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Neutrophil-mediated inflammation in the pathogenesis of *Clostridium difficile* infections

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Abstract

Clostridium difficile is the most important cause of nosocomial infectious diarrhea in the western world. *C. difficile* infections are a major healthcare burden with approximately 500,000 new cases every year and an estimated annual cost of nearly \$1 billion in the U.S. Furthermore, the infections are no longer restricted to health care facilities, and recent studies indicate spread of *C. difficile* infection to the community as well. The clinical spectrum of *C. difficile* infection ranges from asymptomatic colonization to severe diarrhea, fulminant colitis and death. This spectrum results from a complex interplay between bacterial virulence factors, the colonic microbiome and the host inflammatory response. The overall vigor of host inflammatory response is believed to be an important determinant of *C. difficile* disease severity, and a more robust immune response is associated with worse outcomes. Neutrophils are the primary cells that respond to *C. difficile* invasion and neutrophilic inflammation is the hallmark of *C. difficile*-associated disease. In this review, we will focus on the role of neutrophils (infiltration to infected tissue, pathogen clearance and resolution of inflammation) in the immuno-pathogenesis of *C. difficile*-associated disease (CDAD).

Keywords

Clostridium difficile; Neutrophils; Emergency granulopoiesis; Neutrophil recruitment

1. Pathogenesis of *Clostridium difficile*-associated disease

Clostridium difficile is a gram positive, spore-forming and toxin producing anaerobic bacterium that is the leading cause of antibiotic-associated diarrhea in U.S. [1–3]. *C. difficile* is transmitted primarily *via* the fecal-oral route [4]. Major risk factors associated with *C. difficile* infection (CDI) are advanced age, recent hospitalization, gastro-intestinal manipulations, drugs that lead to gastric acid suppression [5] and disruption of normal gut microbial flora by use of anti-microbial and chemotherapeutic agents [5–7].

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Conflict of interests

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A healthy host microbiota resists colonization of *C. difficile* through a number of mechanisms: directly by competing for nutrition and space [8,9] or indirectly by production of molecular mediators that kill *C. difficile* (e.g. Thuricin CD [10]), inhibit *C. difficile* colonization (e.g. bacteriocins and secondary bile metabolites [11]) or enhance intestinal epithelial cell (IECs) survival (e.g. butyrate [12]). Disruption of the host microbiota creates an ideal niche for *C. difficile* colonization, infection and disease [5]. *C. difficile* spores germinate into vegetative forms in the anaerobic environment of colon and release toxins A and B, which are the key virulence determinants of *C. difficile*-associated disease (CDAD). The toxins translocate to cytosol of target cells including IECs, mast cells, fibroblasts, smooth muscle cells, and monocytes [13] through receptor mediated endocytosis. Once endocytosed, toxins inactivate GTP-binding proteins such as Rho, Rac and Cdc42 through glycosylation, resulting in disruption of actin cytoskeleton [14], epithelial cell rounding and eventually cell death and necrosis. This cellular stress response triggers secretion of inflammatory cytokines and expression of leukocyte adhesion molecules (integrins and selectins [15]) which in turn facilitate neutrophil infiltration. In addition to colonic tissue, an increase in neutrophils in the peripheral blood is commonly seen after *C. difficile* infection in both humans and animal models of disease [16,17]. The infiltrated neutrophils are activated at the site of infection and produce bactericidal reactive oxygen intermediates [18], defensins and proinflammatory cytokines and chemokines [19], leading to an intense neutrophil-mediated inflammatory response, which is believed to be one of the key determinants of disease severity [20].

