The immunopathogenesis of *Clostridium difficile* and its toxins

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A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Microbiology, Immunology and Cancer Biology

University of Virginia
December, 2015

Thesis Abstract:

Clostridium difficile is a Gram-positive, spore forming bacillus and the most common cause of antibiotic-associated diarrhea in the United States. Infection with *C. difficile* is associated with disruption of the microbiota, which commonly occurs after administration of antibiotics. Clinical outcomes of *C. difficile* infection (CDI) range from asymptomatic colonization to pseudomembranous colitis, sepsis and death. Recurrent disease is a major problem in treatment of CDI, as approximately 25% of patients who undergo treatment for CDI experience at least one additional infection. Pathogenesis of *C. difficile* is primarily mediated by the action of the Rho-glucosylating Toxins A and B, which cause dramatic host cell death and induce potent pro-inflammatory signaling. Certain strains of *C. difficile* also express a third toxin, known as *C. difficile* transferase or CDT. CDT expression is associated with more severe disease outcome and increased rates of recurrence. In addition to the toxins, multiple innate immune signaling pathways have been implicated in establishing an inflammatory response during infection. The pattern recognition receptors (PRRs), including Toll-like Receptors 2 and 4 (TLR2 and TLR4), are particularly essential in recognition of *C. difficile* and help shape the subsequent inflammatory response. Although multiple studies demonstrate protective immune responses during murine infection, recent clinical data suggests inflammatory markers correlate closely with disease severity. The combination of these findings emphasizes the importance of immune balance during infection and the protective capacity of an immune response which eradicates bacterial threats while preserving tissue integrity and promoting tissue repair. Based on this knowledge, we hypothesized that *C. difficile* toxins A, B, and

CDT play an important role in shifting the immune response towards a pathogenic state which enhances disease severity. We show that all three toxins act synergistically to promote activation of the immune response via a signaling complex known as the inflammasome, and demonstrate that toxin enzymatic activity is required for this process to occur. We also demonstrate that activation of this complex promotes production of the pathogenic cytokine Interleukin-23 in response to *C. difficile*, and clarify the role of Toll-like Receptors in this process. Finally, we demonstrate that intoxication dramatically influences the composition of immune cells during infection by specifically depleting eosinophils. This work suggests that intoxication by *C. difficile* toxins A, B and CDT has profound and synergistic inflammatory consequences which lead to pathogenic signaling and shape the course of disease. Thus, the host immune response is a truly double-edged sword which must be carefully wielded to achieve protection.

Acknowledgements

First and foremost I would like to thank Bill for being an incredible mentor and friend for the past six years. His support and encouragement have been so important to me since I started in his lab as an undergraduate and throughout my time in graduate school. I have always looked up to his positive outlook, his willingness to take risks, his work ethic and his dedication to helping others as a model for the type of scientist I hope to become. Bill, thank you for everything you do – your students care as much about you as you do for them!

I also want to thank Erica for all the ways she has made my time in the lab more fun, for always being there to bounce ideas off of or commiserate with, and for helping me become an immunologist. She is the better half of "Ericarrie" and I will never forget our partnership. Thank you to the other members of Team C. diff, especially Maddie for saving my experiments multiple times, her constant entertainment and impeccable style, and Mahmoud for all of his help with experiments and great sense of humor. Thanks as well to all the other members of the Petri, Mann and Ramakrishnan labs, past and present, who have taught me so much. Special thanks to Katy Ralston for giving me the chance to discover something I'm passionate about and for her patience and guidance in my first years as a scientist.

Thank you to my classmates, who constantly taught me new things, challenged me intellectually and made graduate school so much better than it could have been – Alexandra, Jeff, Awndre and many others. I also want to thank all of our collaborators in the *C. difficile* field, particularly Sarah Kuehne for teaching me so much and for being so

generous, as well as Andrew Dempster for teaching me all of his *C. difficile* culture techniques, Nigel Minton for sharing his expertise in *C. difficile* genetics, Carsten Schwan and Klaus Aktories for their input and for providing purified CDT, Fritz Koch-Nolte for generously providing nanobodies against CDT, Brianna Jackman and Andrew Feig for purifying wild type and mutant Toxin A, and David Lyerly, Bob Carmen and all the wonderful people at TechLab, Inc. for providing purified toxins, toxin ELISA kits and their years of *C. difficile* experience.

Thank you to my committee members, Alison Criss, Jim Casanova, Melissa Kendall and Ulrike Lorenz, for all of the time they have invested in helping me with my project, encouraging me, teaching me and improving my science.

Finally, I want to thank Brendan for his patience while I spent weekends in the lab, his advice and for the hours he spent listening to me talk about science. Special thanks my mother for giving me the science bug early on and my dad and brothers for their support and comedic relief.

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Part of this chapter has been adapted from "Host recognition of *Clostridium difficile* and the innate immune response"

Carrie A. Cowardin and William A. Petri, Jr. 2014. *Anaerobe* 30, 205–209.

1.1 *Clostridium difficile* physiology and epidemiology

Clostridium difficile is a Gram-positive spore forming bacillus and an obligate anaerobe. It is currently the most common cause of hospital acquired antibiotic-associated diarrhea in the United States [1]. Disease is primarily mediated by the action of the Rho-glucosylating Toxins A (TcdA) and B (TcdB), and clinical outcomes of CDI vary from asymptomatic colonization to pseudomembranous colitis, toxic megacolon, sepsis and death. Throughout the last ten years, incidence of *C. difficile* infection (CDI) has increased dramatically in developed countries, including the United States, Europe and Canada. The increase in disease frequency and severity has been at least partially linked to the emergence of a hypervirulent strain known as PCR ribotype 027 [2]. Currently, *C. difficile* is estimated to account for over 1 billion dollars in excess medical costs per year in the U.S. alone[1]. The most common cause of susceptibility to CDI is antibiotic treatment, including exposure to clindamycin, aminopenicillins, cephalosporins and fluoroquinolones. Almost all broad-spectrum antibiotics have been implicated in disruption of the intestinal microbiome, a condition coined as "dysbiosis" which is the underlying cause of increased susceptibility to CDI [3,4]. Certain patient populations are also more predisposed to infection, including those of advanced age as well as patients with inflammatory bowel disease.

Current treatments involve administration of vancomycin, fidaxomicin or metronidazole. However, recurrent infection is seen in 20-30% of patients, and 15% of individuals eventually succumb to disease [1,5-6]. Newer therapies have been developed with the goal of diminishing microbiome disruption or restoring healthy microbiota, most notably fecal microbiota transplant (FMT) [7]. Simultaneously, understanding of the factors

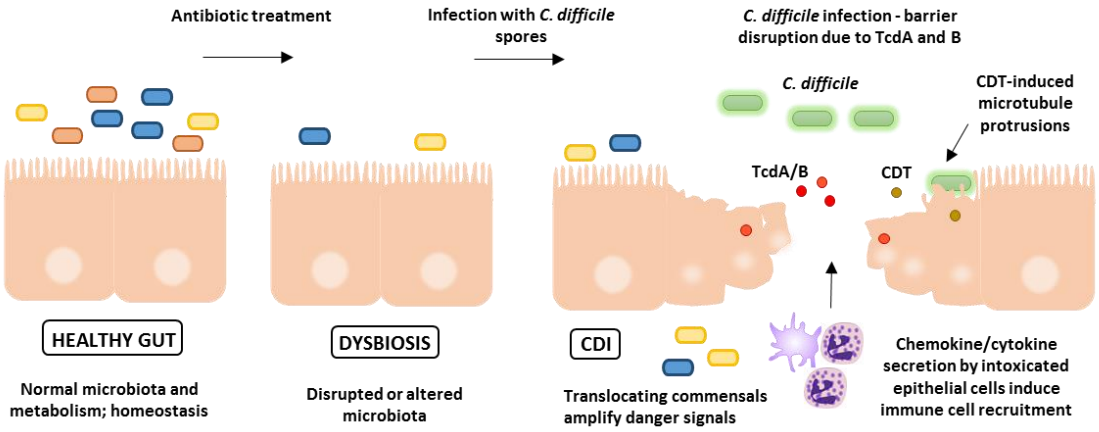
that influence disease severity has also evolved. Recent data suggest that the host immune response to *C. difficile* may play a larger role than previously thought, with *C. difficile* burden less important in determining the eventual outcome of disease. This includes evidence that a single nucleotide polymorphism in the gene encoding IL-8, a cytokine responsible for neutrophil recruitment in humans, results in increased IL-8 production during CDI and predisposes individuals to infection [8]. This suggests that the disease is partially mediated by host factors, and indeed, inflammatory markers correlate more closely to disease severity than pathogen burden [9]. Additionally, increased IL-8 protein levels and CXCL5 and IL-8 message levels have been associated with prolonged disease independent of bacterial burden [10]. Thus, the host immune response may in part be deleterious.

1.2 *C. difficile* pathogenesis

Infection with *C. difficile* spores can occur in the community as well as in the healthcare setting, although disease typically manifests in either setting following disruption of the intestinal microbiome with antibiotics [11]. Spores are transmitted by the fecal-oral route, and once ingested they are capable of surviving passage through the stomach and germinating in the colon and cecum [12]. Once germination occurs, vegetative cells penetrate the mucus layer and colonize by adhering to the epithelial cells of the colon.

Figure 1.1 *C. difficile* pathogenesis.

Healthy individuals possess normal microbiota in homeostasis with the host mucosal immune system. Antibiotics disrupt the microbiome and lead to dysbiosis, with a lack of microbiota diversity specifically linked to susceptibility. Ingested *C. difficile* spores germinate into vegetative cells which produce the major virulence factors, Toxins A and B. Ribotype 027 strains also produce a third toxin, *C. difficile* transferase (CDT), which enhances colonization by inducing microtubule protrusion formation on host cells. Toxins A and B further disrupt the epithelial barrier, triggering pro-inflammatory signaling from epithelial cells and increased immune cell recruitment. Translocation of commensal microorganisms contributes to inflammatory signaling.


Antibiotic treatment
Infection with C. difficile spores
C. difficile infection - barrier disruption due to TcdA and B
C. difficile
CDT-induced microtubule protrusions
TcdA/B
CDT
HEALTHY GUT
DYSBIOSIS
CDI
Normal microbiota and metabolism; homeostasis
Disrupted or altered microbiota
Translocating commensals amplify danger signals
Chemokine/cytokine secretion by intoxicated epithelial cells induce immune cell recruitment

Multiple non-toxin colonization factors have been suggested, although the role of these proteins is much less well understood. The surface layer proteins (SLPs), which form a two-dimensional array on the surface of the bacterium, are thought to mediate attachment to host cells [13]. The true contribution of the SLPs to virulence is not well understood as SLP expression is essential for bacterial survival, although the generation of a host antibody response to these proteins suggests they are immunologically relevant [13,14]. *C. difficile* also encodes multiple Cell Wall Proteins (CWPs) which are thought to contribute to cell adherence as well as degradation of extracellular matrix and bacterial aggregation [15,16]. In addition, *C. difficile* possesses peritrichous flagella which are required for bacterial motility. Interestingly, the flagella appear to inhibit attachment to host cells, as flagellar mutants display increased adherence to intestinal epithelial cell lines *in vitro*. These mutants also express higher levels of Toxins A and B, suggesting that motility and virulence may be opposing phenotypes [17,18].

Following successful colonization, *C. difficile* replicates and produces the enterotoxin Toxin A and the cytotoxin Toxin B. Toxins A and B are thought to be primarily responsible for the abundant tissue damage, epithelial barrier disruption and fluid accumulation seen during disease. Several hypervirulent ribotypes, including ribotype 027 strains, also produce a third toxin termed binary toxin, or *C. difficile* transferase (CDT). This ADP-ribosylating toxin is thought to increase colonization via induction of microtubule protrusions on host epithelial cells [19]. The combined action of Toxins A and B and CDT lead to a robust inflammatory response from host epithelial cells, inducing the production of pro-inflammatory cytokines and chemokines which recruit additional immune cells. A hallmark of *C. difficile* infection is robust neutrophil infiltration, and the

pseudomembranes seen in more severe disease are made up of these cells surrounded by mucin, fibrin and cellular debris [4].

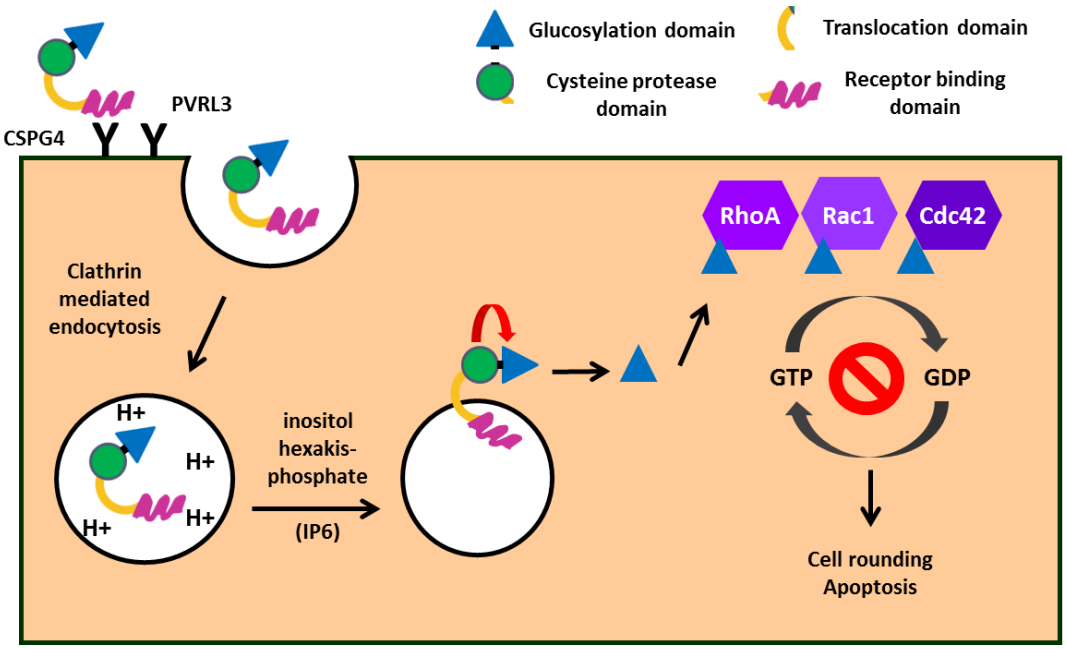
1.2 Intoxication by Toxins A and B

The relative contributions of Toxins A and B to disease pathogenesis are currently topics of intensive study, although it is widely accepted that one or both of these toxins are required for symptomatic infection to occur. Toxin A negative, Toxin B positive isolates have also been identified in the clinic, although strains lacking Toxin B generally are not associated with disease. This data, combined with recent breakthroughs in Clostridial genetics allowing for the generation of isogenic mutants, suggests that while both toxins contribute to pathogenesis, Toxin B may play a dominant role [20,]

Both toxins are chromosomally encoded within a region of the genome known as the "Pathogenicity Locus" or PaLoc. In addition to *tcdA* and *tcdB*, this region contains regulatory and structural genes. *tcdR* encodes an alternative sigma factor which promotes toxin expression, while *tcdC* encodes a negative regulator. Lastly, *tcdE* encodes a holin-like protein which is thought to be required for toxin export [21]. Both Toxin A and Toxin B are ABCD domain containing proteins, where the "A" domain possesses glucosylation activity, the "B" domain contains binding motifs, the "C" domain has cysteine protease (or cutting) activity, and the "D" domain is responsible for translocation [22]. Intoxication occurs following toxin binding to host cell receptors and internalization via receptor-mediated endocytosis (Figure 1.2).

Figure 1.2 Intoxication of host cells with Toxins A and B.

Intoxication with Toxins A and B is initiated by binding of the toxins to host cellular receptors, with evidence for the involvement of both chondroitin sulfate proteoglycan 4 (CSPG4) and poliovirus receptor-like 3 (PVRL3). Receptor-mediated endocytosis results in internalization of the toxin, and endosomal acidification triggers insertion of the translocation domain into the endosomal membrane. Cytoplasmic inositol hexakisphosphate leads to autoprocessing by the cysteine protease domain, resulting in release of the glucosyltransferase domain into the cytoplasm [23]. Once released, the glucosyltransferase domain glucosylates RhoA, Rac1 and Cdc42, preventing their function by blocking the exchange of GDP for GTP. Image adapted from Shen, J Innate Immune, 2012 [22].


Glucosylation domain
Translocation domain
Cysteine protease domain
Receptor binding domain
PVRL3
CSPG4
Clathrin mediated endocytosis
H+
H+
H+
H+
inositol hexakis-phosphate
(IP6)
RhoA
Rac1
Cdc42
GTP
GDP
Cell rounding
Apoptosis

Although much work has been dedicated to identifying the receptors involved in toxin A and B binding, no convincing evidence exists for a single receptor, and in fact multiple receptors have been suggested to play a role in toxin uptake, including poliovirus receptor-like 3 (PVRL3), which is involved in adherens junction formation, and chondroitin sulfate proteoglycan 4, which mediates interactions with extracellular matrix (CSPG4)[24,25]. Following toxin binding and uptake into endosomes, subsequent endosomal acidification triggers the insertion of the translocation domain into the endosomal membrane. This is thought to form a pore through which the glucosyltransferase domain is inserted into the cytoplasmic side of the endosomal membrane. Inositol hexakisphosphate within the cytoplasm triggers autoprocessing of the glucosyltransferase domain by the cysteine protease domain, releasing the glucosyltransferase domain into the cytoplasm. There, the glucosyltransferase domain modifies Rho GTPases via the covalent attachment of a glucosyl residue, preventing the exchange of GDP for GTP and thereby blocking GTPase function. Toxins A and B have been specifically shown to glucosylate the Rho-family GTPases RhoA, Rac1 and Cdc42 [26]. This leads to a loss of integrity in the actin cytoskeleton, resulting in cell rounding and cytotoxicity. There is considerable debate surrounding the type of cell death induced by Toxins A and B, as characteristics of both apoptosis and necrosis have been observed [27].

Multiple cellular signaling pathways are activated by Toxins A and B, many of which are involved in inflammatory gene expression. Toxins A and B are capable of inducing expression of numerous cytokines and chemokines, including IL-1, IL-6, IL-8, IL-12, IL-18, IFN-γ, TNF-α, macrophage inflammatory protein (MIP) 1α, CXCL2 as well as the adipocytokine leptin [28–31]. Although the exact pathways leading to pro-inflammatory

gene expression are unknown, it has been demonstrated that intoxication of cells by Toxins A and B leads to intracellular calcium release and activation of multiple mitogen-activated protein kinase (MAPK) pathways, including p38 MAPK, c-Jun N-terminal Kinase (JNK), and extracellular signal-regulated protein kinase (Erk)1/2. These pathways result in subsequent activation of the transcription factors NFκB and AP-1, known inducers of chemokine and cytokine production [32]. In particular, p38 MAPK phosphorylation has been shown to be essential for NFκB activation in response to Toxin A, and this is thought to occur as a result of mitochondrial oxygen radical generation [27,30]. Both toxins have also been shown to activate MAPK-activated protein kinase (MK2) downstream of p38 MAPK, and this pathway is essential for IL-8 expression [33]. Similarly, production of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PEG_2), thought to be responsible for the fluid accumulation seen in response to toxins, is also dependent on p38 MAPK activation and signaling via mitogen- and stress-activated protein kinase (MSK-1) [34]. Toxin A has also been shown to promote dendritic cell maturation and induce expression of the monocyte and macrophage chemoattractant CX_3CL1 via similar pathways, including p38 MAPK and NFκB [35]. Cell death induced by Toxins A and B also contribute to inflammation by permitting the release of the pro-inflammatory danger signal uridine diphosphate (UDP). UDP signals through the $P2Y_6$ receptor on host cells and enhances NFκB activation and IL-8 production, thereby contributing to the activation of the host immune response [36].

Both toxins are also capable of activating an the adaptor protein apoptosis-associated speck-like protein (ASC)-containing inflammasome, a cellular complex assembled in response PAMPs or danger signals which activates Caspase 1, responsible for processing the cytokines IL-1β and IL-18 into their secreted forms [37]. It is thought that

inflammasome activation by Toxin B requires toxin glucosylation activity, and the full length protein is also necessary. Endocytosis and endosomal acidification are likewise necessary for inflammasome activation by Toxin B [38]. One group identified Rho-glucosylation as the downstream signal required for inflammasome activation which was dependent on the protein Pyrin [39,40]. Pyrin forms a complex with ASC and Caspase 1 to promote IL-1β maturation and secretion, and is thought to recognize modifications to the switch-I region of RhoA. Interestingly, IL-1β and IL-1 receptor signaling pathway genes were also found to be significantly upregulated after toxin challenge in a microarray-based study [31].

1.4 Intoxication by CDT

CDT is a binary toxin comprised of two subunits. CDTb is the binding subunit of CDT, while CDTa is the enzymatic subunit. CDT differs from Toxins A and B both in activity and in mode of uptake. As an ADP-ribosyltransferase, CDT is capable of disrupting the actin cytoskeleton via the ribosylation of free actin subunits, which then act as capping proteins and prevent actin filament elongation [41,42]. This cytoskeletal disruption also leads to microtubule protrusion formation on the surface of intoxicated host cells. Vesicular trafficking is also disrupted following intoxication, leading to shuttling of fibronectin to the microtubules. This process is thought to mediate increased adherence of *C. difficile*, which possesses multiple fibronectin binding proteins, to the host epithelium [19,43].

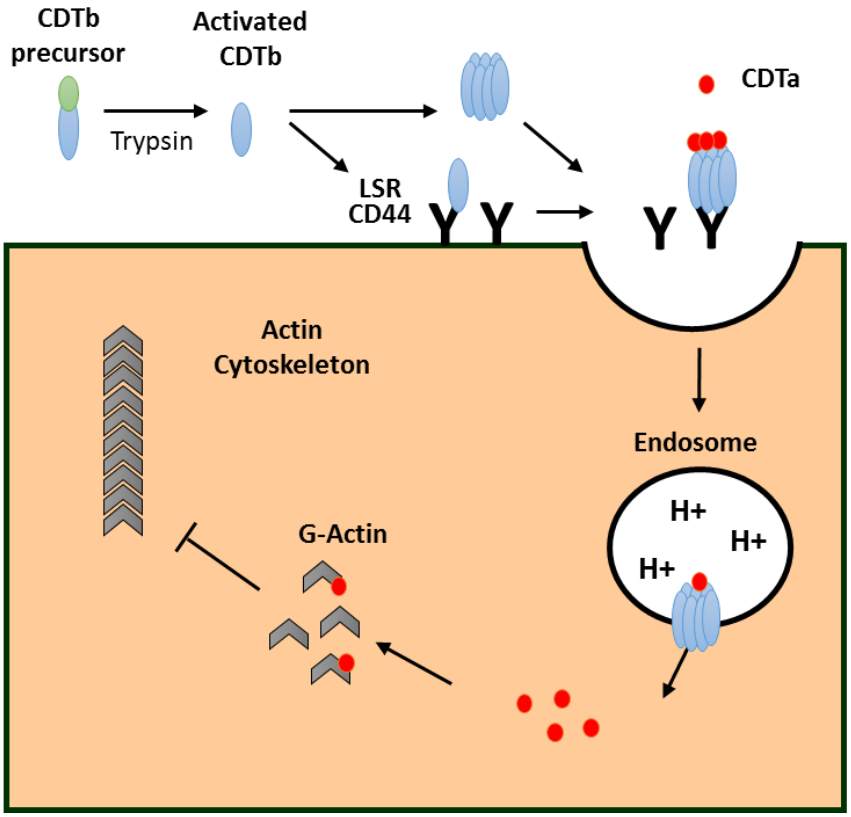
In order to intoxicate host cells, both CDT subunits are required. CDTb initially associates with a receptor on the surface of host cells, thought to be the Lipolysis-

stimulated Lipoprotein Receptor (LSR). CDTb can bind the host receptor as a monomer which subsequently heptamerizes, or as a pre-formed heptamer. CDTa then associates with the CDTb heptamer. The complex is endocytosed, and endosomal acidification triggers insertion of the CDTb-heptamer into the endosomal membrane, at which point the enzymatic domain CDTa is translocated into the cytosol. Once in the cytosol, CDTa carries out its ADP-ribosylation activity by transferring an ADP-ribose moiety from nicotinamide adenine dinucleotide to globular actin. This modification prevents actin polymerization and results in a disrupted host cell cytoskeleton [41,44] (Figure 1.3). Although the LSR which recognizes CDT has been identified as a type I single pass transmembrane protein, its possible functions remains relatively uncharacterized [45,46]. However, it does possess a long intracellular tail which may be involved in intracellular signaling, although this remains to be investigated. LSR is highly expressed both in the liver and large intestine, and has recently been demonstrated to be involved in formation of tricellular tight junctions [47]. A second receptor, CD44, has also been shown to be involved in CDT intoxication, as CD44 knockout mice are partially protected from challenge with the structurally and functionally similar *Clostridium perfringens* iota toxin [48].

Although CDT is not thought to be sufficient for disease pathogenesis, animal models have demonstrated that CDT plays an additive role during infection. A recent study in a hamster model of CDI utilizing isogenic toxin mutants showed that CDT could enhance pathogenesis when Toxin A was present, although no additive effect was observed for Toxin B. Human clinical data also suggests CDT-encoding strains cause more severe disease. CDT expression specifically was found to be associated with increased risk of death, increasing rates of recurrence, and higher peripheral white blood cell count [2,41,49].

Figure 1.3 Intoxication of host cells with CDT.

In order to permit intoxication, the binding subunit of CDT, CDTb, must be cleaved by the protease trypsin from its precursor into its active form. Once cleaved, CDTb associates with a cellular receptor known as the Lipolysis Stimulated Lipoprotein Receptor (LSR), possibly aided by the glycoprotein CD44. CDTb can associate with the receptor as a heptamer, or bind as a monomer which subsequently heptamerizes. CDTa, the enzymatic subunit of CDT, then binds to the CDTb heptamer. The complex is endocytosed, and endosomal acidification induces insertion of the CDTb heptamer into the endosomal membrane. CDTa is then shuttled out of the endosome via a pore within the CDTb heptamer. Inside the cytoplasm, CDTa transfers an ADP-ribose moiety from nicotinamide adenine dinucleotide to globular actin, preventing actin polymerization and disrupting the host cell cytoskeleton. Image adapted from Barth, Microbiology and Molecular Biology Reviews, 2004 [44].


CDTb
precursor
Activated
CDTb
Trypsin
CDTa
LSR
CD44
Actin
Cytoskeleton
Endosome
H+
H+
H+
G-Actin

CDT expression is found in several *C. difficile* ribotypes, although it is most classically associated with ribotype 027 strains, and is encoded within a region known as the "CdtLoc". This region also contains the gene *cdtR*, encoding a response regulator which is required for expression of CDT, although other regulators are thought to exist [50,51].

