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Human neutrophils are resistant to *Clostridioides difficile* toxin B

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ABSTRACT

Objective: The main objective of this study was to evaluate the glucosyltransferase activity of *C. difficile* TcdB on the activity of human PMNs.
Methods: To better understand the interaction between PMNs and TcdB, PMNs were treated with sub-lethal concentrations of TcdB. We evaluated: (i) the glucosylation of GTPases, (ii) the phagocytic and bactericidal activity, and (iii) PMNs activation (through quantification of TNF-α, IL-8, and expression of CD11b cell surface activation marker).
Results: We found that TcdB did not glucosylate RhoA and Rac1 GTPases and did not affect the phagocytic or bactericidal capacity of PMNs. Moreover, TcdB did not increase the production of TNF-α, IL-8, or the expression of activation marker CD11b. The only significant effect of TcdB on PMNs was the partial inhibition of TNF-α and IL-8 production and the diminished expression of CD11b induced by *E. coli*-LPS.
Conclusion: Our results show that human PMNs are resistant to TcdB GTPase glucosyltransferase activity against RhoA and Rac1.

1. Introduction

Clostridiodes difficile infections are a relevant threat to public health due to the increased incidence of outbreaks of in-hospital diarrhea worldwide [1]. *C. difficile* is found in the environment and in the large intestine of humans and animals; therefore, it has been considered a potential zoonotic agent [2–4].

Infection in humans can be asymptomatic or symptomatic, ranging from mild diarrhea to pseudomembranous colitis. The most severe form of the disease presents pseudomembranes with massive infiltration of polymorphonuclear neutrophils (PMNs) [5].

PMNs are short-lived leukocytes considered the first line of defense against pathogens [6]. However, activated PMNs can also cause tissue damage; therefore, regulation mechanisms are required to resolve inflammation [7]. When inflammatory stimuli are continuously present, they can generate aggressive and prolonged responses, causing chronic inflammation [8].

The pathogenicity of *C. difficile* largely depends on the production of toxins A (TcdA) and B (TcdB) [9]. TcdA and TcdB have glucosyltransferase activity, modifying and inactivating small GTPases of the Ras superfamily, including Rho, Rac, Ras, and Cdc42 [10]. Glucosylation of GTPases results in disassembly of the actin cytoskeleton, cell rounding, and cell death. The glucosyltransferase activity of TcdB is considered essential for disease pathogenesis [11], and it is 100 to 10,000 times more potent than TcdA [12]. Thus, TcdB is the primary virulence factor of *C. difficile* and is responsible for inducing the host's innate immune system and inflammatory response [13,14].

The pathogenesis of *C. difficile* is characterized by the influx of PMNs to the intestinal mucosa, and in severe cases, this contributes to the formation of pseudomembranes [15]. The magnitude of the inflammatory response observed in the disease is one predictor of an adverse prognosis [16]. The recruitment of PMNs to the site of infection is mediated by inflammatory molecules, such as tumor necrosis factor-alpha (TNF-α) or interleukin 8 (IL-8) secreted by epithelial cells [17,18], that are produced in response to *C. difficile* toxins.

TNF-α is essential for host resistance to infection since it functions as an immunostimulatory and mediator of the inflammatory response [19]. IL-8 is a member of the chemokine family of chemotactic cytokines. IL-8 regulates adhesion molecules' expression (such as CD11b) and directs the adhesion of PMNs to the vascular endothelium for diapedesis and tissue infiltration [18]. High concentrations of IL-8 in the intestinal lumen are related to a

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greater predisposition to develop symptomatic infections with *C. difficile* [20].

Brito et al. (2002) [21] studied the effect of TcdA on PMNs. They observed that TcdA induced changes in shape, reorganization of the F-actin cytoskeleton, decreased L-selectin expression, increased Mac-1, improved adherence, and decreased oxidative activity. These effects were related to the Rho family GTPases' glucosylation in PMNs [21].

There are other studies on the effect of TcdB on PMNs with discordant results. For example, one study reported that PMNs treated with TcdB showed no significant differences in phagocytic activity or viability [22]. However, in another study, treatment with TcdB reduced phagocytic activity, respiratory burst, and F-actin level in canine PMNs [23]. Moreover, treatment of PMNs with non-cytotoxic fragments of TcdB showed greater activation of PMNs than with the complete toxin of TcdB and TcdA [24].

Here we evaluate the effect of TcdB on human PMNs, considering that: (i) TcdB is the most potent toxin and the main virulence factors of *C. difficile*; (ii) the intensive recruitment of PMNs is considered a significant factor in the pathology of this infection and (iii) the controversy regarding the effect of these toxins on the activity of PMNs.