2. Neutrophil-mediated inflammation: friend or foe?

Neutrophils are short-lived granulocytes emerging from hematopoietic progenitor cells in the bone marrow. Neutrophil numbers in the peripheral blood are maintained at a consistent level (estimated to range between $0.8\text{--}1.0 \times 10^9$ cells/kg of body weight) in healthy adults [21]. Infection-induced neutrophilia is a dynamic process, and systemic and tissue neutrophil numbers are tightly regulated at many levels: generation (granulopoiesis in bone marrow), trafficking (between bone marrow and tissues) and removal at the time of resolution (in tissues) [22,23]. After an infectious challenge, neutrophils migrate to the tissue within minutes and form primary defense against invading pathogens. Neutrophils phagocytose the invading pathogens and elicit a potent inflammatory response including a variety of cytokines and chemokines, reactive oxygen intermediates and anti-bacterial peptides [24,25]. All of these neutrophil actions are primarily aimed at clearing pathogens and restoring tissue homeostasis. While neutrophil-mediated inflammation is essential for host defense, chronic activation of neutrophils exacerbates the inflammatory process and can be detrimental to the surrounding tissues [26] due to the unfortunate lack of target cell specificity of neutrophil response. Such neutrophil-induced tissue damage is seen in several diseases like acute lung injury [27], experimental colitis [28] and rheumatoid arthritis [29]. A dampened neutrophilic response (or delayed neutrophilia) on the other hand can predispose to overwhelming infection. The functional status of neutrophils can also impact the vigor of the initial inflammatory response and eventual resolution. In fact, after *Staphylococcus aureus* infection in mice [30] and endotoxin challenge in humans [31,32], functionally heterogeneous neutrophils subsets have been defined with distinct effects on T-

cell proliferation [32], macrophage activation [30] and varying ability to clear the pathogens [31]. The relative proportion of distinct neutrophil subsets in an inflammatory paradigm could thus influence both pathogen clearance and host tissue damage [30,31]. Thus, neutrophil responses need to be tightly regulated and an imbalance in neutrophil homeostasis can impact disease severity and outcomes.

The exact role of neutrophilic inflammation in the context of CDI remains poorly understood. However, neutrophils do play a central role in disease pathogenesis (Fig. 1). In patients with *C. difficile* colitis, higher neutrophils in the blood are associated with more severe disease and increased 30-day mortality [33]. But studies have also shown an increase in the incidence of CDI (odds ratio, 1.35; $p < 0.001$) in hospitalized leukemia patients with neutropenia [34]. Similarly, in a study of allogeneic hematopoietic stem cell transplant patients, Huang and colleagues showed that individuals with neutropenia are more likely to have recurrent CDI compared to non-neutropenic counterparts [35]. Neutrophil ablation in animal models also elucidates both beneficiary and adverse effects of neutrophils in CDI. For example, inhibition of neutrophil recruitment through intravenous administration of anti-CD18 (leukocyte adhesion molecule) [17] in rabbits or anti-macrophage-inflammatory protein (MIP)-2 [36] in rats resulted in a marked reduction of Toxin-A induced enterotoxicity. On the contrary, ablation of neutrophil recruitment using anti-Gr1 antibody resulted in increased mortality of *C. difficile* infected mice [37]. Collectively these findings reveal the double-edged nature of neutrophils – these cells participate both in re-establishing tissue homeostasis by containing pathogens and can also induce tissue damage by exacerbated release of inflammatory mediators.

2.1. Neutrophil recruitment in response to *C. difficile* infection

C. difficile leads to an intense, early neutrophilic response as evidenced by presence of neutrophils within 1 h in colon mucosa of Toxin-A administered rabbits [38] and within 24–48 h after challenge with *C. difficile* spores [37,39]. After *C. difficile* infection, multiple signaling cascades and neutrophil chemo-attractant proteins are involved in neutrophil recruitment (Table 1). For example, interventions to completely abrogate or diminish P2Y6-CXCL8/IL-8 [40], MyD88-CXCL1 [37], NOD1-CXCL1 [39] and IL-22-STAT3 [41] signaling pathways or neutrophil chemo-attractant proteins GM-CSF [42], CXCL1, CXCL2 (MIP2) [22], IL-23 [43], IL-17 [44], IL-8 [45] and leptin [46] all lead to significantly decreased neutrophil recruitment in mouse models of *C. difficile* infection.

