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Immune responses to *Clostridium difficile* infection

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Abstract

Clostridium difficile is the causal agent of antibiotic-associated diarrhea and is a leading cause of hospital-acquired infections in the US. *C. difficile* has been known to cause severe diarrhea and colitis for more than 30 years, but the emergence of a newer, hypervirulent strain of *C. difficile* (BI/NAP1) has further compounded the problem, and recently both number of cases and mortality associated with *C. difficile*-associated diarrhea has been increasing. One of the major drivers of disease pathogenesis is believed to be an excessive host inflammatory response. A better understanding of the host inflammation and immune mechanisms that modulate the course of disease and control host susceptibility to *C. difficile* could lead to novel (host-targeted) strategies for combating the challenges posed by this deadly infection. This review summarizes our current knowledge of the host inflammatory response during *C. difficile* infection.

Keywords

Pseudomembranous colitis; antibiotic-associated diarrhea; toxic megacolon; chemokines; neutrophils; immune response

Clostridium difficile infection

Clostridium difficile is a Gram-positive, anaerobic, spore-forming, toxin-producing bacterium that was initially identified in 1935 as part of the normal gut flora of neonates [1]. It was initially named *Bacillus difficilis* due to its slow growth and the difficulty encountered in culturing the bacterium [1]. The role of *C. difficile* as a pathogen and as a causative agent for antibiotic-associated diarrhea and pseudomembranous colitis was first defined in 1978 [2, 3]. During the past 30 years, *C. difficile* infection has become one of the most common nosocomial, or hospital-acquired, infections in the U.S. and a major cause of antibiotic-associated diarrhea and pseudomembranous colitis in hospitalized patients and patients in long term care facilities [4, 5]. In fact, the incidence of *C. difficile* infections in some community hospitals is now greater than methicillin-resistant *Staphylococcus aureus* (MRSA) infections [5]. The epidemiology of the disease has also been changing with the emergence of newer, more virulent strains [6, 7] and an increase in the incidence of community-acquired *C. difficile* infections [8, 9].

C. difficile infection is almost always associated with disruption of endogenous gut flora, which is postulated to allow the bacterium to propagate and cause disease [10]. Although some studies had shown that prior treatment with ciprofloxacin, clindamycin, penicillins,

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and cephalosporins are most frequently associated with disease, the use of almost any antibiotic can lead to *C. difficile* infection [11, 12, 14]. The use of drugs to suppress gastric acid production (proton pump inhibitors and H2 blockers) has also been associated with an increased risk of *C. difficile* infection [13, 14]. Apart from treatment with certain drugs, other factors associated with an increased risk of *C. difficile* infection include old age, underlying chronic disease, recent hospitalization, gastrointestinal surgeries, and tube feeds [11, 13, 15, 16, 17]. In the past few years, a new, hyper-virulent strain of *C. difficile* (BI/NAP1/027) has emerged. This strain has been responsible for an epidemic of *C. difficile* that was originally identified in Quebec and has now spread to parts of the US [7, 18]. Although the exact mechanisms of increased virulence are still not clear, this strain is characterized by increased production of toxins A and B, the presence of a binary toxin (CDT), deletion of the gene *tcdC* (a negative regulator of toxin production), and increased resistance to fluoroquinolones [6, 7, 19, 20].

Clinically, patients with *C. difficile* colitis present with abdominal pain, cramps, diffuse watery diarrhea, and leukocytosis. Overall, the disease spectrum can range from asymptomatic colonization to mild diarrhea to severe complicated infections that include fulminant colitis, toxic megacolon, and shock. Many different scoring systems comprising a combination of clinical and laboratory parameters (*e.g.*, altered mental status, fever, abdominal pain, leukocytosis, hypoalbuminemia, and elevated serum creatinine levels) have been studied [21, 22], and although there is no single best predictor of severe disease, the degree of leukocytosis is widely believed to correlate with more severe disease [21, 22].

The mainstay of treatment is discontinuation of the offending antibiotic and administration of metronidazole or vancomycin. Recently, fidaxomicin, a macrocyclic antibiotic, has been shown to slightly reduce the rate of recurrent infections, but the difference was seen in only non-NAP1 strains of *C. difficile* [23]. Other treatment modalities (including the antibiotics nitazoxanide, rifaximin, tigecycline, and teicoplanin), fecal transplant, probiotics, intravenous immune globulin, and the administration of humanized monoclonal antibody against toxin have been tried with limited success and need further evaluation before being broadly used [24]. Despite current therapeutic options, the incidence of *C. difficile*-associated diarrhea has been increasing during the past decade and is now the most common cause of hospital-acquired infection [5].

