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Role of the leukocyte response in normal and immunocompromised host after *Clostridium difficile* infection

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Abstract

Clostridium difficile is the leading cause of healthcare-associated infections in the United States. Clinically, *C. difficile*-associated disease can present as asymptomatic colonization, self-limited diarrheal illness or severe colitis (that may result in death). This variability in disease course and outcomes suggests that host factors play an important role as key determinants of disease severity. Currently, there are several scoring indices to estimate severity of *C. difficile*-associated disease. Leukocytosis and renal failure are considered to be the most important predictors of *C. difficile* disease severity in hosts with a normal immune system. The degree of leukocytosis which is considered significant for severe disease and how it is scored vary amongst scoring indices. None of the scores have been prospectively validated, and while total WBC count is useful to estimate the magnitude of the host response in most patient populations, in immune-compromised patients like those receiving chemotherapy, solid organ transplant patients or hematopoietic stem cell transplants the WBC response can be variable or even absent making this marker of severity difficult to interpret. Other cellular subsets like neutrophils, eosinophils and lymphocytes provide important information about the host immune status and play an important role in the immune response against *C. difficile* infection. However, under the current scoring systems the role of these cellular subsets have been underestimated and only total white blood cell counts are taken into account. In this review we highlight the role of host leukocyte response to *C. difficile* challenge in the normal and immunocompromised host, and propose possible ways that would allow for a better representation of the different immune cell subsets (neutrophils, lymphocytes and eosinophils) in the current scoring indices.

Keywords

Clostridium difficile; Leukocytosis; Disease severity

Clostridium difficile is a gram positive spore forming bacterium that is the leading cause of healthcare-associated infections in the U.S (https://www.cdc.gov/hai/organisms/cdiff/Cdiff_clinicians.html). The principal mode of *C. difficile* transmission is fecal-oral. Host factors including the magnitude of immune response play an important role in disease pathogenesis [1]. The degree of disease severity can vary amongst different type of hosts,

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usually with increased severity in immunocompromised populations. Clinical severity score indices for *C. difficile* infection have emerged as tools to stratify patients into mild or severe forms of disease presentation. By using clinical risk factors included into the scoring index the clinician is able to predict (to some degree) disease prognosis and decide what type of treatment is most appropriate. Currently, there are several scoring indices to estimate severity of *C. difficile*-associated disease (CDAD), however, none of them has been validated in a prospective manner. Most of the scoring indices take into account certain clinical and laboratory variables including total white blood cell count (WBC), serum creatinine and albumin levels and radiographic findings like ascites, ileus, colitis, bowel wall thickening, pneumatosis coli, etc. Notably, the total white blood cell count is part of all available scoring systems. While this is useful to estimate the magnitude of the host response in most patient populations, in certain cases of immune-suppression (cancer chemotherapy, solid organ and hematopoietic stem cell transplant recipients), the WBC response can be inappropriately suppressed or even absent making this marker of severity difficult to interpret.

Here we present a review of the current literature about the role of WBCs (and different cellular sub-populations) in *C. difficile* disease pathogenesis and outcomes in the normal and immunocompromised host. We also propose possible ways that allow for a better representation of the different immune cell subsets into the current available scoring systems.

1. Neutrophils

Neutrophils are the first cells recruited to the colon in response to *C. difficile*, and the neutrophil response is believed to be a determinant of disease severity. Initial migration of neutrophils from the bone marrow into peripheral circulation and recruitment of neutrophils to the site of *C. difficile* infection is mediated by production of neutrophil growth and recruitment factors (for example G-CSF, GM-CSF, IL-17, leptin, etc.) from the inflamed tissue [2–5]. Neutrophils have multiple mechanisms of controlling bacterial infections: release of neutrophil-specific granule components, reactive oxygen species (ROS) production, neutrophil extra-cellular traps production and phagocytosis to name a few. In the presence of *C. difficile* infection, neutrophils can be activated by *C. difficile* toxins, through the formyl peptide receptor-1 (FPR-1) and generate ROS [6]. Neutrophils can also perform phagocytosis of complement and antibody coated *C. difficile* at least *in vitro* [7,8]. However, despite of neutrophil bactericidal response, toxigenic strains of *C. difficile* have evolved mechanisms to resist neutrophil actions, for example, glutamate dehydrogenase secretion from *C. difficile* confers resistance to phagocytosis and neutrophil-induced oxidative stress [9].(See Table 1)