1.5 Host Recognition of *C. difficile*

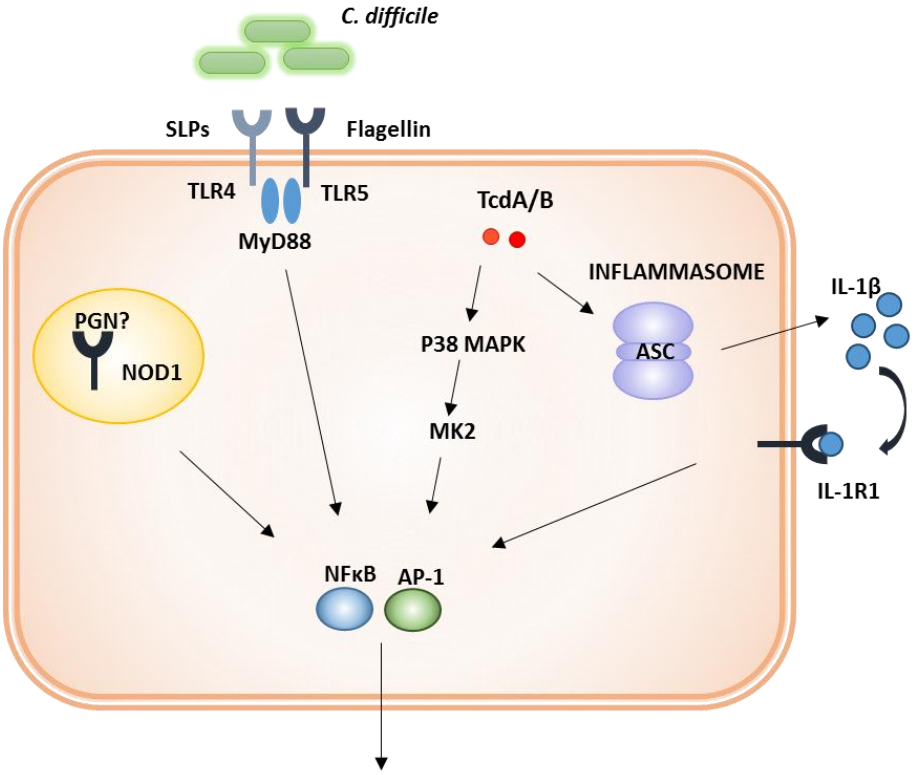
The innate immune system is the first responder to the presence of pathogenic microbes throughout the body, and plays a crucial role in shaping the adaptive response. The innate response is influential during CDI, as multiple innate signaling pathways have been shown to play a role in disease susceptibility. Pattern Recognition Receptors (PRRs) are present on host cells and recognize conserved bacterial signatures (Pathogen Associated Molecular Patterns, or PAMPs) to initiate the immune response [52]. A subset of PRRs, the Toll-like Receptors (TLRs) have been shown to recognize *C. difficile* PAMPs and contribute to the initiation of the host inflammatory response. Specifically, the TLR adaptor protein MyD88 has been shown to be involved in host defense. Mice lacking this molecule and thus, the majority of TLR signaling, show decreased survival during CDI [53,54]. In this context, MyD88-mediated signaling is essential for the production of the chemokine CXCL1, responsible for recruiting neutrophils to the colonic lamina propria. These cells play an important role in preventing the dissemination of commensal microbes to other organs [55]. Toll-like Receptor 4 (TLR4) has also been implicated in recognition of *C. difficile*. Purified surface layer protein (SLP) from *C. difficile* can activate NFκB downstream of TLR4 and induce TLR4 dependent dendritic cell (DC) maturation [54]. SLP-

treated DCs secrete IL-12, IL-23, TNFα, and IL-10, and are able to induce co-cultured T cells to secrete IL-17 and IFN-γ. Additionally, deletion of TLR4 *in vivo* causes an increase in disease severity in a murine model [54]. *C. difficile* flagellin has also been shown to stimulate TLR5, resulting in NFκB and p38 MAP kinase activation and IL-8 secretion. Although large quantities of flagellin are required compared to the more potent *Salmonella typhimurium* flagellin, IL-8 secretion can be prevented using neutralizing antibodies directed against TLR5. IL-8 secretion is also augmented by pre-treatment with Toxin B prior to addition of *C. difficile* flagellin [56] (Figure 1.3). Interestingly, administration of flagellin from *S. typhimurium* prior to infection with *C. difficile* attenuates disease by delaying both growth and toxin production by *C. difficile*. However, deletion of TLR5 in mice does not result in more severe infection, suggesting that this signaling pathway may be less essential for recognition of the pathogen [57].

C. difficile has been shown to signal through nucleotide-binding oligomerization domain 1 (Nod1), an intracellular Nod-like Receptor (NLR) thought to recognize diaminopimelic acid derived from peptidoglycan (PGN) [58]. Although the *C. difficile* PAMP which activates Nod1 signaling remains to be clearly identified, deletion of this receptor impairs production of the neutrophil chemoattractant CXCL1, decreases neutrophil recruitment and results in more severe disease [59]. *C. difficile* infected Nod1$^{-/-}$ mice also displayed elevated levels of lipopolysaccharide from translocating commensals as well as the pyrogenic cytokine IL-1β in their sera, possibly due to reduced clearance of commensals [58].

Figure 1.3 Multiple innate immune pathways contribute to inflammation during CDI.

C. difficile PAMPs, including Surface Layer Proteins (SLPs) and flagellin activate Toll-like Receptor 4 (TLR4) and TLR5 respectively. *C. difficile* can likewise stimulate Nucleotide-binding oligomerization domain-containing protein 1 (Nod1) via an unidentified, secreted PAMP. Toxins A and B also activate inflammatory signaling cascades, including p38 mitogen-activated protein kinase (MAPK). P38 MAPK signals though MAPK-activated protein kinase (MK2) to activate the transcription factors nuclear factor κB (NFκB) and activator protein 1 (AP-1) to induce pro-inflammatory cytokine and chemokine production. The toxins can also activate an ASC-containing inflammasome, leading to IL-1β secretion and NFκB activation via the IL-1 receptor.



IL-6, IL-8, IL-12, IL-18, IFN-γ, TNF-α
MIP1α, CXCL2, PGE_2, CX_3CL1

1.6 Protective and pathogenic features of the inflammatory response to *C. difficile*

The role of inflammation in response to *C. difficile* infection is controversial and multifaceted. Prevailing thought derived from toxin-based models of infection reflects the idea that inflammation is deleterious, as blocking inflammatory responses can prevent some of the tissue damage usually seen after intoxication. Preventing inflammasome activation via deletion of ASC, present in multiple inflammasomes, prevents tissue inflammation and damage following challenge of mice with purified toxins. Similarly, blocking IL-1β and IL-1α signaling with the IL-1 receptor antagonist Anakinra ameliorates toxin damage [38]. Preventing neutrophil recruitment via antibody depletion prior to toxin treatment reduces fluid accumulation, cell death, permeability and histological damage in a rabbit model of intoxication [60]. Neutralization of the pro-inflammatory cytokine IFN-γ has also been shown to protect against Toxin A-induced enteritis in a mouse model, and IFN-$\gamma^{-/-}$ mice are protected from tissue damage and show decreased cytokine production after challenge [61]. Mast cells have also been implicated in a damaging inflammatory response after intoxication, as mast cell deficient mice show decreased inflammation after treatment with Toxin A [62]. The toxins are also able to induce significant levels of the neutrophil chemoattractant CXCL2 in rat ileal loops, and blocking this signal reduces histological damage following intoxication with Toxin A. Similarly, genetic knockout of chemokine MIP1α, or of its receptor CCR1, decreased the damage associated with intoxication by Toxin A [63,64]. Interestingly, the adipokine leptin also appears to play a role in the inflammatory response to Toxin A, as ob/ob leptin deficient mice show reduced

pathology after challenge with Toxin A, including less severe fluid secretion and inflammation [65].

In contrast, similar manipulations in infection models using live *C. difficile* have shown a lack of inflammatory response to be detrimental. Inflammasome deficient $ASC^{-/-}$ mice show decreased survival during infection, suggesting that some inflammation is necessary for bacterial clearance and disease resolution. Interestingly, $ASC^{-/-}$ mice show increased translocation of commensal microbes to organs such as the spleen, liver and lung, suggesting a role for inflammatory pathways in controlling bystander bacteria [66]. Likewise, the cytokine IL-22 has also been shown to play a similar protective role. IL-22 knockout mice were significantly more susceptible to mortality during CDI, and had increased translocation of the pathobionts *Enterococcus faecalis* and *Enterobacter cloacae* to the liver and spleen during infection. Treatment of the mice with ciprofloxacin eradicated the pathobionts while *C. difficile* burden was unchanged, and lead to increased survival of IL-22 knockout animals [67].

Inflammatory signaling by pattern recognition receptors has also been shown to be protective during CDI, as $Nod1^{-/-}$, $MyD88^{-/-}$ and $TLR4^{-/-}$ mice all experience more severe disease [54,55,58]. Leptin signaling also appears to be protective, as leptin deficient mice show higher bacterial burdens [68]. Additionally, several cell subsets have been shown to be essential for protection during disease. Preventing neutrophil recruitment via antibody depletion causes more severe mortality [55], and Innate Lymphoid Cells (ILCs) have also been shown to be protective [69,70]. ILCs respond quickly during infections and help to shape the long term immune response. Although three different subsets of ILCs have been identified, ILC1s and ILC3s specifically play a protective role in a murine model of CDI.

Their associated cytokines, IFNγ and IL-17, are also upregulated during infection, and mixed T_H1/T_H17 responses are induced by *C. difficile*-treated dendritic cells *in vitro* [71]. Interestingly, experiments demonstrating the importance of ILCs were carried out in Rag1 knockout mice, which lack mature B and T cells. These mice demonstrated disease pathology identical to wild type C57BL6 mice, and only $Rag\gamma c^{-/-}$ mice (which lack ILCs as well as B and T cells) showed increased susceptibility. This highlights the importance of the innate response rather than the adaptive response in survival during *C. difficile* infection.

The cytokine Interleukin-23 (IL-23) stands out as an inducer of pathogenic inflammation during infection [72]. IL-23 has been implicated in multiple autoimmune diseases, and is best known for its ability to maintain T_H17 cells and induce production of the cytokines IL-17 and IL-22 [73]. Depletion of IL-23 in a murine model of CDI significantly prevented mortality, suggesting that this cytokine is part of a pathogenic host response to infection. Although the mechanism behind IL-23 pathogenesis is unknown, inflammation in general has long been understood as a balance between eradicating infection while preventing destruction of host tissues, and it may be that IL-23 tips the balance towards pathogenic host damage rather than protective bacterial eradication.

Based on this understanding of the role of *C. difficile* toxins and the immune response during infection, disease outcome is influenced both by the bacterial virulence factors which are expressed during infection, and the type, timing, and intensity of host inflammation generated in response. The goal of my research was to determine what role the toxins play in shaping this immune response, and my overall hypothesis was that Toxins A, B and CDT enhance inflammation and shift the type of response generated by the host to produce pathology during CDI. Specifically, all three toxins contribute to the production of overwhelming Type 17 signals, including IL-1β, IL-6 and IL-23, disrupting the balance of immune signaling required to promote protection.

In this dissertation, I: 1.) demonstrate that Toxins A and B contribute to inflammation by activating the inflammasome to secrete IL-1β, which signals through the IL-1 receptor to enhance production of IL-23; 2.) isolate Rho-glucosylation by Toxin A as essential to inflammasome activation, tissue damage and an inflammatory response in a mouse model of intoxication; and 3.) identify CDT as a virulence factor which contributes to *C. difficile* virulence by enhancing inflammation via activation of NFκB and by suppressing protective colonic eosinophils.

Table 1.1 The role of inflammation in response to *C. difficile* toxins or infection depends on the type of challenge.

Many studies using intoxication as a model (light gray boxes) report that inflammatory pathways are deleterious. However, infection based models (dark gray boxes) have found that certain inflammatory pathways are necessary for survival, with the exception of the pro-inflammatory cytokine IL-23. Thus, the role of inflammation during CDI is likely to be multifaceted and complex.

Challenge	Model	Result	Reference
TcdA/B	$ASC^{-/-}$ Mice Anakinra (IL-1Ra) Mice	Decrease in disease severity	Ng, 2010
TcdA	MIP-2 Neutralized Rat	Decrease in disease severity	Castagliuolo, 1998
TcdA	CCR1-/- Mice MIP1α -/- Mice	Decrease in disease severity	Morteau, 2002
TcdA	Anti-CD18 mAB (neutrophil depletion) Rabbit	Decrease in disease severity	Kelly, 1994
TcdA	IFN-$\gamma^{-/-}$ Mice IFN-γ Neutralized Mice	Decrease in disease severity	Ishida, 2004
TcdA	Mast Cell Deficient Mice	Decrease in disease severity	Wershil, 1998
TcdA	Ob/ob (Leptin Deficient) Mice	Decrease in disease severity	Mykoniatis, 2003
VPI 10463	$Nod1^{-/-}$ Mice	Decreased survival	Hasegawa, 2011
VPI 10463	$ASC^{-/-}$ Mice	Decreased survival	Hasegawa, 2012
VPI 10463	$MyD88^{-/-}$ Mice Neutrophil depleted Mice	Decreased survival	Jarchum, 2012
R13537	$TLR4^{-/-}$ Mice	Decreased survival	Ryan, 2011
VPI 10463	IL-$22^{-/-}$ Mice	Decreased survival	Hasegawa, 2014
VPI 10463	Rag$\gamma c^{-/-}$ Mice	Decreased survival	Abt, 2015
VPI 10463	IL-$23p19^{-/-}$ Mice	Increased survival	Buonomo, 2013

Chapter Two: Toxins A and B enhance IL-23 production via activation of the inflammasome

Part of this chapter has been adapted from "Inflammasome activation contributes to interleukin-23 production in response to *Clostridium difficile*"

Carrie A. Cowardin, Sarah A. Kuehne [1], Erica L. Buonomo [2], Chelsea S. Marie [3], Nigel P. Minton [1], William A. Petri, Jr. 2015. *MBio* Jan 27;6(1)

[1] S.A.K. and N.P.M generated *C. difficile* mutants and provided advice on experimental design
[2] E.L.B assisted with experiments and provided advice on experimental design
[3] C.S.M. assisted with development of NFκB assay and cell culture

2.1 Introduction

Infection with *C. difficile* results in a spectrum of disease ranging from mild diarrhea to severe pseudomembranous colitis, toxic megacolon and death [74]. Antibiotic treatment predisposes individuals to CDI by disrupting commensal microbes in the gut and providing a competitive advantage for *C. difficile* growth as well as impairing the host mucosal immune response [75]. Toxins A and B (TcdA and TcdB) are the major virulence factors of *C. difficile*, responsible for glucosylating host Rho family GTPases [76]. Glucosylation blocks the exchange of GDP for GTP and prevents RhoA, Rac1 and Cdc42 from functioning, resulting in cell rounding and death [77]. Toxins A and B also elicit a robust pro-inflammatory response via activation of multiple MAP kinases which in turn activate the transcription factor Nuclear Factor κB (NFκB)[30,32]. Both toxins activate an ASC-containing inflammasome and can induce secretion of IL-8, TNFα and IL-6 in addition to IL-1β [38,39,78].

C. difficile PAMPs also contribute to the host inflammatory response, including the Surface Layer Proteins (SLPs), which activate Toll-like Receptor 4 (TLR4) and flagellin, thought to be a TLR5 ligand [54,56]. *C. difficile* also possesses uncharacterized Nod1 stimulatory molecules [58]. Although Toxins A and B have been shown to enhance cytokine production resulting from recognition of *C. difficile* PAMPs, it is not well known how this occurs [56]. Innate inflammatory signaling involving Nod1, TLR4, MyD88 and the inflammasome adaptor ASC are protective in mouse models of CDI [54,58,66]. Conversely, human clinical studies have shown that the host response may also be pathogenic, as IL-8 and CXCL5 levels positively predict disease severity whereas *C. difficile* bacterial burden does not [9]. Our lab has previously identified the pro-

inflammatory cytokine IL-23 as a pathogenic mediator during CDI. The importance of IL-23 in these studies suggests that IL-23 may be a key regulator of the balance between bacterial eradication and host tissue damage [72]. IL-23 expression is initiated by the transcription factors NFκB and AP-1 downstream of TLR and IL-1 receptor signaling [79], leading us to hypothesize that *C. difficile* may be able to directly induce IL-23 expression through the action of Toxins A and B. Purified SLPs as well as live *C. difficile* can induce IL-23 production, but the contribution of Toxins A and B to this process has not been extensively studied. Additionally, it is unknown how the toxins contribute to IL-23 production in response to ribotype 027 strains, which are thought to be more pro-inflammatory [78].

We used murine and human dendritic cells to elucidate the regulation of IL-23 in response to *C. difficile.* We found that Toxins A and B increase levels of IL-23, but are not sufficient alone to induce its production. Furthermore, we identified a crucial role for the host inflammasome and IL-1β signaling in toxin-enhanced IL-23 production. Finally, we have shown that IL-1β is increased in the serum of patients with CDI, demonstrating that this cytokine is systemic during human infection and could potentiate IL-23 production in these patients.

2.2 Results

In order to determine how *C. difficile* induces IL-23 expression, we treated murine Bone Marrow-derived Dendritic Cells (BMDCs) with purified Toxins A and B (Figure 2.1A). We found that the toxins, either alone or in combination, were not sufficient to induce IL-23 production (concentrations ranging from 2 pg/ml to 25 μg/ml, Figure 2.1). Toxins A and B were functionally active, as Toxin A induced CXCL1 production and both toxins activated NFκB and caused significant cell death (Figure 2.1). Next, we attempted to induce IL-23 production using fresh toxin-containing culture filtrates from *C. difficile* strain R20291, a ribotype 027 strain responsible for a 2006 outbreak in Stoke Mandeville, UK [80]. Wild type R20291 filtrate induced significant levels of IL-23, while the isogenic toxin mutant R20291 AB- showed greatly decreased IL-23 induction as measured by an anti-IL-23p19 ELISA. Adding 2 ng/ml each of purified Toxins A and B to R20291 AB- culture filtrate restored IL-23 production to levels induced by wild type R20291 (Figure 2.2A). This concentration of the toxins was chosen to mimic physiological levels found in serum during infection [81]. Interestingly, Toxins A and B also increased the amount of IL-23 produced by BMDCs upon exposure to lipopolysaccharide (LPS) from *Escherichia coli*, a PAMP known to induce IL-23 [79]. We concluded that while Toxins A and B alone were not sufficient to induce IL-23, the toxins did enhance IL-23 production in the presence of bacterial products.

Figure 2.1: Toxins A and B cause cell death, activate NFκB and Toxin A induces CXCL1 secretion.

RAW-Blue NFκB Reporter Macrophages (Invivogen) were exposed to purified Toxins A and B as indicated. Cell supernatant was harvested at 8 hours and assayed for Secreted Embryonic Alkaline Phosphatase activity by QUANTI-Blue assay (**A**). RAW-Blue cells were assayed for cytotoxicity by LDH Release (Promega). Cell death is represented as a percentage of total lysis positive control (**B**). BMDCS were exposed to purified Toxins A and B as indicated. CXCL1/KC production was determined by R&D Systems Quantikine ELISA kit (**C**). Student's *t*-test, * = p value < 0.05 compared to untreated, ** = p value < 0.01, *** = p value < 0.001

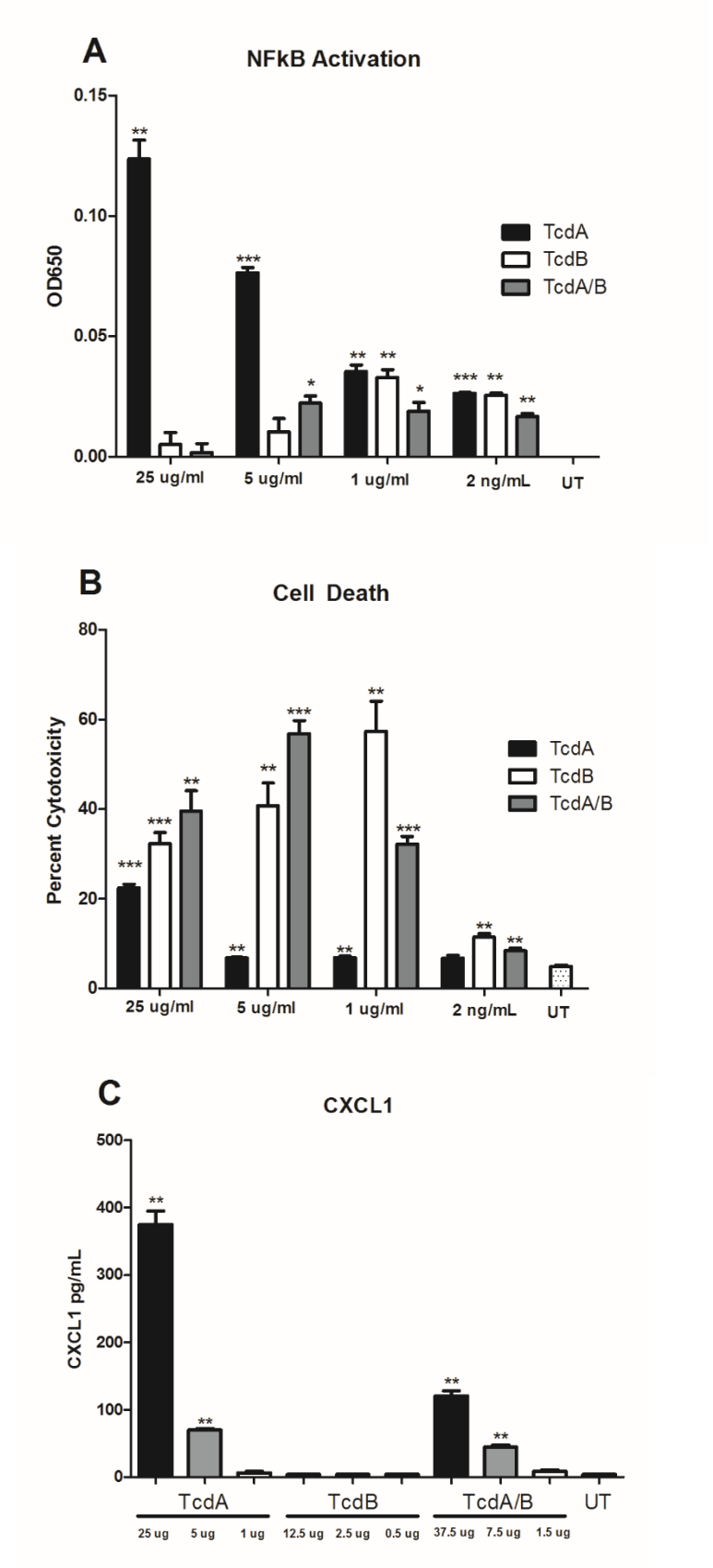
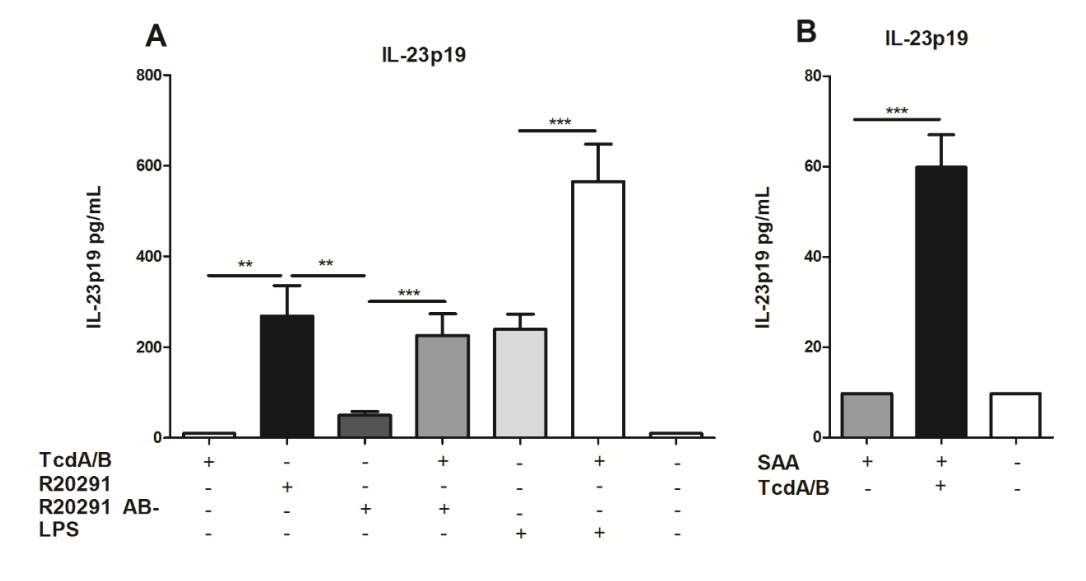

A
NFkB Activation
OD650
0.15
0.10
0.05
0.00
TcdA
TcdB
TcdA/B
25 ug/ml
5 ug/ml
1 ug/ml
2 ng/mL
UT
B
Cell Death
Percent Cytotoxicity
80
60
40
20
0
TcdA
TcdB
TcdA/B
25 ug/ml
5 ug/ml
1 ug/ml
2 ng/mL
UT
C
CXCL1
CXCL1 pg/mL
500
400
300
200
100
0
TcdA
TcdB
TcdA/B
UT
25 ug
5 ug
1 ug
12.5 ug
2.5 ug
0.5 ug
37.5 ug
7.5 ug
1.5 ug

Figure 2.2: *C. difficile* toxins induce detectable IL-23 from Bone Marrow-derived Dendritic Cells only in the presence of danger signals.

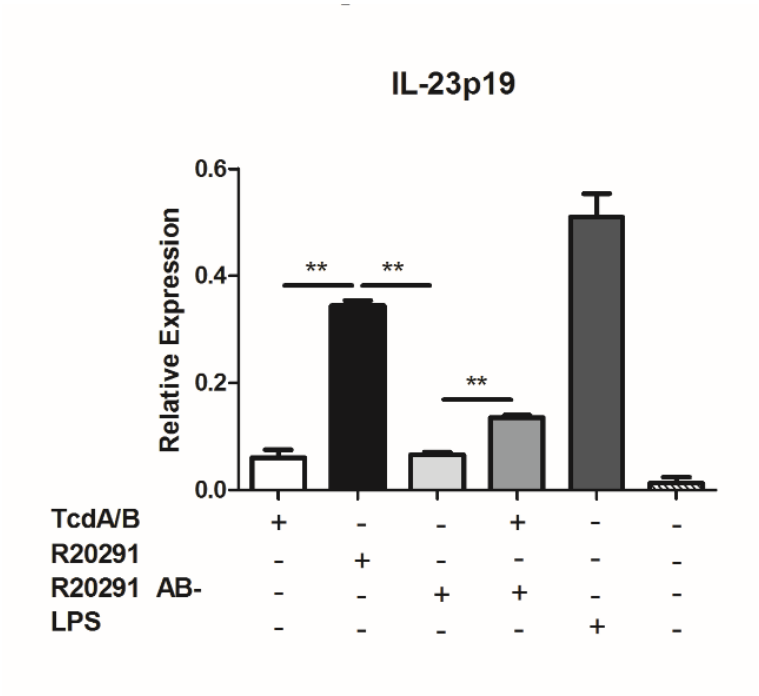
BMDCs were treated for 24 hours with purified Toxins A and B (2ng/ml), LPS (100 ng/ml) or with culture filtrates from *C. difficile* strain R20291 or R20291 AB-. BMDC media was assayed for IL-23 by ELISA. **(A)**. BMDCs were incubated with Serum Amyloid A (500ng/ml) with or without Toxins A and B (2 ng/ml) **(B)**. Student's *t*-test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001. Data shown combined from at least 3 experiments.


A
IL-23p19
IL-23p19 pg/mL
800
600
400
200
0
**
**
***
***
TcdA/B + - - + - + -
R20291 - + - - - - -
R20291 AB- - - + + - - -
LPS - - - - + + -
B
IL-23p19
IL-23p19 pg/mL
80
60
40
20
0
***
SAA + + -
TcdA/B - + -

Although bacterial PAMPs are common priming signals for inflammasome activation, we hypothesized that host-derived danger signals capable of activating NFκB would also potentiate IL-23 production. Serum amyloid A (SAA) is a host-derived inflammatory mediator which is expressed in colonic epithelium, upregulated during CDI, and can play a role in modulating Dextran Sodium Sulfate (DSS) colitis in murine models [82]. Exposing BMDCs to Serum Amyloid A alone did not induce detectable IL-23, but IL-23 production was greatly enhanced when Toxins A and B were added (Figure 2.2B). This suggests that although a danger signal is required for toxin-induced IL-23 production, this signal need not be derived from bacteria.