Two major problems are the largest obstacles to successful treatment of *C. difficile* infections: (i) the emergence of a newer, hypervirulent strain of *C. difficile* (BI/NAP1) that has been associated with more severe disease, including 30 day mortality in approximately 15% of cases [25, 26]; and (ii) higher rates of recurrence [27, 28]. Although the use of fidaxomicin decreases the incidence of recurrence in non-NAP1 strains, the rate is still very high (between 13–15%) [23]. It is important to note that the current standard of practice for treating *C. difficile* infection is the use of antibiotics that further disrupt the microbial flora, which in turn may create a niche for additional *C. difficile* infections (and recurrences). A close look at disease pathogenesis suggests that although targeting the bacterium is important, excessive inflammation also plays an important role in disease pathogenesis. Interestingly, asymptomatic colonization with *C. difficile* is seen in a large number of infants [86, 87] and in about 4% of the adult population [14]. The fact that asymptomatic colonization persists without any overt disease suggests that host-associated factors (immune response and inflammation) may play a critical role in determining the disease outcomes. These intriguing observations suggest that targeting the host inflammation may be a novel adjunctive strategy for combating *C. difficile* infection.

Pathogenesis

Changes to the endogenous microbiome by broad-spectrum antibiotics provide an ideal niche for *C. difficile* infection [12], and infection with *C. difficile* leads to toxin-mediated intestinal inflammation and diarrheal disease [29, 30]. Typically, bacterial spores are transmitted by the fecal-oral route; spores survive the gastric acid barrier and germinate when they reach the anaerobic environment of the colon. The resulting vegetative cells then penetrate the mucus layer and adhere to intestinal epithelial cells. This step is followed by secretion of the large clostridial toxins toxin A and toxin B that are believed to be the major virulence factors. *In vitro* studies have shown that the toxins lead to a characteristic inflammatory response, which includes damage to the intestinal epithelial cells, neutrophilic infiltration, and local chemokine and cytokine secretion [31, 32]. Because it is at least in part a toxin-mediated disease, one of the key determinants of disease severity after infection is the host inflammatory response [33] and blocking the inflammatory response can ameliorate the disease in animal models [31, 34].

C. difficile virulence factors

Clostridial Toxins

Following colonization and replication of the vegetative forms, the bacteria secrete toxin A (TcdA) and toxin B (TcdB). These toxins were identified in the early 1980s [29, 30], and, at least in animal models, administration of the toxins has been shown to replicate all the histopathologic features of *C. difficile* infection [29]. The toxins share 63% amino acid sequence similarity [35] and are members of the clostridial glucosylating toxin family whose activity is due to their monoglucosylation of a threonine residue onto the Rho protein family of GTPases, leading to Rho inactivation [32]. Because Rho proteins are important molecular switches that control actin cytoskeleton re-organization, the inactivation of Rho leads to disruption of the intracellular actin cytoskeleton and, ultimately, cell death [32]. In addition, the toxins induce the release of inflammatory mediators from intestinal epithelial, neuronal, and immune cells via activation of different molecular pathways including TLR4, TLR5, and NOD1 signaling [36, 37, 38, 39].