2. Materials and methods

2.1. Toxins

Native toxins $TcdA_{VPI}$, $TcdB_{VPI}$ were obtained and purified from the reference strain VPI 10463, and $TcdA_{NAP1}$ and $TcdB_{NAP1}$ (NAP1/RT027) from strains isolated from clinical stool samples collected during a CDI outbreak in a Costa Rican hospital [25]. TcdA was used as a comparative effect against TcdB in specific assays considered relevant.

These toxins were supplied by Laboratorio de Investigación de Bacteriología Anaerobia (LIBA) of the Faculty of Microbiology and the Center for Research on Tropical Diseases (CIET) of the University of Costa Rica where they remain at −80 °C in 20 mM HEPES buffer at pH 6.9 supplemented with 50 mM NaCl.

2.2. PMNs isolation

PMNs were obtained from fresh venous blood from one single healthy donor (with normal blood parameters and free from infection or inflammation). Briefly, fresh blood was collected in lithium heparin tubes and diluted with phosphate-buffered saline (PBS, Life Technologies). Using density gradients of polysucrose Ficoll® Histopaque 1119 and 1077 (Sigma-Aldrich), blood was layered by centrifugation without brake. The granulocyte layer was separated, and two washes were performed with PBS. The remaining erythrocytes were lysed with 1X ammonium chloride lysis solution. PMN content percentage was determined by performing a leukocyte differential count on a Wright-stained smear. Purified PMNs (>95%) were resuspended in Hanks' balanced solution buffer (HBSS) and supplemented with calcium, magnesium, and fetal bovine serum or human autologous serum according to the requirements of each experiment.

2.3. Cell culture

HeLa cells (ATCC CCL-2) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were cultured under humid conditions at 37 °C with 5% CO2. These cells were supplied by Centro de Investigación en Enfermedades Tropicales (CIET). To verify the plasma's potential inhibitory effect on the toxin activity, HeLa cells were inoculated with each toxin's sub-lethal concentration in a 24-well plate. After 24 h, cell morphology was observed under an inverted microscope.

2.4. Flow cytometry

The cell death percentage was evaluated in $TcdB_{NAP1}$ exposed PMNs by flow cytometry (Guava easyCyte 5, Merck Millipore) using the fluorescent stains PE Annexin V Stain (Life Technologies) and Alexa-Fluor 488 LIVE/DEAD™ Fixable Green Stain (Thermo Fisher Scientific), following the manufacturer's recommendations. In brief, 500 μl whole blood wells were incubated with different concentrations of $TcdB_{NAP1}$ (50−5000 pM) in a 24-well low cell adhesion plate (Nunclon® Surface) at 37 °C on an exposure time scale (two to 8 h), then staining was performed for 30 min in wet ice and dark conditions. Erythrocytes were subsequently lysed with BD Lysing Buffer.

Data obtained were analyzed with FlowJo (version 10.1). PMNs were selected on a forward vs. lateral scatter plot (Forward vs. Side Scatter) according to their cellular characteristics. PMN population was further gated using anti-CD16b FITC antibody (eBioCB16, eBioscience). The membrane marker expression was measured in arbitrary units (AU) from the fluorescence intensity's median obtained for the selected cell population.

Toxin-generated activation was measured by the expression of cellular activation markers in PMNs. Whole blood samples were incubated with $TcdA_{VPI}$, $TcdB_{VPI}$, $TcdA_{NAP1}$, and $TcdB_{NAP1}$, as described above. As a cell activator and for positive control purposes, we used *E. coli* lipopolysaccharide (LPS) (10 μg/mL) (from now on referred to as LPS). LPS was added after 1 h of toxin incubation. Subsequently, staining was performed using the following activation-related antibodies: PE anti-human CD66b (G10F5, eBioscience), Alexa-Fluor 488 anti-CD35 (E11, eBioscience) and PE anti-CD11b (CBRM1/5, eBioscience). The samples were analyzed by flow cytometry, as detailed above.

2.5. Glucosylation of Rac1 and RhoA

TcdB's ability to glucosylate different monomeric GTPases was examined by a Western Blot assay. Purified PMNs and HeLa cell culture were incubated in 24-well plates with 100 pM of $TcdB_{VPI}$ and $TcdB_{NAP1}$ for 4 h at 37 °C. A cell lysate was obtained with the denaturing agent sodium duodecyl sulfate (SDS) at 2%. The lysed proteins were separated on a 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane by Western Blot. The membranes were blocked with 5% skim milk in 1X PBS Tween and then incubated with the primary antibody overnight at 4 °C.