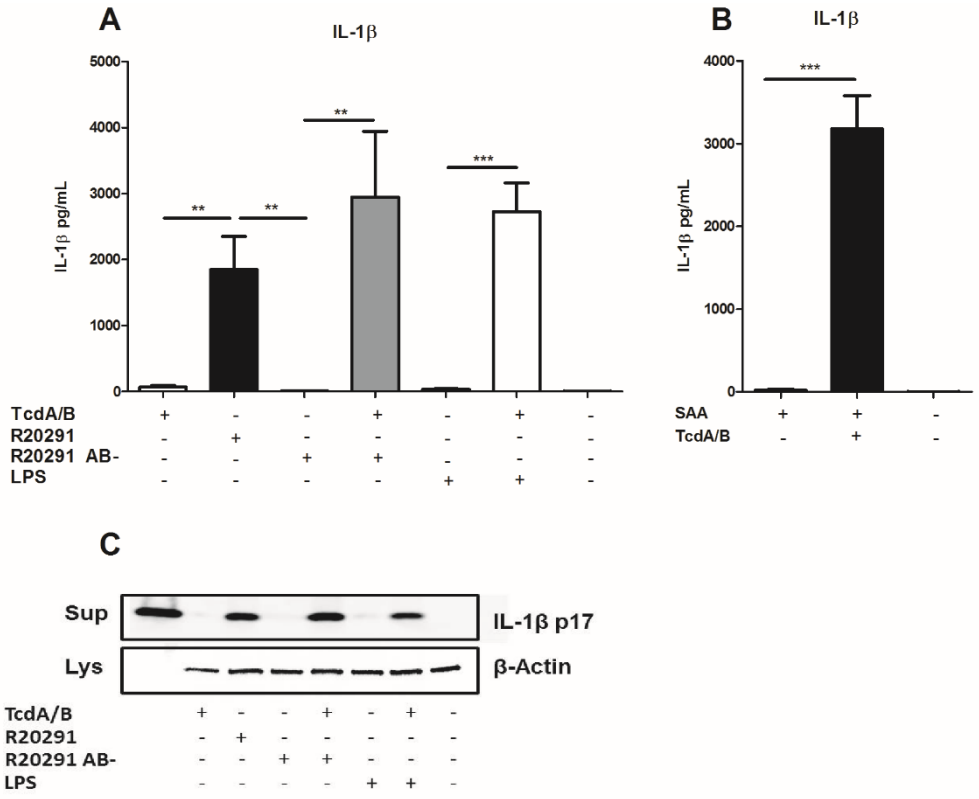
Next we sought to determine whether human dendritic cells respond similarly in the presence of Toxins A and B (Figure 2.3). We generated Monocyte-derived Dendritic Cells (MoDCs) from the peripheral blood monocytes of healthy human volunteers and exposed them to purified Toxins A and B, as well as culture filtrates from *C. difficile* strain R20291. We evaluated *IL-23a* gene expression by quantitative reverse transcription PCR, as human IL-23p19 ELISAs suffer from relatively low sensitivity. Purified Toxins A and B did not induce *IL-23a* gene expression. However, wild type R20291 supernatant did induce significant *IL-23a* expression, and this was lost in the presence of non-toxigenic filtrate from R20291 AB-. The addition of purified Toxins A and B restored some level of *IL-23a* gene expression (Figure 2.3), leading us to conclude that human cells respond similarly to the presence of *C. difficile* toxins and PAMPs.

Figure 2.3: *C. difficile* toxins and PAMPs induce IL-23 expression in human cells. MoDCs were generated from monocytes isolated from healthy human peripheral blood, and were treated for 24 hours with purified Toxins A and B (2ng/ml) or with filter-sterilized culture supernatants from *C. difficile* strain R20291 or an isogenic toxin mutant, R20291 AB-. RNA was isolated and assayed by quantitative reverse-transcription PCR for IL-23 gene expression, shown relative to GAPDH. Student's *t*-test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001. Data shown is representative of three independent experiments.


IL-23p19
Relative Expression
0.6
0.4
0.2
0.0
**
**
**
TcdA/B + - - + - -
R20291 - + - - - -
R20291 AB- - - + + - -
LPS - - - - + -

Toxins A and B have been shown to activate an ASC-containing inflammasome to secrete IL-1β. Both the NLRP3 inflammasome and a Pyrin-dependent inflammasome have been implicated in this process [38,39,78]. Two signals are a well-established requirement for activation of the inflammasome. The first "priming" signal induces NFκB dependent expression of pro-IL-1β, and the second "activation" signal induces assembly of the inflammasome complex and processing of IL-1β into its mature form [83]. Because IL-1β is thought to play a role in the induction of IL-23 [84], we assayed BMDCs for IL-1β production (Figure 2.4). We found that Toxins A and B alone induced low levels of IL-1β production, but treating BMDCs with LPS in combination with Toxins A and B significantly increased levels of IL-1β (Figure 2.4A). Additionally, we found that treating BMDCs with supernatant from strain R20291 also induced significant levels of IL-1β, indicating that this supernatant may be sufficient to provide both priming and activation signals. IL-1β production was lost when BMDCs were exposed to R20291 AB-filtrate, but restored when purified Toxins A and B were added back. We also found that SAA was sufficient to serve as a priming signal to induce increased IL-1β production in the presence of Toxins A and B (Figure 2.4B). Because many IL-1β ELISAs can also detect pro-IL-1β released from dying cells, we confirmed by Western blot that the IL-1β produced in response to Toxins A and B was indeed processed and secreted rather than simply released from intoxicated, nonviable cells (Figure 2.4C). We concluded that danger signals and Toxins A and B synergistically increased IL-1β production by BMDCs.

Figure 2.4: IL-1β secretion by BMDCs in response to *C. difficile* toxins is enhanced by a priming signal. BMDCs were treated for 24 hours with purified Toxins A and B (2ng/ml), LPS (100 ng/ml) or with filter-sterilized culture supernatants from *C. difficile* strain R20291 or an isogenic toxin mutant, R20291 AB-. BMDC media was assayed for IL-1β by ELISA. **(A)**. BMDCs were incubated with Serum Amyloid A (500ng/ml) with or without Toxins A and B (2 ng/ml) (**B**). Western blot of supernatants (Sup) and whole cell lysate (Lys) from treated BMDCs (C). Blots were probed with antibodies directed against IL-1β p17 (supernatant) and β-Actin (lysate). Lane #1 contains recombinant IL-1β as a positive control. (Student's *t*-test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001). Data shown combined from at least 3 independent experiments (**A**-**B**) or one representative experiment from two independent experiments (**C**).

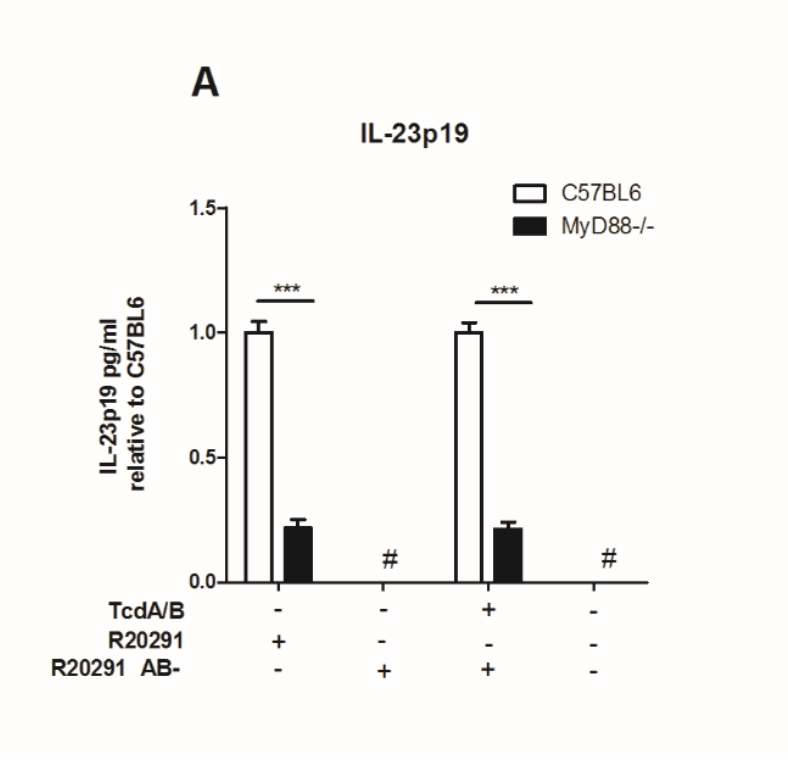

A
IL-1β
IL-1β pg/mL
5000
4000
3000
2000
1000
0
**
**
**
***
TcdA/B + - - + - + -
R20291 - + - - - - -
R20291 AB- - - + + - - -
LPS - - - - + + -
B
IL-1β
IL-1β pg/mL
4000
3000
2000
1000
0
***
SAA + + -
TcdA/B - + -
C
Sup
Lys
IL-1β p17
β-Actin
TcdA/B + - - + - + -
R20291 - + - - - - -
R20291 AB- - - + + - - -
LPS - - - - + + -

Multiple TLRs as well as the IL-1 receptor require the adaptor protein MyD88 in order to successfully activate NFκB to induce downstream cytokine production [85]. In order to determine whether these pathways were required for the induction of IL-23 by *C. difficile*, we utilized BMDCs from MyD88$^{-/-}$ mice. MyD88$^{-/-}$ BMDCs showed significantly reduced IL-23 production in response to R20291 and R20291 AB- supernatant with purified Toxins A and B added (Figure 2.5A). To further delineate the contribution of TLR signaling to this process, we next utilized TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR5$^{-/-}$ BMDCs. TLR4$^{-/-}$ BMDCs, but not TLR2$^{-/-}$ or TLR5$^{-/-}$ BMDCs, showed significantly decreased IL-23 production in response to R20291. Thus the SLPs of *C. difficile*, known to signal through TLR4 [54], are likely a major PAMP involved in IL-23 induction (Figure 2.5B). TLR4$^{-/-}$ BMDCs also demonstrated significantly decreased IL-23 production in the presence of LPS and Toxins A and B, further demonstrating the necessity for a danger signal in addition to the toxins for robust IL-23 production.

Because IL-1β has been shown to contribute to IL-23 production in other models [84], and MyD88 deficient cells did not show an increase in IL-23 in the presence of Toxins A and B, we hypothesized that IL-1β produced in response to Toxins A and B would enhance IL-23 production. To test this hypothesis, we treated BMDCs with filtrate from strain R20291 in the presence of an anti-IL-1β neutralizing antibody or an IL-1 receptor antagonist (Figure 2.6). Both the neutralizing antibody and the receptor antagonist significantly decreased the amount of IL-23 produced compared to an isotype control antibody (Figure 2.6A). As expected, when cells were exposed to R20291 AB-, IL-23 production was minimal.

Figure 2.5: TLR4 and MyD88 signaling contribute to IL-23 production in response to *C. difficile*.

BMDCs from C57BL6/J or MyD88$^{-/-}$ mice (**A**) or C57BL6/J, TLR2$^{-/-}$, TLR4$^{-/-}$, or TLR5$^{-/-}$ mice (**B**) were treated for 24 hours with either LPS, filter-sterilized culture supernatants from *C. difficile* strain R20291 or the non-toxigenic strain R20291 AB-, in the presence or absence of purified Toxins A and B (2 ng/ml). IL-23 protein levels were quantified by ELISA, and data for (**B**) is shown relative to 100% of C57BL6 levels for each condition. Student's *t*-test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001. # = levels below detection. Data shown combined from 3 independent experiments.


A
IL-23p19
C57BL6
MyD88-/-
IL-23p19 pg/ml relative to C57BL6
1.5
1.0
0.5
0.0
***
***
#
#
TcdA/B - - + -
R20291 + - - -
R20291 AB- - + + -

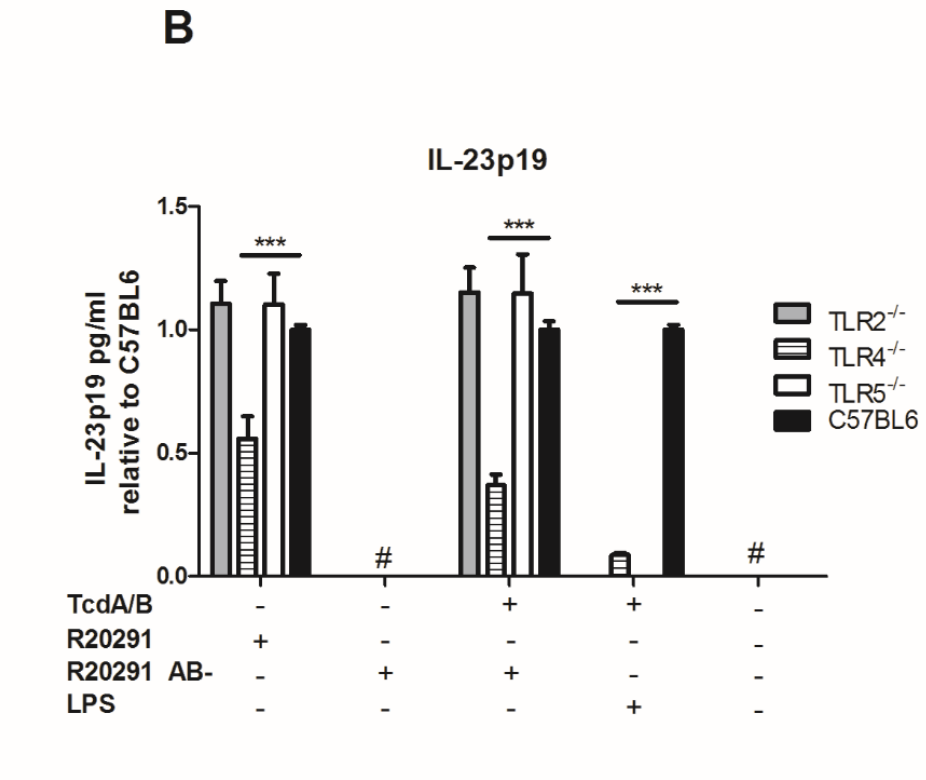
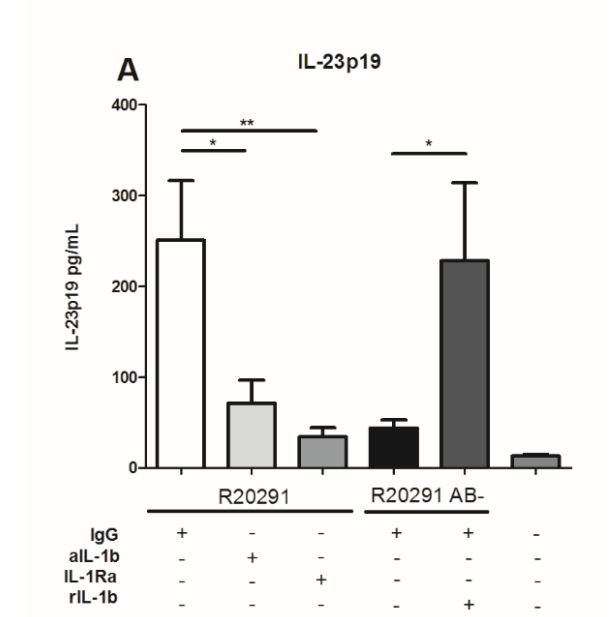
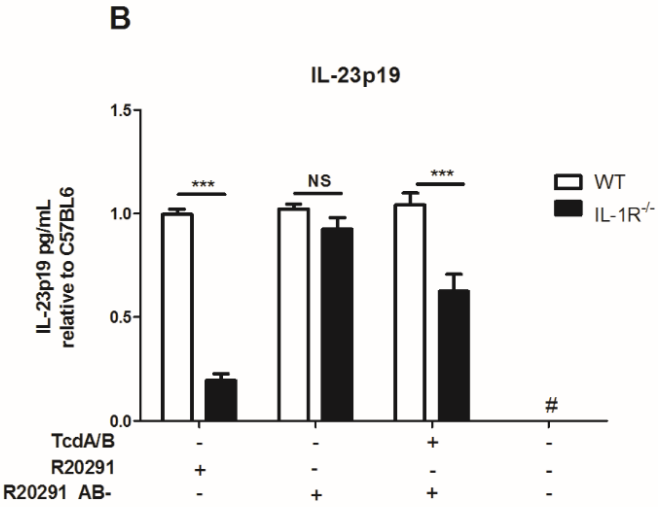

B
IL-23p19
IL-23p19 pg/ml relative to C57BL6
1.5
1.0
0.5
0.0
***
***
***
#
#
TLR2-/-
TLR4-/-
TLR5-/-
C57BL6
TcdA/B - - + + -
R20291 + - - - -
R20291 AB- - + + - -
LPS - - - + -

Figure 2.6: IL-1β signaling contributes to IL-23 production.

BMDCs were treated for 24 hours with filter-sterilized culture supernatants from *C. difficile* strain R20291 or R20291 AB-. Recombinant murine IL-1β (rIL-1β, 50 ng/ml), IL-1β neutralizing antibody (aIL-1β, 1 µg/ml), IL-1 receptor antagonist (IL-1Ra, 500 ng/ml), or IgG isotype control antibody (1 µg/ml) were added as indicated (**A**). BMDCs from C57BL6 mice (white bars) or IL-1R$^{-/-}$ (black bars) were treated for 24 hours with filtrates from *C. difficile* strain R20291 or R20291 AB-, in the presence or absence of purified Toxins A and B (**B**). Data for is shown relative to 100% of C57BL6 levels for each condition. Data shown combined from at least 3 independent experiments. Student's *t*-test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001


A
IL-23p19
IL-23p19 pg/mL
400
300
200
100
0
*
**
*
R20291
R20291 AB-
IgG + - - + + -
aIL-1b - + - - - -
IL-1Ra - - + - - -
rIL-1b - - - - + -

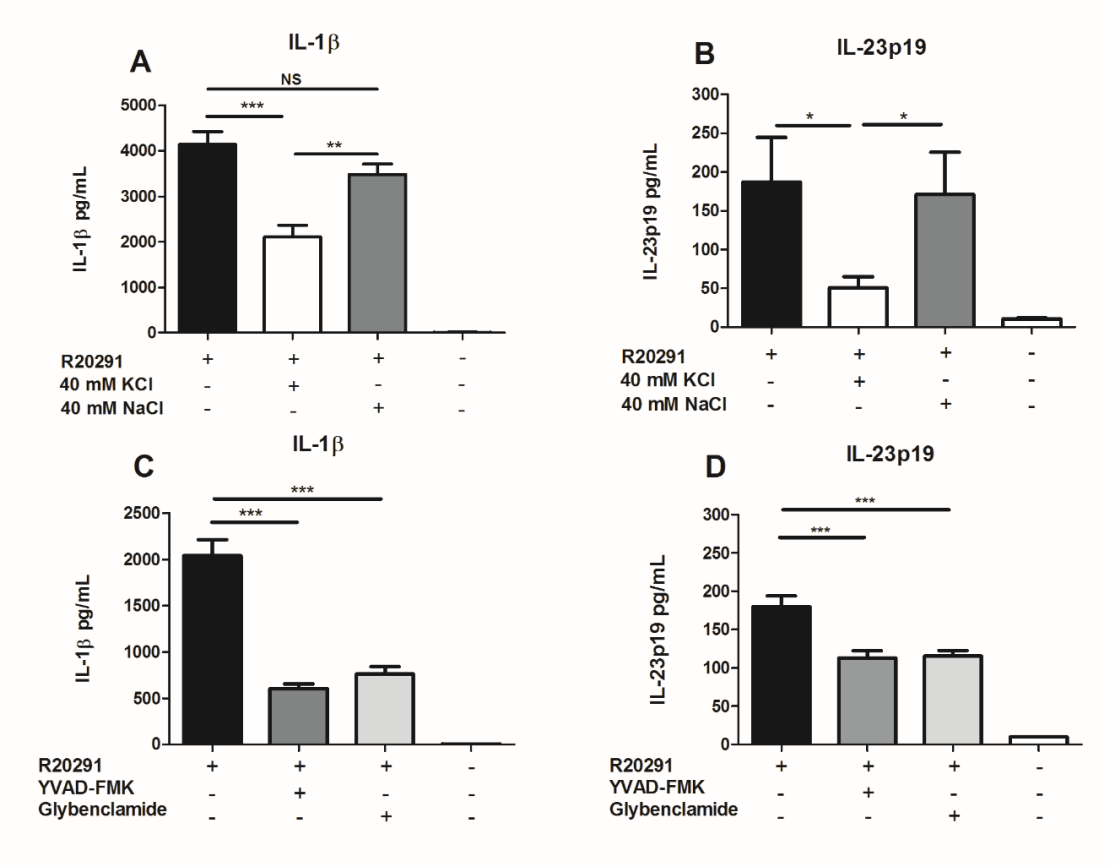

B
IL-23p19
IL-23p19 pg/mL relative to C57BL6
1.5
1.0
0.5
0.0
***
NS
***
#
WT
IL-1R-/-
TcdA/B - - + -
R20291 + - - -
R20291 AB- - + + -

However, IL-23 could be significantly enhanced by adding recombinant IL-1β to R20291 AB-, suggesting that IL-1 receptor signaling could account for the increase in IL-23 observed in the presence of Toxins A and B. Next, we utilized BMDCs from IL-1R$^{-/-}$ mice to further assess the contribution of this receptor to IL-23 production. We found that IL-1R$^{-/-}$ BMDCs displayed significantly reduced IL-23 production in response to R20291 as well as R20291 AB- with added Toxins A and B (Figure 2.6B). As expected, no significant difference was observed for R20291 AB- in the absence of Toxins A and B. These results suggest that following a priming signal, Toxins A and B may have activated the inflammasome to secrete IL-1β, which in turn signaled through the IL-1 receptor to enhance IL-23 production.

Although multiple diverse stimuli have been shown to activate the NLRP3 inflammasome, these stimuli share the downstream requirement for efflux of potassium [86]. Thus the addition of excess extracellular potassium can inhibit NLRP3 inflammasome activation, although this is not specific to NLRP3 and can also inhibit NLRP1 oligomerization [83]. In order to confirm the role of inflammasome-produced IL-1β in enhanced IL-23 production, BMDCs were incubated with culture filtrate from R20291 in the presence or absence of 40 mM KCl compared to 40 mM NaCl as an osmotic control. We found that the addition of extracellular K^+ to BMDCs significantly inhibited the secretion of IL-1β as well as IL-23 (Figure 2.7A, 6B).

Figure 2.7: Inflammasome inhibition reduces IL-23 secretion.

BMDCs were treated with filter-sterilized culture supernatants from strain R20291 in the presence of 40mM KCl, 40 mM NaCl (**A, B**), 20 μM YVAD-FMK or 25 μg/ml Glybenclamide (**C,D**) for 24 hours. IL-1β (**A, C**) and IL-23 (**B, D**) in the cell supernatant were measured by ELISA. Student's *t*-test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001. Data shown combined from at least 3 independent experiments.

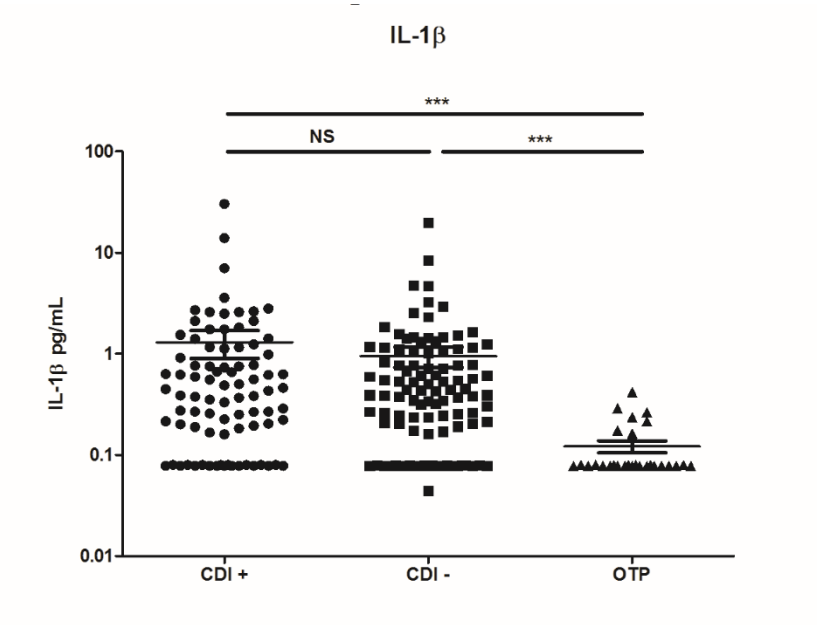

A
IL-1β
NS
***
**
IL-1β pg/mL
5000
4000
3000
2000
1000
0
R20291 + + + -
40 mM KCl - + - -
40 mM NaCl - - + -
B
IL-23p19
*
*
IL-23p19 pg/mL
300
250
200
150
100
50
0
R20291 + + + -
40 mM KCl - + - -
40 mM NaCl - - + -
C
IL-1β
***
***
IL-1β pg/mL
2500
2000
1500
1000
500
0
R20291 + + + -
YVAD-FMK - + - -
Glybenclamide - - + -
D
IL-23p19
***
***
IL-23p19 pg/mL
300
250
200
150
100
50
0
R20291 + + + -
YVAD-FMK - + - -
Glybenclamide - - + -

Similarly, the addition of YVAD-FMK, a Caspase 1/4 inhibitor, or Glybenclamide, an inhibitor of K+ efflux, decreased both the amount of IL-1β and the amount of IL-23 secreted by BMDCs in the presence of R20291 culture filtrate (Figure 2.7C, 2.7D), providing further evidence that inflammasome activation and resulting IL-1 receptor signaling contributed to IL-23 production.

Finally, in order to confirm the presence of IL-1β during human infection, we analyzed sera from CDI positive and CDI negative patients with diarrhea, as well as healthy outpatient controls. Utilizing a high sensitivity IL-1β ELISA specifically designed for cytokine detection in serum (limit of detection 0.16 pg/ml), we found that patients with CDI had significantly higher serum IL-1β than healthy outpatient controls (Figure 2.8). Interestingly, patients with diarrhea from other causes also displayed increased IL-1β, demonstrating that this cytokine is not specific to CDI. These results suggest that inflammasome activation occurs *in vivo* during CDI, and may potentiate the IL-23 production in the lamina propria that we have previously observed [72]. Additionally, the presence of IL-1β in patients with non-CDI diarrhea suggests that this cytokine is present and could be involved in IL-23 production in other models of colitis.

Figure 2.8: IL-1β is increased in the sera of patients with CDI.

Sera from *C. difficile* positive patients with diarrhea (CDI+), *C. difficile* negative patients with diarrhea (CDI-) and healthy outpatients (OTP) were assayed for IL-1β by high sensitivity ELISA (**A**). Each dot represents one patient sample. Mann-Whitney test, * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001


IL-1β
***
NS
***
100
10
1
0.1
0.01
IL-1β pg/mL
CDI +
CDI -
OTP

2.3 Discussion

Toxins A and B are the major virulence factors of *C. difficile* and act via intoxication of Rho family GTPases, including RhoA, Rac1 and Cdc42 [76]. Both toxins are capable of causing epithelial cell death, as well as apoptosis of neurons, endothelial cells and monocytes [27]. Toxins A and B alone can also induce the secretion of IL-8/CXCL1, TNF-α and IL-6 [32].Toxin-induced activation of the inflammasome and IL-1β secretion in primed cells has also been demonstrated [38,39,78]. It is well appreciated that *C. difficile* toxins and other factors contribute to disease severity, as non-toxigenic strains do not cause symptomatic infection [87,88] and genetic manipulation of *C. difficile* sporulation and motility can influence disease outcome [89].

Despite the importance of Toxins A and B during infection, recent findings implicate host-derived inflammation as a contributing factor to the severity of CDI. Therefore, a thorough understanding of *C. difficile* pathogenesis requires examination of both host and pathogen derived mediators. We have previously demonstrated that the cytokine IL-23 is pathogenic during CDI, as mice deficient in IL-23 are significantly protected from mortality [72]. We have also demonstrated that IL-23 is expressed during human infection with *C. difficile*. Although the role of Toxins A and B in the expression of various cytokines has been extensively studied, the induction of IL-23 by *C. difficile* has thus far been incompletely characterized. Thus, the goal of this work was to determine the role of *C. difficile* Toxins A and B in the induction of IL-23.

