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The roles of host and pathogen factors and the innate immune response in the pathogenesis of *Clostridium difficile* infection

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Abstract

Clostridium difficile (*C. difficile*) is the most common cause of nosocomial antibiotic-associated diarrhea and the etiologic agent of pseudomembranous colitis. The clinical manifestation of *Clostridium difficile* infection (CDI) is highly variable, from asymptomatic carriage, to mild self-limiting diarrhea, to the more severe pseudomembranous colitis. Furthermore, in extreme cases, colonic inflammation and tissue damage can lead to toxic megacolon, a condition requiring surgical intervention.

C. difficile expresses two key virulence factors; the exotoxins, toxin A (TcdA) and toxin B (TcdB), which are glucosyltransferases that target host-cell monomeric GTPases. In addition, some hypervirulent strains produce a third toxin, binary toxin or *C. difficile* transferase (CDT), which may contribute to the pathogenesis of CDI. More recently, other factors such as surface layer proteins (SLPs) and flagellin have also been linked to the inflammatory responses observed in CDI.

Although the adaptive immune response can influence the severity of CDI, the innate immune responses to *C. difficile* and its toxins play crucial roles in CDI onset, progression, and overall prognosis. Despite this, the innate immune responses in CDI have drawn relatively little attention from clinical researchers. Targeting these responses may prove useful clinically as adjuvant therapies, especially in refractory and/or recurrent CDI. This review will focus on recent advances in our understanding of how *C. difficile* and its toxins modulate innate immune responses that contribute to CDI pathogenesis.

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Keywords

Clostridium difficile infection; virulence factors; pathogenesis; innate immune response

Introduction

Clostridium difficile (*C. difficile*) is a Gram-positive, spore-forming, toxin-producing, anaerobic rod bacterium. Originally isolated from the meconium of an asymptomatic newborn, *C. difficile* is now recognized as a mammalian enteric pathogen with broad gastrointestinal tissue tropism that is species specific [1]. In the human context, *C. difficile* infection (CDI) is considered the leading cause of hospital and community-acquired antibiotic-associated diarrhea in the western world [1, 2]. This is reflected in the rates of morbidity and mortality with 36,000 cases registered with the UK health protection agency in 2010 alone [3]. The annual incidence of CDI in the USA is more than 3,000,000 cases [4], costing US hospitals an estimated 1-3 billion USD annually [5]. In fact, the incidence of CDI in some community hospitals is now greater than methicillin-resistant *Staphylococcus aureus* infections. Alarming, CDI is increasingly seen in patients with no recent exposure to antibiotics and in young healthy adults [3]. Some have speculated that the increased rates of hospital and community-acquired CDI, and its increased severity, are associated with enhanced *C. difficile* virulence. Indeed, in the past few years, a new, hypervirulent strain of *C. difficile* (BI/NAP1/027) has emerged, which is characterized by increased production of TcdA and TcdB, the presence of binary toxin/CDT, and increased resistance to fluoroquinolones [1]. Antibiotic exposure is the most significant risk factor for CDI [2, 6]. In experimental models of CDI, perturbation of the normal intestinal microbiota is required for *C. difficile* colonization and overt infection [7, 8]. The clinical appearance of CDI is highly variable, from asymptomatic carriage, to mild self-limiting diarrhea, to more severe pseudomembranous colitis that can progress to toxic megacolon, a condition characterized by severe intestinal dilation and inflammatory ileus that often requires surgical intervention [1, 9, 10]. The most common symptom is diarrhea, but other common clinical symptoms include abdominal pain and cramping, increased temperature and leukocytosis [10]. Currently, standard care is the discontinuation of offending antibiotic and administration of metronidazole, vancomycin or the newly developed fidaxomicin [11-13]. Other treatment options currently in clinical development include toxin-absorbing polymer, new antibiotics (e.g. nitazoxanide, rifaximin, tigecycline and teicoplanin), and toxin-specific human monoclonal antibodies [14-17]. Furthermore, three vaccines, respectively from Sanofi, Valneva, and Pfizer, targeting *C. difficile* toxins are in different stages of clinical trials [18-21]. Several other protein or DNA vaccine candidates either targeting *C. difficile* toxins or other virulent factors such as surface-layer protein (SLP), pentasaccharide cell wall repeating unit, cysteine protease and flagellin have been under investigation in animal models [18, 22-28].

Although treatment with metronidazole, vancomycin or fidaxomicin is effective in most patients [11] [12], an estimated 15-35% of those infected with *C. difficile* relapse following treatment [29]. Although it has been reported that fidaxomicin can reduce the rate of recurrence, new therapeutic interventions are required to deal with recurrent and relapsing

CDI [12]. Probiotics and fecal microbiota transplantation (FMT) have been investigated for primary and secondary prophylaxis against CDI, with FMT exhibiting cure rates greater than 90% [30-33]. Despite the success of FMT in the treatment of refractory or recurrent CDI, safety and regulatory issues need to be consolidated across jurisdictions prior to its widespread acceptance as a mainline therapeutic intervention.

