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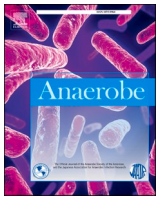

Pathogenesis and toxins

Polymorphonuclear neutrophil depletion in ileal tissues reduces the immunopathology induced by *Clostridioides difficile* toxins

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A R T I C L E I N F O

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A B S T R A C T

Introduction: *Clostridioides difficile*, a leading cause of healthcare-associated infections, causes significant morbidity and mortality. Its pathogenesis centers on TcdA and TcdB toxins, which disrupt intestinal integrity, trigger inflammation, and promote extensive neutrophil infiltration.
Objective: The main objective of this study was to evaluate the role of PMNs in CDI using neutrophil depletion in a murine-ileal-ligated loop.
Methods: Mice were treated with *C. difficile* toxins TcdA, TcdB, and TcdBv, with PMN depletion achieved via intraperitoneal injections of Ly6G/Ly6C antibody. Histopathological analysis, cytokine quantification, and MPO activity assays were performed to assess the inflammatory and tissue damage responses.
Results: PMN depletion significantly reduced histopathological damage and proinflammatory responses. TcdA induced the highest inflammation and epithelial damage, while TcdB showed lower activity, except for MPO. $TcdBv_{NAP1}$'s activity was comparable to that of $TcdB_{NAP1}$ but less than TcdA. The findings indicate that TcdA's enterotoxin effects are more damaging than TcdBs from different strains and confirm the critical role of PMNs in CDI pathogenesis.
Conclusion: Our results show that PMN depletion reduced inflammatory responses and tissue damage, highlighting potential therapeutic strategies targeting PMN regulation. Further research on PMN extracellular traps (NETs) and their role in CDI is necessary to develop comprehensive treatments. Future studies should focus on combined *in vivo* and *in vitro* approaches to fully understand the pathological mechanisms and identify effective biomarkers for CDI therapy.

1. Introduction

Clostridioides difficile is a major cause of healthcare-associated infections (HAIs) and a significant public health threat worldwide [1]. The primary natural reservoir associated with *C. difficile* is the intestinal tract of humans and other varieties of mammals [2,3], which can remain an asymptomatic or symptomatic reservoir. *C. difficile* is not typically harmful to the gut due to other bacteria overseeing *C. difficile* spore germination, growth, and toxin production by controlling primary bile acid levels in the intestinal lumen [4]. Once a host has been administered antibiotics or if other risk factors are present, the likelihood of infection or complications increases significantly. These risk factors are described as a) advanced age, b) chemotherapy, c) proton pump inhibitors or H_2 blockers, d) the presence of comorbidities with functional impairments, e) polymorphism of immune genes (such as IL-8), e) low levels of antibodies against toxin B, and, f) hospitalization [5]. However, antibiotic use is the most common adverse event related to *C. difficile* infection (CDI), which may result in diarrhea through different mechanisms, including osmotic diarrhea and colonization and overgrowth of toxin-secreting C. *difficile* [6].

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C. difficile pathogenesis is similar between strains, but the severity and distribution in the gastrointestinal tract varies widely according to host age and animal species [7]. Subsequently, due to antibiotic treatment, intestinal dysbiosis in the host microbiota can be caused, which commonly *C. difficile* spores are ingested orally by susceptible hosts, followed by C. *difficile* vegetative cells germination, which can opportunistic exponential growth and colonize mammalian colon and induce disease in the intestinal mucus layer [8,9]. After *C. difficile* germination and growth, the bacterium produces two main toxins responsible for the pathogenesis: toxins A (TcdA) and B (TcdB) [10]. Both toxins contain glucosyltransferase activity, but only TcdA targets host Rho GTPases (RhoA, Rac1, and Cdc42) GTPase family proteins [11–13]. GTPase glucosylation leads to cell rounding and death by actin depolymerization [14,15]. In contrast, classical, hypervirulent, and variant TcdBs do not target Rho GTPases but target R-Ras, leading to necrotic cell death independent of glucosylation, proposing a distinct and unique interaction with RAS pathways, exacerbating inflammation and the severity of infection [16]. In addition, CDI has been described as the primary cause of antibiotic-associated diarrhea (AAD) [17]. Still, it is also a disease that can range from mild diarrhea to severe conditions such as pseudomembranous colitis, which leads to a significant clinical and economic burden on hospitals [5].

TcdA and TcdB can activate various immune cells in the mucosa, leading to a secondary phase of inflammatory mediators that release an exacerbated recruitment of neutrophils or polymorphonuclears (PMNs) [18]. PMNs are short-lived blood cells of myeloid origin that function as effector cells in host defense against pathogens, phagocytizing the agent or stimulating inflammation [19,20]. PMNs use oxygen-dependent and -independent methods to kill bacteria. PMNs have a membrane-bound NADPH-dependent oxidase, producing high reactive oxygen species (ROS) levels. Meanwhile, oxygen-independent microbicidal systems utilize antimicrobial peptides and enzymes to facilitate the killing and degradation of ingested microbes [21]. This concerted action of proteolytic enzymes, cationic molecules, and ROS showcases the potent antimicrobial activity of PMNs [22].

TcdA is considered responsible for activating the production of proinflammatory cytotoxicity interleukins, such as IL-8, IL-1β, IL-6, and TNF-α, by human monocytes, as well as Il-8 and IL-6 by epithelial intestinal cells [23–25]. While IL-8 was identified as a potent chemoattractant for the recruitment of human PMNs [26], the orthologue, its counterpart, is absent in mice. Disparate mice express a chemokine known as CXCL1/MGSA [27]. Even IL-6 has been associated with the recruitment of PMNs in ileal loop models, which increases epithelial damage [28,29]. IL-1β plays a crucial role in initiating and amplifying the intestine's inflammatory process [30–32] and is denominated in being activated and secreted by an independent-glucosyltransferase activity process of the toxins [33,34]. Regarding the onset of the pathogenic process, TNF-α is used as an indicator due to its ability to induce the secretion of other proinflammatory mediators [35]. In contrast, TcdB is considered the primary virulence factor of *C. difficile* due to its high toxicity, which is generally more potent (~1000 times) than TcdA [36], particularly in cell culture models [37]. TcdB triggers the release of TNF-α and IL-1β [38].