The relative contributions of toxin A and B in the pathogenesis of *C. difficile* disease remain controversial. Earlier studies had shown that purified toxin A alone, but not B, replicated symptoms of *C. difficile* infection [40]; toxin B was believed to be dispensable, but it was then shown that, in fact, toxin B is essential for virulence and toxin A is dispensable [41]. Using isogenic *tcdA* and *tcdB* mutants in a hamster model, it was shown that toxin B is a key virulence determinant [41]. Most recently, another study [42] used similar methods to show that, in fact, both toxin A and toxin B were important mediators of *in vitro* cytotoxicity and *in vivo* virulence [42]. The differing findings in these two studies could be due to genetic differences in the strains of *C. difficile* used for mutagenesis. Although both groups used derivatives of strain 630, these strains had been independently passaged for many years, and, in fact, the strain used in the latter study [42] produced 3-fold more toxin than the equivalent strain used in the earlier study [41, 43]. The end points used to determine the *in vivo* pathogenicity of the strains were also different in the two studies; death was an end-point in the earlier study [41], and a clinical scoring system comprising weight loss, behavioral changes, and wet tail, followed by sacrifice of moribund and sick animals, was used in the later study [42]. In any case, toxins are essential for disease because toxin A[−]B[−] strains are avirulent [42, 44]. Interestingly, in human *C. difficile* infection, naturally occurring toxin A⁺B[−] isolates have not been reported while toxin A[−]B⁺ variants are being isolated with increasing frequency and have similar disease severity as toxin A⁺B⁺ strains [44, 45]. More studies in mouse models of *C. difficile* infection would help identify the relative role of each toxin in the pathogenesis of *C. difficile* infection.

The genes encoding the *C. difficile* toxins are located within a 19.6 kb region called the pathogenicity locus (PaLoc). Three other genes that regulate the expression and secretion of toxin genes are also present on the PaLoc: *tcdC*, which encodes a regulatory factor; *tcdR*, which encodes a sigma factor for the RNA polymerase; and *tcdE*, which encodes a holin-like protein. TcdC is believed to be a putative negative regulator of the toxin genes, TcdR is an alternative sigma factor important for the expression of toxin genes, and TcdE is believed to be important for secreting toxins from the bacterium [44].

Studies of the hypervirulent NAP1/027 strain of *C. difficile* have shown that it produces 16- to 20-fold higher amounts of toxins A and B [6], as well as a third toxin, binary toxin (CDT) [7]. The binary toxin gene is located on the Cdt locus in the bacterial genome. In addition to the binary toxin, the hypervirulent strain BI/NAP1/027 has a nonsense mutation in the gene encoding the negative regulatory protein TcdC [6] that has been shown to increase expression of toxin A and toxin B genes as well as Vero cell cytotoxicity [6, 46]. The binary toxin induces formation of microtubule-based protrusions that increase bacterial adherence [47]. However, it may play only an adjunct role in *C. difficile*-associated disease; in a rabbit model of *C. difficile*-associated disease, CDT⁺ToxA⁻ToxB⁻ strains led to fluid accumulation and colonization but did not cause death or disease [48].

Other *C. difficile* virulence factors

Beyond the large clostridial toxins, there is a second class of virulence determinants for *C. difficile*, the surface layer proteins (SLPs). Whereas the large clostridial toxins are the major virulence factors, some recent studies suggest that non-toxin *C. difficile* molecules, specifically the surface layer proteins (SLPs), have immunoregulatory roles [49, 36]. *C. difficile* non-toxin proteins include cell surface proteins, adhesins (cwp66), flagellar proteins (FLiC and FLiD), and fibronectin-binding proteins [50]. *In vitro* experiments show that SLPs bind to Vero cells, human epithelial cell lines, and gastrointestinal tissue obtained from biopsy samples [51], and additional *in vitro* studies using mouse bone marrow-derived dendritic cells and human monocyte-derived dendritic cells have shown that purified SLPs induce the production of both proinflammatory [TNF- α , interleukin (IL)-12, IL-23, and IL-1 β] and anti-inflammatory (IL-10) cytokines [49, 36]. In mouse studies, this effect was mediated via TLR4 receptors in an NF κ B-dependent manner [36]. Analysis of serum samples from patients, asymptomatic carriers, and healthy controls has shown specific antibody responses to these SLPs and flagellar proteins [52, 53]; in particular, patient samples had a significantly higher anti-SLP IgG levels as compared to carriers or controls, showing that SLPs are targeted by the host immune responses [53]. The exact role of these antibodies in controlling pathogenesis, however, is still unclear. Flagellar proteins bind to the mucus layer of mouse intestine *in vitro* and play a role in adherence to cecal epithelium *in vivo* [54]. All of these studies suggest that non-toxin *C. difficile* molecules may modify the disease process. However, it is important to note that most of the above referenced studies have been performed *in vitro* using cell lines or biopsy specimens. A more detailed analysis in a mouse model of *C. difficile*-associated diseases (CDAD) would help to define the relative contribution and exact roles of the non-toxin molecules in the pathogenesis of CDAD.