Here we have found that, surprisingly, Toxins A and B alone do not induce production of measurable amounts of IL-23 from BMDCs (Figure 2.2). However, supernatant from the ribotype 027 strain R20291 was capable of inducing IL-23, and IL-

23 production was synergistically increased in the presence of both Toxins A and B and a danger signal (Figure 2.2-2.3). Interestingly, we also found that robust secretion of IL-1β likewise required a priming signal, and *C. difficile* PAMPs were sufficient to prime BMDCs for IL-1β secretion (Figure 2.4). IL-1 receptor signaling following inflammasome activation contributed to the production of IL-23, accounting for the increase in IL-23 observed in the presence of Toxins A and B (Figure 2.5-2.7).

Previously, purified Surface Layer Proteins (SLPs) of *C. difficile* strain R13537 have been shown to induce IL-23 expression in a TLR4 dependent manner [54], demonstrating that *C. difficile* PAMPs can induce IL-23. Additionally, *C. difficile* strains 630 and R20291 also induced IL-23 *in vitro*, and mutants of strain 630 which do not produce Toxins A and B showed significantly decreased IL-23 production [78]. However, mutants of strain R20291 lacking Toxin A and B production were not investigated, although R20291 was shown to induce significantly more IL-23 than strain 630. Additionally, it was unknown whether purified Toxins A and B could induce IL-23. Our data confirm that Toxins A and B enhance IL-23 production by ribotype 027 strains and demonstrate that the toxins alone are not sufficient for IL-23 production (Figure 2.2). The ability of Toxins A and B alone to induce production of IL-8 suggests an underlying difference between this cytokine and IL-23, possibly due to differences in expression level or cell source. Alternatively, IL-8 and IL-23 may have different thresholds of production in response to inflammatory stimuli. Additionally, our results suggest that *C. difficile* PAMPs in combination with Toxins A and B are capable of inducing IL-23 (Figure 2.2), and that both TLR4 and MyD88 signaling are involved in PAMP recognition (Figure 2.5).

Although bacterial danger signals were sufficient to allow IL-23 production in response to Toxins A and B, we also examined the possibility that host derived signals could serve this function. Serum Amyloid A, an endogenous danger signal released from damaged epithelial cells [82], was also sufficient to allow IL-23 production from BMDCs in the presence of Toxins A and B (Figure 2.2). Therefore, the production of IL-23 is not dependent on the presence of bacterial PAMPs. This data provides insight into the inflammatory response to *C. difficile* in germ free mice, which are notably susceptible to CDI [90]. Under germ free conditions, *C. difficile* PAMPs or host derived danger signals in combination with Toxins A and B could be sufficient to invoke a robust inflammatory response involving both IL-1β and IL-23.

The ability of *C. difficile* toxins to activate the inflammasome is well known; however, the specific inflammasome components involved are still in question. Originally, Toxins A and B were thought to activate the NLRP3 inflammasome in an ASC-dependent manner [38]. However, recently work has demonstrated that inflammasome activation by these toxins may be NLRP3 independent and instead involve activation of the Pyrin inflammasome [39,78]. Although we have not examined a necessity for specific inflammasome components, we have found that BMDCs are capable of robust IL-1β production in response to Toxins A and B when a priming signal is provided (Figure 2.4). Interestingly, potassium efflux appears to be involved in this process as excess extracellular potassium decreases both IL-1β and consequently, IL-23 production (Figure 2.6). However, potassium efflux is not specific to NLRP3 inflammasome activation and does not rule out involvement of other inflammasomes.

The ability of IL-1β to influence IL-23 signaling has been demonstrated previously in the context of autophagy, where inhibition of the autophagosome increased IL-23 production in response to TLR agonists, and this increase was mediated by IL-1 receptor signaling [84]. Similarly, we found that IL-1 receptor signaling contributes to IL-23 production in the presence of *C. difficile* toxins and bacterial products (Figure 2.4, Figure 2.6). The use of anti-IL-1β neutralizing antibody or IL-1 receptor antagonist decreased the levels of IL-23 produced under inflammasome-activating conditions (Figure 2.6). Recombinant IL-1β increased the amount of IL-23 produced, demonstrating that IL-1 receptor signaling can account for much of the increase in IL-23 found in the presence of Toxins A and B. However, we cannot rule out additional roles of the toxins which may also contribute to IL-23 production. For example, disruption of the epithelial barrier by toxins *in vivo* may lead to sensing of *C. difficile* and commensal DAMPs by basolateral host PRRs, which would typically be unresponsive or tolerogenic in the healthy host.

We have also found that IL-1β was elevated in the serum of patients with CDI (Figure 2.8), which had been previously shown in stool [91]. Detection of serum IL-1β required the use of a high sensitivity ELISA. Although we have not examined the presence of Toxins A and B in patient serum samples, the toxins have been shown to be present in the serum of infected animals, suggesting that inflammasome activation may be a systemic response to CDI [81]. Increased serum IL-1β was also noted for patients with diarrhea from other causes, suggesting that elevation of this cytokine is a common response to diarrheal illness. Indeed, IL-1β release is triggered in response to other bacterial infections, as well as increased in cells from patients with Inflammatory Bowel

Disease [92,93]. IL-1β may therefore play a role in the induction of IL-23 in other diarrheal illnesses, an interesting possibility of which more investigation is warranted.

The role of IL-1β during CDI is complex, as reduction in IL-1β production in $ASC^{-/-}$ mice has been demonstrated to be protective during challenge with Toxins A and B [38] but pathogenic in an infection model [66]. IL-1β is thought to play an essential role in the induction of CXCL1 and recruitment of neutrophils, which may be pathogenic during challenge with toxins alone, but play an essential role in controlling infection with live *C. difficile* [55-60]. Our data further demonstrate that IL-1β likely plays a multifaceted role during CDI by inducing the pathogenic cytokine IL-23.

In conclusion, we suggest that *C. difficile* is initially recognized in a TLR4 and MyD88-dependent manner resulting in low level expression of IL-23. Intoxication of primed host cells by Toxins A and B leads to robust IL-1β secretion, likely due to inflammasome activation. IL-1β then signals through the IL-1 receptor to further enhance the production of IL-23. This model therefore suggests that two signals are required for a robust inflammatory response to *C. difficile*: detection of bacterial or host derived danger signals, as well as intoxication of host cells. Our work demonstrates that although Toxins A and B are essential virulence factors of *C. difficile*, additional signals play a role in shaping the inflammatory response during CDI. Therefore, targeting the innate signaling pathways of TLR4 and the IL-1 receptor could present novel therapies to disrupt pathogenic host signaling during disease.

2.4 Methods

Bacterial Strains and Growth Conditions. *C. difficile* strain R20291 AB- was generated using the ClosTron system and functional inactivation of the targeted genes confirmed by Western blot as previously described [87]. Strains were inoculated onto BHI agar and incubated at 37°C overnight in an anaerobic work station (Shel Labs). Single colonies were inoculated into TY medium [94] and grown anaerobically overnight at 37°C. *C. difficile* cultures were prepared for cell stimulation by resuspending each culture to an OD corresponding to $2x10^7$ CFU/ml. Cultures were spun down and the supernatant removed and sterilized through a 0.2 μM filter.

Mice and Cells. C57BL6, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR5$^{-/-}$ and IL-1R$^{-/-}$ mice were purchased from the Jackson Laboratory. Mice were males aged 8-12 weeks for all experiments. All animals were housed under specific pathogen free conditions at the University of Virginia's animal facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. BMDCs were generated as previously described with minor modifications [95]. Briefly, femurs and tibia were removed and bone marrow flushed with PBS. Cells were counted and viability assessed by Trypan Blue staining and resuspended in RPMI 1640 media (Life Technologies) containing 10% Fetal Bovine Serum, 2mM L-glutamine and 100 U/ml Penicillin and 100 U/ml Streptomycin. Media was supplemented with 10 ng/ml GM-CSF (Peprotech) and 55 μM β-mercaptoethanol (Gibco), and $3x10^6$ cells were seeded into a T75 vent cap tissue culture flask. Cells were cultured for 7 days, and supplemented with fresh media on days 2 and 4. On day 7 cells were harvested for stimulation. Human MoDCs were generated as described previously [96]. Briefly, peripheral blood mononuclear

cells were isolated from the blood of healthy human volunteers by Ficoll-Paque density gradient centrifugation. Mononuclear lymphocytes were collected and resuspended in RPMI 1640 containing 10% Fetal Bovine Serum, 2mM L-glutamine and 100 U/ml Penicillin and 100 U/ml Streptomycin. PBMCs were adjusted to $2x10^6$ cells/ml and 10ml of cell suspension added to each T75 tissue culture flask. PBMCs were incubated at 37°C for 3 hours and suspension cells were aspirated. Adherent monocytes were washed thoroughly and detached with gentle scraping. Monocytes were counted and assessed for viability by Trypan Blue exclusion. Monocytes were adjusted to $1x10^6$ cells/ml and 50 ng/ml recombinant human GM-CSF (Peprotech), 50 ng/ml recombinant human IL-4 (Peprotech) and 55 μM β-mercaptoethanol added. Monocytes were differentiated for 6 days, with addition of fresh media on days 2 and 4.

Cell Stimulation. BMDCs or MoDCs were harvested and resuspended in complete RPMI 1640. $2x10^5$ cells per well were seeded into 48 well tissue culture plates. *C. difficile* filtrate was diluted 1:20 into the BMDC cell suspension and remaining volume made up with complete culture media (containing Toxins A and B where indicated). Purified Toxins A and B were a kind gift from Techlab, Inc. (Blacksburg, VA). Recombinant Serum Amyloid A (Peprotech), TLR-ligand tested lipopolysaccharide (Sigma), anti-mouse IL-1β monoclonal antibody (clone B122, eBioscience), IgG isotype control (eBioscience), recombinant IL-1 receptor antagonist (R&D Systems), recombinant murine IL-1β (R&D Systems), YVAD-FMK (Enzo Life Sciences) and Glybenclamide (Invivogen) were diluted in RPMI 1640 and added as indicated. For incubation of BMDCs with excess potassium, 0.8 M stock solutions of KCl and NaCl were prepared and filter sterilized, and diluted 1:20 into the BMDC cell suspension.

Stimulated cells were incubated for 24 hours at 37°C with 5% CO_2. Cells were spun down at 300 x *g* for 5 minutes and supernatant harvested and frozen down at -80°C. Remaining cells were washed in 1x PBS once and lysed in an appropriate volume of Buffer RLT (Lysis Buffer, RNeasy Kit, Qiagen). RNA-containing lysates were frozen at -80°C until RNA isolation.

Detection of Cytokines. IL-1β and IL-23 were detected in protein supernatants from BMDCs using the Mouse IL-1β Ready-Set-Go! ELISA kit (eBioscience) and the Mouse IL-23p19 DuoSet kit (R&D Systems) according to manufacturer's instructions. IL-1β processing was confirmed by Western blot. To generate supernatant samples, protein supernatant from stimulated BMDCs was harvested and concentrated by methanol precipitation. Whole cell lysate was generated from the same BMDCs by washing cells once in ice cold PBS and adding 100 µl Lysis Buffer to each well (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 ml HALT Protease Inhibitor (Pierce)). Lysates for each condition were pooled and shaken at 4°C for 30 minutes before spinning down at 12,000 rpm at 4°C. Supernatant and whole cell lysate protein samples were run on BioRad Mini-Protean TGX gradient gels (4-15%) and transferred to Amersham Hybond-C Nitrocellulose. Blots were probed with either rabbit polyclonal anti-IL-1β antibody (supernatant samples; Abcam ab9722) or rabbit polyclonal anti-β-Actin loading control (whole cell lysates; Abcam ab75186) followed by LI-COR anti-rabbit 800nm secondary antibody. Fluorescent protein bands were detected using an Odyssey Infrared Imaging System (LI-COR Biosciences). Image manipulation was limited to adjustment of brightness. MoDC IL-23 production was assessed by quantitative reverse transcription PCR. RNA was isolated using the RNeasy isolation kit (Qiagen). Contaminating genomic

DNA was digested using the Turbo DNA-free kit (Ambion) and RNA reverse transcribed with the Tetro cDNA synthesis kit (Bioline) according to manufacturer instructions. The resulting cDNA was purified using Qiagen's PCR purification kit. *IL-23a* gene expression was quantified by Quantitect Primer assay (Qiagen) using Sensifast SYBR & Fluorescein Mix (Bioline) using the Quantitect 2-step amplification protocol. Gene expression was normalized to β-Actin (forward primer - ATTGCCGACAGGATGCAGAA, reverse primer – GCTGATCCACATCTGCTGGAA).

Detection of Human Serum IL-1β. Serum samples collected at the time of diagnosis of *C. difficile* diarrhea from 85 patients at the University of Virginia, as well as 104 with diarrhea but negative for *C. difficile*, and 28 healthy outpatients and stored at -80C until assayed. IL-1β in the serum samples was quantified using the eBioscience High-Sensitivity ELISA kit according to manufacturer instructions. The study was approved by the University of Virginia Institutional Review Board for Health Sciences Research.

Chapter Three: Glucosylation by Toxin A drives innate inflammation

Carrie A. Cowardin, , Brianna M. Jackman [1], Zannatun Noor [2], Stacey L. Burgess [2], Andrew Feig [1], William A. Petri, Jr.

[1] B.M.J. and A.F. provided toxin and mutants and assisted with experimental design
[2] Z.N. and S.L.B assisted with mouse experiments

3.1 Introduction

As the primary virulence factors of *C. difficile*, both Rho-glucosylating Toxins A and B cause cell death and induce inflammation. Although the toxins are structurally similar, remarkable differences in their ability to intoxicate and kill cells have been noted. In a cecal injection model of intoxication, Toxin A has been shown to induce a dramatic inflammatory response leading to fluid accumulation and cytokine production. In contrast, Toxin B is less effective at inducing inflammation in this model [97], leading to the hypothesis that disruption of tissue by Toxin A may be a prerequisite for Toxin B activity. Toxin A and B activities also differ *in vitro*, where Toxin B is more effective at eliciting cell death at low concentrations [98].

Although both toxins are thought to share a similar mechanism of intoxication, the role of each step of this process in eliciting cell death is not well understood. To intoxicate cells, both toxins bind to host cell receptors which are then internalized via receptor-mediated endocytosis. Acidification of the endosomal compartment leads to insertion of the translocation domain into the endosomal membrane. The glucosylation domain is then translocated out through a pore formed by the translocation domain. The cysteine protease domain is responsible for autoprocessing of the glucosyltransferase domain which is released into the cytoplasm, where it modifies Rho GTPases by covalently attaching a glucosyl moiety. This modification prevents the GTPase function of RhoA, Rac1 and Cdc42 [26].

Recent reports indicate that autoprocessing of the cysteine protease domain is dispensable for the induction of cell death. Recombinantly expressed toxin containing inactivating mutations in the cysteine protease domain were capable of killing cultured

epithelial cells [27]. The same study also found that glucosyltransferase deficient Toxin B (TcdB D270N) was able to cause caspase 3/7 activation and LDH release from epithelial cells, suggesting that glucosyltransferase activity was also not required for cell death to occur. Evidence also suggests Toxin B is capable of inducing two distinct phenotypes, with separate mechanisms. Low concentrations of Toxin B lead to cell rounding due to Rho glucosylation, while high concentrations lead to pyknotic cell death characterized by shrinkage of cells and chromatin condensation [99]. Thus, cell rounding and cell death may be glucosyltransferase dependent and independent, respectively [100]. However, this distinction is less well understood for Toxin A. Although Toxin A also causes cell death, this process seems to be primarily dependent on glucosyltransferase activity as mutation of the glucosyltransferase domain resulted in diminished cellular apoptosis[101,102]. Therefore, it appears that Toxins A and B may activate distinct cell death pathways, with Toxin A dependent cell death requiring glucosylation while Toxin B elicits glucosylation-independent cell death at high concentrations.

Interestingly, differences in the ability of Toxin A and B to cause an inflammatory response have also been documented. While both toxins are able to activate the inflammasome to secrete IL-1β, Toxin B is able to induce inflammasome activation at much lower concentrations than Toxin A [38], mirroring the cell death phenotype. Conflicting results have been reported on the ability of Toxin B to induce inflammasome activation in the absence of glucosyltransferase activity, with one group reporting that glucosyltransferase deficient Toxin B was able to induce IL-1β secretion in the human monocytic cell line THP-1. A second group reported that glucosyltransferase deficient Toxin B was unable to induce IL-1β secretion in murine primary bone marrow derived

macrophages [39]. Although both groups used similar mutations to prevent glucosylation activity, they were not identical, perhaps explaining the difference in results obtained. Alternatively, there may be differences in the response to the toxins between murine and human cells. The role of glucosylation in inflammasome activation by Toxin A is less well understood. Although it has been reported that Toxin A glucosylation activity is necessary for TNFα production, [103] inflammasome activation is a distinct process which requires two separate signaling events to occur. The first step, referred to as "priming", involves recognition of a danger signal which activates the transcription factor NFκB and induces pro-IL-1β gene expression [83]. The second "activation" step leads to assembly of the inflammasome and activation of Caspase 1. Caspase 1 is responsible for the processing of pro-IL-1β into its mature, secreted form. Although multiple inflammasomes which recognize diverse stimuli have been identified, evidence suggests that the Pyrin inflammasome is primarily responsible for IL-1β secretion in response to Toxins A and B [39]. Indeed, this inflammasome is thought to be activated in response to Rho GTPase modification, lending support to the idea that glucosylation is required for inflammasome activation, particularly with regard to Toxin B.

In order to clarify the role of Rho glucosylation by Toxin A in inflammasome activation, as well as to further investigate glucosylation *in vivo*, we utilized purified Toxin A (WT-TcdA) as well as an enzymatically inactive mutant of Toxin A (NVN-TcdA) generated by Andrew Feig and Brianna Jackman at Wayne State University. Originally developed by Teichert et al, NVN-TcdA possesses two mutations (D285/287N) which together reduce glucosylation of Rho family proteins by 6,900 fold compared to the wild type toxin [104]. These mutations have been used extensively in the literature to investigate

glucosylation-dependent phenotypes and the mutant toxin is capable of entering cells to a similar extent as wild type Toxin A [103]. We use this mutant to show for the first time that inflammasome activation by Toxin A is enhanced by Rho glucosylation, and that glucosylation is absolutely required for tissue damage and an inflammatory response *in vivo*.

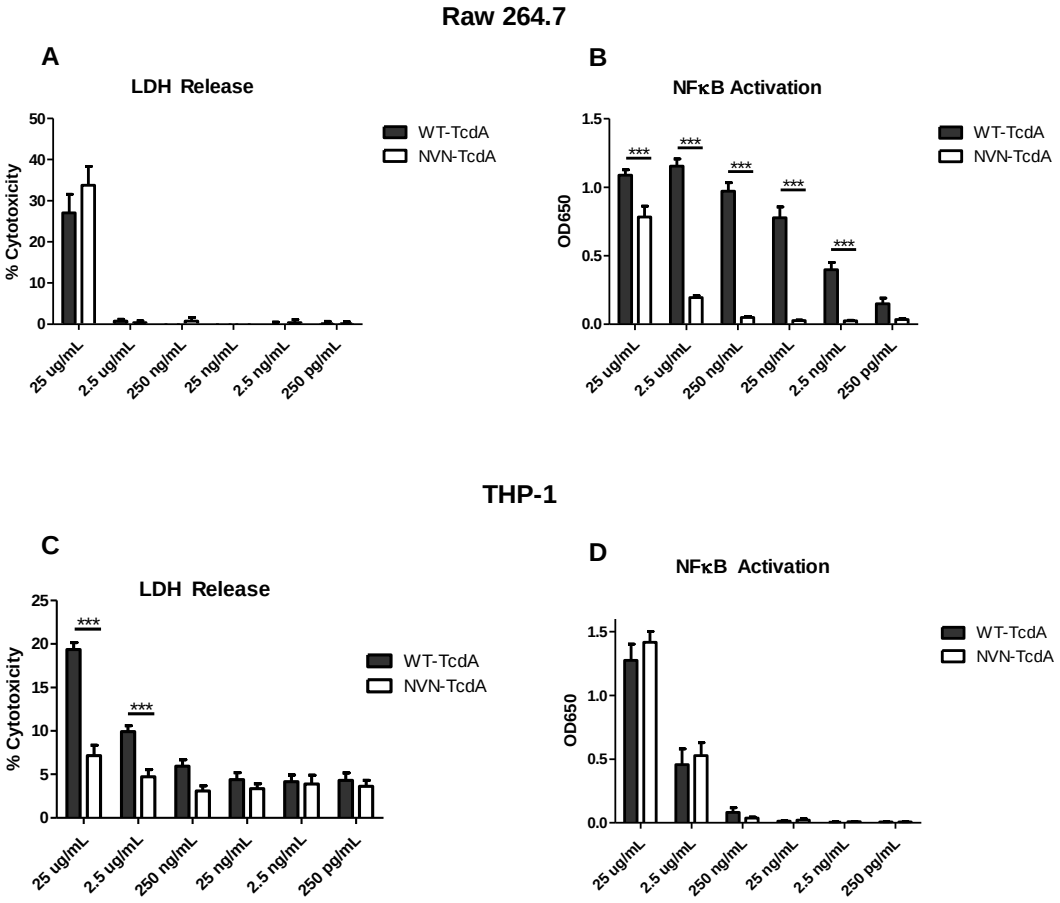
3.2 Results

To begin to assess the role of glucosylation in the response to Toxin A, we first examined the ability of WT-TcdA and NVN-TcdA to cause cell death and activate the transcription factor NFκB. We utilized two distinct cell lines which both express a gene encoding the colorimetric reporter Secreted Embryonic Alkaline Phosphatase (SEAP) expressed by a promoter containing multiple NFκB binding sites. Cell death was assessed by measuring Lactate Dehydrogenase (LDH) release into the culture media which occurs following plasma membrane damage. Upon treating the cells with WT-TcdA and NVN-TcdA, we noted distinct differences in cell death and NFκB activation between the two cell lines. In the human monocytic cell line THP-1, WT-TcdA induced significantly more cell death at the two highest concentrations compared to NVN-TcdA (Figure 3.1A). However, in the murine macrophage cell line Raw 264.7, no significant differences in cytotoxicity were noted (Figure 3.1C). In contrast, no difference in the ability of WT-TcdA and NVN-TcdA to activate NFκB were observed in THP-1s, while in Raw cells, WT-TcdA was able to induce significantly more NFκB activation at almost all concentrations tested (Figure 3.1B and 3.1D). These findings reinforce previous findings which indicate that different cell

types respond differently to intoxication with Toxins A and B [100], and suggest that NFκB activation and cell death may involve distinct pathways.

Figure 3.1: The contribution of Toxin A enzymatic activity to cell death and NFκB activation is cell type dependent.

Raw Blue NFκB reporter macrophages were exposed to either wild type or NVN TcdA at the indicated concentrations for 24 hours. Lactate dehydrogenase release into the culture media was assessed by fluorometric assay (**A**). SEAP levels in the culture media were quantified at 24 hours by QUANTI-Blue assay (**B**). THP-1 monocytes were exposed to wild type or NVN TcdA at the indicated concentrations. LDH (**C**) and SEAP (**D**) levels in the culture media were quantified at 24 hours. Data is shown combined from three independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.

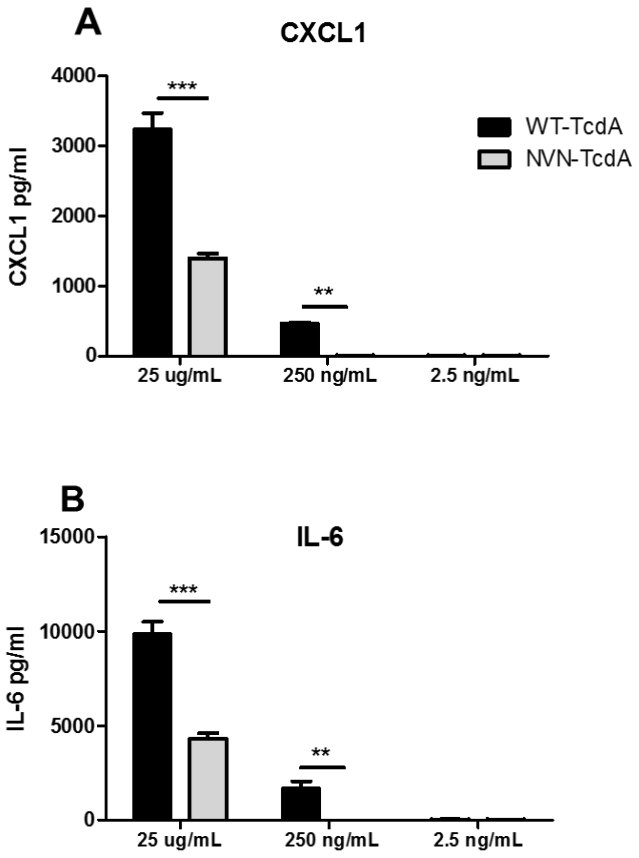

Raw 264.7
A
LDH Release
% Cytotoxicity
WT-TcdA
NVN-TcdA
25 ug/mL
2.5 ug/mL
250 ng/mL
25 ng/mL
2.5 ng/mL
250 pg/mL
B
NFκB Activation
OD650
***
THP-1
C
LDH Release
D
NFκB Activation

Next, we investigated the ability of NVN-TcdA to induce secretion of cytokines, including IL-6 and CXCL1. IL-6 is a pro-inflammatory cytokine with numerous diverse functions, and has been found to be highly upregulated during *C. difficile* infection [105,106]. CXCL1, a chemokine responsible for recruiting neutrophils in mice and a murine homolog of human IL-8, is also dramatically increased during infection [107]. Using primary murine bone marrow derived dendritic cells (BMDCs) we found that WT-TcdA was able to induce significantly higher levels of both IL-6 and CXCL1 than NVN-TcdA at two different concentrations (Figure 3.2A and 3.2B).

Because these results suggested that glucosylation contributed to cytokine production, we next investigated the ability of NVN-TcdA to induce IL-1β secretion. We found that, alone, neither WT-TcdA nor NVN-TcdA induced significant levels of IL-1β (Figure 3.3A). This was expected, as Toxin A able to activate but not prime the inflammasome. However, when lipopolysaccharide was added as a priming signal in combination with WT or NVN TcdA, significant IL-1β release was detected. WT-TcdA induced higher levels of IL-1β secretion than NVN-TcdA from the murine BMDCs, suggesting that glucosylation does contribute to inflammasome activation in response to Toxin A. We verified this phenotype in human THP-1 cells by measuring Caspase 1 in the culture media, which is responsible for the cleavage of pro-IL-1β and is secreted upon inflammasome activation [108]. In human monocytes, significantly more Caspase 1 was detected in the BMDC supernatants in response to WT-TcdA than NVN-TcdA (Figure 3.3B), supporting the observation that glucosylation contributes to inflammasome activation.

Figure 3.2: Toxin A enzymatic activity enhances cytokine secretion.