As the incidence of CDI continues to increase, interest has been renewed in the development of non-antibiotic and adjunct approaches that target the pathogenic host inflammatory response [34]. Several excellent reviews on immune responses to *C. difficile* infection have been available [35-38]. The important role of adaptive immunity in defending CDI has been appreciated for many years. Antibodies to TcdA or TcdB are found in up to 60% of healthy adults and older children. Serum anti-toxin antibodies play an important role in protection against CDI recurrence. *C. difficile* flagellar proteins FliC and FliD and surface layer proteins are also immunogenic. In the current review, we will discuss key aspects of CDI pathogenesis including the risk factors associated with disease; the virulence factors that contribute to CDI pathogenesis and a detailed summary of the mechanisms through which TcdA and TcdB and other *C. difficile* virulence factors trigger inflammation through activation of the innate immune system. An illustration on the overall CDI pathogenesis/Host-pathogen interactions is presented in Figure 1.

CDI pathogenesis – Host factors that combat CDI

The current dogma suggests that the risk for CDI increases with age, the duration of antibiotic use and hospital stay and is also elevated in individuals with compromised immune status [36, 39-41]. Over the past decade, additional risk factors have been described including treatment with proton-pump inhibitors, H₂ antagonists and methotrexate, and the presence of underlying gastrointestinal pathologies, such as the inflammatory bowel diseases [42]. More recently, rates of community-acquired CDI have increased and those affected are often healthy individuals. Chitnis *et al.* [43] reported that nearly a third of patients with community-acquired CDI were not receiving antibiotics and nearly 20% had no exposure to the health care setting. Interestingly, these individuals were more likely to be exposed to household member with CDI or an infant. Taken together, these data highlight the need for increased monitoring/reporting in order to better understand the factors that increase the risk for CDI.

Despite one recent study showed that nearly a third of community-acquired CDI occurs in the absence of antibiotic treatment[43], the community-acquired cases are still a significant minority of all CDI. The current paradigm suggests that alterations in the composition or diversity of the intestinal microbiome provide an ideal niche for the expansion of *C. difficile* in the gastrointestinal tract. In addition to directly competing for specific space and resources, the normal intestinal microbiota may suppress *C. difficile* invasion of the intestinal tract via the transformation of bile acids, which have profound effects on spore germination and vegetative growth of *C. difficile in vitro* [44]. Human synthesize two main primary bile acids, cholate and chenodeoxycholate (CDCA), which are conjugated to an amino acid (glycine or taurine) [45]. The microbiota plays two important roles in bile acid transformation. First, bile salt hydrolase enzymes secreted by the intestinal microbiota

deconjugate the bile acids from their amino acid in the intestinal lumen. Second, the microbiota can transform primary bile acids to secondary bile acids via the enzyme 7-dehydroxylase, converting cholate and CDCA into deoxycholate and lithocholate, respectively [45]. Cholate stimulates the germination of *C. difficile* spores, while CDCA has a strong inhibitory effect on spore germination [46]. It has been hypothesized that antibiotic treatment and the subsequent change in diversity may reduce the members of the microbiota that generate cholate or alter the ratio between cholate and CDCA. In support of this hypothesis, antibiotic treatment can alter the ratio of cholate to CDCA in humans and animals [47] and small intestinal/cecal contents from antibiotic-treated mice were able to support *C. difficile* spore germination at higher levels than control mice [47].

In addition to the intestinal microbiota, additional host factors also contribute to protection from CDI. The mucus layer covering the intestinal epithelium creates a dynamic defense barrier against luminal bacteria including *C. difficile*. In addition, antimicrobial peptides, defensins, and cathelicidins secreted by specialized cells may provide another layer of defense against *C. difficile* [48]. In fact, cathelicidin and defensins have been demonstrated to significantly reduce tissue damage and inflammation caused by *C. difficile* toxins [49, 50]. However, *C. difficile* has developed a well-characterized antimicrobial peptide resistance mechanism, d-alanylation of teichoic acid, in response to antimicrobial peptide exposure [51].

Should perturbation of the microbiota allow for sufficient colonization by germinated *C. difficile*, it can pass through the mucus layer and adhere to the intestinal epithelial cells using the cysteine protease Cwp84 [52] [53] and adhesions such as the S-layer p36 and p47 proteins [54-56], a 66 kDa cell-wall protein Cwp66 [57], the GroEL heat-shock protein [58], a 68kDa fibronectin-binding protein [59], and the flagella components FliC and FliD [60].

Recently a novel host defense mechanism was reported by Savidge et al. Authors demonstrated that *C. difficile* toxins are S-nitrosylated by the infected host and that S-nitrosylation attenuates virulence by inhibiting toxin self-cleavage and cell entry. Notably, inositol hexakisphosphate (InsP₆)- and inositol pyrophosphate (InsP₇)- induced conformational changes in the toxin enabled host S-nitrosothiols to transnitrosylate the toxin catalytic cysteine, which forms part of a structurally conserved nitrosylation motif. Moreover, treatment with exogenous InsP(6) enhanced the therapeutic actions of oral S-nitrosothiols in mouse models of *C. difficile* infection [61].

