]. The importance of antibodies to protect the host from CDI is also supported by low levels of serum antibodies directed against toxins A and B observed in patients with recurrent CDI compared to controls that do not relapse [18,67,68]. In fact, new immunotherapy vaccination strategies that promote anti-toxins antibodies are being investigated to treat CDI and there are currently three clinical trials in Phase II or Phase III that have demonstrated promising results in preventing disease relapse [73]. Thus, the ability of the microbiota to aid in antibody responses is likely another mechanism by which the microbiota-immune response axis influences disease outcome.

As previously described, immune mediators can play either a pathogenic or protective role during CDI. An intriguing avenue to pursue is investigation into how the microbiota may affect the presence and absence of established immune mediators that are important in dictating disease outcome. For instance, there is evidence to support that expression of IL-25 is dependent on the microbiota and this cytokine signals inversely of the pathogenic mediator IL-23 [69–71]. Germ-free and antibiotic mice had reduced IL-25 expression and enhanced IL-23 levels in the intestine [69]. This association prompts numerous hypotheses for researchers studying the immune response to CDI. Firstly, are cytokines which signal inversely of deleterious immune mediators, such as IL-25, protective during CDI? Secondly, does antibiotic treatment have a secondary role in contributing to pathogenesis by influencing the immune responses, like IL-25 and IL-23, that dictate disease severity and host outcome.

6. Remaining questions

Current therapy for CDI involves stopping the offending antibiotic that rendered the patient susceptible and beginning vancomycin, fidaxomicin, or metronidazole treatment. This treatment remains inadequate, as made apparent by high rates of relapse and mortality in CDI patients. Fecal transplantation is being utilized more frequently for cases of CDI relapse [72]. Although, FMT is successful at preventing relapses in approximately 90% of cases, it is of unknown effectiveness for primary CDI. Despite the ability of FMT to prevent relapse, the understudied possibility of long-term risks associated with recolonization of unknown bacteria remains a disadvantage of FMT. A powerful therapy for CDI would be the development of defined bacterial cocktails that effectively displace *C. difficile* while limiting long-term deleterious side effects. Emerging evidence supporting the role of specific immune mediators to shape the outcome of disease provides an interesting area to consider in future therapeutic interventions. Furthermore, facilitating targeted bacterial cocktails strategies to reconstitute commensal bacteria based on their ability promote protective immune responses while suppressing deleterious immune mediators may provide an efficient strategy to treat both primary and secondary CDI. Therefore, understanding which mediators of both the microbiota and the immune response regulate a balanced and beneficial host response to favor eradication of *C. difficile* burden while preventing tissue destruction will provide new and innovative therapeutic approaches.

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- Clostridium difficile infection (CDI) causes a wide range of patient symptoms
- Microbiota outcompetes *C. difficile* for space and nutrients and regulates bile acids to prevent pathogen outgrowth
- The immune response to CDI can influence disease severity
- An altered microbiota shapes mucosal immune responses

Figure 1.

A healthy microbiota is important to inhibit host susceptibility to *Clostridium difficile* infection. Antibiotic treatment reduces microbial abundance and diversity which results in enhanced nutrients and regulation of primary and secondary bile acids that favor *C. difficile* outgrowth. Once established in the colon, *C. difficile* releases toxin that lead to cell death, disruption to the epithelial barrier, and induction of proinflammatory responses.

Molecule	Role during CDI	Reference
TLR4 and MyD88	Reduce morbidity in mice	Ryan et al (2011), Jarchum et al (2011), Lawley, 2009
IL-22/complement	Control bacterial translocation; reduce mortality in mice	Hasegawa et al (2014)
Innate Lymphoid Cells (ILCs)	Ablation of ILCs lead to severe mortality in mice	Abt et al (2015), Geiger et al (2014)
IL-23	Increases mortality/morbidity	Buonomo et al (2013)
IL-8 / CXCL5	Predicts poor prognosis better than <i>C. difficile</i> burden in humans	El Feghaly et al (2013A &B)

Figure 2.

Multifaceted role of the immune response during *Clostridium difficile* infection. There is evidence to support both pathogenic and protective roles for the immune response during CDI. Thus, an immune response which controls infection while limiting off target effects that lead to tissue pathology is likely beneficial to disease outcome