Host immune response during *Clostridium difficile* infection

The spectrum of disease following *C. difficile* infection ranges from asymptomatic colonization to mild diarrhea to fulminant colitis and death. Such variability in disease outcome is believed to be at least partly dependent on host factors including age, co-morbidities, and the immune responses generated by exposure to the bacterium. Following *C. difficile* infection, both the adaptive and innate arms of the immune system are activated.

Because toxins are a major *C. difficile* virulence factor, and administration of toxin A was shown to replicate all the histopathologic features of *C. difficile* infection in animal models, rabbit and hamster ileal loop models of *C. difficile* toxin A challenge have been employed to study the host response. The toxins disrupt the actin cytoskeleton and activate the inflammasome and NF κ B-mediated pathways that lead to proinflammatory cytokine and chemokine production. The actions of *C. difficile* toxins can be broadly divided into cytopathic effects characterized by disruption of the cellular barrier and enterotoxic effects with the initiation and propagation of an inflammatory response.

Innate immune responses

The innate defense mechanisms against *C. difficile* infection include the endogenous microbial flora (Box 1), the mucus barrier, intestinal epithelial cells, and the mucosal immune system. The *C. difficile* toxins have multiple effects on all of these innate immune defenses of the body, including stimulating the release of multiple proinflammatory mediators (cytokines, chemokines, neuro-immune peptides) and the recruitment and activation of a variety of innate immune cells (Table 1).

Box 1

Role of the microbiome in *C. difficile* infection

The diverse and complex endogenous gut microbial flora likely plays a key role in preventing *C. difficile* infection. Colonization resistance is a defense mechanism by which the normal, endogenous gastrointestinal flora prevents the establishment of infection, and colonization resistance plays a major role in defense against *C. difficile*. Disruption of the endogenous gut micro flora is one of the prerequisites for establishing infection. A large percentage of young infants (who do not have an established microbiome) are colonized with *C. difficile* [86], and as the microbial flora of infants matures colonization with *C. difficile* is reduced and is almost gone by 2 years of age. It is important to note that despite high levels of colonization, clinical infection in infants is rare, suggesting that host responses are important determinants of disease pathogenesis [86]. Recent studies have also shown that the presence or absence of *C. difficile* colonization during early infancy is associated with specific alterations in the gut microbiome [87]. In adults, changes in the composition and diversity of the microbial flora by the use of antibiotics makes the host more susceptible to infection [11, 12], and experiments in animal models show that a single dose of antibiotics changes the composition and diversity of microbial flora and makes the host more susceptible to *C. difficile* infection for at least 10 days [88]. Specifically, changing the microbial environment from Firmicute-dominant to Proteobacteria-dominant using different combinations of antibiotics was associated with clinical disease manifestation in mice [89].

The complex interplay of the metabolic pathways influenced by changing the gut microbiome can play a role in *C. difficile* disease as well. One such area of study is the role of gut flora in influencing bile salt metabolism. Bile salts play an important role in regulating the transformation of *C. difficile* spores to vegetative forms. Specifically, studies have shown that some primary bile salts (cholate, taurocholate, glycocholate) can stimulate the germination of spores *in vitro* [90], whereas others (chenodeoxycholate) can inhibit germination [90]. Interestingly, it has been postulated that one of the mechanisms by which the microbiome could influence disease pathogenesis is by changing the composition of bile salts in the colon, which can then effect the germination of *C. difficile* spores [91].

The importance of the microbiome during *C. difficile* infection and disease is further highlighted by reports of successful intestinal microbiota transplantation as a therapy for recurrent infections where standard therapy has failed [92]. Although this therapeutic option has not been extensively studied and is not a standard of care, multiple case reports have shown that this can be used as a treatment for severe, recurrent *C. difficile* infection when standard treatment has failed. A recent review of 27 clinical reports and case series shows that intestinal microbiota transplantation has high success rates (92%) in resolving disease [92]. However, there is a lack of any randomized controlled trial and the case series and reports vary in terms of patient characteristics, the amount and route of fecal instillation, as well as differences in follow-up to define the cure rates, just to name a few distinction. And although unproven at this time, there is a potential risk of transferring pathogens from donor to recipient. Overall, even though the concept is interesting and shows early promise, more studies are needed before intestinal microbiota transplantation can be used regularly as a therapeutic option for patients with *C. difficile* infection.