CDI pathogenesis – The role of *C. difficile* virulence factors

Although a healthy and diverse intestinal microbiota and functional intestinal mucosal barrier afford protection against CDI, perturbation of intestinal homeostasis can allow for *C. difficile* colonization. Following this, the vegetative cells secrete TcdA and TcdB, *C. difficile*'s two major virulence factors [62-64], and trigger the pathogenic host responses that are characteristic of CDI, including epithelial barrier disruption, inflammatory mediator release, immune cell infiltration and altered mucosal secretory responses [65-68]. TcdA and TcdB, two large clostridial toxins (LCTs; 308 and 270 kDa, respectively), inactivate host monomeric G-proteins of the Rho and ras families through glucosylation [69]. Both toxins

share similar multi-domain structures that include an N-terminal catalytic glucosyltransferase domain (GTD), an autolytic cysteine proteinase domain (CPD), a central translocation domain (TM), and a C-terminal receptor-binding domain (RBD) [70, 71] (Figure 2). The mechanism of cellular intoxication can be summarized in the following four steps: 1) receptor binding and toxin internalization, 2) pore-formation and translocation of the GTD across the vesicular membrane, 3) autocleavage of the toxins and the release of the GTD into the cytosol, and 4) glucosylation of host Rho and ras GTPases.

The C-termini of TcdA and TcdB consist of highly repetitive structures termed combined repetitive oligopeptides (CROPs) that are composed of multiple 19-24 amino acid short repeats (SRs) and 31 amino acid long repeats (LRs) [69][72]. The CROPs form cell wall binding motifs which contribute to toxin binding, however they are not the sole component involved in the binding process. It has been shown that removing the CROPs from TcdA or TcdB attenuates, but does not eliminate cytopathicity, suggesting the existence of an additional binding domain outside the CROPs [73-75] which may be encoded in the region preceding the C-terminal repeats.

TcdA and TcdB enter the cell via clathrin-mediated endocytosis [76], however, the receptor(s) that initiate this process have yet to be completely elucidated. Although a series of carbohydrate receptors, such as the alpha-Gal epitope, Lewis Y, Lewis X and Lewis I [9] have been reported to act as surface receptors for TcdA on the brush border of intestinal epithelial cells, to date, no surface receptor for TcdB has been identified. Once the toxins are internalized into the endosome, the GTD is delivered across the membrane following endosomal acidification. This process triggers dramatic rearrangements within the toxins allowing the central hydrophobic translocation domain to insert into the endosomal membrane. Pore formation and translocation are thought to be mediated by the central delivery domain (~aa 801-1831). Recent studies indicate that residues 1500-1850 of the delivery domain constitute a minor receptor binding domain [74].

Following the translocation of the GTD through the endosomal membrane into the target cell cytosol, the CPD undergoes autoproteolytic cleavage at a conserved leucine residue to release the GTD into the cytosol [77]. The induction of this cleavage event is mediated by inositol hexakisphosphate (Insp6) [78, 79]. Interestingly, the induction of autoproteolytic cleavage is not equivalent between the toxins, as the TcdB is more sensitive than TcdA to InSP6-induced cleavage in *in vitro* reactions [79].

Following its release into the cytosol of the host cell, the N-terminal GTD [80] utilizes UDP-glucose as a substrate and glucosylates small Rho and ras family GTPases, which act as master regulators of a number of vital cellular process including cell cycle progression, cell-cell adhesion, cytokinesis, secretion and maintenance of the cytoskeleton [64, 65, 69, 81]. Toxin-induced glucosylation of the monomeric G-proteins RhoA, Rac1 and Cdc42 disrupts the actin cytoskeleton, reducing cell-cell contacts, inducing cell death and ultimately disrupting intestinal epithelial barrier [64, 82]. In addition to these effects, TcdA and TcdB stimulate the release of inflammatory mediators, triggering immune cell infiltration, fluid accumulation and extensive tissue destruction[83-88].

There has been considerable debate as to whether TcdA or TcdB is responsible for the inflammatory response and tissue damage associated with CDI. Purified TcdA possesses potent enterotoxic and pro-inflammatory activities, as determined in ligated intestinal loops and intrarectal instillation studies in animals [89]. Interestingly, TcdB has been previously reported to exhibit no enterotoxic activity in animals [90, 91], but recent studies have described enterotoxic and proinflammatory activities in human intestinal xenografts in immunodeficient (SCID) mice [92]. Reports suggest that TcdB may synergize with TcdA, enhancing its ability to trigger intestinal inflammation and cause tissue damage [91, 93]. Since TcdB alone causes little effect in animal models of toxin exposure, yet TcdA and TcdB exhibit synergy in the same systems, it has been proposed that the receptor for TcdB exists on the basolateral surface of the intestinal epithelial cell [9], however this has yet to be elucidated.