Murine bone marrow derived dendritic cells were incubated for 24 hours with wild type or NVN TcdA at the indicated concentrations. CXCL1 (**A**) and IL-6 (**B**) protein levels within the culture supernatants were assessed by ELISA. Data is shown combined from three independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.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CXCL1 pg/ml
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3000
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WT-TcdA
NVN-TcdA
25 ug/mL
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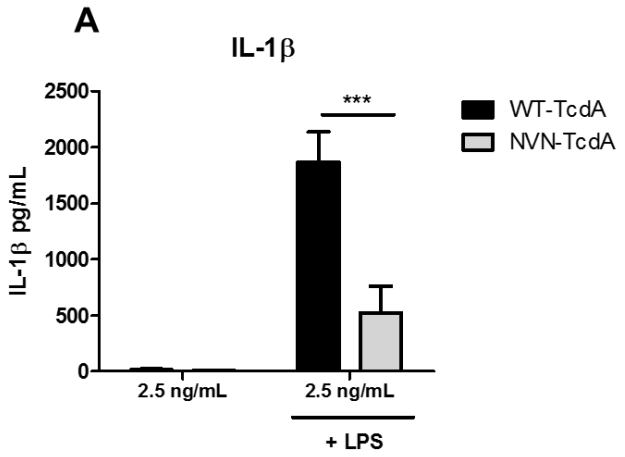
2.5 ng/mL
B
IL-6
IL-6 pg/ml
15000
10000
5000
0
***
**
25 ug/mL
250 ng/mL
2.5 ng/mL

Figure 3.3: Toxin A enzymatic activity enhances inflammasome activation.

Murine bone marrow derived dendritic cells were incubated with 2.5 ng/ml wild type or NVN TcdA for 24 hours in the presence or absence of LPS as a priming signal. Inflammasome activation indicated by IL-1β secretion was assessed by ELISA (**A**). Human THP-1 monocytes were incubated for 24 hours with wild type or NVN TcdA and Caspase 1 release was evaluated by ELISA (**B**). Data is shown combined from three independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.


A
IL-1β
2500
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WT-TcdA
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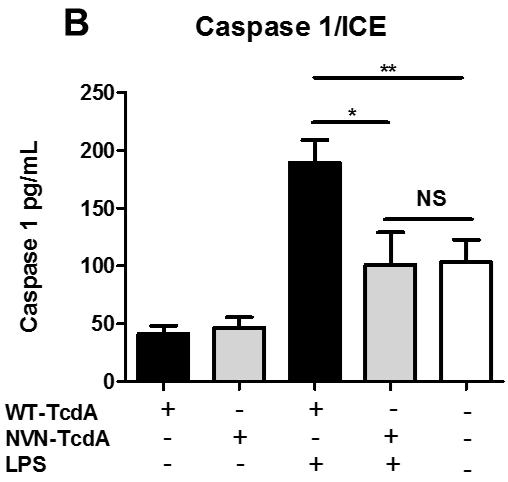
+ LPS


B
Caspase 1/ICE
250
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NS
WT-TcdA + - + - -
NVN-TcdA - + - + -
LPS - - + + -

Because glucosylation appeared to be key to the induction of inflammation, we next tested whether this enzymatic activity contributes to inflammatory processes *in vivo*. To answer this question, we used a cecal injection model of intoxication. In this procedure, mice are anaesthetized and undergo laparotomy during which purified toxins are injected directly into the ceca. Eight hours following injection of the toxins, tissue damage was assessed by examining haemotoxylin and eosin stained sections of the cecal tissue. We found that WT-TcdA induced dramatic tissue disruption after injection of purified toxin, while NVN-TcdA treated ceca appeared similar to mock treated controls (Figure 3.4A to C). Sections were assessed for epithelial disruption, inflammatory infiltrate, submucosal edema, mucosal thickening and luminal exudate. Quantification of the histological scores revealed a significantly higher damage score for WT-TcdA treated mice compared to NVN-TcdA treated mice, demonstrating that glucosylation activity is critical for tissue disruption and inflammation *in vivo* (Figure 3.4D). In order to quantify inflammatory mediators following intoxication, we assessed cytokine levels within the cecal tissue of these mice. IL-6, CXCL1 and IL-1β closely mimicked the trend observed with histological scores, with WT-TcdA inducing significantly more of these cytokines compared to mock treated and NVN-TcdA treated groups, which were roughly equivalent (Figure 3.5A to C). Thus glucosylation is necessary both for tissue damage and for an inflammatory response during intoxication, raising the idea that disruption of glucosylation could provide a viable therapy for CDI.

Figure 3.4: Toxin A enzymatic activity is required for tissue damage.

15 µg of equivalent buffer (Mock, **A**) recombinant Wild-Type TcdA (**B**), or mutant TcdA (**C**) was administered intracecally via laparotomy. The incision was closed and animals were monitored for 8 hours before being humanely euthanized. Cecal sections were fixed in Bouin's solution for 18 hours before undergoing paraffin embedding, sectioning and haemotoxylin & eosin staining. Samples were scored blinded based on 5 parameters (submucosal edema, inflammatory infiltrate, epithelial disruption, luminal exudate and mucosal thickening). Data shown represents the cumulative score for each section (**D**). Data shown is representative of two independent experiments (n=13). * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.

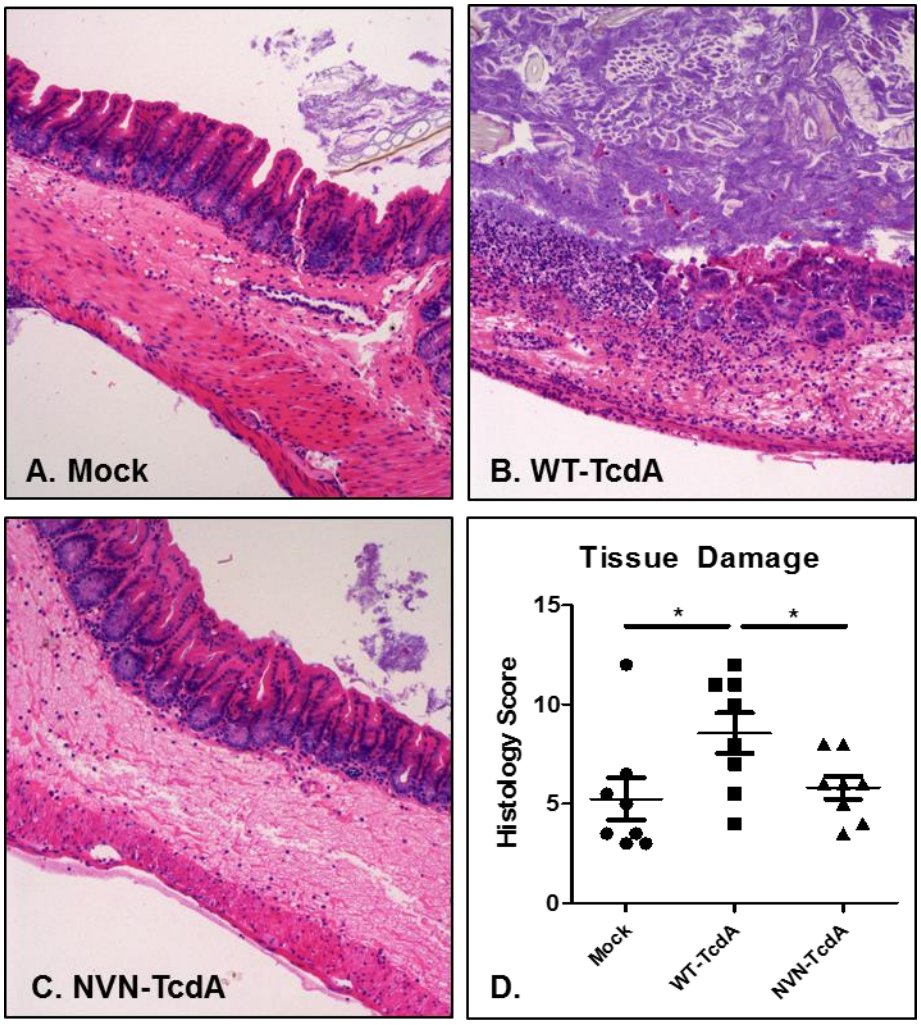
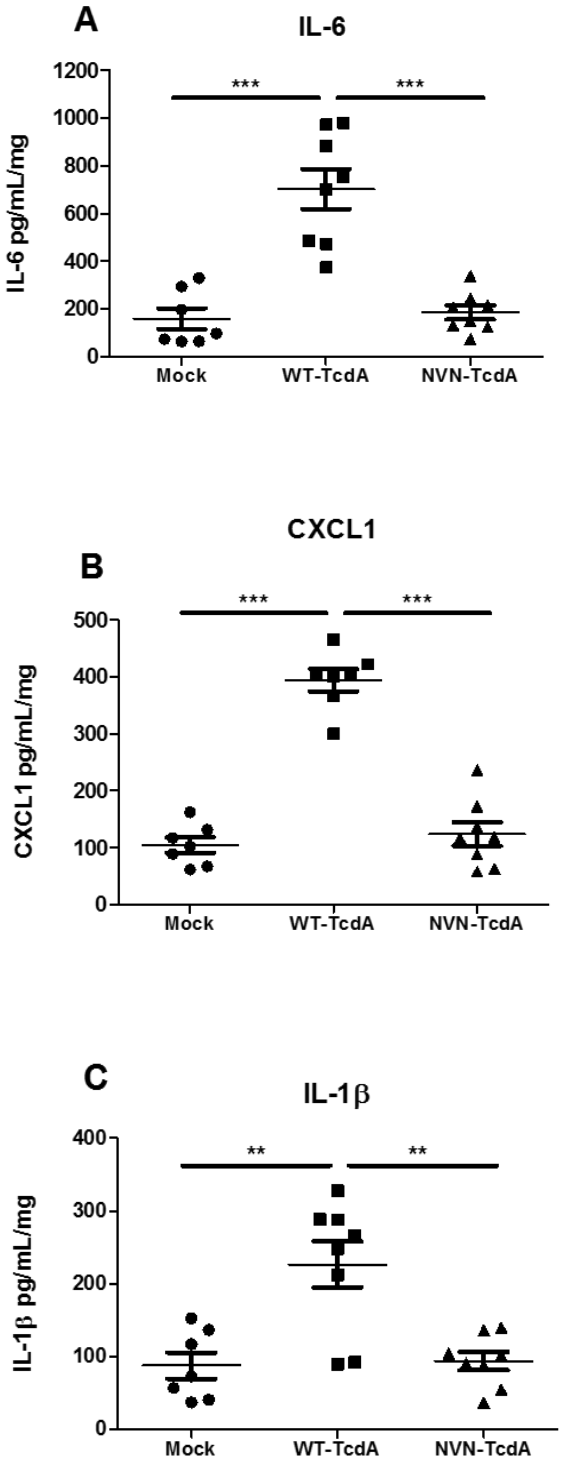

A. Mock
B. WT-TcdA
C. NVN-TcdA
Tissue Damage
Histology Score
15
10
5
0
*
*
Mock
WT-TcdA
NVN-TcdA
D.

Figure 3.5: Toxin A enzymatic activity is required for inducing an inflammatory response *in vivo*.

Mice were challenged with 15 µg wild type TcdA or NVN TcdA injected directly into the cecum via laparotomy. Cecal cytokine protein levels were assessed 8 hours following injection by lysing tissue and subsequent ELISA for IL-6 (**A**), CXCL1 (**B**) and IL-1β (**C**). Data shown is representative of two independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.


A
IL-6
IL-6 pg/mL/mg
1200
1000
800
600
400
200
0
***
***
Mock
WT-TcdA
NVN-TcdA
B
CXCL1
CXCL1 pg/mL/mg
500
400
300
200
100
0
***
***
Mock
WT-TcdA
NVN-TcdA
C
IL-1β
IL-1β pg/mL/mg
400
300
200
100
0
**
**
Mock
WT-TcdA
NVN-TcdA

3.3 Discussion

As essential virulence factors for *C. difficile*, Toxins A and B are well understood to cause significant tissue damage and inflammation. Although the mechanisms are less clear, both toxins are able to kill cells and induce inflammatory signaling. The role of glucosylation in activation of the inflammasome by Toxin B has been investigated previously, with contradictory results reported. The contribution of glucosylation by Toxin A is less well characterized. Here we sought to determine whether Rho-glucosylating activity following intoxication with Toxin A was essential for cytotoxicity, activation of the inflammasome and cytokine secretion. Treatment of cells with WT-TcdA and NVN-TcdA revealed dramatic differences in cytotoxicity and NFκB activation which were cell type dependent. Intoxication of human monocytes with WT-TcdA induced dose-dependent cell death which was dramatically attenuated in the absence of glucosylation activity. However, both WT-TcdA and NVN-TcdA displayed a similar ability to activate NFκB in these cells. Conversely, in murine macrophages, there was no difference between WT-TcdA and NVN-TcdA with regards to cell death, while WT-TcdA was significantly more effective at activating NFκB.

These results suggest that not only are the consequences of intoxication different depending on the cell type in question, but also that cell death and activation of cytokine production may follow different pathways. Both outcomes may be influenced by the magnitude of the signal generated, and this may also be influenced by cell type. Indeed, Raw macrophages are known to be deficient in an essential inflammasome adaptor protein, Apoptosis-associated speck-like protein or ASC [109]. This differential expression of ASC could influence both cytotoxicity and NFκB activation following intoxication.

Alternatively, differences in Toxin A receptor expression could account for some of the dissimilarities observed. Finally, another potential factor complicating this interpretation may be the presence of bacterial PAMPs within the toxin preparation, which could influence the activation of NFκB and be recognized differently by cell types expressing different PRRs. Indeed, purified Toxin B is frequently contaminated with *C. difficile* genomic DNA, although this has not been demonstrated for Toxin A [110]. Although differential consequences of intoxication depending on cell type have been reported previously, our data emphasizes the implications this may have for the immune response to *C. difficile* by suggesting that the type of immune cells recruited during an inflammatory response could profoundly shape the consequences of intoxication.

We have also identified a role for glucosylation by Toxin A in the inflammatory response *in vivo* as well as in primary murine bone marrow derived dendritic cells. Glucosylation was necessary for tissue damage to occur following cecal injection of the toxin. Similarly, production of IL-6, CXCL1 and IL-1β *in vivo* and *in vitro* also depended on glucosylation. Therefore, we conclude that glucosylation by Toxin A is a key step leading to the profound tissue damage and inflammation observed during infection with *C. difficile*. This work also highlights the idea that disruption of glucosylation using therapeutic inhibitors could be a viable strategy for treatment of CDI. Current treatments involve use of the same antibiotics which render patients susceptible, perpetuating a state of dysbiosis. Disruption of toxin activity is an attractive alternative therapy which would theoretically have minimal impact on protective host microbiota. Future work is needed to address the inhibition of glucosylation as a promising potential treatment for this increasingly common and poorly managed disease.

3.4 Methods

Mice and Cells. C57BL6 mice were purchased from the Jackson Laboratory. Mice were males aged 8-12 weeks for all experiments. All animals were housed under specific pathogen free conditions at the University of Virginia's animal facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. BMDCs were generated as previously described with minor modifications [111]. Briefly, femurs and tibia were removed and bone marrow flushed with PBS. Cells were counted and viability assessed by Trypan Blue staining and resuspended in RPMI 1640 media (Life Technologies) containing 10% Fetal Bovine Serum, 2mM L-glutamine and 100 U/ml Penicillin and 100 U/ml Streptomycin. Media was supplemented with 10 ng/ml GM-CSF (Peprotech) and 55 μM β-mercaptoethanol (Gibco), and approximately $3x10^6$ cells were seeded into a T75 vent cap tissue culture flask. Cells were cultured for 7 days, and supplemented with fresh media on days 2 and 4. On day 7 cells were harvested for stimulation. THP-1 Blue (#thp-nfkb) and Raw Blue (#raw-sp) cells were obtained from InvivoGen and grown according to the vendor's instructions.

Cell Stimulation. BMDCs were harvested and resuspended in complete RPMI 1640. $2x10^5$ cells per well were seeded into 96 well tissue culture plates. Toxins or buffer controls were added at the indicated concentrations. Stimulated cells were incubated for 24 hours at 37°C with 5% CO_2. Cells were spun down at 300 x *g* for 5 minutes and supernatant harvested and frozen down at -80°C. Raw Blue and THP-1 Blue cells were harvested and resuspended in complete media, and $1x10^5$ cells per well were seeded into 96 well tissue culture plates. Toxins or buffer controls were added at the indicated

concentrations and cells were incubated for 20 hours at 37°C with 5% CO_2. Cells were spun down at 300 x *g* for 5 minutes and supernatant harvested, except for positive controls for cytotoxicity, which were lysed with Triton X-100 according to manufacturer's instructions prior to supernatant aspiration.

Cecal Injection and Histology. Mice were anesthetized with ketamine-xylazine before surgery, and mid-line laparotomy was performed. The cecum was located and injected with 15 ug of purified WT-TcdA, NVN-TcdA, or "mock" control buffer in 100 ul of 0.9% normal saline as previously described [31]. Incisions were sutured and mice were monitored during recovery. Animals were monitored throughout the course of the 8 hour time period and humanely euthanized if moribund. To generate histological sections, mice were sacrificed at 8 hours and the ceca were removed and a sample placed in Bouin's Solution (Sigma) for 24 hours. Tissue samples were moved to 70% Ethanol before paraffin embedding and sectioning. Sections were mounted on slides and stained with haemotoxylin and eosin prior to microscopic examination. Slides were scored blinded, with a score from 0 to 3 assigned based on 5 parameters: epithelial disruption, submucosal edema, inflammatory infiltrate, mucosal thickening and luminal exudate. Scores were added for each sample and total score plotted for each animal.

Detection of Cytokines, NFκB Activation and Cell death. Cytotoxicity was measured immediately following supernatant removal by LDH release assay according to the manufacturer's instructions (Promega). NFκB activation was also measured immediately following supernatant removal by adding cell supernatant to Quanti-Blue reagent according to manufacturer's instructions. IL-1β, IL-6 and CXCL1 were detected in protein supernatants from BMDCs using the Mouse IL-1β and IL-6 Ready-Set-Go!

ELISA kits (eBioscience) and the Mouse CXCL1 DuoSet kit (R&D Systems) according to manufacturer's instructions. To measure cecal cytokines following cecal injection, total cecal lysate was generated by removing the ceca and rinsing gently with PBS. Tissue was bead beaten for 1 minute in 400 ul of Lysis Buffer I (1x HALT Protease Inhibitor (Pierce), 5 mM HEPES). 400 ul of Lysis Buffer II was added (1x HALT Protease Inhibitor (Pierce), 5 mM HEPES, 2% Triton X-100) and tubes inverted gently. Tissue samples were incubated on ice for 30 minutes, followed by a 5 minute spin at 13,000 x *g* at 4°C. Supernatant was removed to a fresh tube, and total protein concentration was assessed by BCA assay according to manufacturer's instructions (Pierce). Cytokine concentration is shown relative to total protein concentration.

Chapter Four: CDT expression enhances virulence by skewing the innate immune response

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[1] E.L.B, M.S., M.W. and S.L.B assisted with mouse experiments
[2] S.A.K. and N.P.M. generated mutants and provided advice on experimental design
[3] C.S. and K.A. purified CDT and provided advice on experimental design
[4] F.KN. provided anti-CDT nanobodies and advice on experimental design

4.1 Introduction

In addition to Toxins A and B, certain strains of *C. difficile* also express a third toxin. *C. difficile* transferase, or CDT (frequently referred to as "Toxin C" or binary toxin), is distinct from Toxins A and B and performs a separate enzymatic function. Although CDT is most notably expressed by ribotype 027 "hypervirulent" strains, other ribotypes, including the emerging ribotype 078, also express CDT. In fact, CDT expressing strains have become increasingly common over the last ten years, paralleling the overall increase in incidence and severity of CDI [112]. CDT+ strains now account for up to 20% of isolates in the hospital setting [113], and CDT expression has been associated with higher mortality, increased peripheral white blood cell count, and elevated risk of recurrence in clinical studies [2,49,114].

CDT belongs to a class of binary exotoxins and shares structural and functional similarity with *Clostridium perfringens* iota toxin and *Clostridium botulinum* C2 toxin [115,116]. These toxins consist of two subunits which act cooperatively to intoxicate cells. CDTb, the binding subunit of CDT, is produced as a precursor protein and requires proteolytic cleavage via trypsin prior to receptor binding. Following cleavage, CDTb associates with a cellular receptor, which is thought to be the lipolysis stimulated lipoprotein receptor, or LSR [117,118]. This receptor is highly expressed within the liver, small intestine, colon and various other tissues, and is involved in the uptake and removal of lipoproteins [41]. LSR has also been shown to be involved in the formation of tricellular tight junctions. Despite possessing a long cytoplasmic tail, a role for LSR in signaling has not been demonstrated [47]. Activated CDTb can bind as a heptamer, or as a monomer which subsequently heptamerizes. Following CDTb binding of the host receptor, the enzymatic

subunit CDTa associates with the CDTb heptamer. This complex is endocytosed, and endosomal acidification triggers insertion of the CDTb heptamer into the endosomal membrane, where it is thought to form a pore through which CDTa is released into the cytoplasm [44]. Once within the cytoplasm, CDTa transfers an ADP-ribose moiety from nicotinamide adenine dinucleotide to globular actin. ADP-ribosylated actin then acts as a capping protein and prevents actin filament elongation. The end result of intoxication by CDT is collapse of the actin cytoskeleton. One consequence of cytoskeletal disruption is the formation of long microtubule protrusions on the surface of host cells, which have been shown to modify vesicular trafficking from the endoplasmic reticulum and shuttle fibronectin to the surface of host cells [19,43]. As *C. difficile* possesses multiple fibronectin binding proteins, this process has been suggested to increase adherence of *C. difficile* to the colonic epithelium.

Although CDT expression has been associated with more severe disease outcome in multiple clinical studies, the mechanism underlying this increase is not well understood. Increased colonization via enhanced adherence of *C. difficile* to the host epithelium in the presence of CDT is one potential mechanism. However, recent clinical studies have suggested that the intensity of the host inflammatory response correlates more closely with disease outcome than bacterial burden [10]. Based on these findings, we hypothesized that CDT may play additional roles during infection by influencing the host inflammatory response. Mouse models have revealed that the type of immune response generated by the host during infection with *C. difficile* is critical in determining disease outcome. Innate "Type 17" responses, characterized by IL-1β, IL-6, and IL-17 production and neutrophil recruitment, are thought to be detrimental during infection with *C. difficile*. Our group has

demonstrated previously that Toxins A and B play a role in shifting the immune response towards this pathogenic inflammatory state. Therefore, we hypothesized that CDT may additionally enhance the production of a pathogenic host inflammatory response.

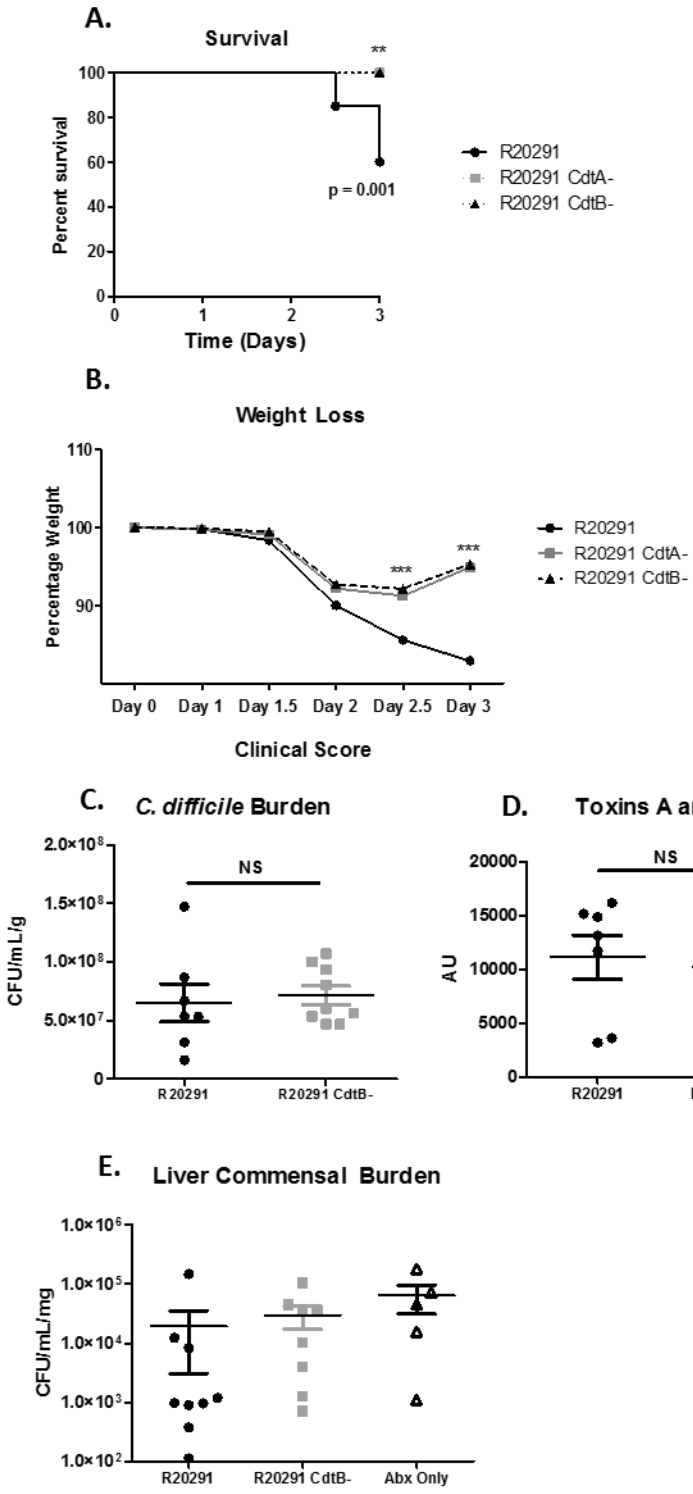
In order to investigate the role of CDT during infection, we utilized isogenic mutants of strain R20291 in a mouse model of *C. difficile* colitis. This isolate is a ribotype 027 strain expressing Toxins A and B as well as CDT. In this system, we show that CDT is a true virulence factor capable of driving pathogenesis independent of its colonization role. We found that CDT enhanced virulence by inducing pathogenic host inflammation via a novel Toll-like Receptor 2 dependent pathway, which resulted in suppression of a protective host eosinophilic response during infection.

4.2 Results

In order to isolate the role of CDT in virulence, we utilized the ribotype 027 strain R20291 in a murine model for severe infection [119]. This strain expresses Toxins A and B as well as CDT, allowing us to isolate the role of CDT while leaving Toxin A and B expression unchanged. Mice were infected with R20291 or one of the isogenic mutants R20291 CdtB- (lacking the binding subunit of CDT) or R20291 CdtA- (lacking the enzymatic subunit of CDT), originally generated by Kuehne et al [87].

Figure 4.1 CDT expression enhances the virulence of a ribotype 027 *C. difficile* isolate in a murine model of infection.

8 week old C57BL6/J mice underwent an antibiotic regimen prior to infection with 10^7 CFU of vegetative *C. difficile* strain R20291 or the isogenic mutants lacking the enzymatic domain of CDT (R20291 CdtA-) or the binding domain of CDT (R20291 CdtB-). Animals were monitored and weighed twice daily throughout the course of infection (**A**) and survival recorded (**B**). Animals were sacrificed at the peak of infection (day three) and *C. difficile* burden in the cecal contents enumerated anaerobically on BHI plates (**C**). Toxins A and B in the cecal contents were also assessed on day three via ELISA (**D**) and total liver bacterial burden was determined by plating liver homogenate on non-selective BHI and incubating aerobically overnight (**E**). Data shown is representative of at least two independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Kaplan-Meier survival analysis (**A**) and Student's T-test (**B**-**E**).