The challenge of ileal loops with *C. difficile* toxin A leads to an intense inflammatory response characterized by fluid accumulation, edema, increased mucosal permeability, mast cell degranulation, epithelial cell death, and neutrophil recruitment. In colonic epithelial cells, toxins have been shown to induce fluid secretion, upregulate production of reactive oxygen intermediates and IL-8 from epithelial cells [55, 56], induce cytokine and chemokine production [56, 57], and downregulate mucin exocytosis from mucin-producing colon cells [58]. *In vitro* challenge of epithelial cell lines and primary human colonic epithelial cells with toxin A led to cellular rounding, detachment, and apoptosis [55]. Similarly, exposure of lamina propria cells to toxin A *in vitro* induced apoptosis in macrophages, eosinophils, and T cells [59]. Notably, another study has shown that neutrophils are resistant to *C. difficile* toxin A-mediated apoptosis [60].

The toxins induce the production of multiple proinflammatory cytokines and chemokines including IL-12, IL-18, IFN- γ , IL-1 β , TNF- α , MIP-1 α , MIP-2, IL-8, and leptin [61, 62, 63], which propagate inflammation and may be responsible for host damage and many of the histopathologic features of CDAD. IFN- γ -deficient mice had less severe enteritis as compared with wild type mice following toxin A challenge, which manifested as decreased fluid secretion, decreased cellular edema and damage, and decreased myeloperoxidase production (indicating decreased neutrophil activity) [62]. Of note, IFN- γ deficiency was associated with attenuated TNF- α and chemokine secretion [62]. The chemokines RANTES and MIP1 α propagate the inflammatory response to toxin A [61]. Blocking RANTES and MIP1 α signaling by use of a RANTES inhibitor in MIP1 α -deficient mice or CCR1-deficient mice (CCR1 is the common signaling receptor for RANTES and MIP1 α) led to increased inflammatory responses following toxin A challenge [64]. Similarly, the promoter of the human IL-8 gene (functionally equivalent to the mouse neutrophil-attracting chemokine KC/CXCL1) has a particular polymorphism that is associated with increased IL-8 production as well as increased risk of recurrent *C. difficile* infection [65]. By contrast, mice deficient in the fractalkine receptor (*CX3CR1*^{-/-}) had increased inflammation following toxin A challenge [66]. The protective role of this pathway in toxin A-mediated inflammation is likely via induction of heme oxygenase-1 expression in CX3CR1-expressing macrophages [66].

C. difficile toxins activate both surface and intracellular innate immune sensors, including the inflammasome and the TLR4, TLR5, and NOD1 signaling pathways [36, 37, 38, 39]. TLR4- and MyD88-dependent signaling pathways lead to enhanced inflammatory response [36], and blocking of these pathways leads to increased bacterial burden and worsening of

the disease [36]. In the case of TLR5 signaling, although deficiency was not associated with any change in survival, exogenous stimulation of TLR5 signaling with flagellin was protective against *C. difficile* infection [37]. The TLR5-mediated protection is likely secondary to protective effects on the intestinal epithelial layer [37].

The intracellular innate immune sensors NOD1 and the IL-1 β /inflammasome are also activated after *C. difficile* infection [38, 39]. *C. difficile*-induced NOD1 activation led to increased chemokine production and *NOD1*^{-/-} mice have lower chemokine production, less neutrophil recruitment, and more severe disease [38]. Similar to *TLR4*^{-/-} mice, *NOD1*^{-/-} mice have a higher *C. difficile* burden [38]. *C. difficile* toxins stimulate IL-1 β release by activating inflammasomes in both mouse macrophages and human colon biopsy specimens [39]; however, unlike TLR4 and NOD1 signaling, blocking both the inflammasome signaling by using IL-1 receptor antagonist or *ASC*^{-/-} mice is associated with less toxin-mediated inflammation and damage [39]. It is important to note that whereas the studies looking at the role of TLR4, TLR5, and NOD1 signaling pathways used an infection model of *C. difficile*, the IL-1 β /inflammasome pathway was studied in the context of purified toxin injection in an ileal loop model.