In the context of CDI, PMNs may cause tissue damage. Their regulation is limited to the site of inflammation, where a massive neutrophil infiltration is required to solve the pathology [39–41]. It has been described that the toxins TcdA and TcdB [39] induce a cellular response leading to massive PMN infiltration, ultimately destroying the intestinal epithelium. Also, neutrophilia correlates with a reserved prognosis [42]. Then, an exaggerated inflammatory response is mainly responsible for the pathology associated with *C. difficile* infections [43]. Furthermore, continuous exposure to aggressive and prolonged inflammatory stimuli during CDI generated chronic inflammation in the host [44].

Through genomic and clinical descriptions, epidemic strains have been detected, mainly belonging to Clade 2 and with the NAP1/RT027/ST01 genotype. This is why a toxin from this genotype was selected to study the role of neutrophils during CDI [45–48]. Previous studies reported that TcdA was the primary virulence factor, while TcdB played a lesser role in the disease [49,50].

However, due to *in vivo* animal model approaches, such as animal ileal loops, a more relevant role is given to TcdB [51]. This is in tune with the emergence of natural variant strains of *C. difficile* that produce TcdB but not TcdA (TcdA- TcdB+), which also causes severe CDI in humans [52]. Subsequently, Wershil et al. [53], using a murine ileal loop model, reported that *C. difficile* toxins stimulate mast cells, which participate in neutrophil infiltration. Utilizing murine models is imperative for advancing the comprehension of disease pathophysiology *in vivo* and the function of specific cells, such as PMN, in these pathologies.

Despite the vital importance of PMNs in response to pathogens, the effect of *C. difficile* toxins TcdA and TcdB on pathology has mainly been studied *in vitro* but yielded inconsistent results [54–59]. A recent *in vitro* study discusses the immunopathogenesis of human PMNs and their resistance to TcdB's glucosyltransferase activity on small GTPases belonging to the superfamily of Ras proteins, including RhoA and Rac1 [60]. Some *in vivo* studies are available on the immunopathogenic effects of PMNs in CDI [33–35,49,53,59,61–63]; however, to our knowledge, no study has utilized PMN depletion strategies to evaluate the role of neutrophilic inflammation in the context of CDI in the absence of neutrophils [29]. To understand the role of PMNs during CDI and how toxins can affect their functioning under natural physiological conditions, it is crucial to study the activity of *C. difficile* toxins in *in vivo* models. As a result, we evaluated the role of PMNs in the pathogenesis produced by *C. difficile* toxins A (TcdA) and B (TcdB) in a neutropenic murine model.

2. Materials and methods

2.1. Toxins

C. difficile native toxins $TcdA_{VPI}$, $TcdB_{NAP1,}$ and $TcdBv_{NAP1}$ were purified from the strains VPI 10463/RT087 (ATCC 43255) [TcdA], and NAP1/RT027/ST01 [TcdB], and NAP1/RT019/ST67 [TcdBv], both TcdBs were obtained from clinical isolates collected during a CDI outbreak in Costa Rica tertiary hospital, respectively [64]. The Laboratorio de Investigación en Bacteriología Anaerobia (LIBA) of the Faculty of Microbiology and the Center for Research on Tropical Diseases (CIET), University of Costa Rica, supplied all the toxins used in this study. Toxins remained at −80 °C in 20 mM HEPES buffer at pH 6.9 supplemented with 50 mM NaCl until use.

2.2. Animals

Male Swiss mice of the Hsd: ICR (CD-1®) strain, aged between 5–8 weeks and with a body weight of 18–25 g, were supplied by the Servicio Nacional de Salud Animal, Ministry of Agriculture, Costa Rica. Mice were grouped in polycarbonate cages and maintained under controlled conditions and a photoperiod of 12 h. All mice were housed under specific pathogen-free conditions and had free access to standard rodent food and water *ad libitum*.

2.3. Mice neutrophil depletion protocol

Mice were depleted by an intraperitoneal treatment with 100 μg of Ly6G/Ly6C [Gr-1] anti-mouse antibody (InVivoMab, BioXCell) suspended in PBS solution (Cell culture, Gibco) at a final volume of 100 μL as previously described [65]. Non-depleted mice (control mice) were inoculated only with PBS solution. PMN depletion was verified at 24 h after Gr-1 or PBS treatment in the blood (hematological and flow cytometry analyses) and ileal segments (histological analysis). All mice were anesthetized with ketamine (60 mg/kg), xylazine (5 mg/kg), and blood collected from the retro-orbital sinus in EDTA tubes. Mice were euthanized by cervical dislocation after blood collection. Subsequently,

ileal segments were collected for histological analysis.

2.4. Hematological and flow cytometry analyses

To assess the number of PMNs remaining in whole blood in depleted mice, a complete hematological analysis was performed using the automated hemocytometer VetScan HM5 Hematology Analyzer, following the manufacturer's instructions. In addition, PMNs were labeled with the monoclonal antibody FITC anti-Ly6G (RB6-8C5) (Invitrogen, ThermoScientific), following the manufacturer's recommendations [65]. The percentage of PMNs was quantified by flow cytometry using the guava easyCyte™ (Merk-Millipore™). The PMN population was selected on Side Scatter and Green Fluorescence according to its cellular characteristics. The analysis of the populations of interest was performed using the FlowJo cytometry software package (version 10.1).

2.5. Experimental design and murine-ligated-ileal loop model (MLIL)

Mice were classified into ten experimental groups of ten individuals each as follows: 1) PBS, 2) PBS + GR-1, 3) TcdA, 4) TcdA + GR-1, 5) TcdB, 6) TcdB + GR-1, 7) TcdA + TcdB, 8) TcdA + TcdB + GR-1, 9) TcdBv, and 10) TcdBv + GR-1. Briefly, mice were treated with Gr-One antibody (depleted) or PBS (non-depleted) for 24 h, according to the experimental groups. Then, all mice were fasted overnight. After 24 h of depletion, mice were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg). An ileal loop was ligated through a midline laparotomy, and 300 μL [1 μg/μL] of the corresponding toxin or PBS was injected into the ileal loop (depending on the experimental group). Mice were euthanized for 4 h by cervical dislocation after injection, and the length and weight of the intestinal loops were recorded [66,67]. To evaluate pathological and inflammatory responses elicited by the toxins in each experimental group, we performed histopathological analysis, measured the MPO activity, and quantified cytokines production in ligated ileal loops.