A.
Survival
**
Percent survival
100
80
60
40
20
0
p = 0.001
0
1
2
3
Time (Days)
R20291
R20291 CdtA-
R20291 CdtB-
B.
Weight Loss
Percentage Weight
110
100
90
***
***
Day 0
Day 1
Day 1.5
Day 2
Day 2.5
Day 3
R20291
R20291 CdtA-
R20291 CdtB-
Clinical Score
C.
C. difficile Burden
CFU/mL/g
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0
NS
R20291
R20291 CdtB-
D.
Toxins A and B
AU
20000
15000
10000
5000
0
NS
R20291
R20291 CdtB-
E.
Liver Commensal Burden
CFU/mL/mg
1.0×10^6
1.0×10^5
1.0×10^4
1.0×10^3
1.0×10^2
R20291
R20291 CdtB-
Abx Only

We found that an infectious dose of 10^7 colony forming units of R20291 lead to a mortality rate of 40% in this model, while the same dose of R20291 CdtA- or CdtB- resulted in 0% mortality by day 3 of infection (Figure 4.1A). Interestingly, this dose is significantly higher than the dose used in our lab to infect mice with strain VPI 10463 ($1x10^5$ CFU), which has been used historically in the murine model of CDI. However, VPI 10463 is known to produce Toxins A and B at very high levels, which could explain the lower infectious dose required. Overall, the animals infected with wild type R20291 lost significantly more weight than the groups infected with R20291 CdtA- or CdtB- (Figure 4.1B). By day three of infection, the CDT mutant infected groups began to recover weight and showed decreased clinical symptoms, indicating recovery from disease. At the same time point, groups infected with R20291 expressing CDT displayed increased weight loss and a high mortality rate. Thus, the mutants lacking CDT were attenuated at this dose.

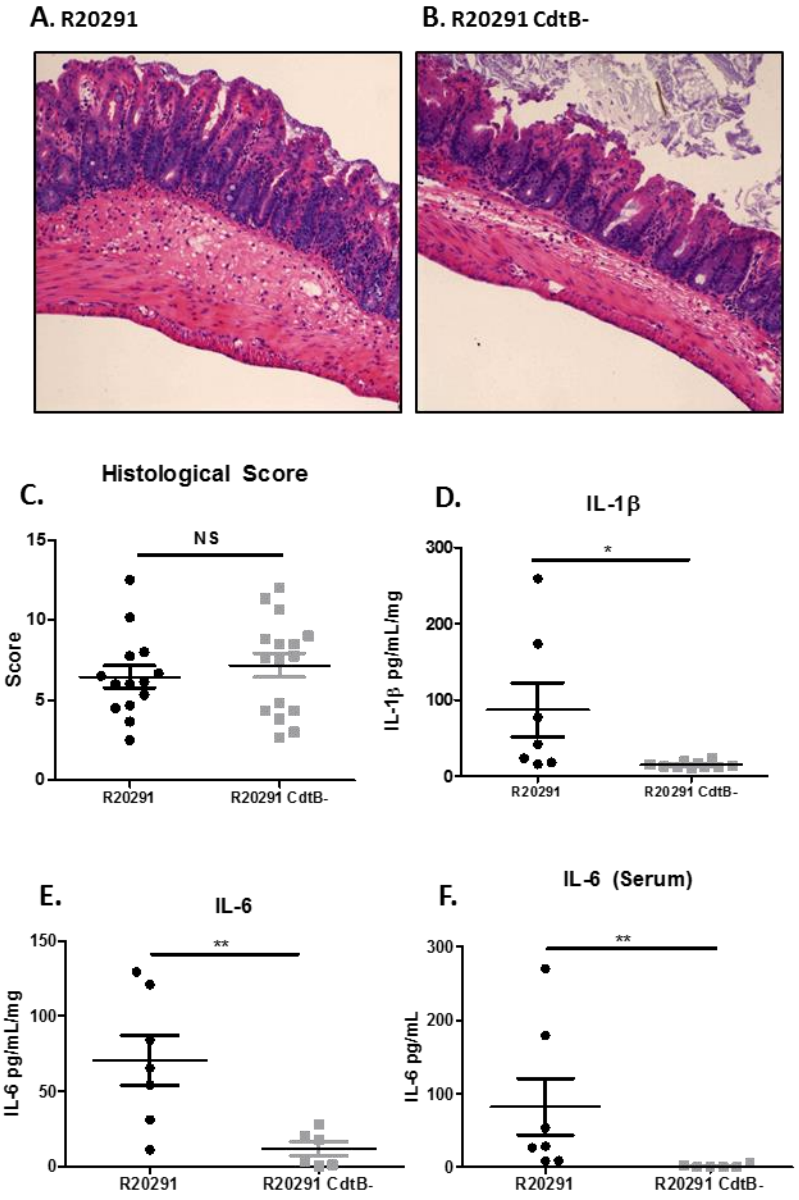
To begin to understand why the CDT mutant strains displayed less severe infection, we first measured *C. difficile* burden in cecal contents at the peak of infection (day three). Because CDT is thought to increase *C. difficile* colonization, we hypothesized that wild type R20291 would show increased burden compared to the CDT mutants. However, there was no difference in *C. difficile* burden at this time point (Figure 4.2C). Although this does not rule out differences in colonization at earlier time points, it does suggest that bacterial burden is not driving the high rates of mortality observed at this time point. We also examined the production of Toxins A and B, as different levels of these virulence factors could underlie disease severity. However, we did not observe differences in production of Toxins A and B (Figure 4.2D). Additionally, we examined the translocation of commensal microbes to the liver, which has recently been suggested to contribute to mortality during

CDI (Figure 4.2E). However, we did not observe a difference in the total number of bacteria isolated from the liver on non-selective media incubated aerobically, although this does not rule out differences in liver burden of species which do not grow under these conditions, such as anaerobic bacteria or non-culturable organisms. We also did not find a significant increase in the amount of translocating commensals over a control group which was treated with antibiotics but not infected, suggesting that mortality due to infected with R20291 is not driven by commensal translocation.

To further characterize the differences in disease manifestation in the presence or absence of CDT, we examined H&E stained histopathological sections of the ceca from groups infected with R20291 or R20291 CdtB- on day three post infection. The slides were scored according to five parameters (inflammatory infiltrate, submucosal edema, epithelial disruption, mucosal thickening, and lumenal exudate) by three independent, blinded observers. Surprisingly, we did not observe a difference in the total pathology score between groups infected with R20291 or R20291 CdtB- (Figure 4.2A.-C., n = 14 per group). Similarly, we did not note a statistical difference in any of the five parameters individually. Although a small number of mice infected with R20291 succumbed to disease on day 2 and were not included in this analysis, all surviving animals (including those observed to be moribund) were assessed. These results suggest that the mortality observed during infection with R20291 was not due to tissue damage, although some survival bias may have been present at this time point.

Figure 4.2 CDT expression does not enhance tissue pathology but influences inflammatory signaling on day 3 post-infection.

Cecal sections were fixed in Bouin's solution for 18 hours before undergoing paraffin embedding, sectioning and haemotoxylin & eosin staining. Samples were scored blinded based on 5 parameters (submucosal edema, inflammatory infiltrate, epithelial disruption, luminal exudate and mucosal thickening). Data shown represents the cumulative score for each section, averaged between three independent, blinded observers (**A**-**C**). Cecal cytokines were assessed by lysing whole cecal sections and quantifying protein via ELISA and are shown normalized to total protein concentration (**D**-**E**). Serum cytokines were measured via Luminex assay (**F**). Data shown is representative of at least two independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.

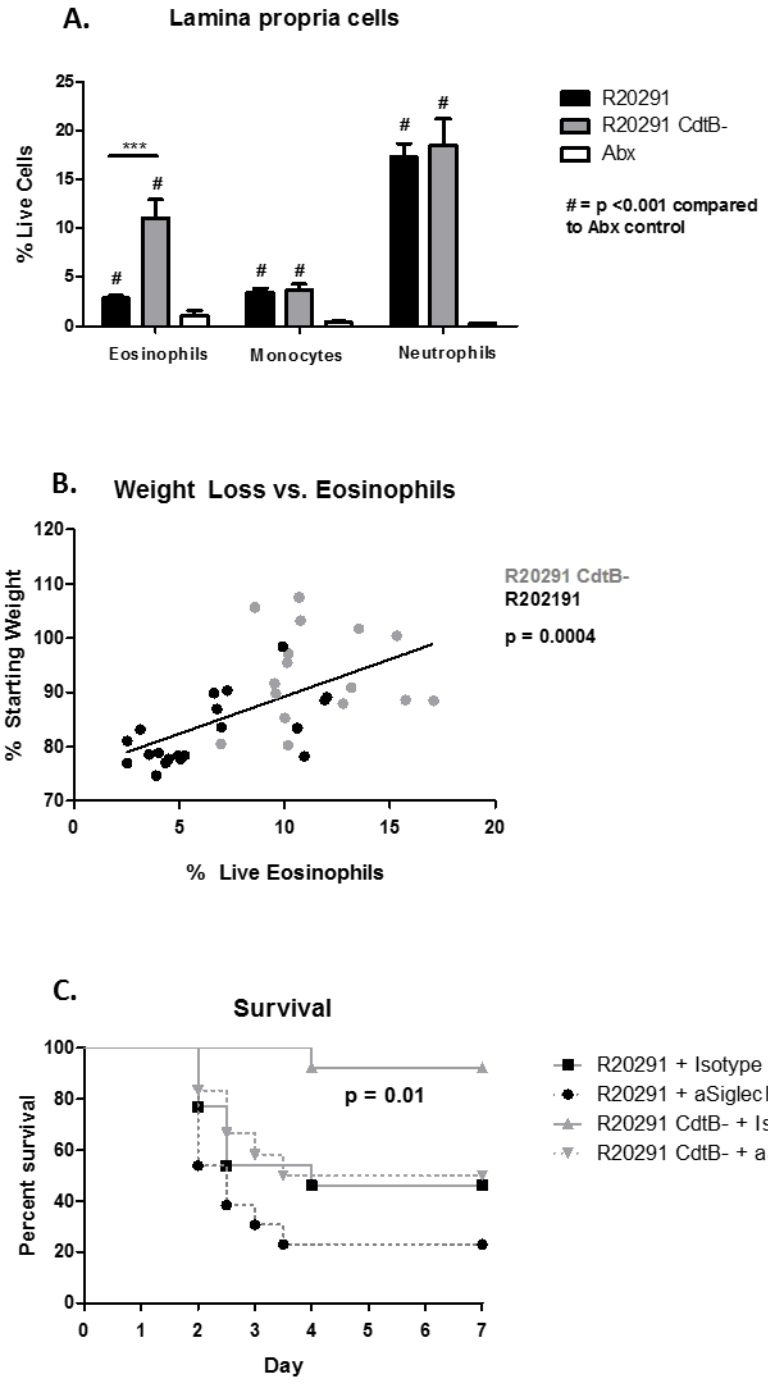

A. R20291
B. R20291 CdtB-
C.
Histological Score
NS
Score
15
10
5
0
R20291
R20291 CdtB-
D.
IL-1β
*
IL-1β pg/mL/mg
300
200
100
0
R20291
R20291 CdtB-
E.
IL-6
**
IL-6 pg/mL/mg
150
100
50
0
R20291
R20291 CdtB-
F.
IL-6 (Serum)
**
IL-6 pg/mL
300
200
100
0
R20291
R20291 CdtB-

Based on clinical data demonstrating a correlation between the host inflammatory response and disease severity, we hypothesized that a systemic inflammatory response could be contributing to mortality during infection with R20291. To investigate this possibility, we quantified the inflammatory cytokines IL-1β and IL-6 within cecal tissue. Both IL-1β and IL-6 are known to be highly upregulated during CDI and play influential roles in determining the type of immune response which develops. We found that R20291 expressing CDT induced significantly more cecal IL-1β and IL-6 than R20291 CdtB-. Similarly, when we assayed systemic IL-6 within the serum, we observed this cytokine to be significantly elevated. These results suggest R20291 expressing CDT is capable of inducing a stronger local as well as systemic inflammatory response within the murine host.

Because cytokine production shapes the immune response by influencing cell development, recruitment, proliferation and survival, we next examined the recruitment of various effector cells to the colon by flow cytometry. As expected, neutrophils (CD45+ CD11b+ Ly6G+ Ly6C+), which are strongly associated with CDI, dominated the innate compartment of both infected groups, representing roughly 20% of live cells in the colon. Monocytes (CD45+ CD11b+ Ly6C^{hi} Ly6G-) were also significantly elevated in the colon of both infected groups compared to uninfected, antibiotic treated controls (Figure 4.3A.) However, no difference was observed between neutrophils and monocytes in R20291 versus R20291 CdtB- infected groups, suggesting that differences in neutrophil and monocyte recruitment were not responsible for differences in survival. In contrast, eosinophils were significantly elevated in R20291 CdtB- infected mice compared to R20291 infected animals.

Figure 4.3 Protective colonic eosinophils are decreased in response to the CDT producing strain, R20291.

Mice infected with the indicated strain, or uninfected and treated with antibiotics were harvested on day three and colonic lamina propria processed to a single cell suspension and stained for flow cytometry. Eosinophils (CD45+ CD11b+ SiglecF+), Monocytes (CD45+ CD11b+ Ly6Chi) and Neutrophils (CD45+ CD11b+ Ly6G+) were quantified. Representative data from one independent experiment is shown (**A**) Weight loss and percent live eosinophils were compared using data from three independent experiments (**B**) Colonic eosinophils were depleted using 40 ug of an anti-SiglecF targeted antibody or an isotype control antibody one day prior and one day following infection with *C. difficile*. Animals were monitored for clinical symptoms and humanely euthanized when required. Data shown combined from two independent experiments (n = 13) (**C**). Data shown is representative of at least two independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Two-way ANOVA (**A**), linear regression (**B**) and Kaplan-Meier survival analysis (**C**).


A.
Lamina propria cells
% Live Cells
25
20
15
10
5
0
***
#
#
#
#
#
#
Eosinophils
Monocytes
Neutrophils
R20291
R20291 CdtB-
Abx
= p <0.001 compared to Abx control
B.
Weight Loss vs. Eosinophils
% Starting Weight
120
110
100
90
80
70
0
5
10
15
20
% Live Eosinophils
R20291 CdtB-
R202191
p = 0.0004
C.
Survival
Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Day
p = 0.01
R20291 + Isotype
R20291 + aSiglecF
R20291 CdtB- + Isotype
R20291 CdtB- + aSiglecF

As with neutrophils and monocytes, eosinophils were significantly increased in both groups compared to uninfected, antibiotic treated controls. Because the protected, R20291 CdtB- infected group showed increased eosinophils compared to R20291, we hypothesized that eosinophils may correlate with protection from mortality. To examine this more closely, we compared the amount of weight lost by each animal as a measure of disease severity against the percentage of eosinophils isolated from the colon on day three of infection. There was a significant correlation between increased weight loss and lower eosinophil counts, suggesting that eosinophils were associated with protection from disease (Figure 4.3B.).

To determine whether eosinophils themselves were protective, or simply associated with protection, we depleted eosinophils in mice infected with R20291 or R20291 CdtB- using an anti-SiglecF targeted antibody which has previously been shown to induce eosinophil apoptosis or an isotype control antibody [120] (Figure 4.3C.). As expected, animals infected with R20291 CdtB- and treated with an isotype control antibody survived infection. In contrast, depletion of eosinophils with anti-SiglecF prior to and during infection with R20291 CdtB- significantly enhanced mortality. In R20291 infected mice, depletion of eosinophils did not significantly enhance mortality, likely because eosinophil counts are already fairly low in this group. Overall, these findings demonstrate that an eosinophilic response is protective during CDI and that a ribotype 027 strain expressing CDT has a suppressive effect on eosinophils in the colon.

Next, we investigated the cause of the decrease in eosinophils observed in R20291 infected mice. Although we initially hypothesized that eosinophil growth factors or recruitment signals would be different between the groups, this proved not to be the case.

In fact, both R20291 and R20291 CdtB- infected mice produced equivalent levels of the eosinophil-specific chemokines eotaxin-1 and eotaxin-2 (Figure 4.4D.-E.). Eosinophil-promoting growth factors, such as IL-5, GM-CSF, IL-13, IL-33, IL-25 and TSLP, were unchanged between groups or elevated in R20291 infected mice, suggesting they were not responsible for the decrease observed *in vivo* (Figure 4.5A.-F.) [121–123]. As an alternative explanation, we hypothesized that differences in receptor expression could influence responsiveness to equivalent signals. Because expression levels of CCR3, the cognate receptor for the eotaxins, has been shown to influence eosinophil chemotaxis [124], we evaluated expression of this receptor during infection with R20291 and R20291 CdtB- (Figure 4.4A.-C.)

Figure 4.4 Fewer CCR3+ eosinophils are found in the cecum of mice infected with CDT+ *C. difficile*, while levels of eotaxins are unchanged.

Mice infected with the indicated strain, or uninfected and treated with antibiotics were harvested on day three. Colonic lamina propria was processed to a single cell suspension and stained for flow cytometry. Eosinophils were identified as CD45+ CD11b+ SiglecF+ SSC^{hi} cells (**A**) Eosinophils from mice infected with R20291 showed significantly less CCR3+ staining than eosinophils from mice infected with R20291 CdtB- (**B**) and the latter group had more CCR3+ eosinophils overall (**C**) Eotaxin-1 and Eotaxin-2 were quantified in cecal lysates by ELISA (**D**-**E**) and are shown normalized to total protein concentration. Data shown is representative of at least two independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.

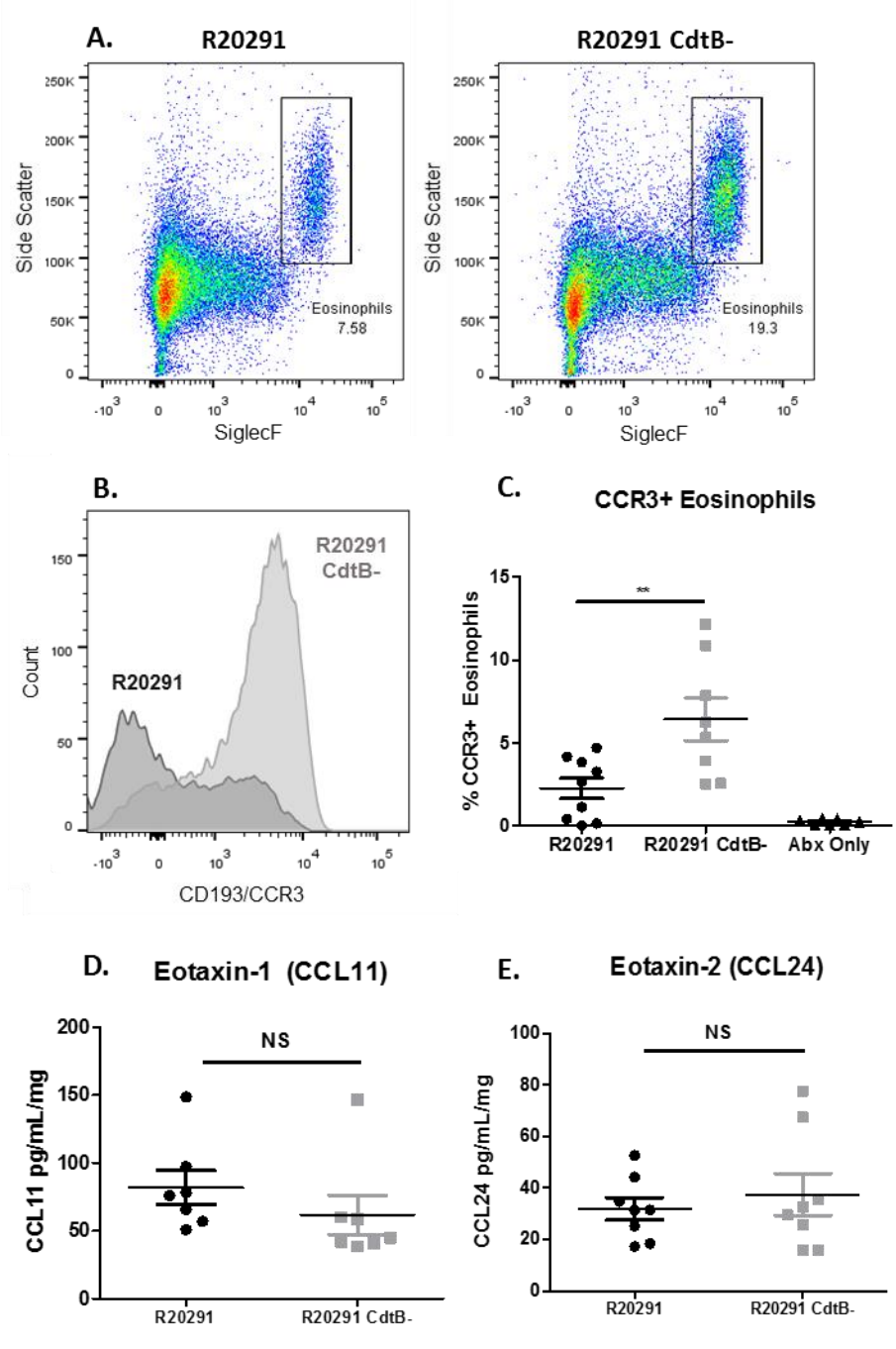
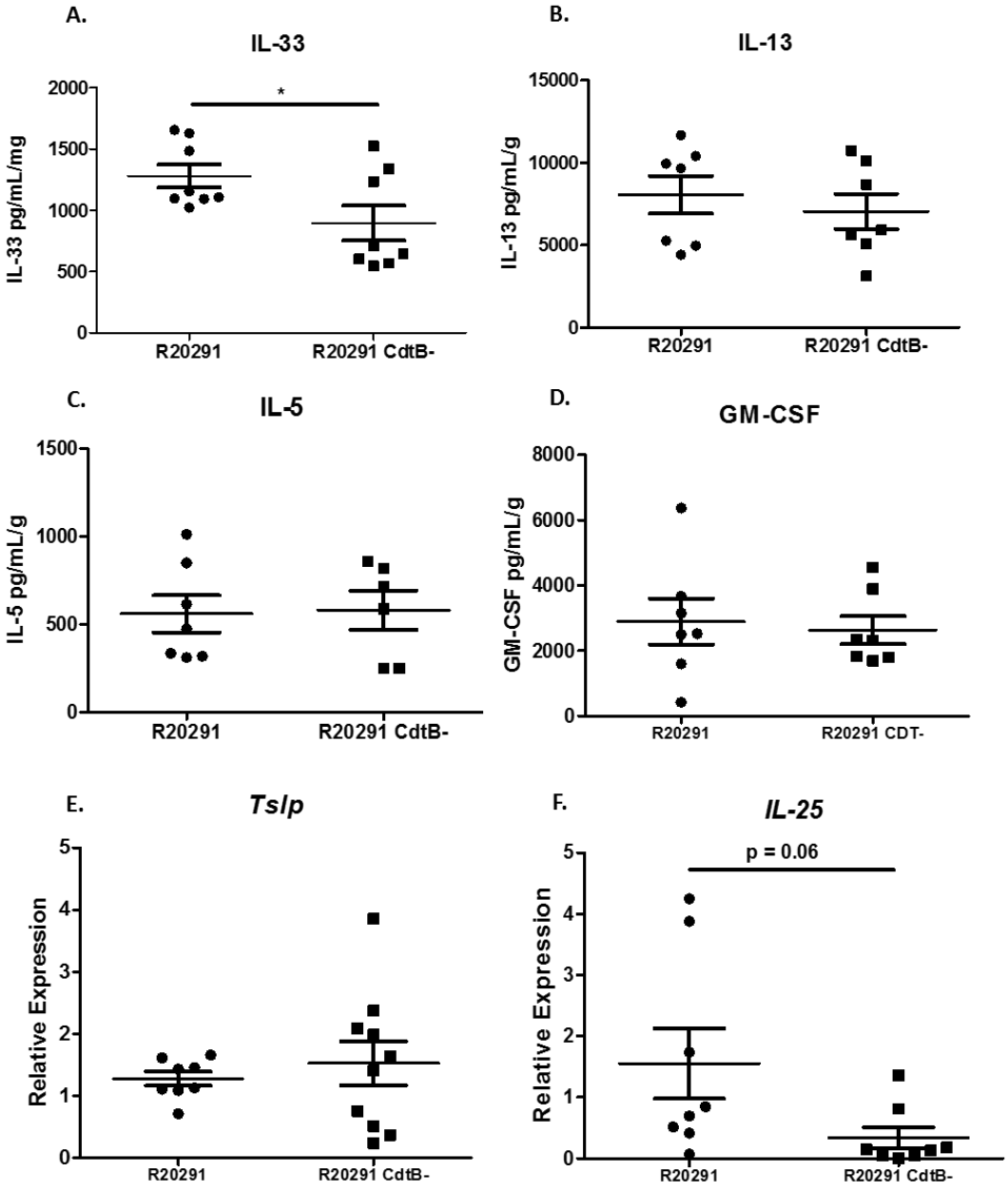

A.
R20291
R20291 CdtB-
Side Scatter
SiglecF
Eosinophils
7.58
Eosinophils
19.3
B.
R20291
R20291
CdtB-
Count
CD193/CCR3
C.
CCR3+ Eosinophils
% CCR3+ Eosinophils
**
R20291
R20291 CdtB-
Abx Only
D.
Eotaxin-1 (CCL11)
CCL11 pg/mL/mg
NS
R20291
R20291 CdtB-
E.
Eotaxin-2 (CCL24)
CCL24 pg/mL/mg
NS
R20291
R20291 CdtB-

Figure 4.5 Changes in Type 2 cytokines are not responsible for the decrease in eosinophils during infection with CDT+ *C. difficile*.

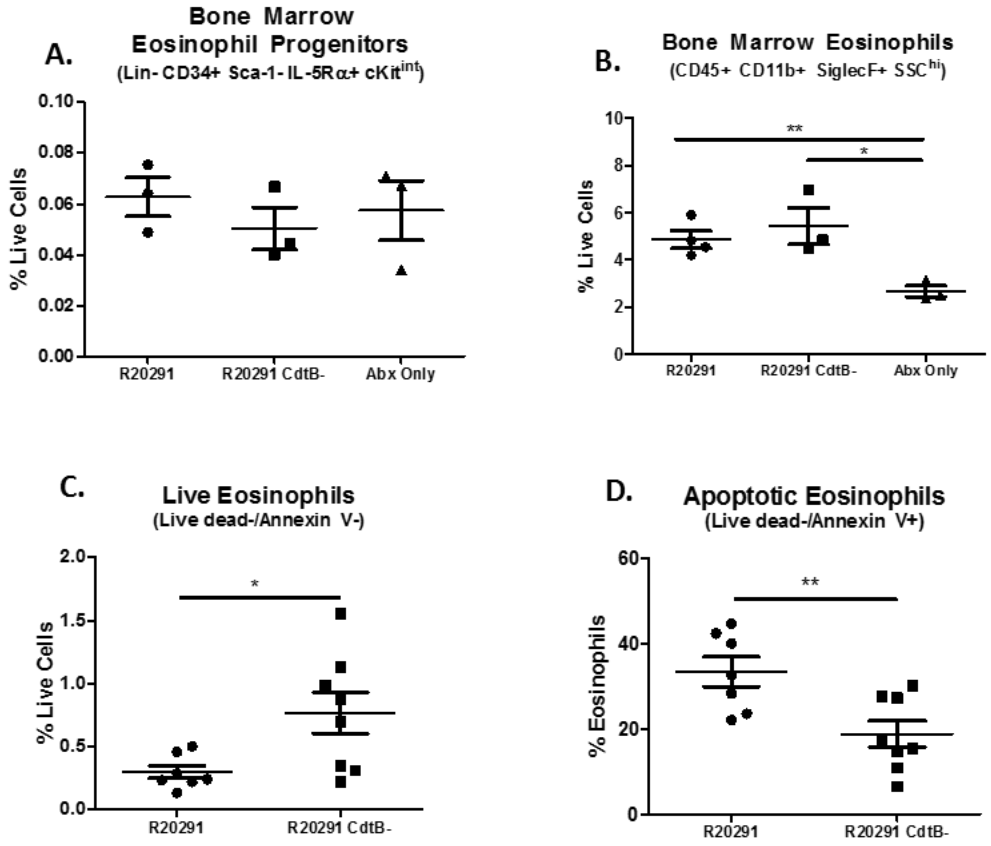
IL-33, IL-13, IL-5 and GM-CSF cytokine levels were assessed by ELISA (**A, D**) or Luminex assay (**C, E**) in whole cecal lysate from mice infected with R20291 or R20291 CdtB- on day 3 post infection and are shown normalized to total protein concentration. *Tslp* and *IL-25* gene expression was assessed by qRT-PCR (**E-F**) in whole cecal tissue from mice under the same conditions and expressed relative to GAPDH. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.