Activation of the innate immune sensors and the release of cytokine and chemokine mediators is followed by an intense local neutrophilic infiltration [31]. This neutrophilic infiltration is one of the major pathologic findings after *C. difficile* infection, and it is believed that neutrophils play a major role in disease pathogenesis. Both local recruitment and systemic proliferation of neutrophils is seen in CDAD [22, 31]. Antibody-mediated blocking of neutrophil migration in rabbits significantly reduced disease severity following challenge with *C. difficile* toxin A [31]. Similarly in rats, induction of neutropenia (loss of neutrophils) was associated with less severe disease [34]. These studies using toxin A-mediated enteritis would suggest that neutrophils enhance the inflammatory response and lead to host damage.

Intestinal mast cells also play an important role in the toxin-mediated inflammatory responses. Both toxins A and B lead to activation, degranulation, and the release of inflammatory mediators from mast cells [67]. *In vitro* studies showed that inhibiting mast cell degranulation and blocking mast cell-derived histamine was associated with a decrease in inflammatory responses to toxin A [68]. Mast cell-deficient mice have less severe inflammation and neutrophilic infiltration as compared with wild-type mice in response to *C. difficile* toxin A [69]. These studies suggest that like neutrophils, mast cells propagate the inflammatory response in CDAD. At least part of the toxin A-mediated neutrophil recruitment in rat ileal loops is dependent on mast cell activation [69].

The role of other immune cells, including macrophages, monocytes, and dendritic cells, has generally been extrapolated from *in vitro* and *ex vivo* studies using human and mouse cell lines, human monocytes, and monocyte-derived dendritic cells. These studies have shown that *C. difficile* toxins can stimulate the release of proinflammatory cytokines and chemokines from these cells in a MAPK- and p38-dependent manner [70, 71], and toxin A induces NF κ B-mediated IL-8 production from human monocytes [72]. Whereas all of these studies using *ex vivo* toxin challenge would suggest that inflammatory cell recruitment is associated with worse disease, at least one recent study has shown that lack of neutrophils could lead to poor control of bacterial burden and thus more *C. difficile*-mediated disease [38].

Another interesting facet of *C. difficile* toxin A-mediated inflammation is the induction of a strong neuroinflammatory response via secretion of various neuropeptides: neurotensin (NT), substance P (SP), calcitonin gene-related peptide (CGRP) [73], corticotropin-releasing

hormone (CRH) [74], and melanin-concentrating hormone [75]. Blocking enteric neural transmission by the local administration of lidocaine or systemic administration of hexamethonium abrogated the toxin A-mediated inflammatory response [76]. This was further shown to be dependent on the release of SP, CGRP, and NT in the intestine [73, 76]. Both an SP receptor antagonist (SR-48,692) and an NT antagonist (CP-96,345) reduced *C. difficile* toxin A-mediated intestinal inflammation, mast cell degranulation, and proinflammatory cytokine secretion [73, 76].

Adaptive immune responses

Antibodies to *C. difficile* toxins are naturally present in up to 60% of healthy adults and older children [53, 77]. In experimental models, passive immunization of mice and hamsters with antibodies to *C. difficile* can be protective [78, 79]. In the case of patients infected with *C. difficile*, systemic antibodies can be detected against both the toxins and various non-toxin antigens [53, 80, 81]. The presence of anti-toxin A antibodies is strongly associated with asymptomatic carriage of *C. difficile*, and levels of anti-toxin A IgM during an acute episode of *C. difficile* infection correlate with the risk of developing recurrent disease [80, 81] (Table 1). Patients that had recurrent infections had lower anti-toxin A antibody levels during the initial episode as compared to patients with a single episode of disease [81]. Recent studies have shown that this holds true for anti-toxin B antibodies as well—lower levels of anti-toxin B antibodies correlate with a higher risk of developing recurrent infections [82]. This suggests that an antibody-mediated immune response to *C. difficile* toxins has an important role in determining asymptomatic carriage and predisposition to recurrent infections. Furthermore, passive immunization studies in both humans [83] and hamsters [84] have shown that anti-toxin antibodies reduce recurrence (human studies and animal models) and enhance protection in primary disease (animal models).