2.6. Histopathological analysis

For histopathology, murine-ligated-ileal loops samples were fixed in 10 % neutral buffered formalin, routine processed and stained with hematoxylin and eosin (H&E). These preparations were evaluated for neutrophilic infiltration, epithelial damage, and lamina propria and submucosa inflammation using a histopathological scoring (HS) system that ranged from 0 (absence of alterations) to 4 (severe) as previously reported [68–70]. A certified pathologist performed all histopathological assays in a single-blinded setting.

2.7. Myeloperoxidase (MPO) assay

A colorimetric MPO activity assay estimated PMN degranulation and migration in homogenized ligated ileal tissues [71]. Briefly, ligated ileal tissues (approx. 56 mg) were homogenized in hexadecyltrimethylammonium bromide (HTAB) (Sigma-Aldrich™) buffer (PBS, HTAB 50 % w/v, and H_2O_2 0.1 % v/v) and cleared by centrifugation at 5000 g for 7 min at 4 °C. The resulting supernatants were incubated with a 0,017 % o-dianisidine solution (Sigma-Aldrich™), and after 5 min, the absorbance was determined at 450 nm. Results were reported as MPO/100 mg of ligated ileal tissue.

2.8. Quantification of CXCL-1, TNF-α, IL-6, IL-1β, and IL-10

The concentration of proinflammatory interleukins (CXCL-1, TNF-α, IL-6, IL-1β, and IL-10) in the ligated ileal tissue homogenized in Phosphate-Buffered-Saline (PBS) solution estimated with commercial enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions (Invitrogen).

2.9. Ethics statement

The ligated-ileal loop surgery procedure was performed as previously described [64]. This procedure was approved by the "Comité Institucional para el Cuido y Uso de los Animales" from the Universidad de Costa Rica (under executive order No. 26668 with folio No. CICUA-043-2021) and performed under the corresponding law "Ley de Bienestar de los Animales de Costa Rica" (Law 9458 on animal welfare). Furthermore, the project was also approved by the "Consejo Nacional de Investigación en Salud" (CONIS) under official letter No. UNA-PCVET-OFIC-087-2021.

2.10. Statistical analyses

Data from the animal models are presented as mean ± standard deviations (S.D.) or medians. Means and median were compared using Students' *t*-distribution and one-way ANOVA, and two-way ANOVA tests with Bonferroni correction or Kruskall-Wallis tests followed by Dunn's multiple comparison test, respectively. p values of <0.01 (**) and $*< 0.05$ (*) were considered statistically significant.

3. Results

GR-1 antibody treatment significantly decreases the PMN population in peripheral blood, lamina propria, and submucosa.

To verify the capacity of the GR-1 monoclonal antibody to induce PMN depletion in our mice model, we treated mice with GR-1 or PBS (as a negative control). We then evaluated whether GR-1 treatment induced a statistically significant decrease of PMNs in peripheral blood, lamina propria, and intestinal submucosa.

First, we performed a complete hematological analysis to quantify the number of PMNs and other parameters in peripheral blood (Table 1). Accordingly, GR-1 induced a statistically significant decrease in PMNs ($p < 0.05$). PMN depletion did not affect the other hematological parameters evaluated since GR-1 did not cause a statistically significant alteration in the different values compared to the control group ($p > 0.05$). A flow cytometric analysis was performed in whole blood to quantify the percentage of remaining PMNs after depletion using a FITC anti-Ly6G marker. Accordingly, the PMN population was selected and quantified on an SSC vs. Green Intensity diagram. As observed, the non-depleted group had 10,3 % of PMNs, while the PMN-depleted group had only 0.38 % PMNs (Fig. 1A). This analysis confirmed the significant decrease of PMNs in peripheral blood observed in the hematological analysis.

Since our model is performed in the ileal tissue, we also assess whether the treatment with GR-1 antibody also induced a reduction of the PMN population in lamina propria and intestinal submucosa. Therefore, we quantified the number of PMNs in these tissues in PMN-depleted and non-depleted mice by direct microscopic observation. Fig. 2 shows the abundant presence of PMNs (yellow arrows) in non-depleted mice, while PMN-depleted mice showed a decreased number presence of these cells. Treatment with GR-1 antibody or PBS did not induce pathological histological alterations in any of the tissues evaluated. Fig. 1B shows a significant difference ($p < 0.01$) in the number of PMNs between both experimental groups.

3.1. PMN depletion reduces the histopathological damage induced by *Clostridioides difficile* toxins

We then wanted to evaluate whether PMN depletion positively impacted the severity of the histopathological damage induced by *C. difficile* toxins. Therefore, we inoculated the ileal loops with each toxin individually or combined in both experimental groups. The histopathological patterns observed in the ileal loops were characterized by (i) epithelial damage in TcdA and TcdA/TcdB combinations and (ii) inflammation (with neutrophilic infiltration) in lamina propria and

Table 1
Hematological values of CD1 mice after 24 h of i.p administration of PBS (non-depleted) or 100 μg Gr-1 anti-mouse antibody (PMN-depleted).

Parameter	Unit	Depleted		Non-depleted		*p* value
		Mean	CI 95 %	Mean	CI 95 %	
Leucocytes	10^9/L	5.77	[3.03–8.52]	7.95	[7.13–8.77]	0.0680
Lymphocytes	10^9/L	5.57	[2.97–8.16]	7.40	[6.58–8.21]	0.0985
Monocytes	10^9/L	0.09	[-0.01 – 0.19]	0.13	[0.00–0.27]	0.5350
Neutrophils	10^9/L	0.12	[0.03–0.20]	0.42	[0.15–0.69]	0.0169
Erythrocytes	10^{12}/L	9.97	[8.16–11.78]	10.02	[9.78–10.26]	0.9366
Hemoglobin	g/dL	14.70	[11.76–17.64]	15.30	[14.75–15.85]	0.5930

CI: Confidence interval.