The importance of TcdA in human CDI has been called into question since some strains of *C. difficile* do not produce TcdA can still cause pseudomembranous colitis [94]. Recent genetic analyses of isogenic toxin mutants suggest that TcdB is required for virulence in a hamster model of CDI [95]. However, this has not been a consistent finding. While TcdA⁺ TcdB⁻ mutants were less virulent in studies performed by Lyras *et al.* [96], the equivalent mutants in studies performed by Kuehne *et al.* [95] were as virulent as wild-type. Interestingly, TcdA⁻ TcdB⁺ strains have been reported clinically worldwide while no clinical TcdA⁺TcdB⁻ has been isolated [94]. In addition, several isoforms of TcdB have been reported [97].

In addition to TcdA and TcdB, a limited number of *C. difficile* isolates, including the epidemic NAP1/027 strain, produce a binary toxin/CDT that exhibits ADP-ribosyltransferase activity [98-100], but its role in the development of human disease is not well understood [101]. Interestingly, although CDT is found in approximately 10% of clinical isolates [1], recent epidemiological analyses showed that patients infected with strains producing CDT had 60% higher fatality rates compared to those infected with CDT-deficient strains [102]. CDT is composed of two separate subunits, CDTa (49 kDa, ADP-ribosyltransferase) and CDTb (99 kDa, receptor binding/translocation domains) [103]. CDTa-CDTb complex induces cell rounding and cell death in Vero cells, and the uptake of CDT into cells also requires endosomal acidification [38, 104]. Following proteolytic activation on the target cell surface, the binding domain oligomerizes and binds to unknown cell receptors. Binding results in receptor-mediated endocytosis. During acidification of the endosome, the binding domain undergoes conformational rearrangements, leading to pore formation and translocation of the ADP-ribosyltransferase domain into the target cell cytosol in an Hsp90-dependent manner [105]. The ADP-ribosyltransferase ribosylates monomeric G-actin, which blocks actin polymerization and ultimately leads to the disruption of the actin cytoskeleton. In addition to these effects, CDT may increase adherence of *C. difficile* to target cells, by the formation of microtubule protrusions [106].

Relapse constitutes one of the most significant issues facing clinicians in the management of CDI. The role of toxins in the pathogenesis of relapse has not been studied due to lack of animal models. Because TcdB is more potent and less immunogenic to mount an antibody

response, which is crucial for protection against relapse [107, 108], we propose that TcdB will likely play a more important role in CDI relapse.

In addition to toxin production and release, several other factors may contribute to disease pathogenesis, such as fimbriae and other molecules facilitating adhesion, capsule production, and hydrolytic enzyme secretion [109-111]. Recent studies have shown that the SLP of *C. difficile* may contribute to bacterial colonization [55, 112]. The SLP is a crystalline structure made up of two peptide subunits that encoded by a single gene, *slpA* [54]. It comprises a large proportion of the total cell protein, and is the major surface antigen to which the immune system is exposed on the intact cell. The low-molecular-weight subunit varies substantially in amino acid sequence, and is the more immunogenic peptide in infected individuals. The larger and more conserved high-molecular-weight peptide is involved in ligand binding and may therefore be important in colonization of the gut [55]. SLPs have been identified in all *C. difficile* isolates, and their recognition by Toll-like receptors (TLRs) results in the engagement of adaptor molecules and subsequent signaling to activate inflammatory responses which may contribute to CDI pathogenesis [113]. However, no significant differences were found in the activation induced in monocytes and monocyte-derived dendritic cells by SLPs from hypervirulent and epidemic or non-hypervirulent and epidemic *C. difficile* strains [114].

C. difficile are highly motile through the function of their flagella. Flagellin, a structural component of flagella, can elicit inflammatory responses by activating TLR5 on intestinal epithelial cells, a response that plays a pivotal role in bacterial infections of the large intestine [115]. *C. difficile* flagellin triggers the production of CXCL8/IL-8 and CCL-20 through the TLR5-dependent activation of NF- κ B and p38 MAP kinase pathways [115]. However, whether this response contributes to CDI pathogenesis has yet to be fully characterized. Interestingly, administration of purified *Salmonella*-derived flagellin, proved protective in a mouse model of CDI by delaying *C. difficile* growth and toxin production in the colon and cecum, a process that required TLR5 expression [116].

CDI pathogenesis - Induction of the innate immune response

Much of our early understanding of CDI pathogenesis and the role of the innate immune response was generated from animal models involving the injection of purified TcdA or TcdB into surgically generated ileal loops [117, 118]. This approach allowed investigators to assess the inflammatory response and tissue damage evoked by toxin exposure in a variety of species, including rabbits, rats and mice. More recently, a non-surgical toxin exposure model was developed which involves the intrarectal instillation of purified toxins into the mouse [119]. Data from this new model parallel the inflammatory profile and tissue damage initially described by investigators using the ileal loop surgery model. The intrarectal model is especially attractive because it removes the requirement for a skilled small animal surgeon and increases animal throughput, the latter being especially important for large-scale pre-clinical studies. In addition to toxin-exposure models, live-infection models have been developed to study CDI pathogenesis [120]. Early live-infection experiments utilized the Syrian hamster, a rodent that is extremely sensitive to antibiotic-associated enterocolitis caused by *C. difficile* [121] [122]. Since then, a number of small animals have been used in