Antibody responses to non-toxin components of *C. difficile*, such as the SLPs, are also observed in *C. difficile* disease. However, the role of antibodies to non-toxin components in protecting against *C. difficile* infection is unclear. Although serum IgG and IgM antibodies to SLPs have been observed in both *C. difficile* patients and asymptomatic carriers [52, 53], there was no difference in the antibody response between colonized patients with or without symptomatic disease. Cases of *C. difficile* diarrhea did have a higher anti-SLP IgG levels as compared to asymptomatic carriers and controls, showing an increased antibody response during infection; however, the exact role of this response in controlling the disease manifestations is still unclear. Recent data from animal models suggests that immunization of hamsters using Cwp84 protease as an antigen increased protection against *C. difficile* infection [85], but more studies are needed to clarify the role of SLPs and the immune response to these antigens during infection.

Concluding remarks

With the widespread use (and misuse) of antibiotics and emergence of the hypervirulent NAP1 strain, *C. difficile* has become a major nosocomial pathogen. The bacterium remains susceptible to metronidazole and vancomycin, but the incidence of recurrent infections is on the rise and novel therapeutic strategies are urgently needed. The hallmark of *C. difficile* infection is an intense inflammatory response characterized by recruitment of polymorphonuclear lymphocytes to the colon. However, it remains unclear whether the inflammatory response (Table 1, Figure 2) is beneficial or harmful to the host. It is also very interesting that the enteric nervous system and neuropeptides play an important role in directing inflammatory responses during this disease (Table 1). The studies discussed in this review suggest that whereas the adaptive immune response (in the form of antibodies to toxins and non-toxin antigens) may have a beneficial effect on the outcome of infection, the innate immune responses may enhance disease by initiating and propagating an

inflammatory cascade. Many lines of evidence suggest that dampening the host response can ameliorate the severity of *C. difficile* toxin-mediated disease. However, some recent data from animal models of *C. difficile* infection suggests that dampening the immune response could lead to a higher pathogen burden. This could partly be a feature of the model systems used: toxin A-mediated enteritis versus infection with *C. difficile*. One can postulate that in the absence of bacterium (toxin A-induced enteritis) blocking the robust inflammatory response helps control host damage, whereas during infection with *C. difficile* blocking the immune response limits immune-mediated host damage but this control exposes the host to a higher bacterial burden, leading to bacteria-mediated damage.

Given the current morbidity and mortality associated with *C. difficile* disease despite antibiotic therapy, one could argue that it is time to take a different approach. Targeting specific pathways in the inflammatory cascade that follows *C. difficile* infection could be an adjunct to antibiotic therapy. A clearer understanding of the nature and character of the host immune responses to *C. difficile* will be critical step in developing novel host-targeted therapies for this truly difficult to treat disease.

Glossary

Colonization resistance	defines the concept that indigenous bacterial flora (particularly gut anaerobes) limit the growth and concentration of potentially pathogenic bacterial species
Pathogenicity locus	a 19.6 kb region in the <i>C. difficile</i> genome that encodes for various toxins and virulence factors
Large clostridial toxins	are a family of related exotoxin molecules that act as major virulence factors. These include Toxins A and B of <i>C. difficile</i> , lethal and hemorrhagic toxin of <i>C. sordellii</i> , and the α -toxin of <i>C. novyi</i>
Toll-like Receptors (TLRs)	are extracellular pathogen recognition receptors that recognize harmful stimuli and play an important role in innate immune host defense
NOD signaling pathway	nucleotide-binding oligomerization domain (NOD) proteins are part of the innate immune system and bind to NOD-like receptors (NLRs), which are cytoplasmic receptors that triggers various inflammatory and apoptotic pathways
Inflammasome	a multi-protein complex whose formation is triggered by specific stimuli, promoting the maturation of proinflammatory mediators interleukin (IL)-1 β and IL-18
Leukocytosis	above average white blood cell count in the blood, often indicative of an inflammatory response
Lamina propria	thin layer of connective tissue below the surface cellular layer (epithelium) of mucosal surfaces of the body
Pseudo-membranous colitis	infection of the large intestine that presents with yellow exudates (pseudo-membranes) in the colon, usually associated with severe <i>C. difficile</i> infection
Fulminant colitis	severe, rapidly progressive infection of the large intestine (colon) that often leads to systemic shock and illness