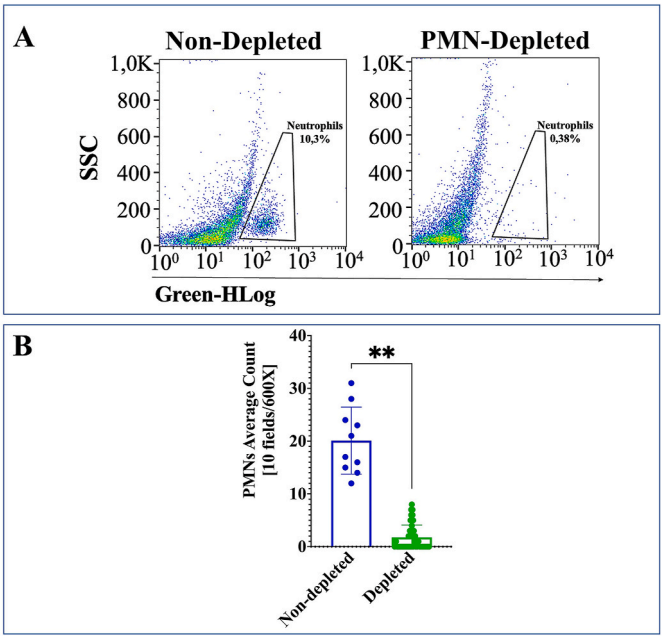

Fig. 1. Evaluation of PMN depletion in peripheral blood and intestinal lamina propria and submucosa. PMN depletion was assessed in (A) peripheral blood by flow cytometry using a FITC anti-Ly6G antibody and in (B) tissue sections (lamina propria and submucosa) counted in 40X fields. Results are expressed as mean PMN count per field ± S.D., with comparisons between groups showing significant differences ($p < 0.01$, **).

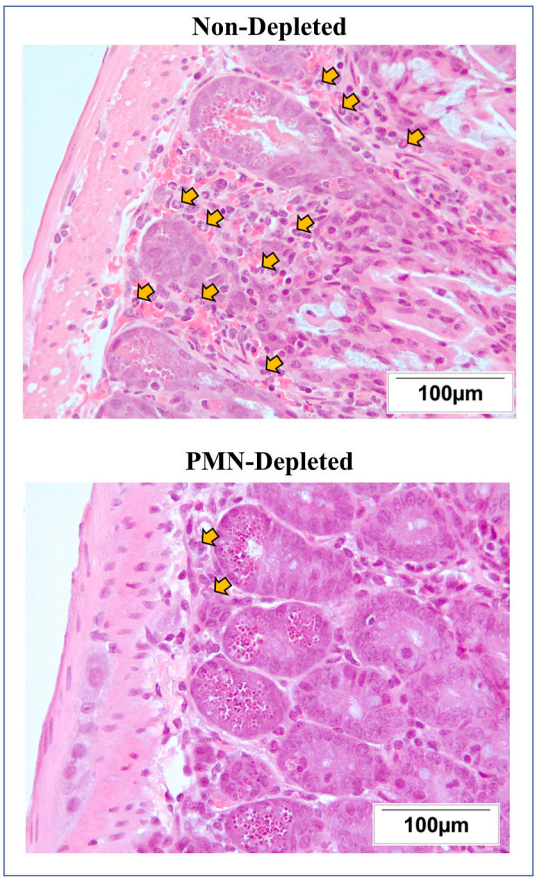

Fig. 2. Histopathological images from non-depleted and PMN-depleted ileal tissues. PMN depletion was evaluated in tissue sections (lamina propria and submucosa) stained with H&E. PMNs (yellow arrows) in 40X fields.

submucosa. Inflammation was more conspicuous in TcdA and TcdA/TcdB combinations compared to TcdB or TcdBv treatments (Fig. 3A, C, 3E, and 3G). Tissue alterations were reduced (to a greater or lesser extent depending on the toxins) in the PMN-depleted group (Fig. 3B, D, 3F, and 3H).

Tissue alterations were scored using a histopathological scale. As observed in Fig. 4, the TcdA toxin induced the most significant histopathological damage, followed by the combination of TcdA + TcdB toxins. Mainly, TcdA caused epithelial damage (Fig. 4A) and inflammation in lamina propria (Fig. 4B) and submucosa (Fig. 4C). Likewise, in the mice treated with TcdA and depleted of PMNs, the most significant decrease in the scale of histopathological damage was observed in comparison to the non-depleted mice ($p < 0.01$) (Fig. 4).

TcdB and TcdBv toxins did not induce epithelial damage (Fig. 4A); however, they did show some inflammatory response and lamina propria (Fig. 4B) and, to a lesser extent, in the submucosa (Fig. 4C). As a general outcome, PMN depletion induced a significant decrease in the scale of histopathological damage in all toxin treatment groups compared to the non-depleted group of mice (Figs. 3 and 4).

3.2. Upon treatment with C. difficile toxins, PMN-depleted mice displayed decreased cytokine production

To evaluate and characterize the inflammatory response elicited by *C. difficile* toxins, we quantified CXCL-1 (as a PMN recruiter), TNF-α, IL-1β, and IL-6 (as proinflammatory cytokines) and IL-10 (as an anti-inflammatory-cytokine) in ileal tissues. Overall, TcdA induced the highest cytokine production (Fig. 5). However, similar to the histopathological response, all the cytokines induced by this toxin significantly decreased in the PMN-depleted group ($p < 0.01$). The reduction in cytokine production was also observed in the TcdA + TcdB combination. Nevertheless, it is worth noticing that despite TNF-α, TcdA + TcdB combination induced a lower level of cytokine production compared to TcdA treatment alone ($p < 0.01$) (Fig. 5A, B, 5D, and 5E, respectively).