live-infection experiments, including rats, rabbits, guinea pigs, prairie dogs and, more recently, mice [123]. Although toxin-exposure models allow for the targeted study of TcdA- and TcdB-dependent effects and their contributions to CDI pathogenesis, live-infection models allow for the study of additional aspects, including host-microbe interactions, *C. difficile* colonization dynamics, CDI recurrence/transmission, strain-dependent disease severity and testing of new antimicrobial therapeutics [123-125]. Furthermore, live-infection models have provided significant insight into the role that the innate immune response plays in tissue damage associated with CDI, but ultimately protects the host by enhancing *C. difficile* clearance and protecting against infection-associated systemic dissemination of enteric bacteria, concepts that will be discussed in the following sections.

The inflammatory response observed in CDI, primarily driven by the activation of the innate immune system, is initiated mainly by the actions of TcdA and TcdB on the intestinal epithelial cells, although other components of the bacterium, such as the SLP and flagellin, can contribute to this response through the activation of pattern recognition receptors [113, 115]. As discussed in the previous sections, the pathogenesis of CDI is thought to involve inflammation-associated tissue damage secondary to the intoxication of the intestinal epithelial cells. Following the breakdown of the intestinal epithelial barrier, immune cells within the mucosa (resident or recruited macrophages; resident mast cells) are exposed to TcdA and TcdB, triggering a “second-wave” of inflammation and tissue damage. The contributions of macrophages and mast cells to toxin-induced mucosal inflammation and tissue damage have been confirmed by a number of reports, suggesting this “second-wave” is important in the pathogenesis of CDI. The following sections will highlight the key inflammatory events that contribute to CDI pathogenesis, and outline the signaling pathways activated by TcdA and TcdB in the intestinal epithelial cell, mucosal macrophages and tissue mast cells that initiate and propagate the inflammatory response to *C. difficile*, through the release of inflammatory mediators, induction of immune cell recruitment and initiation of cell death pathways. The pathognomonic feature of CDI is the influx of neutrophils into the mucosa and, in severe cases, their movement into the lumen where they contribute to the formation of the pseudomembranes observed in pseudomembranous colitis [9]. In experimental models of CDI, the administration of TcdA triggers substantial neutrophilic infiltration and associated tissue damage including epithelial sloughing, edema and increased intestinal epithelial permeability [9]. Interestingly, in the clinical setting, the magnitude of the inflammatory response observed in CDI, but not the overall toxin burden within the intestine, is the best predictor of poor outcomes highlighting the important role that inflammatory events play in CDI pathogenesis [126]. However, it is important to note that impairment of the innate immune response in transgenic mice renders them more susceptible to CDI in live-infection models, through their inability to clear the initial infection and appropriately handle commensal bacteria that have translocated across the damaged intestinal epithelial barrier [127-130]. Taken together, these data would suggest that a robust inflammatory response in CDI is necessary to cope with microbial insult associated with the barrier dysfunction caused by *C. difficile* toxins, but must be appropriately controlled in order to limit collateral tissue damage.

The induction of the inflammatory response in CDI begins with the intoxication of the intestinal epithelial cell. In addition to modulating cytoskeletal function and disrupting cell shape and cell-cell contacts, TcdA and TcdB can activate a variety of intracellular signaling cascade responsible for the induction of gene transcription and the production and release of inflammatory mediators. Signaling pathways activated by TcdA and TcdB in intestinal epithelial cells are summarized in Figure 3. Many features of toxin-induced cytokine and chemokine release have been identified, however the proximal receptor(s) initiating these events has yet to be identified. TcdA and TcdB have been shown to trigger the production and release of CXCL8/IL-8, a potent neutrophil chemoattractant, from intestinal epithelial cells through a variety of mechanisms. Mahida *et al.* (1996) [131] were the first to describe the production of CXCL8/IL-8 in a variety of commonly studied intestinal epithelial cell lines, including HT-29 and T84, in response to TcdA treatment. Interestingly, although the authors associated the induction of apoptosis with the production and release of CXCL8/IL-8, TcdA-treated Caco-2 cells exhibited increased sensitivity to toxin-induced cell death but failed to release measurable quantities of CXCL8/IL-8. Branka *et al.* (1997) subsequently reported that TcdA triggered the release of CXCL8/IL-8 from HT-29-Cl.16E cells, a sub-clone of the HT-29 cell line that exhibits goblet cell-like properties. The concentration-effect relationship for TcdA-induced CXCL8/IL-8 release in these cells was bell-shaped, exhibiting maximal effect at 10^{-9} mol/L but nearly absent at 10^{-8} mol/L; the concentration at which the authors observed cell detachment and reduced cellular DNA suggesting that TcdA was cytotoxic at this concentration [132]. More recently, our group has reported that TcdB, but not TcdA, triggers the release of CXCL8/IL-8 from Caco-2 cells [133]. This was associated with TcdB-induced cell death, but no cytotoxicity triggered by TcdA [133]. Mechanistically, toxin-induced CXCL8/IL-8 production from intestinal epithelial cells precedes the detection of Rho glucosylation, suggesting it may not be driven by the enzymatic activities of TcdA and TcdB. He *et al.* (2002) reported that the activation of NF- κ B, one of the transcription factors involved in the induction of CXCL8/IL-8 production, occurred within the 30 minutes of exposure to TcdA, before Rho glucosylation could be detected [134]. Interestingly, the activation of NF- κ B appeared to be driven by alterations in mitochondrial function and the generation of reactive oxygen species following intoxication of the intestinal epithelial cell, since both TcdA-induced CXCL8/IL-8 reporter activity and NF κ B-DNA binding was blocked by antioxidant treatment [134]. Indeed, others have reported that TcdB can alter mitochondrial function by activating membrane channels that trigger hyperpolarization, mitochondrial swelling and cytochrome C release, the latter of which drives caspase-9 dependent apoptosis in this system [135]. Taken together, these data suggest that toxin-induced cell stress may be an initiator of CXCL8/IL-8 release from intestinal epithelial cells, but the signaling events proximal to NF- κ B were not addressed in these studies.