A.
IL-33
IL-33 pg/mL/mg
2000
1500
1000
500
0
*
R20291
R20291 CdtB-
B.
IL-13
IL-13 pg/mL/g
15000
10000
5000
0
R20291
R20291 CdtB-
C.
IL-5
IL-5 pg/mL/g
1500
1000
500
0
R20291
R20291 CdtB-
D.
GM-CSF
GM-CSF pg/mL/g
8000
6000
4000
2000
0
R20291
R20291 CDT-
E.
Tslp
Relative Expression
5
4
3
2
1
0
R20291
R20291 CdtB-
F.
IL-25
p = 0.06
Relative Expression
5
4
3
2
1
0
R20291
R20291 CdtB-

We observed a significant decrease in the number of CCR3+ eosinophils in the colon of R20291 infected mice compared to R20291 CdtB- infected animals. These results could indicate that eosinophils in R20291 infected mice are less responsive to eotaxins in the gut, thereby impacting recruitment to the gut. However, CCR3 upregulation is thought to be responsible for eosinophil egress from the bone marrow, and high levels of expression are associated with mature eosinophils [125]. Therefore, a decrease in CCR3+ eosinophils could also reflect a decrease in the number of mature eosinophils in the colon due to cell death.

To examine this possibility, we sought to determine where the eosinophil defect began in R20291 infected mice. Eosinophils develop within the bone marrow from eosinophil progenitors (EoPs), defined as Lineage-, CD34+, Sca-1-, IL-5Rα+, cKit^{int} cells [120,126–128]. Consistent with the similarities in eosinophil specific growth factors between groups, we did not observe a significant difference in the number of EoPs in R20291 or R20291 CdtB- infected mice (Figure 4.6A.). When we examined mature eosinophils within the bone marrow, we noted that both infected groups had significantly more eosinophils than uninfected, antibiotic treated mice, similar to eosinophil increases observed in the colon during infection (Figure 4.6B.). However, there were no differences in the number of mature eosinophils between the infected groups within the bone marrow. In contrast, when we examined eosinophils in the peripheral blood, we observed a decrease in live eosinophils in the R20291 infected group compared to those infected with R20291 CdtB- (Figure 4.6C.). This mirrored the phenotype observed in the colon and indicated that the eosinophil defect was systemic, although did not extend into the bone marrow itself.

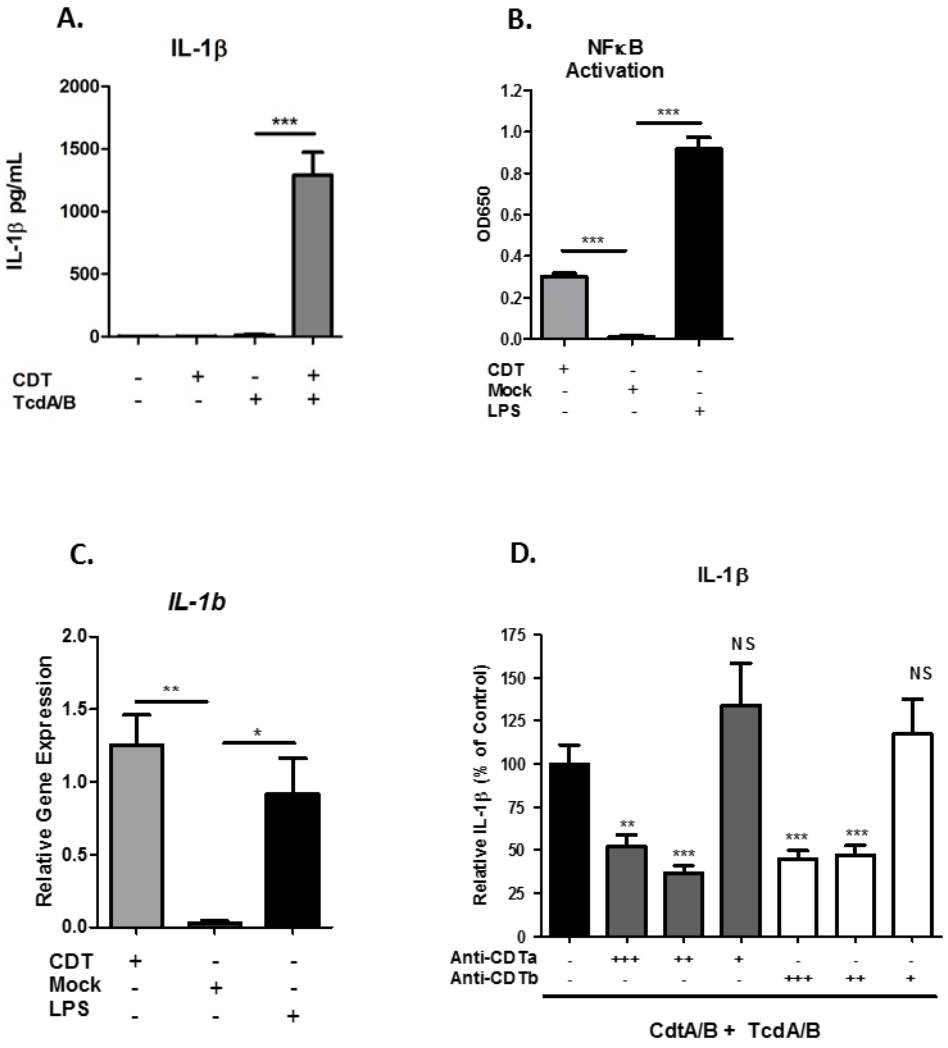
Figure 4.6 Blood eosinophils undergo apoptosis during infection with a CDT-producing strain while eosinophil progenitors in the bone marrow are unaffected. Mice were humanely euthanized on day 3 and bone marrow harvested for flow cytometry. Eosinophil progenitors (Lin- CD34+ Sca-1- IL-5Rα+ cKit^{int}) were quantified (**A**.) Mature bone marrow eosinophils (CD45+ CD11b+ SiglecF+ SSC^{hi}) were increased in infected groups compared to uninfected controls (**B**.) Blood eosinophils were assessed on day three of infection by flow cytometry following cardiac puncture, red blood cell lysis, and staining for flow cytometry. Live eosinophils were identified at CD45+ CD11b+ SiglecF+ Live dead- (**C**.) Apoptotic eosinophils were identified as CD45+ CD11b+ SiglecF+ cells which stained positive for Annexin V and negative for Live Dead viability dye (**D**.). Data shown is representative of at least two independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.


A.
Bone Marrow
Eosinophil Progenitors
(Lin- CD34+ Sca-1- IL-5Rα+ cKit^int)
% Live Cells
0.10
0.08
0.06
0.04
0.02
0.00
R20291
R20291 CdtB-
Abx Only
B.
Bone Marrow Eosinophils
(CD45+ CD11b+ SiglecF+ SSC^hi)
% Live Cells
10
8
6
4
2
0
**
*
R20291
R20291 CdtB-
Abx Only
C.
Live Eosinophils
(Live dead-/Annexin V-)
% Live Cells
2.0
1.5
1.0
0.5
0.0
*
R20291
R20291 CdtB-
D.
Apoptotic Eosinophils
(Live dead-/Annexin V+)
% Eosinophils
60
40
20
0
**
R20291
R20291 CdtB-

We also found that the eosinophils in circulation in mice infected with R20291 more frequently stained positive for Annexin V, a marker of apoptosis, which could explain the defect in mature eosinophils (Figure 4.6D.). Taken together, these results suggest that eosinopoiesis functions similarly within both groups of infected mice, but the mature eosinophils which exit the marrow in the R20291 infected group are more likely to undergo apoptosis, resulting in reduced eosinophilia in both the blood and colon of these animals.

In order to understand why eosinophils were dying in the mice infected with CDT+ R20291, we sought to determine how CDT signaling directly influences the immune response. To investigate this, we utilized bone marrow derived dendritic cells (BMDCs) as a model for cytokine production. We treated these cells with purified CDT in the presence or absence of Toxins A and B, and measured the amount of IL-1β secreted into the culture medium to assess inflammasome activation (Figure 4.7A). We chose to examine IL-1β initially because this cytokine was significantly elevated during *in vivo* infection with CDT+ R20291. Additionally, as we and other groups have shown, Toxins A and B are sufficient to activate the inflammasome, but a prior "priming" signal is required for robust IL-1β secretion. Therefore, understanding the relationship between CDT and inflammasome activation would be particularly informative to determining the impact of this toxin on immune signaling. We found that CDT alone does not induce IL-1β secretion, but CDT does enhance the secretion of IL-1β in the presence of Toxins A and B, suggesting that CDT acts as a priming signal prior to inflammasome activation.

Figure 4.7 Purified CDT primes the inflammasome by activating NFκB. Bone marrow derived dendritic cells were treated with 200 ng/mL CDTa and 200 ng/mL CDTb (CDT) or 2 ng/mL Toxin A and 2 ng/mL Toxin B (TcdA/B) for 24 hours. Secreted IL-1β was measured by ELISA (**A**). NFκB activation was detected in a Raw Blue NFκB reporter cells by measuring Secreted Embryonic Alkaline Phosphatase (SEAP) in the culture media (**B**) IL-1β gene expression in BMDCs was assessed by qRT-PCR following 24 hours of exposure to 200 ng/mL CDTa and 200 ng/mL CDTb. (**C**) BMDCs were treated with 200 ng/mL CDTa and 200 ng/mL CDTb plus 2 ng/mL Toxin A and 2 ng/mL Toxin B in combination with decreasing amounts of anti-CDTa nanobody or anti-CDTb nanobody as indicated. +++ = 200 ng/mL, ++ = 20 ng/mL, + = 2 ng/mL. Secreted IL-1β was measured by ELISA (**D**) Data shown combined from independent experiments. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Student's T-test.

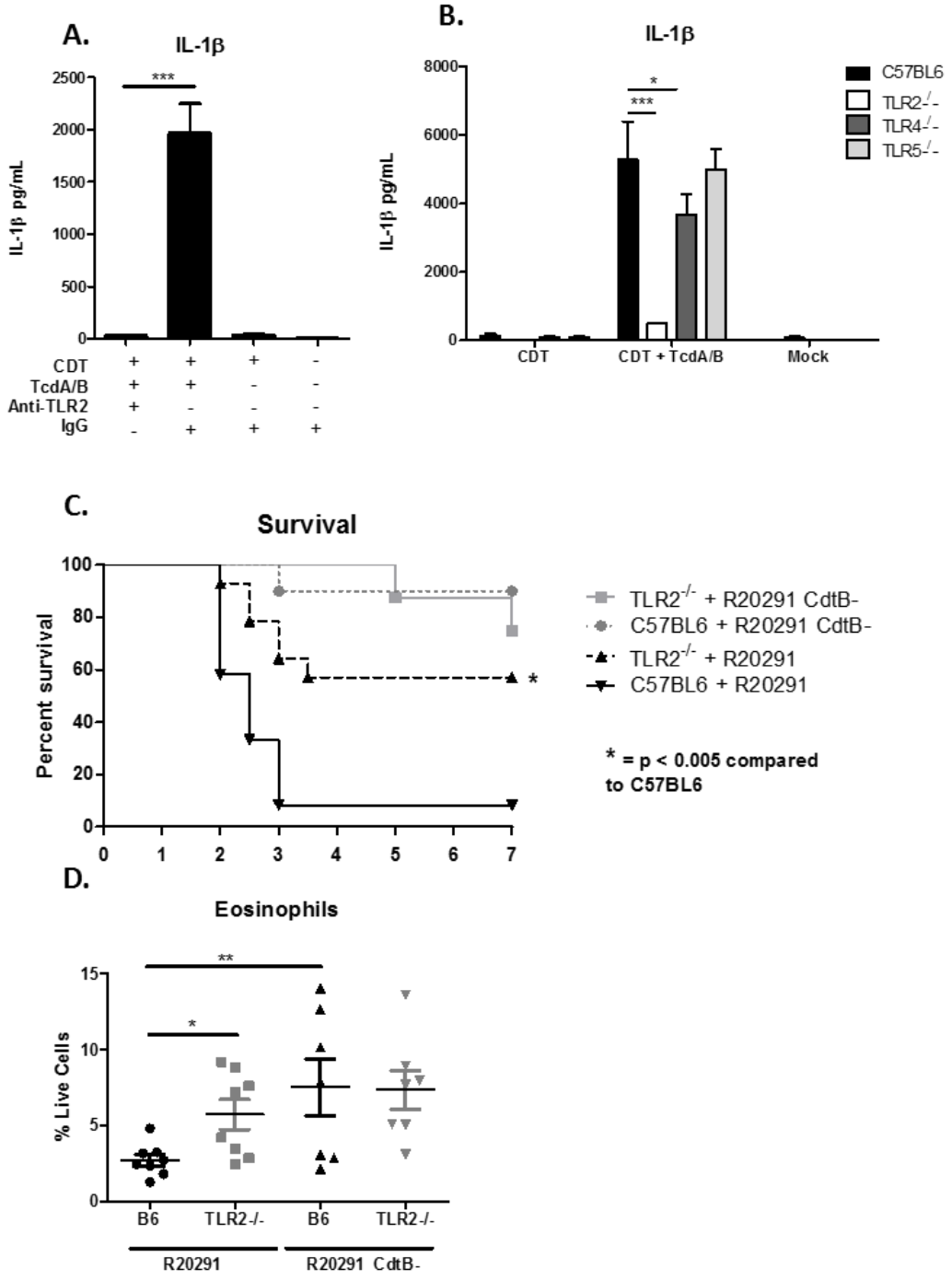

A.
IL-1β
IL-1β pg/mL
2000
1500
1000
500
0
***
CDT - + - +
TcdA/B - - + +
B.
NFκB Activation
OD650
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
CDT + - -
Mock - + -
LPS - - +
C.
IL-1b
Relative Gene Expression
2.0
1.5
1.0
0.5
0.0
**
*
CDT + - -
Mock - + -
LPS - - +
D.
IL-1β
Relative IL-1β (% of Control)
175
150
125
100
75
50
25
0
NS
NS
**
***
***
***
Anti-CDTa - +++ ++ + - - -
Anti-CDTb - - - - +++ ++ +
CdtA/B + TcdA/B

To investigate this further, we examined the ability of CDT to activate NFκB in a Raw 264.7 macrophage reporter cell line, by measuring the colorimetric reporter Secreted Embryonic Alkaline Phosphatase (SEAP). CDT alone was able to significantly activate NFκB over mock treated cells (Figure 4.7B.). We confirmed this phenotype by examining pro-IL-1β gene expression in BMDCs following treatment with CDT, and found that CDT can induce significant pro-IL-1β gene expression (Figure 4.7C.). In order to determine whether this phenotype was specific to CDT activity, we utilized anti-CDT targeted nanobodies which have previously been demonstrated to block ADP-Ribosylation by CDT [129]. At concentrations of 200 ng/mL and 20 ng/mL, the anti-CDTa and anti-CDTb nanobodies both significantly decreased the amount of IL-1β secreted by the BMDCs upon exposure to CDT and Toxins A and B, demonstrating that this phenotype is specific to CDT and requires both subunits to be functional (Figure 4.7D.). Thus, we concluded that exposure to CDT was sufficient to serve as a priming signal for inflammasome activation.

Because activation of NFκB is classically associated with Pattern Recognition Receptor (PRR) signaling, we next questioned whether these signaling pathways were involved in recognition of CDT to activate NFκB and mediate inflammasome priming. We utilized primary murine BMDCs from TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR5$^{-/-}$ mice, and interrogated their ability to secrete IL-1β following exposure to CDT and Toxins A and B. We found that TLR2$^{-/-}$ BMDCs were unable to respond to CDT, suggesting that this pathway may be involved in CDT recognition (Figure 4.8A.). To confirm this phenotype, we treated wild type C57BL6 BMDCs were CDT and Toxins A and B in the presence of an anti-TLR2 neutralizing antibody or an isotype control. The anti-TLR2 antibody abolished the release of IL-1β observed in the isotype control treated group (Figure 4.8B.).

Figure 4.8 TLR2 mediates CDT recognition and is required for eosinophil suppression.

TLR2, TLR4, and TLR5 knockout BMDCs were treated with 200 ng/mL CDTa and 200 ng/mL CDTb (CDT) and 2 ng/mL Toxin A and 2 ng/mL Toxin B (TcdA/B) for 24 hours and IL-1β was assessed by ELISA (**A**) BMDCs were treated with 200 ng/mL CDTa and 200 ng/mL CDTb (CDT) and 2 ng/mL Toxin A and 2 ng/mL Toxin B (TcdA/B) for 24 hours in the presence of a TLR2 neutralizing antibody or an isotype control. IL-1β was assessed by ELISA (**B**) 8 week old TLR2 knockout mice or C57BL6/J mice were cohoused for two weeks to normalize microbiota prior to infection with R20291 and the isogenic CDT mutant R20291 CdtB-. Animals were humanely euthanized when necessary (**C**) Mice were sacrificed on day three of infection and colonic eosinophils were measured by flow cytometry following tissue processing and staining (**D**). Data shown combined from three independent experiments (**A**-**B**) or representative of at least two independent experiments (**C**-**D**). * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 by Two-way ANOVA (**A**), Student's T-test (**B**, **D**), or Kaplan-Meier Analysis (**C**).


A.
IL-1β
IL-1β pg/mL
2500
2000
1500
1000
500
0
***
CDT + + + -
TcdA/B + + - -
Anti-TLR2 + - - -
IgG - + + +
B.
IL-1β
IL-1β pg/mL
8000
6000
4000
2000
0
*
***
C57BL6
TLR2-/-
TLR4-/-
TLR5-/-
CDT
CDT + TcdA/B
Mock
C.
Survival
Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
*
TLR2-/- + R20291 CdtB-
C57BL6 + R20291 CdtB-
TLR2-/- + R20291
C57BL6 + R20291
* = p < 0.005 compared to C57BL6
D.
Eosinophils
% Live Cells
15
10
5
0
**
*
B6
TLR2-/-
B6
TLR2-/-
R20291
R20291 CdtB-

To determine whether this interaction occurs *in vivo*, we next infected TLR2$^{-/-}$ mice alongside C57BL6 controls with R20291 or R20291 CdtB-. As expected, neither TLR2$^{-/-}$ nor B6 mice experience significant mortality after infection with R20291 CdtB-. However, TLR2$^{-/-}$ mice were significantly protected from mortality after infection with CDT-producing R20291, suggesting that TLR2 signaling enhances *C. difficile* virulence in the presence of CDT (Figure 4.8C.). Finally, we asked whether TLR2 may be involved in the pathogenic suppression of eosinophils observed during infection with R20291. To answer this question, we assessed eosinophilia in the colonic lamina propria via flow cytometry, and found that TLR2$^{-/-}$ mice displayed a trend towards increased eosinophilia after infection with R20291. No difference was noted after infection with R20291 CdtB-, suggesting that the increase in colonic eosinophils in TLR2$^{-/-}$ mice specifically occurs in the presence of CDT (Figure 4.8D.). Overall, these results suggest that infection with CDT+ R20291 leads to enhanced systemic and local inflammation during infection, and CDT directly or indirectly suppresses eosinophilia in the blood and colon via TLR2.

4.3 Discussion

Despite understanding the enzymatic function of CDT, the role of this toxin in enhancing *C. difficile* virulence remains incompletely characterized, and the influence of CDT on the host immune response has not been examined. We found that CDT was able to enhance the virulence of a ribotype 027 *C. difficile* strain independent of bacterial colonization, Toxin A and B production, or translocation of commensals to other organs (Figure 4.1). Instead, CDT enhanced production of Type 17 cytokines, including IL-1β and IL-6, and suppressed protective eosinophils within the colon (Figure 4.2-4.4). This defect

in eosinophil response was not due to a lack of Type 2 survival signals (Figure 4.4) and was also present systemically, although it did not extend into the bone marrow (Figure 4.5). Instead, we found that peripheral blood eosinophil apoptosis was elevated in the presence of CDT+ R20291 (Figure 4.5). We also examined the impact of purified CDT on immune cells and found that the toxin was able to enhance IL-1β secretion and serve as a priming signal for inflammasome activation (Figure 4.7). This required TLR2 signaling, and TLR2 deficient mice were protected from the CDT-producing strain R20291 *in vivo* and showed increased colonic eosinophils compared to wild type mice (Figure 4.8).

Although we were unable to identify a colonization defect in the strains lacking CDT, this does not rule out a contribution of this toxin to *C. difficile* adherence. More work is required to thoroughly assess this possibility, including an examination of lumenal and epithelial associated *C. difficile* CFU throughout different time points of infection. It is conceivable that CDT enhances colonization at earlier time points, allowing the infection to establish before an appropriate host inflammatory response is generated. This could result in increased inflammation at later time points as the immune response overreacts to this increased burden. Alternatively, similar levels of colonization could result in differential immune responses if *C. difficile* interacts differently with epithelial or immune cells in the presence of CDT. We have also not conclusively ruled out a role for CDT in disrupting the host epithelial barrier, which would again require examination of the progression of disease over time. In addition to liver commensal translocation, other markers of gut permeability need to be assessed. This could be accomplished by FITC-Dextran assay, which measures the ability of fluorescently conjugated Dextran to exit the gut into the bloodstream.

Although we have demonstrated that CDT is able to activate NFκB and enhance inflammatory cytokine production, it is unclear at which step this signaling occurs *in vivo*. CDT may be sensed by epithelial cells to promote recruitment of immune cells, which in turn enhance the inflammatory response. Alternatively, CDT may act directly on immune cells to enhance NFκB activation. It is also unclear how CDT signaling results in apoptosis of eosinophils. CDT signaling could potentially directly induce eosinophil apoptosis via a TLR2-dependent or independent mechanism. Another possible explanation is that CDT signaling may shape the inflammatory environment to support eosinophil apoptosis. Although Type 2 survival signals are not influenced by the presence of CDT, the toxin clearly enhanced production of Type 17 cytokines in a TLR2-dependent manner. Although is it unclear if these Type 17 signals can influence eosinophil survival, TLR2-dependent eosinophil suppression is not unprecedented, as multiple groups have reported in different murine models of allergic inflammation [130–132]. Administration of a TLR2 agonist has been shown to reduce eosinophilia by inducing T cell apoptosis in a model of allergic conjunctivitis, as well as by enhancing T regulatory cells and inducing Type 1 cytokines in murine asthma models. It remains to be investigated whether these mechanisms underlie eosinophil suppression during CDI, which takes place in an innate context where T cell involvement is unlikely.

Finally, the role of LSR in mediating an immune response to CDT is also unclear. *In vitro* studies suggest that this receptor is required for intoxication by CDT. Based on our findings that TLR2 mediates NFκB activation by CDT, it is possible that LSR and TLR2 mediate separate pathways in the response to the toxin. Alternatively, these receptors may cooperate to permit recognition and intoxication by CDT. Some evidence also exists that

CD44, a surface expressed glycoprotein, promotes intoxication with CDT [48]. CD44 has also been shown to associate directly with TLR2 [133], presenting the intriguing possibility that an interaction between TLR2 and CD44 may be involved in toxin recognition. Supporting the idea of interaction between these receptors, LSR has been shown to cluster into lipid rafts following CDTb binding, and evidence also exists for clustering of CD44 and TLR2 into lipid rafts following stimulation [134–138].

Understanding the mechanism by which CDT enhances *C. difficile* virulence is essential to understanding the virulence of ribotype 027 strains, as well as other "hypervirulent" strains which express CDT. These isolates are increasingly common and their spread is likely contributing to the overall increase in CDI incidence and severity. Our data suggests that targeting CDT in the development of vaccines and therapeutic inhibitors is essential to successfully treating these strains, and highlights the protective role eosinophils play during CDI. Our knowledge of protective and pathogenic immune responses during CDI continues to evolve, presenting new potential drug targets for treating this common and life threatening infection.

4.4 Methods

Bacterial Strains and Culture: *C. difficile* strains R20291 CdtA- and R20291 CdtB- was generated using the ClosTron system and functional inactivation of the targeted genes confirmed by Western blot as previously described (Kuehne, 2013). To prepare the infection inoculum, strains were inoculated onto BHI agar from frozen stocks and incubated at 37°C overnight in an anaerobic work station (Shel Labs). Single colonies were inoculated into BHI medium supplemented with cycloserine and cefoxitin (Sigma) and grown anaerobically overnight at 37°C. The next day, cultures were spun down for 1 minute at 6,000 x *g* and washed twice in anaerobic PBS. The optical density of the cultures were measured and culture density adjusted to $1x10^8$ CFU/mL in sterile, anaerobic PBS. Syringes were loaded with the inoculum and sealed in airtight bags before and during transport to the infection facility. To enumerate *C. difficile* in cecal samples, cecal contents were resuspended by weight in pre-reduced PBS. Resuspended cecal contents were serially diluted in PBS and plated on BHI agar supplemented with 1% Sodium Taurocholate and 1 mg/mL D-cycloserine and 0.032 mg/mL cefoxitin (Sigma) before incubating overnight anaerobically at 37ºC. Liver bacterial burden was determined by homogenizing whole liver samples in sterile, aerobic PBS. Liver homogenate was serially diluted and plated on non-selective BHI agar before incubating aerobically overnight at 37ºC. Liver CFU are reported according to liver sample weight.

Cell culture: BMDCs were generated as previously described with minor modifications (38). Briefly, femurs and tibia were removed and bone marrow flushed with PBS. Cells were counted and viability assessed by Trypan Blue staining and resuspended in RPMI 1640 media (Life Technologies) containing 10% Fetal Bovine Serum, 2mM L-glutamine

and 100 U/ml Penicillin and 100 U/ml Streptomycin. Media was supplemented with 10 ng/ml GM-CSF (Peprotech) and 55 μM β-mercaptoethanol (Gibco), and $3x10^6$ cells were seeded into a T75 vent cap tissue culture flask. Cells were cultured for 7 days, and supplemented with fresh media on days 2 and 4. On day 7 cells were harvested for stimulation. For stimulation, BMDCs were detached with a cell scraper and resuspended to $1.1x10^6$ cells/mL in fresh media. 180 ul of cell suspension was added per well of a 96 well plate. Cells were stimulated with the indicated concentrations of CDT or Toxins A and B in a total volume of 20 ul complete media. After the indicated incubation time, cells were spun down and supernatant removed and frozen at -80 ºC for later analysis. Cells were washed once in PBS and lysed in buffer RLT prior to RNA extraction (Lysis Buffer, RNeasy Kit, Qiagen). For cell stimulation, TLR-ligand tested lipopolysaccharide (Sigma) was used as a positive control. Anti-TLR2 antibody and IgG isotype control were obtained from Invivogen and eBioscience, respectively. Anti-CDT nanobodies were generated as previously described (Unger, 2015). Raw Blue NFκB reporter cell were obtained from Invivogen and grown in DMEM supplemented with 4.5 g/L glucose and 10% FBS. For stimulation, cells were detached using a cell scraper and resuspended to a density of 5.5 x 10^5 cells/mL in fresh media. 180 ul of cell suspension was added per well of a 96 well plate, and cells were stimulated with CDT as indicated. SEAP secretion was quantified by spinning down cells, removing the culture supernatant, and incubating 50 ul of supernatant with 150 ul of Quanti-Blue detection media (Invivogen) for 30 minutes at 37ºC prior to reading in a spectrophotometer.

Toxins: Purified CdtA and CdtB were obtained as previously reporter (Schwan, 2009). CdtB was previously activated by protease cleavage (0.2 μg of trypsin/μg of protein for 30

min at 37°C) prior to addition of trypsin inhibitor. Mock buffer used contained identical concentrations of trypsin and trypsin inhibitor to mimic the purified proteins. Purified Toxins A and B were a kind gift from Techlab, Inc. (Blacksburg, VA). Toxins were detected within cecal contents using the *C. difficile* TOXA/B ELISA according to manufacturer instructions, also kindly donated by Techlab, Inc.