TcdB and TcdBv cytokine profiles were very similar. Both toxins induced significant production of TNF-α (similar to TcdA and TcdA +

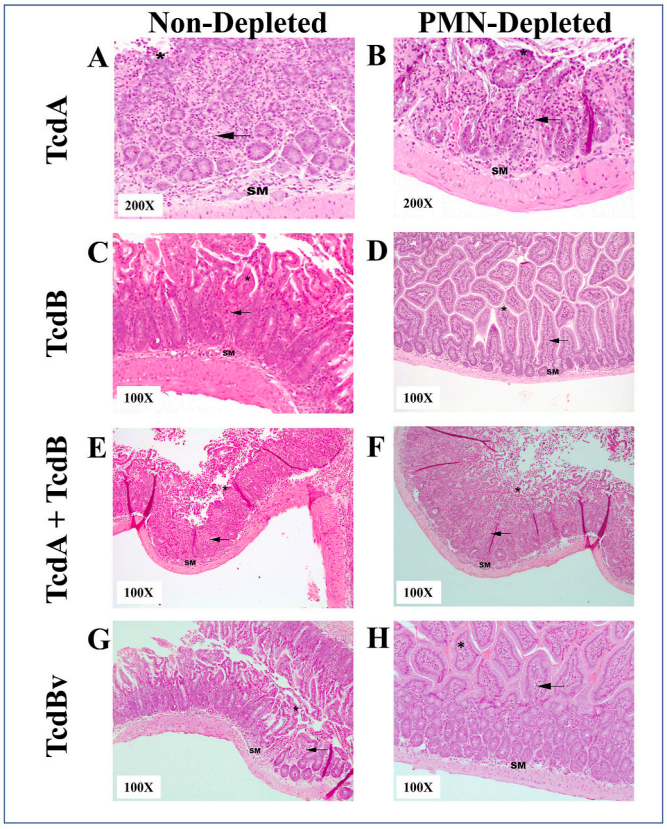

Fig. 3. Histopathological images from ileal-ligated-loop tissues treated with TcdA, TcdB, TcdBv, or combinations of TcdA + TcdB in non-depleted and PMN-depleted mice. Tissues were fixed in 10 % formalin, stained with H&E, and analyzed for histopathological changes. Tissue from cases treated with toxin TcdA (A, H&E 200x) and combinations of TcdA + TcdB (E, H&E 100x) exhibit the most relevant histopathological alterations with higher degrees of epithelial necrosis (*) and inflammatory infiltrated through the lamina propria (arrow) and into the submucosa (SM) (E, H&E, 100x). Non-depleted cases showed less severe inflammatory and necrotic changes (H&E, B, 200x; D, F, H, 100x).

TcdB combinations) with a statistical reduction ($p < 0.01$) in the PMN-depleted group (Fig. 5C). A similar result was observed in IL-10 production with TcdB treatment but not with TcdB since no IL-10 production was observed (Fig. 5E). CLCX-1, IL-6, and IL-1β production was almost absent upon TcdB and TcdBv treatments (Fig. 5A, B, 5E). In general, a significant decrease in the production of cytokines (proinflammatory, inflammatory, and anti-inflammatory) was observed after toxin treatments in the PMN-depleted group.

3.3. PMN depletion reduced myeloperoxidase (MPO) activity induced by the $TcdB_{NAP1}$ toxin

The MPO activity was measured as a surrogate indicator of neutrophil migration and degranulation to assess the PMN inflammatory response induced by *C. difficile* toxins in ileal tissues. As observed in Fig. 6, TcdB was the only toxin inducing a significant increase in the MPO activity. However, similar to the previous results, MPO activity decreased in the PMN-depleted group ($p < 0.01$). TcdA, TcdB, or the combination TcdA + TcdB did not induce a significant MPO activity in any of the groups.

4. Discussion

Neutrophils play a critical role in the host body's immune response as a first defense line against bacterial infections [72]; however, a massive PMN response and infiltration can also cause tissue damage, contributing to the infection's severity [73]. Given that PMNs play a critical role in the immunopathogenesis of *C. difficile* infections, it has been proposed that PMNs may increase the pathology induced by these bacteria [74]. Contributing to this query, here we show that, by generating a significant PMN reduction in peripheral blood and ileal tissues, the immunopathology induced by *C. difficile* toxins TcdA, TcdB, TcdA+TcdB, and TcBv was reduced. This study is the first *in vivo* investigation using PMN depletion in a *C. difficile* murine infection model, showing PMN depletion in both blood and ileal tissues, and also involving two toxin groups: conventional TcdB and variant TcdB from clinically hypervirulent strains that target different GTPases (Fig. 1A and B).

Our results confirmed that TcdA induces lamina propria (LH) and submucosa (SM) inflammatory responses, recruitment of PMNs, and extensive tissue damage (Fig. 2A, B, 3A, 3B, and 3C) after a short incubation period. The same inflammatory process has been described in several animals with intestinal loop models, cell cultures, and *in vitro* assays [75–78]. TcdA also inhibits the migration and proliferation of intestinal epithelial cells, which are essential for maintaining and repairing the epithelial barrier [79]. In addition, we show that combinations of toxins TcdA+TcdB induces the most significant alteration in the intestinal mucosa, inflammatory response, and exacerbated PMN infiltration (Fig. 2E, F, 3A, 3B, and 3C). These results correlate to Hirota et al.'s intrarectal instillation mouse model study [80].

$TcdB_{NAP1}$ induced minor lamina propria (LH) and submucosa (SM) inflammatory responses and PMN recruitment compared to those induced by TcdA (Fig. 2C and D). It has been described that TcdB does not induce acute enterotoxicity in animal intestinal loop models [76,77, 81–83], and it is considered a toxin with bimodal cell death mechanisms [84]. TcdB induced similar histopathological results as $TcdB_{NAP1}$. These results confirm that TcdBv and TcdB induce less tissue damage and PMN recruitment in the intestine than TcdA. Our results are in tune with another study that reported, using murine loop models, cell cultures, and *in vitro* assays, that *C. difficile* $TcdB_V$ is equivalent to other classical TcdBs ($TcdB_{NAP1}$ and $TcdBv_{NAP1}$) strains [85].

Except for the experimental group $TcdA_+$ TcdB, all histopathological analyses of the PMN-depleted group showed a significant reduction in epithelial damage, LH, and SM inflammation compared to the non-depleted groups. A previous report using a murine rectal instillation model with a mixture of recombinant TcdA and TcdB showed only subtle acute inflammation at the histopathological level [80]. These histopathological results correlate with the notion that PMNs, involved in host defense, can also be detrimental to an organism when dysregulation occurs, contributing to tissue damage and the inflammatory response induced by *C. difficile* toxins [29]. This phenomenon is known as the paradox of PMNs [86].