Neutrophil-mediated inflammation can act as a double-edged sword and neutrophil actions themselves can lead to immune-mediated damage of host tissues. In animal models of *C. difficile*, ablation of neutrophil response can either have beneficial or deleterious effects [2,10]. In case of *C. difficile* Toxin A-induced intoxication, depleting neutrophils decreases edema and colonic disease at the microscopic levels [11]. However, in a mouse model of *C. difficile* infection, depletion of neutrophils while associated with decreased colonic

inflammation was associated with higher mortality, likely due to inability to control translocation of commensal gut microbes [2]. Similar dichotomy is seen in patients with *C. difficile* colitis as well: while leukocytosis (albeit without discrimination of cellular components) has been associated to increased mortality, neutropenia has also been associated with an increased incidence and recurrence of *C. difficile* associated diarrhea [12–14]. Thus, a well-balanced and controlled neutrophil response is needed for best outcomes after *C. difficile* infection. Neutrophils are also known to set stage for eventual disease resolution [15], by clearance of bacteria and secretion of anti-inflammatory and pro-resolving intermediates. However, the role of neutrophil-mediated disease resolution after *C. difficile* infection has not been well studied in either animal models of in patient cohorts.

Interestingly, clinical factors like age, steroids and chemotherapy, which have been associated to *C. difficile* infection are also known to modify the normal neutrophil response. Thus, while most of the clinical studies and scoring indices focus on total WBC count, we think that further studies should be focused on studying neutrophils as disease modifying mediators. We postulate that the magnitude of the neutrophil response varies amongst hosts, and could be a good predictor of the *C. difficile* clinical outcomes. It is also important to consider how such clinical scoring indices should be adjusted for neutropenic populations. Current studies would suggest that neutropenia should also be considered a risk factor similar to leukocytosis.

2. Eosinophils

Eosinophils are granulocyte leukocytes that at homeostasis are present in the gut [16]. Eosinophils are involved in presentation of antigens through MHC II, expression of pattern recognition receptors like TLR2, NOD1, NOD 2, response to immunomodulatory mediators like IL-33, IL-25, TGF β and IL-17A and secretion of IL-10 and TGF β [16]. Eosinophils from the Lamina Propia in the intestinal mucosal surface have been shown to induce differentiation of regulatory T cells [17], as well as to be important for development and maintenance of mucosal IgA plasma cells. Thus, a protective and regulatory role for Lamina Propia eosinophils has been proposed [18]. Normal eosinophil counts in the blood vary from 50 to 350 cells/microliter (0–6% of total WBCs). In patients with *C. difficile*, a recent meta-analysis comparing Vancomycin vs Fidoxamicin for the treatment of CDAD, showed that low eosinophil counts in the blood was an independent predictor of persistent diarrhea and death in the first 12 days of therapy. The same effect was not observed later in the course of disease (days 13–40) [19]. In animal studies, Buonomo et al. demonstrated a protective role of tissue eosinophils in mice, and this effect was mediated by IL-25 secretion. In this study, restoration of IL-25 levels in a murine model of *C. difficile* infection led to reduced mortality, whilst eosinophil depletion resulted in loss of the protection mediated by IL-25 [20]. In another study by the same group, *C. difficile* Binary toxin CDT (*C. difficile* transferase) was able to induce inflammation in a murine model by binding to eosinophil Toll-like receptor 2 (TLR-2), resulting in activation of NFκB and suppression of eosinophil response via indirect induction of eosinophil apoptosis. This experiment suggests a protective role from eosinophils in *C. difficile* infection [21].

Eosinophils are known to play an important role in asthma inflammatory response as well as a protective role in helminth infections [22,23]. But so far, there is lack of evidence that suggests that they offer *in vivo* antibacterial activity against *C. difficile*. The exact mechanisms by which eosinophils provide protection against *C. difficile* are not clearly understood, and further study of eosinophil immune response to *C. difficile* in animal models is needed to better understand the possible protective role of this cellular subset. However, based on current studies, the incorporation of eosinophil values into *C. difficile* scoring indices may improve prediction of disease severity. We propose categorizing absolute eosinophil counts into low, normal and high values in *C. difficile* infected patients in order to determine if there is an association with disease severity.