Myeloperoxidase	enzyme that is expressed at high levels in neutrophilic granules and participates in host defense mechanisms against pathogens
Neutrophil	most abundant type of white blood cells in the body which play an important role in innate responses
Mast cell	granular cells of the immune system that reside in various tissues and mediate early inflammatory responses

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Box 2**Outstanding Questions**

- a. Is the *C. difficile* toxin-induced inflammatory response beneficial or harmful to the host?
- b. Are there differences between the host response to *C. difficile* toxin A-mediated disease and infection with bacterium?
- c. What is the role of innate immune responses during *C. difficile* colitis?
- d. Can we target the host inflammatory response as an adjunct therapy to ameliorate *C. difficile*-associated disease?
- e. Can the gut microbial communities be manipulated as a novel strategy for curing *C. difficile* infection?

Figure 1. Compound tomography (CT) scan of patient with *C. difficile* colitis showing diffuse thickening along the entire colon

CT findings in patients with *C. difficile* colitis include colonic thickening, peri-colonic stranding edema, and 'accordion sign' (oral contrast material trapped between edematous haustral folds).

Figure 2.
Pathogenesis of *C. difficile*-associated disease.

Table 1List of innate and adaptive immune responses to *C. difficile*

Innate Immunity	Role in <i>C. difficile</i> infection	Effect of inhibition	Refs
Immune Cells	Neutrophils increase the immune response to <i>C. difficile</i> toxin A	Blocking neutrophil recruitment or induction of neutropenia decrease Toxin A mediated disease severity	31, 34
	Mast cells increase activation by <i>C. difficile</i> toxins	Mast cell-deficient mice show decreased inflammation in response to Toxin A	68, 69
Cytokines	IFN γ expression is induced by Toxin A	IFN $\gamma^{-/-}$ mice are protected from toxin A-mediated enteritis	62
Chemokines and chemokine receptors	A high level of IL-8 production is associated with increased risk of recurrent disease		65
	CX3CR1 induces heme oxygenase-1 expression after Toxin A challenge	CX3CR1 $^{-/-}$ mice have increased fluid accumulation and neutrophil recruitment, as well as more pathological cellular structures, as shown by histology	66
	Both CCR1 and MIP1 α induce Toxin A mediated inflammation	CCR1 $^{-/-}$ or MIP1 $\alpha^{-/-}$ mice are protected from toxin A-mediated enteritis	64
	RANTES induces Toxin A mediated inflammation	RANTES inhibition (met-RANTES) protects from toxin A-mediated enteritis	64
	MIP2 induces neutrophilic infiltration after Toxin A exposure in rat ileal loops	Anti-MIP2 antibody decreased neutrophil recruitment after toxin A challenge	63
Innate immune receptors	TLR4 induces cytokine production in response to SLPs	TLR4 $^{-/-}$ mice have worse disease and higher <i>C. difficile</i> burden	36
	TLR5 stimulation decreases <i>C. difficile</i> -induced injury	TLR5 agonist (flagellin) protects from <i>C. difficile</i> colitis and death	37
	NOD1 recognizes <i>C. difficile</i> and induces neutrophil recruitment	NOD1 $^{-/-}$ mice have worse disease and higher bacterial burden	38
Neuroinflammatory mediators	Neurotensin (NT) and Substance P induce Toxin A-mediated mast cell activation	NT receptor antagonist inhibits Toxin A-induced enteritis	73
		SP antagonist reduces Toxin A-induced fluid secretion and neutrophil recruitment	69, 73
Other mediators	Toxins A and B induce inflammasome activation and signaling in THP-1 cells and mouse macrophages	ASC $^{-/-}$ and IL-1R antagonism protect from toxin A- and B-mediated intestinal injury, inflammation, and neutrophil recruitment	39
	Heme oxygenase 1 protects from Toxin A-mediated enteritis	HO-1 inhibition (tin-protoporphyrin-IX) enhances Toxin A-mediated enteritis	66
	Leptin enhances Toxin A-induced enteritis	db/db (<i>LepR</i> $^{-/-}$) mice are protected from Toxin A-mediated enteritis	61
Adaptive immunity	Mediators	Role in immune response	
Antibodies to toxins	Anti-Toxin A and anti-Toxin B antibodies	Associated with protection, carrier state, decreased recurrence	81, 82
Antibodies to non-toxin components	Anti-SLP antibodies	No difference between colonized patients with or without disease	52, 53