The cytokine quantification of ileal loops obtained agreed with the role of PMNs in histopathology's inflammatory response. Most cytokines showed a systematic decrease in the PMN-depleted group. The quantification of CXCL-1 revealed low values compared to other cytokines (Fig. 5). This phenomenon has been described in a previous study by Sawant et al., that relatively low concentrations of CXCL-1 in their mouse infection models could be due to a phenomenon described during active PMN recruitment since CXCL-1 levels can vary spatially and temporally by many orders of magnitude related to its concentration. Our histopathological data also suggests that a deficiency in CXCL-1/KC, rather than CXCL-2/MIP2, is likely the main factor for the lack of neutrophil infiltration observed by Fukata et al. [87].

IL-1β is highly associated with the immune response induced by toxins and other components secreted by *C. difficile*, such as the independent activation of the glycosyltransferase activity [88,89]. In addition, IL-1β and IL-8 have been described as innate inflammatory mediators that are elevated in the tissue of patients with ICD [90]. TcdA was the only toxin that induced the production of high quantities of IL-1β together with intestine inflammation, fluid accumulation, PMN

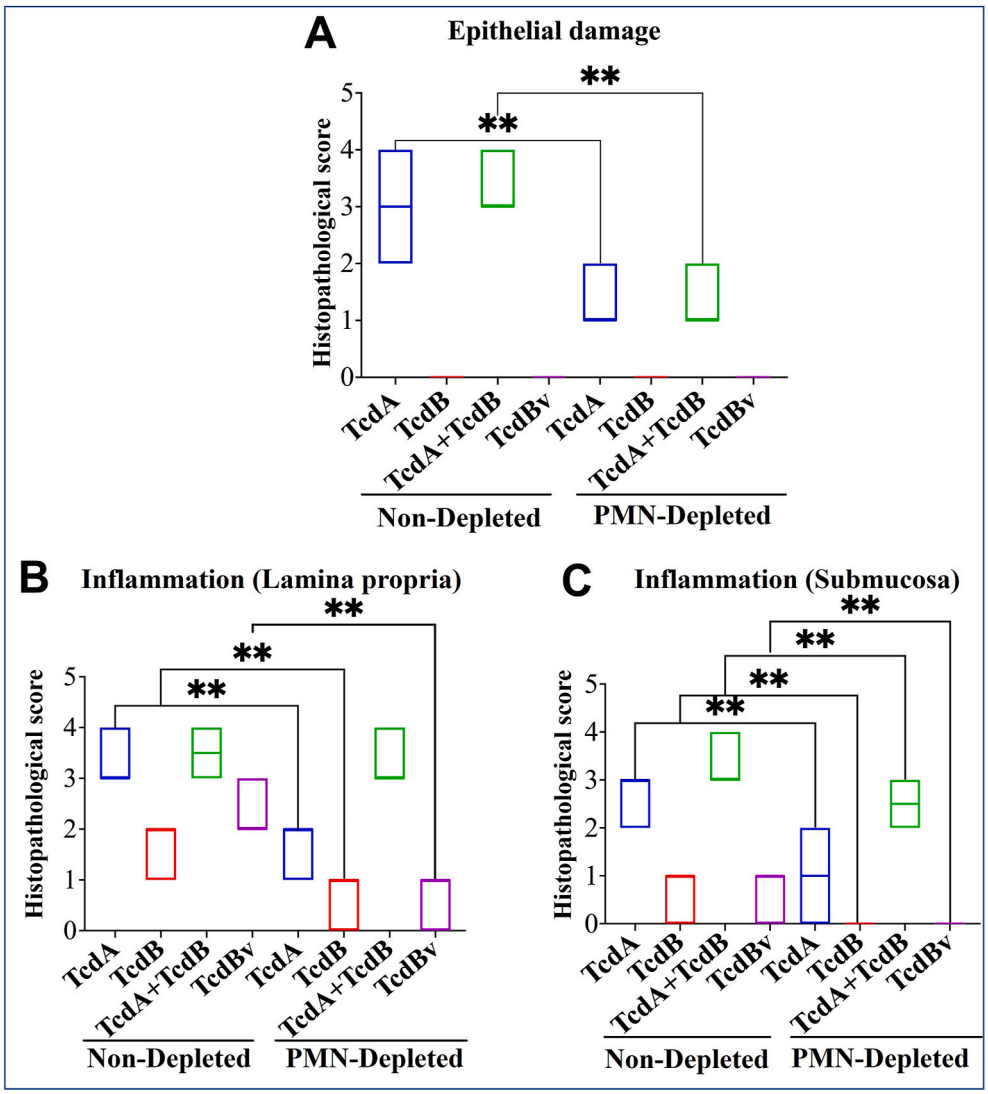

Fig. 4. Histopathological alteration scores observed in ileal-ligated-loop tissues treated with *C. difficile* toxins in non-depleted and PMN-depleted mice. Formalin-fixed tissues stained with H&E were scored on a histopathological scale from 1 (mild) to 4 (severe). Parameters assessed included (A) epithelial damage, (B) lamina propria inflammation, and (C) submucosa inflammation. Results are expressed as median ± extreme values, with significant differences ($p < 0.01$, **) between non-depleted and PMN-depleted groups.

infiltration, and tissue damage in the ileal loops. These findings correlate with previous studies where the activity of TcdA and TcdB was evaluated separately in murine ileum [76,78].

In addition, the production of other inflammatory mediators such as TNF-α, IL-6, and IL-10 was also detected, all associated with CDI (Fig. 5B, C, and 5E) [90–93]. In the ligated ileal model, TNF-α has been used as an indicator of the initiation of a pathogenic process [91]. The observed lower quantities of TNF-α and IL-6 compared to other studies likely reflect variability in inflammatory responses, and experimental conditions can influence [45]. Such conditions may include the strain type of *C. difficile* used, differences in animal models (e.g., age, sex, or genetic background), the specific toxin variants employed, the dosage and timing of toxin exposure, as well as variations in environmental factors such as diet or housing conditions during the study [16]. Other factors include the high production of IL-10 detected in the TcdA group (Fig. 5E) [91].

In contrast, our results showed no expression of IL-10 in the TcdBs. Recent studies reported that *in vivo* (mouse cecal epithelial cells) and *in vitro* (human intestinal cells [HCT-8]) early stimulation of mRNA expression is capable of positively controlling two adenosine receptors induced by short-term effects (2–6 h) by *C. difficile* toxins [94,95]. These adenosine receptors, A_{2A} and A_{2B}, are located in immune cells rather than in epithelial cells in the intestinal tract [96]. In macrophages, it has been described that A_{2A} receptor activation decreases the secretion of inflammatory substances (cytokines) such as TNF-α and IL-6 and increases IL-10 [97,98].