In a healthy state, the large storage pool of mature neutrophils is retained within bone marrow by a mechanism involving interactions of neutrophil CXCR4 and stromal cell-derived factor (SDF-1 α) in bone marrow [22]. These neutrophils can be readily mobilized to circulation in response to infection or inflammatory event. G-CSF generated at the site of inflammation, leads to a reduction of CXCR4 and SDF-1 α expression on neutrophils and stromal cells, respectively, and thus enhances the migration of neutrophils from the bone marrow [47]. Intra-peritoneal injection of G-CSF induces a surge in circulating neutrophils but fails to recruit neutrophils to the peritoneum [48] indicating that the main role of G-CSF is in egress of neutrophils from the bone marrow. Neutrophil recruitment from blood to the infected- or inflamed-tissue on the other hand, requires additional signaling from

chemokines like CXCL1 and CXCL2 [37,49,50]. Notably, a surge in G-CSF levels is seen both in patients with *C. difficile* infection [51] and mice challenged with *C. difficile* [52]. This surge may facilitate neutrophil egress from bone marrow in response to *C. difficile* infection and manifest as leukocytosis which is commonly seen in patients with *C. difficile* infection. G-CSF knockout mice could be a useful model in identifying the role of G-CSF in neutrophil recruitment after challenge with *C. difficile*.

GM-CSF is another important growth factor that plays a key role in granulopoiesis [53] and neutrophil recruitment during pulmonary and intestinal inflammation [54] [55]. Recent studies have shown an important role for GM-CSF in *C. difficile* infection as well. Antibody-mediated neutralization of GM-CSF in *C. difficile* infected mice reduced both colonic neutrophil infiltration and expression of pro-inflammatory cytokines (IFN γ , IL-1 β , IL-6, TNF α) and chemokines (CXCL1 and CXCL2) [42]. Whilst this data indicates that GM-CSF is involved in neutrophil recruitment, it is also possible that neutralization of GM-CSF causes extra-intestinal effects such as defective granulopoiesis resulting in a reduction of total available neutrophils.

IL-23 is an inflammatory cytokine which plays an important role in host defense against bacterial infections and is present in colon biopsy specimens [56] and stool samples of *C. difficile* infected patients [51]. In murine models of *C. difficile* infection, defective IL-23 signaling (genetic deficiency and antibody-mediated ablation) significantly reduces neutrophil infiltration to the colon compared to wild type controls [43]. Thus, IL-23-mediated neutrophil recruitment contributes to intestinal histopathology in CDI. Ablation of IL-23 signaling also reduces levels of neutrophil recruiting chemokines: CCL3, CXCL1, and CXCL2 in colon mucosa. These studies are in agreement with an earlier study which reported reduced morbidity and mortality in IL-23-deficient *C. difficile* infected mice [56], an outcome attributed to reduced neutrophil infiltration and inflammation.

In addition to inflammatory cytokines and chemokines, recent data has shown that metabolic mediators like leptin play a role in host protection against *C. difficile* pathogens [46,57,58]. Mice with complete leptin receptor deficiency (*db/db*) were partially protected against toxin A-induced intestinal inflammation [58], whilst both *db/db* mice and S1138 mice (with abrogated leptin-STAT3 pathway) exhibited reduced signs of inflammation and poor control of pathogen burden in *C. difficile* infection [46]. Concurrently, S1138 mice had a significant reduction in infiltrated inflammatory cells including neutrophils [46], which suggests that leptin-mediated neutrophil recruitment enhances disease.