Cytokine detection: IL-1β and IL-6 were detected in protein supernatants from BMDCs and tissue lysates using the Mouse IL-1β and IL-6 Ready-Set-Go! ELISA kit (eBioscience) according to manufacturer's instructions. IL-6 in serum was assessed by Luminex assay. Eotaxin-1 and Eotaxin-2 were detected in cecal lysates using R&D Systems DuoSet ELISA kits according to manufacturer's instructions. BMDC pro-IL-1β production was assessed by quantitative reverse transcription PCR. RNA was isolated using the RNeasy isolation kit (Qiagen). Contaminating genomic DNA was digested using the Turbo DNA-free kit (Ambion) and RNA reverse transcribed with the Tetro cDNA synthesis kit (Bioline) according to manufacturer instructions. The resulting cDNA was purified using Qiagen's PCR purification kit. *IL-1b* gene expression was quantified by Quantitect Primer assay (Qiagen) using Sensifast SYBR & Fluorescein Mix (Bioline) using the Quantitect 2-step amplification protocol. Gene expression was normalized to the S14 housekeeping gene (forward primer - TGGTGTCTGCCACATCTTTGCATC, reverse primer – AGTCACTCGGCAGATGGTTTCCTT).

Mice and infection: C57BL6, TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR5$^{-/-}$ mice were purchased from the Jackson Laboratory. C57BL6 mice were ordered from room AX4 when possible. Mice were males aged 8-12 weeks for all experiments. TLR2-/- and C57BL6 were cohoused for each experiment by exchanging bedding between each cage every two days for two weeks

prior to the start of the infection procedure. All animals were housed under specific pathogen free conditions at the University of Virginia's animal facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. Mice were infected using a modified version of a previously published model for CDI (Chen, 2008, Buonomo, 2013). Briefly, mice were given an antibiotic cocktail consisting of 45 mg/L Vancomycin (Mylan), 35 mg/L Colistin (Sigma), 35 mg/L Gentamicin (Sigma), 215 mg/L Metronidazole (Hospira) ad libitum for 3 days (Days -6 to -4). Mice were then switched back to regular drinking water for two days (Days -3 onward), followed by an IP injection (0.032 mg/g) of Clindamycin (Hospira) on Day -1. On Day 0, mice were gavaged with 10^7 CFU of *C. difficile*. Mice were monitored twice daily throughout the course of the infection and immediately euthanized if severe illness developed according to clinical scoring parameters.

Tissue Protein and Histology: Mice were humanely euthanized and tissue was immediately removed for analysis. Total cecal lysate was generated by removing the ceca and rinsing gently with PBS. Tissue was bead beaten for 1 minute in 400 ul of Lysis Buffer I (1x HALT Protease Inhibitor (Pierce), 5 mM HEPES). 400 ul of Lysis Buffer II was added (1x HALT Protease Inhibitor (Pierce), 5 mM HEPES, 2% Triton X-100) and tubes inverted gently. Tissue samples were incubated on ice for 30 minutes, followed by a 5 minute spin at 13,000 x *g* at 4°C. Supernatant was removed to a fresh tube, and total protein concentration was assessed by BCA assay according to manufacturer's instructions (Pierce). Cytokine concentration is shown relative to total protein concentration. To generate histological sections, cecal samples were placed in Bouin's Solution (Sigma) for 24 hours. Tissue samples were moved to 70% Ethanol before

paraffin embedding and sectioning. Sections were mounted on slides and stained with haemotoxylin and eosin prior to microscopic examination. Slides were scored blinded, with a score from 0 to 3 assigned based on 5 parameters: epithelial disruption, submucosal edema, inflammatory infiltrate, mucosal thickening and luminal exudate. Scores were averaged between 3 independent, blinded observers and each parameter score added for a given sample, with total score plotted for each animal.

Eosinophil Depletion: Eosinophils were depleted using an anti-mouse SiglecF monoclonal antibody (R&D Systems). Control groups received Rag IgG2A isotype control antibody (R&D Systems). Both groups were given 40 ug of antibody per mouse on day -1 and day +1 of infection (80 ug total per mouse). Eosinophil depletion was evaluated by flow cytometry, which showed an 82% average reduction in colonic eosinophils.

Flow Cytometry: Colonic lamina propria was prepared for flow cytometry by thoroughly rinsing the tissue in Hank's Balanced Salt Solution (HBSS) supplemented with 5% FBS and 25 mM HEPES. Epithelial cells were removed by gentle shaking for 40 minutes at 37ºC in HBSS with 15 mM HEPES, 5 mM EDTA, 10% FBS and 1 mM DTT. Halfway through the incubation, colon samples were transferred to fresh buffer. Next, colon samples were thoroughly chopped using scissors and digested in RPMI 1640 containing 0.17 mg/mL Liberase TL (Roche) and 30 ug/mL DNase (Sigma). Samples were digested for 40 minutes at 37ºC with gentle shaking. Samples were then spun down at 300 x *g* and resuspended in HBSS with 5% FBS and 25 mM HEPES before passage through a 100 uM cell strainer followed by a 40 uM cell strainer (both Fisher Scientific). Cells were counted and density adjusted to $1x10^7$ cells/mL. 100 ul of cell suspension were aliquoted per well of a 96 well plate for antibody staining. For staining, cells were

initially blocked with TruStain fcX (anti-mouse CD16/32 antibody, BioLegend) for ten minutes at room temperature. Cells were spun down and resuspended in LIVE/DEAD Fixable Aqua (Life Technologies) for 20 minutes at room temperature. Cells were washed twice and stained with fluorochrome conjugated antibodies. Flow cytometry was performed on an LSR Fortessa cytometer (BD Biosciences) and all data analysis performed via FlowJo (Tree Star Inc.).

Antibodies used were as follows:

Fluorochrome	**Antibody**	**Source**	**Clone**
Brilliant Violet 421	CD11c	BioLegend	N418
AlexaFluor 488	CD125/IL-5Rα	BD Biosciences	T21
PE	SiglecF	BD Pharmingen	E50-2440
PeCy7	CD11B	BioLegend	M1/70
APC-CY7	CD45	BioLegend	30-F11
AlexaFluor 647	CD193/CCR3	BD Biosciences	83103
PerCP-Cy5.5	Gr1	BioLegend	RB6-8C5
AlexaFluor 488	Annexin V	Life Technologies	
PE	c-Kit/CD117	BioLegend	2B8
AlexaFluor 700	CD34	BD Biosciences	RAM34
PeCy7	Sca-1	BioLegend	D7
PerCP-Cy5.5	TCRβ	BioLegend	H57-597
PerCP-Cy5.5	CD3ε	BioLegend	17A2
PerCP-Cy5.5	CD49b	BioLegend	DX5
PerCP-Cy5.5	B220	BioLegend	RA3-6B2
PerCP-Cy5.5	CD11c	BioLegend	N418
PerCP-Cy5.5	CD11b	BioLegend	M1/70
FITC	LY6C	BD Biosciences	AL-21
PE-CY7	LY6G	BD Biosciences	1A8

Chapter 5: Conclusions and Future Directions

Part of this chapter has been adapted from "Host recognition of *Clostridium difficile* and the innate immune response"

Carrie A. Cowardin and William A. Petri, Jr. 2014. *Anaerobe* 30, 205–209.

5.1 Toxins A and B initiate pathogenic inflammation

Prior to the development of a mouse model, the role of the host immune response to *C. difficile* was evaluated using intoxication based systems, where purified Toxins A and/or B were injected directly into the cecum or ileum. Although this system was useful for isolating a response to the toxins alone, it did not always represent what happens during infection with the live bacterium. Indeed, in intoxication models, the inflammatory response was consistently observed to be pathogenic, and disruption of innate signaling pathways or preventing inflammatory cell recruitment tended to improve tissue pathology and survival. However, many studies using the mouse model of infection with *C. difficile* have since demonstrated that the immune response is not always detrimental. In fact, multiple innate pathways including TLR4, which recognizes *C. difficile* surface layer proteins, MyD88, which mediates TLR and IL-1 receptor signaling, and ASC, an essential adaptor protein for multiple inflammasomes, are all required for protection from *C. difficile* colitis [54,55,66]. Nucleotide oligomerization domain 1 (Nod1) also plays a protective role, as do Type 1 Innate Lymphoid Cells [58,139], indicating that some level of immune response is needed to prevent mortality.

As with most aspects of the immune system, it is likely that a balanced response to infection is required in order to eradicate the bacterial threat while protecting host tissues. This requires exquisite control of inflammatory machinery in order to prevent aberrant inflammation and further tissue pathology. It is interesting to note that many of the same signals which promote protection from *C. difficile* colitis also participate in the induction of a pathogenic response, culminating in IL-23 production and mortality. To explain these

seemingly disparate findings, we propose a context-dependent model of *C. difficile*-induced inflammation, where initial bacterial recognition by PRRs promotes an innate inflammatory response, and subsequent intoxication of epithelial and immune cells provokes a shift towards an extremely pro-inflammatory, destructive form of inflammation.

This hypothesis is supported by our findings that Toxins A and B are able to enhance production of pathogenic IL-23 from host immune cells. Treatment of host cells with non-toxigenic *C. difficile* supernatants leads to low levels of IL-23 production, and adding back Toxins A and B leads to a dramatic increase in IL-23. This process is mediated by production of IL-1β resulting from inflammasome activation and subsequent IL-1 receptor signaling. It seems likely that the role of IL-1β during infection is multifaceted, as in addition to inducing IL-23, a lack of inflammasome signaling in ASC deficient mice leads to increased mortality. IL-1β is a ubiquitous danger signal which contributes to protection from bacterial infection such as with *Bordetella pertussis* [140] but also leads to multiple inflammatory diseases, including inflammatory osteomyelitis [141] and psoriasis [142,142]. Within colitis models, including those for Inflammatory Bowel Disease (IBD), the role of IL-1β is even more complex, with multiple groups reporting disparate findings as to whether depletion of IL-1β leads to protection or pathogenicity [143,144,145]. Differences in the microbiota composition of these mice are likely at least partially responsible for the different roles of IL-1β observed during IBD, a factor which almost certainly contributes to different findings in *C. difficile* models as well. Because susceptibility to *C. difficile* is tightly linked to microbiome composition, it is widely acknowledged in the field that certain phenotypes may be microbiome dependent. For this reason, mice of different

genetic backgrounds are now routinely co-housed prior to infection to normalize their microbiota. More detailed description of the origin of mice, including the room in which they were bred, may help clarify some of these issues. Sequencing the fecal microbiota for all infected mice is a major undertaking which could help shed light on species associated with protection and susceptibility; however, unless methods are in place to normalize microbiota between diverse facilities, the cost effectiveness of this approach remains dubious.

Another complicating problem is determining the temporal requirement for IL-1β signaling, which may initially be protective while simultaneously helping to shape a later, IL-23-dependent response which proves to be pathogenic to the host. In both situations, the context in which IL-1 signaling occurs is essential to determining the ultimate outcome. For example, different microbiota compositions may provide different stimulatory backgrounds upon which IL-1 signaling is imposed, and an IL-1β signal received during the early innate response may result in a different reaction than one received later on. Finally, the levels of IL-1β produced are also likely to influence the overall immune tone, with high levels resulting in a stronger inflammatory response. More research is needed to answer these questions and clarify the different roles of IL-1 signaling during *C. difficile* infection.

In addition to determining the overall role of IL-1β during infection, many questions also remain concerning the cellular processes that trigger activation of the inflammasome following intoxication. We found that IL-1β production was critically dependent on the enzymatic activity of Toxin A, confirming similar results reported for Toxin B [39]. This lends credence to the idea that Rho-glucosylation is the ultimate signal

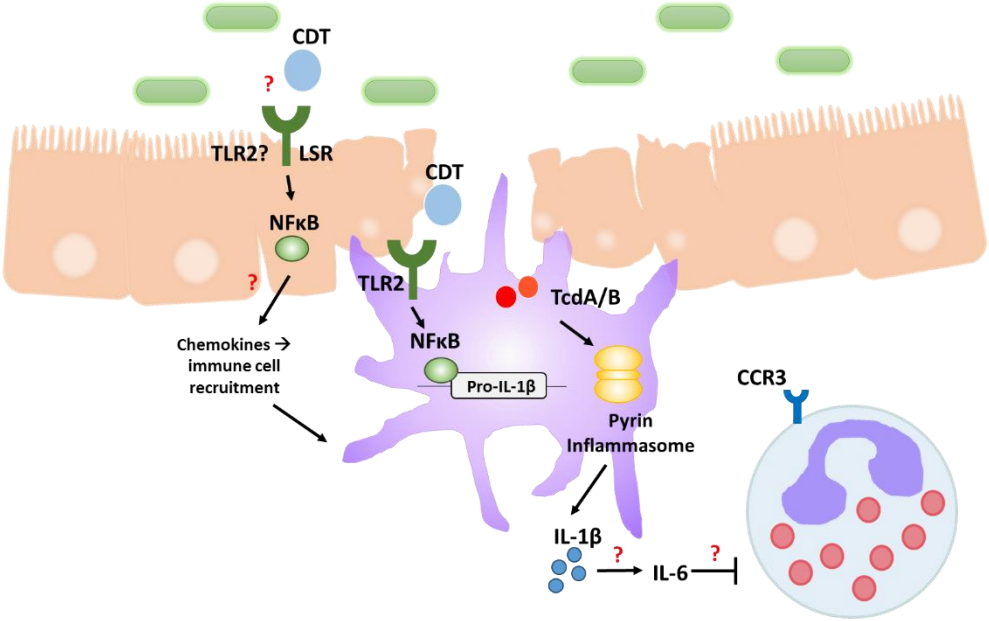
required for Pyrin-dependent inflammasome activation [39]. However, evidence for the activation of the NLRP3 inflammasome also exists. This inflammasome recognizes a wide variety of danger signals, including pore-forming toxins, ATP, viral and fungal ligands as well as particulate matter [40,146,147]. It is conceivable that both inflammasomes contribute in some way during *C. difficile* infection. Interestingly, we noted that the glucosyltransferase-deficient Toxin A had retained some ability to activate the inflammasome, although this may be due to low level glucosylation activity (particularly at higher doses). It is conceivable that this residual activation may be due to contamination of the purified toxin with bacterial PAMPs, a possibility which warrants further investigation by confirming these findings in cells lacking PRRs (such as HEK cells). We also found that the glucosyltransferase activity of Toxin A was absolutely required for tissue pathology and IL-1β induction *in vivo*. Because tissue damage and cytokine production are both intertwined and likely interdependent in this model, teasing out the cause and effect of these pathways should provide fascinating information on how inflammation progresses in response to the Rho-glucosylating Toxins A and B. In the context of previous findings on *C. difficile* toxins, our results suggest that Toxins A and B help to shape host inflammation by providing an additional danger signal which skews towards a pathogenic response characterized by IL-23 production. Glucosylation by the toxins is likely required for this to occur, suggesting that targeting this process could help to treat severe CDI by preventing tissue pathology and an aberrant immune response to infection.

5.2 Role of CDT in enhancing inflammation

The incidence and severity of *C. difficile* infection has increased dramatically over the past 15 years, and in the United States, CDI is now the most common hospital acquired infection, surpassing Methicillin Resistant *Staphylococcus aureus* [148]. Multiple factors are likely contributing to the increased incidence of CDI, including host factors, such as advanced age and inflammatory bowel disease, both of which are increasingly common and enhance susceptibility to CDI. Certain ribotypes of *C. difficile* have also acquired fluoroquinolone resistance, which is thought to have contributed to their global spread [149]. The most classic of these ribotypes is 027, although ribotype 078 strains have also been associated with outbreaks. Some studies have also suggested that increased rates of sporulation may also contribute to the spread of these isolates, but this has not been demonstrated consistently. There are also multiple suggested mechanisms for the increased severity of disease, including increased production of Toxins A and B, as well as CDT production. As with sporulation, production of Toxins A and B does not consistently follow virulence, although some evidence does suggest that ribotype 027 strains produce toxins earlier than their less virulent counterparts [150]. We have found that CDT also contributes to *C. difficile* virulence in a ribotype 027 isolate by enhancing the inflammatory response and shifting it away from protective eosinophil recruitment (Figure 5.1). However, it remains to be determined whether increased colonization due to CDT also contributes to the increase in inflammation, and our findings do not rule out additional roles for CDT which culminate in enhanced virulence.

Figure 5.1 CDT enhances inflammatory during infection and suppresses protective eosinophils.

Initial recognition and intoxication by CDT may occur at the epithelium or on recruited immune cells, or both. TLR2 likely plays a role in the recognition of CDT, leading to activation of NFκB and increased inflammatory cytokine production. NFκB activation also serves as a priming signal for the inflammasome, which is then activated by Toxins A and B. It is unclear whether CDT directly induces eosinophil apoptosis, or contributes to increased inflammation which then suppresses eosinophils.


CDT
?
TLR2?
LSR
NFκB
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Chemokines →
immune cell
recruitment
CDT
TLR2
NFκB
Pro-IL-1β
TcdA/B
Pyrin
Inflammasome
CCR3
IL-1β
?
IL-6
?

One likely, non-mutually exclusive alternative is that CDT expression provides a competitive advantage for *C. difficile,* either directly or by influencing the inflammatory state in such a way that is beneficial for *C. difficile* survival or spread. Indeed, recent work has demonstrated that the spread of ribotype 027 strains is more likely to be due to increased infectivity and clinical severity than direct competition with other *C. difficile* isolates [151]. Although we suggest that CDT expression contributes to the severity of ribotype 027 strains, other factors, such as early Toxin A and B production, may also contribute. The ability to manipulate clinical isolates with recently discovered genetic tools will aid in the further characterization of CDT as a virulence factor, as other CDT-expressing strains could be investigated to confirm our findings. Although expression of CDT in strains which do not naturally produce this toxin would be fascinating, the possibility of generating a hypervirulent spore-forming organism should give us pause. To this end, exogenous administration of purified CDT could be explored as an alternative. Despite all that remains to be examined, our results suggest that CDT is a virulence factor which could underlie some of the increase in disease severity observed over the last fifteen years. Because CDT expression had previously been associated with poor outcome in clinical studies, these findings increase our mechanistic understanding of pathogenesis and suggest new avenues for targeted therapy.

5.3 Protective mechanism of eosinophils

The role of eosinophils in CDI is a fascinating area of study, as two different models have demonstrated these cells to be important. The protective role of eosinophils was

initially identified by Erica Buonomo, who discovered that administration of the Type 2 cytokine IL-25 protected mice from mortality due to the VPI 10463 strain of *C. difficile*. Her work went on to demonstrate that this protection was mediated by eosinophils (Buonomo, unpublished). Building on this observation, we found that depletion of eosinophils increases disease severity during infection with the ribotype 027 strain R20291. There are multiple potential mechanisms by which these cells could play a beneficial role. Eosinophils express cytotoxic granules which contain Major Basic Protein, Eosinophil Peroxidase, Eosinophil-derived Neurotoxin, and Eosinophil Cationic Protein, and these granule components contribute to allergic inflammation and are capable of bacterial killing. Erica did not observe a difference in *C. difficile* burden when eosinophils were depleted, suggesting these cells do not protect by eradicating the pathogen. However, Erica did find that protected mice with high levels of eosinophils did have fewer commensals translocating to the liver, which may suggest that these cells can help control commensal microbes which escape the gut during infection [152,153]. Interestingly, we did not observe a difference in translocation of commensals between CDT+ R20291 infected mice, who have few eosinophils, and R20291 CdtB- infected mice, who have significantly more eosinophils, suggesting that these cells may also fulfill other protective functions. Eosinophils may contribute to tissue repair via secretion of IL-4 and TGF-β, and may be required for the regeneration of the epithelial barrier which is profoundly disrupted during CDI [154]. Thirdly, eosinophils can help balance the type of immune response generated by producing Type 2 cytokines and suppressing Type 1 and Type 17 signals [155]. In this way, eosinophils could help shift the immune response away from a pathogenic Type 17 response. This remains to be determined by further characterizing the inflammatory

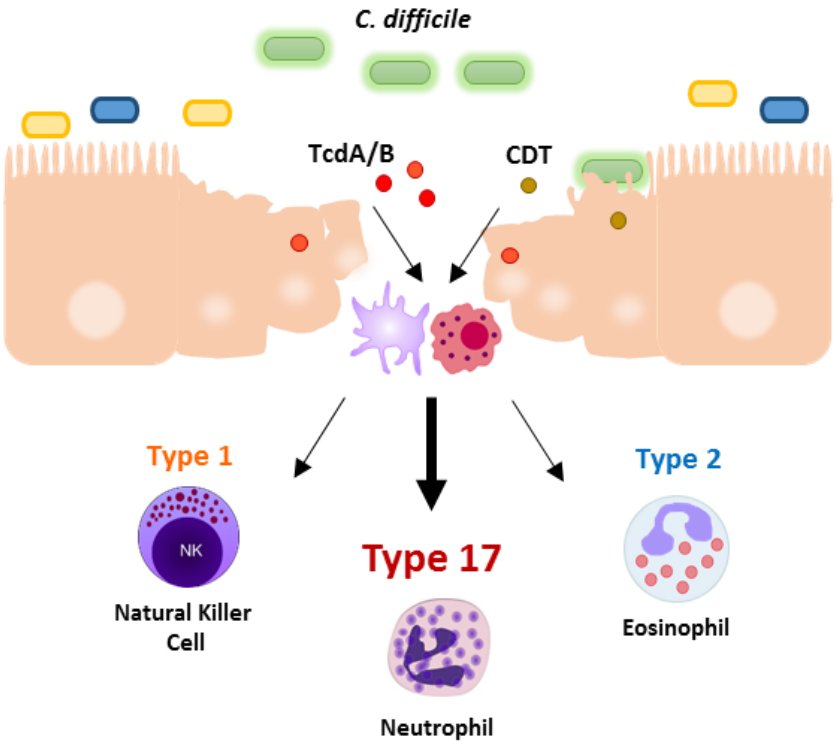
signaling which occurs in the presence and absence of eosinophils during infection. However, eosinophils also play a deleterious role in other models of colitis, including IBD, highlighting the importance of cellular function, such as production of inflammatory cytokines and eosinophil peroxidase [120]. These functions, rather than simple presence or absence, may determine the eventual outcome of disease, and it remains to be determined what protective function eosinophils perform in our models of CDI.

As part of a characteristic Type 2 response, eosinophils play into the larger question of what type of immune response best promotes protection during CDI. Evidence from our lab suggests that characteristics of a Type 17 response, including IL-1β, IL-6, and IL-23, may promote a pathogenic response during infection. Interestingly, neutrophils have been shown to be required for protection from *C. difficile,* [55] although these cells are also thought to be the primary cell type associated with a Type 17 response. This again recalls the issue of balance, suggesting that a complete lack of neutrophils may be as detrimental as an overabundance of neutrophilic inflammation. Other groups have shown that Type 1 responses, critically dependent on IFNγ produced by type 1 Innate Lymphoid Cells (ILC1), are required for protection [70]. This is in contrast with our findings that Type 2 responses promote protection. In a natural infection, Type 17 responses may dominate due to the intensely pro-inflammatory and damaging stimulus provided by the toxins, and preventing balance by abrogating Type 2 or Type 1 responses serves to enhance this pathology. In this way, seemingly contradictory results concerning the immune response may be reconciled. This hypothesis suggests the principle of immune balance is broadly applicable to the host response during infection, with no single pathway standing alone to promote protection but

each type of response functioning interdependently to eradicate the infection and heal host tissue (Figure 5.2).

Figure 5.2 *C. difficile* toxins cooperatively enhance Type 17 responses to disrupt intestinal immune balance.

Initial recognition of *C. difficile* by the immune system is followed by intoxication with Toxins A and B, which induces a dramatic and inappropriate inflammatory response characterized by Type 17 cytokines and neutrophil recruitment. CDT, when present, further enhances the Type 17 response. Overwhelming inflammatory signaling likely also recruits Type 1 and Type 2 effector cells (Natural Killer cells and eosinophils, respectively) but the survival of these cells is impaired by the Type 17 signaling which dominates the inflammatory environment. Complete disruption of Type 1 and Type 2 pathways by depletion of Type 1 ILCs or eosinophils enhances pathology by further shifting immune balance. Enhancing Type 1 and Type 2 responses may act to balance the robust Type 17 response to prevent a pathogenic immune response.


C. difficile
TcdA/B
CDT
Type 1
NK
Natural Killer Cell
Type 17
Neutrophil
Type 2
Eosinophil

5.4 Role of the microbiota in shaping a protective immune response

These characteristics of the immune response are now understood to be intimately linked to the composition of the host microbiota. In fact, gut bacteria can shape immune responses in disparate organs, including the lung and bone marrow, a prime example of this is the ability of segmented filamentous bacteria (SFB) to induce T_H17 responses [156,157]. The role of the microbiota in controlling susceptibility to CDI is also widely appreciated, with much effort put forth to understand how and why this occurs. Fecal Microbiota Transplantation, or FMT, is becoming a more common treatment for recurrent cases of CDI, and appears to be highly effective [158]. In general, the ability of the host microbiota to prevent infection with pathogenic microbes is known as colonization resistance [75]. Direct colonization resistance refers to the ability of certain microbes to prevent pathogenic infection by competition for nutrients or by inhibiting pathogen growth via secretion of molecules which are toxic to other species. In the case of indirect colonization resistance, beneficial microorganisms prevent infection by activating or skewing host immune responses. Recent efforts to isolate and identify species underlying host colonization resistance to *C. difficile* have borne fruit. A major player in this process is the secondary bile-salt producing species *Clostridium scindens,* which was shown to be associated with protection both in mouse models and in human patients [139], although these interactions appear to be largely mediated by direct colonization resistance. As with all microbiota research, the major challenge facing this approach is demonstrating mechanism rather than association, as many species identified by 16S sequencing are unculturable and cannot be easily transplanted into germ free animals. Additionally, it remains to be seen what type of

role the immune system, through indirect colonization resistance, plays in protection from or susceptibility to CDI.

Understanding how the microbiome shapes the host immune response will be essential to fully appreciating the role of the immune system in infection, and may also lead to greater understanding of how the balance of inflammatory processes shapes the course of disease. This will provide clues towards preventing infection by reducing the immunological effects of microbiome disruption which lead to susceptibility and will open the door to numerous potential therapies targeting host signaling during CDI.

5.4 Closing statements

Current treatment options for *C. difficile* invoke an interesting paradox: the same antibiotics used to eradicate *C. difficile* also disrupt the host microbiota and can lead to reinfection or recurrent infection [3,11,159]. As an alternative, FMT is highly effective to prevent relapses and is currently being tested in treatment-resistant acute disease. However, it may not be appropriate for all patients, and safety concerns about its use do exist. Until a defined mix of beneficial bacteria is isolated and extensively tested, it is difficult to predict negative effects following FMT. With more evidence emerging that the microbiota can influence diverse disease states, including obesity, allergy and cancer, the need to fully define risks and benefits is paramount. These issues highlight the need for alternative therapies, and as a major driver of disease pathogenesis, manipulation of the host immune response is a promising avenue for intervention. Indeed, many drugs are already on the market to treat autoimmune or inflammatory diseases which may prove beneficial during CDI, such as ustekinumab, which targets IL-23 and IL-12 and is approved for the treatment

of psoriasis. However, targeting the host immune response is not without its challenges. As a complex system with many moving parts, achieving a balanced immune response to CDI is a formidable task. Thus, the challenge facing *C. difficile* immunology is twofold: determining how to shape a protective versus pathogenic immune response, and harnessing these findings to combat disease. This will require integrating different findings into a comprehensive model of host and pathogen interactions during disease, determining what shape an appropriate immune response may take, and isolating immune mediators which prevent or modify this appropriate response to cause pathogenesis. Our work has demonstrated the role of the major bacterial immune modulators, Toxins A and B and CDT, in initiating a pathogenic immune response, providing understanding of how bacterial virulence factors interact with the host to cause disease. This knowledge sheds light on previously unidentified areas of *C. difficile* pathogenesis, providing crucial clues for future researchers to pursue.

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