Like IL-1, IL-6 was only detected in the groups treated with TcdA and the TcdA+TcdB mixture. The TcdBs did not induce the production of IL-6 (Fig. 5B). Moreover, IL-6 is a master cytokine secreted in epithelial and immune cells at the intestinal level. This cytokine is induced by *C. difficille* toxins or other metabolites from supernatants free of bacteria via NOD and NFκβ-dependent pathways [88,99]. In addition, IL-6 has even been associated with the recruitment of PMNs in models of the ileal loop, which increases epithelial damage [92,100]. The differences in cytokine expression between the PMN depletion model and other studies using purified toxins in cell cultures and intestinal loops could stem from the absence of direct host-pathogen interactions [101]. In the PMN depletion model, the immune system's response to *C. difficile* toxins is altered due to the removal of critical immune cells, such as neutrophils, which play a crucial role in pathogen recognition and signaling [102]. Without the involvement of immune cells, the body's signaling pathways for triggering cytokines like IL-6 and TNF-α may differ significantly

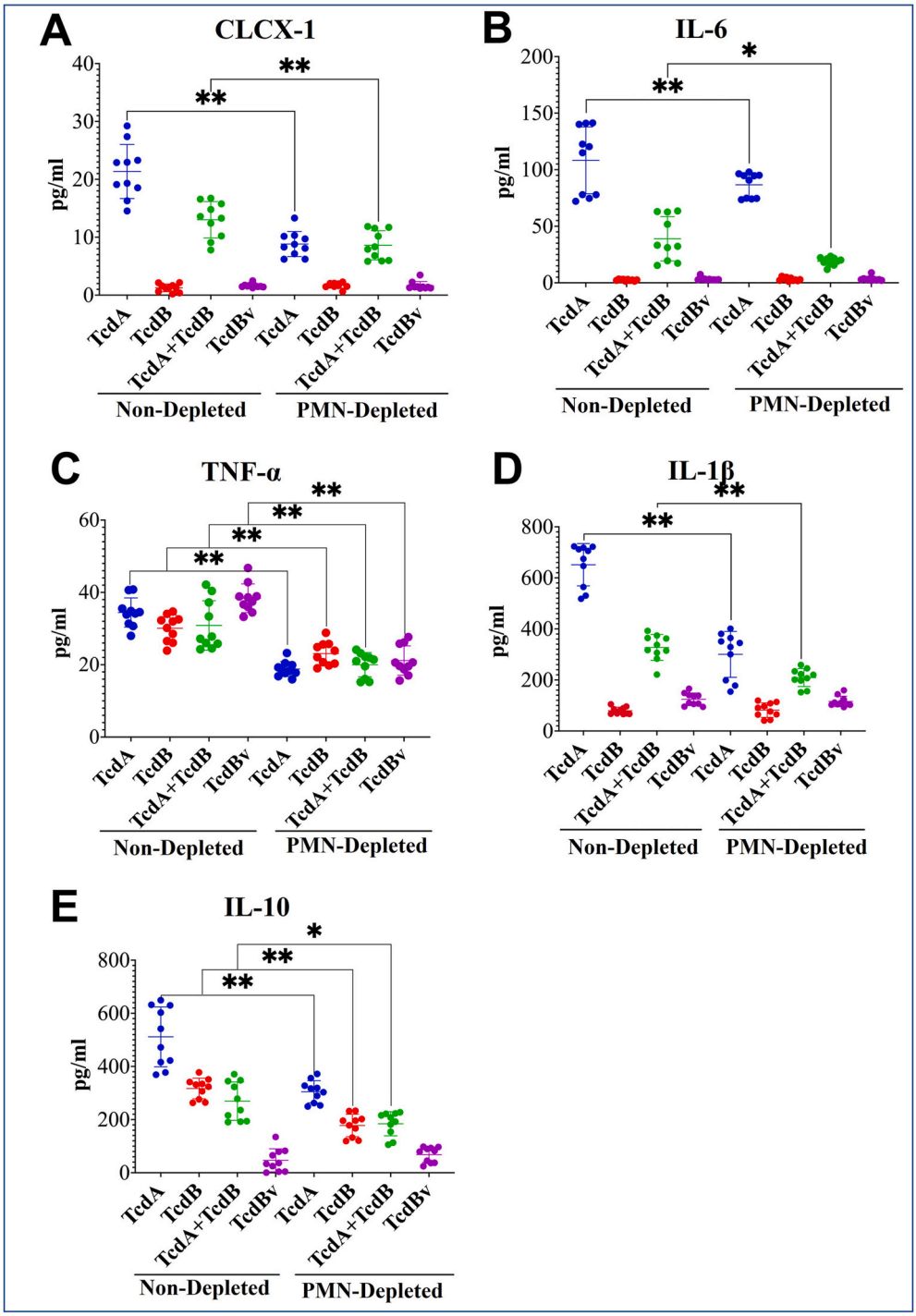

Fig. 5. Quantification of CXCL1, TNF-α, IL-6, IL-1β, and IL-10 in the murine ileal-ligated-loop tissues treated with *C. difficile* toxins in non-depleted and PMN-depleted mice. The production of (A) CXCL1, (B) TNF-α, (C) IL-6, (D) IL-1β, and (E) IL-10 was measured by ELISA from ileal-ligated-loop tissue homogenized in PBS. Bars represent mean ± S.D., with gray shading indicating control group S.D. Significant differences ($p < 0.05$ (*) and $p < 0.01$ (**)) were observed between non-depleted and PMN-depleted groups.

from scenarios where toxins directly interact with host cells or tissues [103].

All cytokines quantified in the non-depleted group showed significantly higher levels than those in the depleted group, which had higher production in the groups treated with TcdA. This result supports two precepts: (i) TcdA as an enterotoxin produces more significant histopathological damage and proinflammatory response than TcdB and TcdBv, and (ii) by significantly reducing the presence of PMNs, a reduction of the inflammatory response is obtained. Accordingly, regulating the number of neutrophils or macrophages has been proposed as a new strategy for treating inflammatory disorders [104].