A variety of proximal signaling events can trigger the production of CXCL8/IL-8 in intestinal epithelial cells through the activation of AP-1- or NF- κ B-dependent gene transcription. Na *et al.* (2005) examined the role of MAP kinase signaling in the induction of TcdB-induced CXCL8/IL-8 release [136]. They reported that TcdB treatment triggered the activation of ERK1/2 through the transactivation of the EGFR, events that required the activity of an MMP and production of TGF- α . Lee *et al.* (2007) reported that TcdA triggered

the production and release of CXCL8/IL-8 in HT-29 cells through JNK- and p38 MAP kinase-dependent activation of AP-1 [137]. Our lab recently identified a mechanism through which proximal P2Y₆ receptor activity leads to NF-κB-dependent CXCL8/IL-8 production in Caco-2 cells [133]. As in the studies discussed in the previous section, TcdB exposure triggered cell stress and the release of UDP, a putative “danger signal” and ligand for the P2Y₆ receptor, which triggered the production and release of CXCL8/IL-8. Upon pharmacological blockade of the P2Y₆ receptor, toxin-induced CXCL8/IL-8 release was completely abolished [133].

In addition to CXCL8/IL-8, a variety of other inflammatory mediators can be released from intestinal epithelial cells following exposure to *C. difficile* toxins and contribute to the induction of the innate immune response. Kim *et al.* (2002) reported that TcdA triggered the rapid release of GROα and MCP-1 from HT-29 and Caco-2 cells [138]. This was followed by the delayed release of ENA-78 nearly 18 hours after TcdA-stimulation. Interestingly, the stimulation of polarized Caco-2 cells with TcdA triggered the release of GROα and MCP-1 in the basolateral directional. Furthermore, co-treating these cells with TNF-α and IFN-γ enhanced this response. Interleukin-6 (IL-6) can also be released from CCL-241 human intestinal epithelial cells treated with TcdA or TcdB [139]. Furthermore, TNF-α and IL-1β, two mediators released from mucosal macrophages upon exposure to TcdA and TcdB, trigger substantial IL-6 release from intestinal epithelial cells, providing an additional mechanism through which this mediator may contribute to the inflammation and tissue damage associated with CDI. In a similar fashion, TcdA can trigger production of MIP-1 from intestinal epithelial cells, both *in vitro* and *in vivo*, the latter of which probably involves the actions of TNF-α released during toxin exposure [140].

Following the breakdown of the intestinal epithelial barrier, TcdA and TcdB can enter the mucosa where they can stimulate a variety of cells including macrophages/monocytes [84, 88, 141-144], dendritic cells [145], mast cells [146-148] and neutrophils [149]. Macrophages are an abundant source of inflammatory cytokines and release large amounts of IL-1β, CXCL8/IL-8 and TNF-α upon exposure to *C. difficile* toxins. Rocha *et al.* (1998) reported the induction of IL-1β release from rat peritoneal macrophages treated with TcdA [143]. Mechanistically, Warny *et al.* (2000) reported that the early and sustained activation of p38 MAP kinase and the transient activation of ERK1/2 were involved in the production and release of CXCL8/IL-8 and IL-1β [88]. Ng *et al.* (2010) implicated the important role of inflammasome, an intracellular innate immune signaling complex, in the release of IL-1β from TcdA- and TcdB-treated THP-1 and mouse peritoneal macrophages [141]. Although, toxin-driven inflammasome-dependent production of IL-1β was inhibited by reagents that prevented endosomal acidification, this response occurred independent of the toxin's enzymatic function, requiring the intact toxin; as neither the binding domain nor the pre-cleaved catalytic domain could elicit caspase-1 activation and IL-1β release. In both studies, the mechanisms driving the release of inflammatory cytokines were also involved in the late stage induction of cell death. Furthermore, inhibition of p38 MAP kinase signaling or blockade of inflammasome function afforded protection against toxin-induced inflammation and tissue damage in mouse models of toxin exposure. A very recent breakthrough on how TcdB induces inflammasome activation was reported by Xu *et al.* They showed that Pyrin

mediates caspase 1 inflammasome activation in response to Rho-glucosylation activity of TcdB [150].