Despite numerous studies demonstrating significant leukocytosis and neutrophilia in both patients with *C. difficile* and animal models of infection, little is known about the mechanism by which host immune system meets the high demand for neutrophils. Emergency granulopoiesis is a well-known phenomenon in response to infections and is characterized by accelerated cell cycle of granulocyte progenitors, increased frequency of granulocytic precursor cells and a rapid surge in systemic neutrophil count in response to noxious challenge [59,60]. Mouse models of inflammatory peritonitis (intra-peritoneal injection of alum [61] and lipopolysaccharide [62]) have clearly shown that the high demand for neutrophils is met by emergency granulopoiesis in the bone marrow. *C. difficile* infection

and subsequent trigger of inflammation results in significant increase in the levels of growth factors and molecules that influence emergency granulopoiesis (e.g. GM-CSF, G-CSF, IL-23, ROS) [60,61,63,64], thus it is likely that emergency granulopoiesis plays a key role in *C. difficile* disease pathogenesis. However, the exact contribution of emergency granulopoiesis and neutrophil turnover during *C. difficile* infection has not been studied by rigorous *in vivo* experimentation.

2.2. Neutrophil functions in *C. difficile* infection and resolution

Neutrophils are present in a highly activated state after infection with *C. difficile* as shown by increased levels of calprotectin and lactoferrin in stool samples from patients with CDI as compared to controls [51]. Toxins produced by *C. difficile* (mainly TcdB) can activate neutrophils through formyl peptide receptor-1 (FPR-1) and generate bactericidal concentrations of reactive oxygen species (ROS) [65]. In addition to the bactericidal effects, ROS also activates anti-bacterial defensins [66] including human α -defensin 5, which can directly inactivate *C. difficile* Toxin B *in vivo* [67], and kill *C. difficile* in *in vitro* culture systems [68]. Direct phagocytosis of the *C. difficile* bacteria by neutrophil is another important mechanism of pathogen clearance. Neutrophils can ingest complement or anti-*C. difficile* antibody coated *C. difficile* bacteria [69], and ablation of neutrophils using anti-Ly6G antibody results in increased *C. difficile* pathogen burden [39]. However, despite such potent anti-microbial activity from neutrophils, *C. difficile* can still survive and cause disease. This can be attributed to bacterial factors including toxins and glutamate dehydrogenase (GDH): toxigenic strains of *C. difficile* are more resistant to phagocytosis than the non-toxigenic strains [69] and GDH secreted by *C. difficile* [70] enables the bacterium to resist neutrophil-induced oxidative stress.

Neutrophil extracellular traps (NETs) are structures composed of granules and nuclear constituents released by neutrophils to disarm, trap and kill bacteria [71]. Release of NETs is predicted to trap pathogens and limit their dissemination while creating a contained environment to achieve effective concentration of neutrophil microbicidal effectors. NETs have been identified in killing and clearance of both gram-positive (e.g. *S. aureus*) and gram-negative (e.g. *Shigella flexneri*) [71–73]. Presence and activity of NETs have been recently identified in non-infectious inflammatory diarrhea e.g. ulcerative colitis [74] as well. Appearance of NETs have been strongly associated with ROS formation [72] and with strong evidence of ROS formation and overall neutrophil activation in response to *C. difficile*, we speculate that NETs may contribute to clearance of *C. difficile* bacterium as well. But the role of NETosis during *C. difficile* infection remains to be further elucidated.

In bacterial infections of the gut, neutrophils are involved in healing and restoring tissue integrity by phagocytosis of pathogens and cell debris [75], secretion of factors that enhance mucosal healing [76] and by secreting anti-inflammatory molecules [77] that inhibit further neutrophil recruitment (Fig. 1). Resolution of the inflammatory response is critical since persistent inflammation is associated with worse outcomes such as longer time-to-diarrhea resolution [78,79] and weight loss [80] in *C. difficile* infected patients. Contrary to the early belief that resolution is a passive process, it is now known that resolution is an active

biochemical and metabolic process involving an array of pro-resolution molecules [76]. Importantly, neutrophils are a key component of the resolution process.