Our results suggest that a therapeutic strategy reducing the number of PMNs or their agonists (extracellular traps of PMNs [NETs]) in the surrounding tissue could improve the exacerbated inflammatory response induced by PMNs and the continuous inflammatory stimuli that trigger chronic inflammation.

The activity of the MPO enzyme increased only in the non-depleted TcdB-treated mice (Fig. 6). This MPO activity also decreased upon PMN

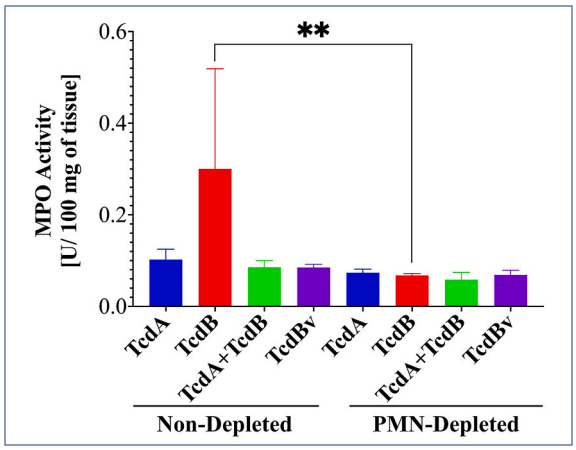

Fig. 6. Quantification of MPO activity in ligated-loop tissues treated with *C. difficile* toxins in non-depleted and PMN-depleted mice. MPO activity was measured colorimetrically by mixing tissue homogenates in HTAB solution with o-dianisidine and recording absorbance changes at 450 nm. Results are expressed as mean ± S.D., with gray shading indicating control group S.D. Significant differences ($p < 0.01$, **) were observed between non-depleted and PMN-depleted groups.

depletion, which coincides with PMNs (primary producers of MPO) participating in the host defense against *C. difficile* toxins. In contrast, TcdBv and TcdA expressed MPO values within the control group. These results correlate with previous reports using the murine-ligated ileal loop model but using higher quantities of toxins. Another study, using a mouse cecal injection model treated with recombinant TcdA and TcdB, reported similar data to our findings regarding TcdB and MPO activity [105]. In this study, Peritore-Galve et al. emphasized that severe inflammatory and oxidative responses are associated with mucosal damage caused by a variant of the TcdB toxin. They also mentioned that inflammation may contribute to epithelial damage in specific models, leading to conflicting results that are difficult to correlate with findings from other authors. Our result suggests that MPO production is a signature enzyme of PMNs in mice and humans [97] and is an essential marker of exacerbated inflammatory response, leukocyte degranulation process, and PMN recruitment induced by TcdB. All these effects have been described as the leading causes of epithelial damage [106].

Additionally, the substantial difference in MPO production by TcdB and TcdBv suggests that TcdB variants could enter the cell similarly to TcdA but may have altered binding affinities for different receptors as previously described [107] and also indicates that TcdBv may be dependent on glucosylation. Classical TcdBs, such as those from hypervirulent strains (e.g., NAP1/027), can interact with a broader range of receptors, including Frizzed proteins (FZDs) and chondroitin sulfate proteoglycan 4 (CSPG4) as two major host receptors, and potentially others, allowing for more efficient internalization [107,108]. Also, these results highlight that classical TcdB toxin stimulates significant neutrophil migration and degranulation, marked by increased myeloperoxidase (MPO) production [23], which decreases when neutrophils (PMNs) are depleted, suggesting TcdB's pivotal role in neutrophil-driven inflammation. Furthermore, the literature describes that TcdA primarily induces oxidative stress involving reactive oxygen species (ROS) and nitric oxide, contributing to enteritis and colitis [109]. TcdB directly engages neutrophils without significant ROS involvement [109–111].

It is essential to highlight that both TcdA and TcdB are crucial for the pathogenesis of *C. difficile*. Our ileal loop model shows that TcdA induces a more severe proinflammatory and histopathological than TcdB; however, both were significantly reduced in the PMN-depleted groups. Previous studies using intestinal loop models have also demonstrated that rabbits, hamsters, and mice respond more actively to TcdA proinflammatorily and histopathologically than TcdB [112,113]. This discrepancy suggests that toxin behaviors *in vivo* and *in vitro* models can vary.

In summary, we conclude that partial removal of PMNs, peripherally and in ileal tissues, reduced the pathogenesis induced by *C. difficile* by decreasing proinflammatory and inflammatory response and histopathological damage. This reduction was more evident in TcdA compared to TcdB and TcdB. Further research is required better to characterize the role of PMNs in this pathological process, particularly evaluating cytotoxic molecules or substances released by PMNs during infection. These molecules or substances could be used as biomarkers for therapies against the pathology caused by *C. difficile* toxins. It is also interesting to investigate the role of PMN extracellular traps (NETs) and the immunological function of NETosis, as there is a lack of information on this topic in *C. difficile* research.

5. Conclusions

Using the systemic PMN depletion model in mice and murine ligated ileal loop model, it was evidenced that the absence of PMNs in the intestinal lumen during CDI reduces the pathogenic and inflammatory potential induced by *C. difficile* toxins TcdA, TcdB, and TcdBv. We hypothesized that the recruited PMNs in the intestinal lumen exacerbate the pathogenesis of infection caused by *Clostridioides difficile* toxins.

CRediT authorship contribution statement

Montoya-Torres Brayan: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alfaro-Alarcón Alejandro:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Carlos Quesada-Gómez:** Writing – review & editing, Validation, Resources, Methodology, Funding acquisition, Data curation, Conceptualization. **Esteban Chaves-Olarte:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Barquero-Calvo Elías:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

MILL	Murine-Ileal-Ligated-Loop
CXCL-1	Chemokine (C-X-C motif) ligand 1
CDI	Clostridioides difficile infection

IL	Interleukin
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP	North American pulsotype
NOD	Nucleotide-binding oligomerization domain
PaLoc	Pathogenicity locus
PAMPs	Pathogen associated molecular patterns
PMNs	Polymorphonuclears
PRRs	Pattern recognition receptors
ROS	Radical oxigen species
RT	Ribotype
ST	Sequence type
TCD	Binary toxin
TcdA	Toxin A
TcdB	Toxin B
TcdBv	Variant toxin B
TLRs	Toll-like receptors

Data availability

Data will be made available on request.

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