3. Lymphocytes

Lymphocytes are a regular anatomic component of the gut. Lymphocyte subsets present in the intestinal tissue include B cells, plasma cells, $CD4^+$, $CD8^+$ and gamma-delta T cells, Natural Killer cells and Innate Lymphoid cells [24–26]. Lymphocytes migrate from the peripheral circulation into the intestine and harbor in Peyer's patches. In the intestinal lymphoid tissue, they can become activated if they are exposed to the appropriate antigens. Lymphocytes go back to the systemic circulation and later return to the intestinal Lamina Propia where they execute effector functions [27].

In humans, the role of B cells seems to be related to disease severity and infection recurrence. Clinical data of *C. difficile* infection in humans suggest that while there is no role of antibody response in protection against colonization, asymptomatic infection carriers have greater levels of anti-Toxin A IgG, compared to patients who develop diarrhea [28]. Most recently a randomized, double-blind, placebo-controlled study of two neutralizing, fully human monoclonal antibodies against *C. difficile* toxins A (CDA1) and B (CDB1) in patients with symptomatic *C. difficile* diarrhea demonstrated decrease recurrence of *C. difficile* infection [29]. Toxin B is essential for disease while Toxin A may not be [30], a recent multivariate analysis of the placebo arm of the aforementioned trial found that in this study the main predictor for *C. difficile* infection recurrence was age >65 (OR 3.93, p 0.09), meanwhile, antibodies to toxin B were found to be protective against disease recurrence (OR 0.11, p 0.05) [31].

The role of T cells is less clear in *C. difficile* infection. After infection with *C. difficile*, $CD4^+$ T cell-deficient mice are unable to produce an IgG antibody response (which is thought to be dependent on $CD4^+$ T cells), but generate anti-toxin IgA responses, as compared to wild type mice who produce both IgG and IgA antitoxin antibodies [32]. However, $CD4^+$ T cell-deficient mice (which lack an IgG antibody response) are able to recover from primary infection, and upon re-challenge, they do not developed diarrhea and have normal colon histology [32]. Thus, it is possible that both IgG and IgA anti-toxin responses play a role in controlling disease after *C. difficile* challenge. The protective role of humoral immunity in humans may also be supported by incidental findings in a study assessing the use of anti-peristaltic agents in *C. difficile* infection in cancer patients [33]. In this study 303 Multiple Myeloma patients received chemotherapy and 43 developed *C. difficile* associated diarrhea. Out of all Immunoglobulin Myeloma isotypes, IgA Multiple

Myeloma seems to have a protective effect against *C. difficile* diarrhea (RR 0.35; 95% CI 0.13–0.93, *p* 0.04) [33]. The mechanism of protection is not clear but is likely that hyper-secretion of mucosal IgA prevents *C. difficile* infection. In humans, anti-toxin IgG immunity actively acquired during *C. difficile* infection appears to decrease disease recurrence [34].

Further studies of *C. difficile* in the context of T-cell deficiency, for example, in HIV infected patients could provide useful insights into the role of T-cell mediated immune responses after *C. difficile* infection. Measurement of Immunoglobulin isotypes in serum and stool in this type of model may allow us to identify potential immunoglobulin deficiencies that contribute to more severe presentations of *C. difficile* infection.

4. Leukocytes and scoring systems

In the normal host with CDAD, leukocytosis can manifest in different patterns. It can coincide with the onset of symptoms in the setting of recent antibiotic administration. It can present as worsening leukocytosis with non-specific *C. difficile* symptoms during or following antibiotic administration and lastly; can present as unexplained leukocytosis associated to fever, abdominal pain and systemic symptoms while receiving antibiotic therapy, and diarrhea will occur later in the course of the disease [35]. Unexplained leukocytosis ≥15,000/mm^3, has been reported to be associated with *C. difficile* infection, in a study by Musher et al., 58% of patients with unexplained leukocytosis in a tertiary care center in the United States were found to have a positive assay for *C. difficile* toxin A, later followed by appearance of symptoms [36]. The degree of leukocytosis in CDAD can range from none in immunocompromised hosts to leukemoid reactions, the latter being associated with high in-hospital mortality [37,38]. Leukocytosis is usually associated to *C. difficile* infection, nonetheless, leukocyte response can be highly variable, follow different patterns and change based on intrinsic characteristics of the host immune response.