In addition to the intestinal epithelial cell and monocytes/macrophages, intestinal mast cells and substances released from the enteric nervous system may also play a key coordinated role in the induction of the innate immune response to *C. difficile* toxins. Mast cells can be directly activated by TcdA and TcdB, triggering degranulation and the release of inflammatory mediators, including histamine and leukotrienes [146]. TcdB can activate a variety of intracellular signaling pathways in mast cells including the p38 MAP kinase and ERK1/2 signaling cascades, the former contributing to prostaglandin production and release, an event that drives the autocrine signaling that enhances degranulation [146]. Toxin-exposure studies in animals have reinforced the role that mast cells play in the inflammatory response driven by TcdA. In the rat, mast cell degranulation occurs very rapidly, within 15-30 minutes, following TcdA exposure [151, 152]. Wershil *et al.* (1998) reported that mast cell-deficient mice were protected from TcdA-induced enteritis, an effect that was lost when these mice were reconstituted with mast cells [147]. Furthermore, the fluid accumulation and neutrophil influx was associated with increased levels of tissue substance P [147]. Interestingly, intestinal mast cells are often located in close proximity to substance P-containing sensory neurons [153]. Inhibition of substance P signaling can attenuate a portion of the mast cell-dependent inflammation induced by TcdA [147]. Furthermore, ablation of sensory neuron contents with capsaicin can significantly reduce TcdA-induced fluid accumulation and neutrophil influx [152], highlighting the complexity of the innate immune response to *C. difficile* toxins. Although nerve/mast cell-dependent mechanisms have been linked to toxin-induced inflammation and tissue damage, to date, little is known about how these responses contribute to the pathogenic or host-defense processes during a true CDI. Signaling pathways activated by TcdA and TcdB in mucosal immune cells and enteric nerves are illustrated in Figure 4.

CDI – Targeting the innate immune response

As outlined in the previous sections, toxin-induced immune cell activation contributes to the inflammatory response and tissue damage associated with CDI. In experimental toxin-exposure models, strategies that inhibit immune cells activation and/or inflammatory mediator release are protective. Furthermore, blockade of the cytokine signaling, such as in the case of IL-1 β [141], or the influx of neutrophils, through the use of neutralizing antibodies [154], attenuates toxin-induced tissue damage *in vivo*. These data would suggest that inhibiting the innate immune response or blocking the activity of its associated inflammatory mediators may prove an effective treatment for CDI. Indeed, in other inflammatory gastrointestinal pathologies, such as Crohn's disease and ulcerative colitis [155-157], strategies that target inflammation are effective in the clinical setting. In both CDI and other inflammatory gastrointestinal pathologies, inflammatory responses play key roles in contributing to the disease symptoms, though the former is a pathogen-induced relatively acute disease, while the latter is more like a immune-aberrant chronic inflammation. However, more recent data using the live-infection model in the mouse, in contrast to toxin-exposure models, call into question the rationale for developing therapies that target the inflammatory response to treat CDI. Hasegawa *et al.* (2011) reported that

Nod1-deficiency rendered mice more susceptible to CDI in the live-infection model, an effect that was associated with impaired CXCL1 production, reduced neutrophil recruitment, impaired clearance of *C. difficile* and enhanced translocation of commensal bacteria [127]. Subsequent to this report, two studies confirmed the importance of innate immune signaling in the clearance of *C. difficile* during an active infection. Ryan *et al.* (2011) reported that a functional TLR4 signaling cascade was required to detect and mount an efficient response to *C. difficile* through the recognition of its SLP [128]. TLR4- and MyD88-deficient mice were markedly more susceptible to CDI in the live-infection model, an effect that was associated with impaired production of IFN- γ and IL-17 [128]. Jarchum *et al.* (2012) reported that MyD88-deficiency impaired neutrophil recruitment to the colonic lamina propria, an effect that was attributed to reduced CXCL1 expression, and decreased survival during CDI [129], an observation supporting the previous finding by Hasegawa *et al.* (2011) [127]. Lastly, Hasegawa *et al.* (2012) found that the inflammasome played a protective role in the live-infection model through the initiation of an innate immune response that effectively cleared *C. difficile* and prevented the propagation of the intestinal mucosal inflammation by preventing the translocation of commensal bacteria [130]. Interestingly, these data appear to directly contrast the previous findings by Ng. *et al.* (2010), where the authors reported that inflammasome activation and subsequent IL-1 β production contributed to toxin-induced inflammation and tissue damage [141], presumably implicating this process in the pathogenesis of CDI.