In mouse models of islet cell transplant a distinct population of pro-angiogenic neutrophils that expresses high levels of matrix metalloproteinase-9 (MMP-9) has been identified [81]. This CD11b⁺Gr1⁺CXCR4^{hi} population of neutrophils expresses 10 times higher levels of MMP-9 than neutrophils that are recruited to an inflammatory stimulus and are believed to enhance angiogenesis and thus aid in tissue healing and survival of the transplanted tissue. Different neutrophil phenotypes have been identified in experimental endotoxemia [31,32], systemic lupus erythematosus [82], and animal models of *S. aureus* infection [30] as well. These subsets are believed to execute different functional roles based on the severity and stages of infection. For example, in human volunteers challenged with LPS (2 ng/kg i.v.), a population of CD16^{low}ROS^{low} neutrophils that has a reduced capacity to generate oxidative burst is detected in blood at 3 h after challenge [31]. Thus, distinct neutrophil sub-populations can play a role in directing both inflammation and healing. Even though not experimentally evidenced yet, the possibilities of having similar neutrophil subsets managing different stages of inflammation and disease resolution in *C. difficile* infection cannot be ruled out at the moment.

Removal of neutrophils from the site of infection and inflammation is important in controlling ongoing inflammation and promote healing. Lipid mediators including resolvins [83] and lipoxins [76,84] assist in disease resolution by increasing phagocytosis of apoptotic neutrophils by macrophages and inhibiting further neutrophil recruitment. Annexin A1 is a phospholipid binding protein that is increased on neutrophils, IECs and dendritic cells during resolution phase. Annexin A1 increases neutrophil apoptosis and enhances phagocytosis of apoptotic neutrophils by macrophages [85] and inhibits further tissue accumulation of neutrophils by reducing neutrophil attachment and transmigration to the site of inflammation [85]. This alters the tissue macrophages to a proresolving phenotype which culminates in resolution of the inflammatory response in experimentally induced mouse models zymosan-induced peritonitis [86]. Similar mechanisms of macrophage-neutrophil interactions culminating in pro-resolution environment may exist in *C. difficile* infection but have not yet been investigated.

3. Conclusion

Neutrophil responses play an inevitable role in host immune response to *C. difficile* infection. Neutrophils are recruited to colonic mucosa after *C. difficile* infection and they lead a robust immune response to contain the disease that includes phagocytosis of *C. difficile* and secretion of an array of inflammatory mediators and bactericidal peptides. While the driver of the host inflammatory response could be either *C. difficile* (and its toxins) or exposure to other colonic microbial products during CDAD, an overexuberant neutrophil response is clearly associated with tissue damage and persistent clinical disease. The degree of initial neutrophilia, kinetics of neutrophil recruitment to the site of infection, subsets of the infiltrating cells and eventual clearance of neutrophils, could all alter the clinical outcomes of *C. difficile*-associated disease. It is thus important to understand the mechanisms that maintain the right balance in neutrophil responses after *C. difficile*

challenge, and focus future studies on better elucidating the mechanisms of disease resolution and mucosal healing.

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Abbreviations

CDI	<i>Clostridium difficile</i> infection
CDAD	<i>Clostridium difficile</i> associated diseases
IECs	Intestinal epithelial cells
IL	interleukins
IFN	interferon
GM-CSF	granulocyte-macrophage colony stimulating factor
G-CSF	granulocyte-colony stimulating factor
CXCL	chemokine (C-X-C motif) ligand
CCL	chemokine (C-C motif) ligand
ROS	reactive oxygen species

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Fig. 1. Pathogenesis of *C. difficile*-associated disease

In normal gut, host microbial communities resist *C. difficile* colonization. Disruption of gut microbiota creates an ideal niche for *C. difficile* colonization. In *C. difficile* infection, toxins disrupt the epithelial integrity resulting in elevation of inflammatory cytokines and chemokines followed by both systemic and tissue neutrophilia. Infiltrated neutrophils generate inflammatory mediators, perform phagocytosis of pathogen and toxins, and in severe cases lead to pseudomembranous colitis. Neutrophil response is almost immediately followed by presence of macrophages and dendritic cells in systemic circulation as well as tissue. Apoptosis of neutrophils and subsequent phagocytosis by macrophages kicks in the resolution phase. Phagocytosis of apoptotic neutrophils coincides with surge in pro-resolution molecules and reduction in inflammatory cytokines and chemokines leading to tissue and systemic homeostasis.