Leukocytosis has been used as a surrogate marker for *C. difficile*-associated disease severity, as well as, a risk factor for mortality [39]. Usually, higher total white blood cell counts have been associated with increased disease severity. Therefore, leukocytosis is included in different severity scoring systems like CDC, Beth Israel, UPMC, Hines, University of Calgary, etc. The degree of leukocytosis which is considered significant for severe disease and how it is scored vary amongst scoring systems [40,41]. The Infectious Diseases Society of North America recommends using peak leukocyte counts and, as well as, the European Society of microbiology uses a leukocyte count >15,000 cells/mm^3 to define severe *C. difficile* disease [42,43]. Also, the timing of leukocytosis assessment as a clinical risk marker for mortality differs between authors. In a systematic analysis by Bloomfield et al., the peak white blood cell count within a week of diagnosis seems to be the most used [39]. The Hines severity score index appears to be the best predictor of CDAD severity [44]. In this index, 5 factors are taken into account including presence of fever, radiologic findings, blood pressure and WBC count; each factor can obtain a score from 0 to 2, a score of 3 represents severe *C. difficile* diarrhea [40]. Determining CDAD severity in special populations like solid organ transplant patients, cancer patients and Hematopoietic stem cell transplant recipients, can be challenging with the current scoring systems. Total white blood cell counts may not be a reliable indicator of severity as it may be affected by factors like

immunosuppression and chemotherapy. In a study by Sullivan et al., involving 192 Liver transplant recipients with a rate of 14% *C. difficile* infection, white blood cell count greater than 12,000 cells/mm^3 was only present on 7% of the patients, other signs of severity like temperature more than 38 °C were present in 26% of the patients, signs like elevated lactate, renal failure and shock requiring vasopressors were uncommon [45]. Also, in a retrospective study involving 603 kidney transplant recipients with a total of 24 (6.1%) *C. difficile infected* patients, peak white blood cell count and nadir absolute lymphocyte counts within the first 30 days post-transplant were collected, a white blood cell count >20,000 cells/mm^3 was only present on 9 of the infected patients. Most of the patients who were infected were also found to be lymphopenic. On the overall case analysis, cases had a mean white blood cell count of 18,696 cells/mm^3 and controls 15,520 cells/mm^3 with no statistical difference p 0.06. On the other hand, mean nadir absolute lymphocyte counts at 30 days were statistically significantly lower on the cases compared to the control group 96.6 vs 285.6 cells/mm^3 respectively, p 0.02 [46]. Findings seem to be similar in patients receiving simultaneous kidney and pancreas transplantation, with overall no statistical difference in white blood cell counts between cases and controls, but with significant difference in disease incidence p 0.038 in cases with white blood cell counts <1000 cells/mm^3 compared to controls. Use of granulocyte stimulating factor did not appear to play a significant role between groups [47]. Other solid organ transplant recipients seem to follow a similar pattern; a descriptive analysis at a single center of patients with *C. difficile* infection involving recipients of Heart, Lung, Liver, Kidney and multi-organ, mean white blood cell counts in all the groups did not appear to be > 10,000 cells/mm^3 [48].

In cancer patients, chemotherapy has been reported as a risk factor for developing *C. difficile* infection. Platinum based regimens, Cisplatin, Bleomycin, Vinblastine, Placlitaxeland, Doxorubicin are amongst the regimens identified as a risk factor for developing disease [49–51]. Other studies have reported lack of association after controlling for antimicrobial therapy [52]. Increased incidence of *C. difficile* has been associated to leukemia patients with neutropenia [53], also in a study with allogenic hematopoietic stem cell transplant patients, individuals with neutropenia were more likely to have *C. difficile* infection recurrence compared to non-neutropenic individuals [12]. As neutropenia appears to be a risk factor for developing *C. difficile* infection as well as a marker of disease severity in patients receiving chemotherapy [13,54], a recent case control study of 144 patients receiving chemotherapy for hematologic malignancies with *C. difficile* diarrhea compared to controls with *C. difficile* negative diarrhea, presence of neutropenia was not associated with higher mortality [13]. Similar findings have been previously reported in a prior study also comparing patients with hematological malignancies receiving chemotherapy who developed *C. difficile* diarrhea, compared to immunocompetent individuals without malignancy. In this study 62% of the patients on the chemotherapy group received colony-stimulating factor for neutropenia. No significant differences were observed regarding ICU admission (p 0.24) or the need for vasopressors (p 1.0), and mean peak white blood cell counts amongst the 2 groups were not statistically significant (p 0.94) [52]. Data must be interpreted carefully as there is a scarce amount of these type of studies and sample sizes as well as co-morbidity indexes amongst patients may play an important role at the time to draw conclusions.