It is now increasingly apparent that the induction of an efficient innate immune response, involving the release of inflammatory mediators and neutrophil recruitment, is required for swift clearance of *C. difficile* and protection against the translocation of the commensal bacteria. Furthermore, some have proposed that recurrent CDI may be caused by the failure of the innate immune system to clear the initial infection [158]. Treating mice with immunosuppressives renders them more susceptible to fulminant and recurrent CDI [125] and relapsing diarrheal episodes have been reported in patients with functional neutrophil defects [159]. Despite these mechanistic links, our understanding of recurrent CDI pathogenesis is limited and requires more attention in future studies.

Although required for pathogen clearance, prolonged inflammation, in the context of refractory CDI or relapsing disease, may prove pathogenic. It should be pointed out that the dual role (i.e., beneficial /harmful) of the inflammatory response during infection is not specific to *C. difficile* infection [160]. Thus targeting both the infectious agent and carefully modulating the innate immune response triggered by its virulence factors may provide improved clinical outcomes. This notion, akin to a paradigm shift, suggests it prudent that we reassess the conclusions of the seminal studies that have implicated the toxin-induced inflammatory response in the pathogenesis of CDI. Although we, and others, have published reports characterizing a variety of receptors and signaling cascades that contribute to the inflammatory response and resulting tissue damage triggered by TcdA and TcdB, we have yet to reassess these findings in the context of an active CDI, and determine whether modulating these responses will prove beneficial in experimental models. Given the contrasting conclusions of Ng *et al.* (2010) [141] and Hasegawa *et al.* (2012) [130], utilizing the toxin-exposure and infection models respectively, we feel it imperative that we continue

to assess the role of the innate immune response in the pathogenesis and/or protective host response in CDI.

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Highlights

This review focuses on recent advances in our understanding of how *C. difficile* and its toxins modulate innate immune responses that contribute to CDI pathogenesis

Figure 1. CDI pathogenesis/Host-pathogen interactions – outlining the innate signaling pathways activated by *C. difficile*

Host defense mechanisms are marked in red. Neutrophil infiltration on one hand leads to intestinal inflammation and tissue damage; on the other hands phagocytoses the infecting *C. difficile* and other pathogens. (SLPs: Surface-layer proteins; CDT: *Clostridium difficile* transferase; MAPK: mitogen-activated protein kinase; TLR4: toll-like receptor 4; TLR5: toll-like receptor 5; Nod1: nucleotide-binding oligomerization domain 1).

Figure 2. Structure of *C. difficile* toxins – a schematic depiction of the key domains within TcdA and TcdB

TcdA and TcdB consist of four domains: N-terminal glucosyltransferase domain (GT), the newly identified autocatalytic cysteine proteinase domain (CPD), the central translocation domain (TMD) covering a minimal pore-forming region (PFR), and the C-terminal receptor binding domain (RBD) consisting of two regions RBD1 covering clostridial repetitive oligopeptides (CROPS) and RBD2. RBD2 encodes an additional binding activity, since removal of the CROPS of TcdB only partially reduces cytopathic potency, truncation to 1-1500 or 1-1529 completely attenuates cytopathicity. The binding role of RBD2 is also indicated in TcdA, since TcdA lacking CROPS is able to intoxicate cells, albeit with 5-10 fold less potency than full-length toxin. The DXD motif and a conserved tryptophan present on the GT domain are involved in Mn^{2+} , UDP and glucose binding. The conserved DHC catalytic triad in the CPD domain mediates toxin autocleavage.

Figure 3. Signaling pathways activated by TcdA and TcdB in intestinal epithelial cells

The current paradigm suggests that toxin A (TcdA) binds to a surface on the apical surface of the intestinal epithelial triggering its endocytosis. Following liberation of the catalytic domain from the endosome, TcdA inhibits monomeric G-proteins (GTPase) and disrupts mitochondrial function to trigger reactive oxygen species (ROS) production. These events trigger disruption of the actin cytoskeleton, loss of cell-cell contacts and apoptosis, ultimately leading to disruption of intestinal epithelial barrier function. Following the breakdown of the barrier toxin B (TcdB) can bind to its basolateral receptor and enter the cell. TcdA and TcdB can activate a number of intracellular signaling pathways including p38 MAP kinase (p38), ERK1/2 and c-jun N-terminal kinase (JNK) which leads to the production and release of inflammatory mediators through NF- κ B- and AP-1-dependent gene transcription. The exact mechanisms that link TcdA and TcdB to the aforementioned signaling pathways have yet to be completely elucidated. Toxin-induced activation of the epidermal growth factor receptor (EGFR) and P2Y₆, through the release of uridine 5'-diphosphate (UDP) have been reported to initiate NF- κ B-dependent CXCL8/IL-8 production.

Figure 4. Signaling pathways activated by TcdA and TcdB in mucosal immune cells and enteric nerves

Following toxin-induced perturbation of the intestinal epithelial barrier, toxin A (TcdA) and toxin B (TcdB) can directly activate mucosal immune cells, such as macrophages and mast cells, leading to the “second wave” of inflammatory mediator release, and neutrophil influx. Toxin-induced substance P release from enteric sensory neurons, a response that can be augmented by mast cell interactions, recruits additional inflammatory cells into the mucosa, contributing to toxin-induced intestinal fluid accumulation, inflammation and tissue damage.