Table 1

The role of different signaling pathways in neutrophil recruitment in infection models.

Pathway	Species/model/pathogen	Proposed mechanism of action on neutrophil recruitment	Observed effect (time point)		Reference
GM-CSF-GM-CSFR	C57BL6 mice Anti-GM-CSF <i>C. difficile</i> (630)	GM-CSF is vital in neutrophil recruitment	•	Reduced neutrophil count in colon (Day 4)	[42]
			•	Reduced <i>cxc11</i> and <i>cxc12</i> expression in colon (Day 4)	
Leptin	C57BL/j6 <i>db/db</i> and S1138 mice <i>C. difficile</i> (VPI 10463)	Robust LepR-STAT3 signaling enhances neutrophil recruitment	•	Reduced neutrophil count in lamina propria (Day 2)	[46]
			•	Reduced <i>cxc19</i> , <i>cxc110</i> , <i>ccl3</i> , and <i>ccl4</i> expression in colon (Day 2)	
IL-23	C57BL6 mice, IL-17a ^{-/-} and p19 ^{-/-} (IL-23KO) <i>C. difficile</i> (VPI 10463)	IL-23 mediated modulation of neutrophil chemokines resulting in increased neutrophil recruitment.	•	Reduced neutrophil count in colon (Day 2)	[43]
			•	Reduced <i>cxc11</i> , <i>cxc12</i> , <i>ccl3</i> , <i>csf3</i> expression in colon (Day 2)	
P2Y ₆ -CXCL8	C57BL6 mice, P2Y ₆ antagonist (MRS2578) <i>TcdA/B</i> toxins	P2Y ₆ dependent expression of CXCL8 leading to increased neutrophil recruitment	•	Reduced neutrophil activity (measured as MPO activity) (4 h)	[40]
			•	Reduced <i>cxc18</i> expression (<i>in vitro</i>)	
MyD88-CXCL1	C57BL6 mice (MyD88 ^{-/-}) <i>C. difficile</i> (VPI 10463)	MyD88-dependent expression of CXCL1 enhances neutrophil recruitment	•	Reduced neutrophil count in cecum and colon (Day 2)	[37]
			•	Reduced <i>cxc11</i> expression in cecum (Day 2)	
NOD1-CXCL1	C57BL6 mice (Nod1 ^{-/-} and Nod2 ^{-/-}) <i>C. difficile</i> (VPI 10463)	Nod1-dependent expression of CXCL1 enhances neutrophil recruitment	•	Reduced neutrophil count in cecum and colon (Day 2)	[39]
			•	Reduced <i>cxc11</i> expression in serum (Day 1) and in cecum (Day 2)	
IL-22-STAT3	C57BL6 mice (anti-IL-22 & anti-CD-160) <i>C. difficile</i> (630)	IL-22-CD160-dependent phosphorylation of STAT3 and subsequent modulation of neutrophil chemokines enhances neutrophil recruitment	•	Reduced neutrophil count in cecum and colon at (Day 4)	[41]
			•	Reduced <i>cxc11</i> , <i>ccl2</i> , <i>cxc19</i> , <i>cxc110</i> expression in colon (Day 4)	
IFNγ-IDO1	C57BL6 mice (IDO-1 ^{-/-}) <i>C. difficile</i> (VPI 10463)	IDO-1 mediated tryptophan catabolism reduces	•	Increased neutrophil accumulation in cecum (Day 2)	[87]

Pathway	Species/model/pathogen	Proposed mechanism of action on neutrophil recruitment	Observed effect (time point)	Reference
		neutrophil accumulation	•	Increase in IFN γ and IL-4 expression (Day 2)

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