5. Conclusion

The immune response to *C. difficile* is complex and involves elements of both the innate and adaptive immune systems, however it is important to note that the current scoring indices account for only total leukocytosis. While leukocytosis makes part of all available severity scoring indices and is of importance in assessing *C. difficile* infection severity, recurrence risk, disease complications and treatment failure [14,55], it is certainly not a perfect predictor and poses several barriers to determine disease severity. First, early or late diagnosis of *C. difficile* infection could potentially influence total white blood cell counts, and response to disease and therapy can vary from patient to patient and total WBC counts can be influenced by other factors like comorbidity status, concurrent infections and medication use. Second, current use of leukocytosis does not account for differences in immune response amongst special populations like transplant, chemotherapy and immunosuppressed patients. Third, cut-off leukocyte values that define disease severity vary between different scoring systems, and prospective validation is needed to determine which values and in what type of populations are these parameters most useful. Finally, *C. difficile* is a colonic pathogen and the immune response and associated pathology is predominantly seen at the tissue level. Thus, scoring systems that take into account only peripheral leukocytosis may not accurately reflect what is happening at the tissue level.

In our opinion, total leukocyte counts fail to provide a dynamic representation of the host immune response to *C. difficile* infection and could misrepresent the role from other cellular lines that play an important role in the inflammatory response. Neutrophil response can either be beneficial or detrimental to the host. It is clear that neutrophilia is associated to disease severity. On the other hand, the association between neutropenia and severe *C. difficile* disease seems to be inconsistent as not all neutropenic patients have increased mortality or more severe disease presentations when compared to immunocompetent hosts [13,52]. More recently a hypothetical protective role of eosinophils has emerged, in humans low eosinophil levels were found to be predictor of persistent diarrhea and death, however further validation of this findings is needed. In kidney transplant recipients, lymphocyte depletion has been associated to *C. difficile* infection, however, there is not enough use of lymphocytes as markers of disease to draw conclusions regarding this specific cell subset. Antibody response has shown to be important as asymptomatic infection carriers have greater levels of anti-Toxin A IgG, compared to patients who develop diarrhea, and antibodies to toxin B seem to be protective against disease recurrence.

We propose that the differential cell count be incorporated into severity scoring systems as they could provide a more accurate picture of the host immune response to *C. difficile* infection and thus could improve determination of disease severity. Further, scoring systems should be designed to account for unique factors in different patient populations like host comorbidities and immune status (chemotherapy, transplant, etc.).

Future areas of research

Further questions remain regarding assessing severity of *C. difficile* infection.

1. Should scoring systems be modified for use in special populations?

2. Should we measure disease predictors like (leukocytes, creatinine, albumin, etc.) at different points during disease to have a more dynamic view of disease that could represent the host immune response?

3. Does the use of different cell lines improve prediction of disease severity?

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Table 1

Leukocyte cut-off values for the commonly used *C. difficile* severity score indices

Each severity index (left column) assigns different leukocyte values (middle column) to determine disease severity. The weight of leukocyte values into each scoring index is represented in points, which are the values in parenthesis. After adding up the points given to different clinical variables (additional clinical variables not represented on this table) the clinician is able to define severe disease (right column). Severe disease is associated with worse clinical outcomes. (SHEA) Society for Healthcare Epidemiology of America, (IDSA) Infectious Disease Society of America, (UPMC) University of Pennsylvania medical center.

Severity Index	White blood cell counts (cells/mm^3)	Score defining severe disease
UPMC [56]	<1500 or >20,000 (1 point)	≥2 points
Hines VA [57]	≥15,000 to < 30,000 (1 point) or ≥ 30,000 (2 points)	≥3 points
Beth Israel [58]	>20,000 (1 point)	>4 points
University of Illinois [59]	≥15,000 (1 point)	≥2 points or Pseudomembranous Colitis on endoscopy or treatment in intensive care unit.
SHEA-IDSA [43]	≥15,000	≥15,000 cells/mm^3 or a creatinine level greater than or equal to 1.5 times the premorbid level.