Glucosylation of Rac1 and RhoA was determined with anti-Rac1 (clone 102/Rac1; BD Transduction Laboratories) and anti-RhoA (clone 1B12; Abcam) monoclonal antibodies, which recognize unmodified (non-glucosylated) isoforms. As a loading control, β-actin was detected using a rabbit anti-actin antibody (A2066; Sigma-Aldrich). After three washes with PBS Tween, they were incubated for 1 h with the conjugated secondary antibody (horseradish peroxidase-conjugated secondary antibodies mouse/rabbit (Rockland).

Proteins were detected with Lumi-Light Western Blotting Substrate (Sigma-Aldrich), and signals were recorded using ChemiDoc XRS+ Gel Imaging System (BioRad).

2.6. PMNs phagocytic capacity and bactericidal activity

For the phagocytic capacity assay, the TcdB exposure model was repeated, and 3.0−3.4 μm latex particles (BD CompBeads) were

added after 3 h of toxin exposure. Blood smears were performed one and 2 h after adding the latex particles and stained with Wright's stain. Three cell counts of 100 PMNs per smear were averaged, differentiating PMNs that had phagocytosed at least one latex particle. The results were expressed as phagocytosis percentage.

The Green et al. (2017) [26] protocol was used as guidance to determine the bactericidal activity. 5×10^6 purified PMNs (suspended in 500 μL of HBSS) were previously exposed to different toxins (100pM) for 4 h, and they mixed with bacteria (*Staphylococcus aureus*, 1×10^8 bacteria/mL) opsonized with 10% human autologous serum [26]. The bacteria survival was determined at 10 and 20 min of incubation. PMNs were lysed with 1X PBS 0.5% Tween 20, washed twice with 1X PBS, following serial dilutions, and were inoculated onto trypticase soy agar (ATS) (BD) plates in 20 μL triplicate drops. The ATS plates were incubated overnight at 37 °C, and colonies were counted. The phagocytosis percentage was calculated from these results means [26].

2.7. TNF-α and IL-8 quantification

From the previous toxin and LPS exposure assays in whole blood, plasma was obtained by centrifugation from each condition and stored at −20 °C for later analysis. To evaluate TNF-α and IL-8 levels in these samples, anti-TNF-α and anti-human IL-8 sandwich ELISA assays (eBioscience) were performed following the manufacturer's instructions. The results were expressed in pg/mL of TNF-α or IL-8.

2.8. Statistical analysis

The results obtained were reported as means (± standard deviation) of at least three repetitions of each experiment. The differences between the averages of the samples that received treatment and the control group were analyzed with parametric ANOVA and *t*-test to determine their significance. Statistical analyses were made in the JASP program (version 0.7.5.5), and a level of statistical significance of $p < 0.05$ was admitted.

3. Results

3.1. Determination of the sub-lethal concentration of TcdB

High concentrations of TcdB induce necrotic cell death related to the production of reactive oxygen species (ROS) [27] and glucosyltransferase independent effects [24]. For this reason, it is critical to test sub-lethal but cytotoxic concentrations (determined on susceptible cells such as HeLa).

To validate a sub-lethal intoxication procedure for PMNs with $TcdB_{NAP1}$, we treated PMNs with different toxin concentrations (at various incubation times). This validation allowed us to determine the maximum toxin concentration inducing the least cell death (sub-lethal concentration) of PMNs at a given exposure time.

First, the percentage of PMN cell death was quantified using a high toxin concentration (5000 pM). Then, samples were analyzed every 2 h up to a maximum of 8 h. After 4 h of incubation, we observed a progressive increase in cell death in the PMN population. Therefore, we defined 4 h as the maximum time of incubation (Fig. 1A).

Then, increasing concentrations of TcdB (from 50 to 5000 pM) were evaluated at 4 h of incubation to determine the maximum concentration of toxin inducing the lowest percentage of cell death. We observed a gradual increase in the rate of cell death as the toxin concentration increased. A low percentage of cell death (less than 5%) was observed at 4 h of incubation with 100 pM of TcdB. These working conditions were established for subsequent experiments (Fig. 1B).

3.2. TcdB does not glucosylate Rac1 and RhoA GTPases in PMNs

Given that glucosylation of Rac1 and RhoA GTPases by TcdB is responsible for the induction of a cytopathic effect, we evaluated the glucosylation activity of TcdB on PMN. We treated purified PMNs with $TcdB_{VPI}$ and $TcdB_{NAP1}$, and glucosylation activity was assessed by Western Blot. Glucosylation of Rac1 and RhoA was evidenced as a decrease in the intensity of the bands since we used anti-Rac1 and anti-RhoA antibodies that only recognize the non-glucosylated form of GTPases [12].

In HeLa cells (Fig. 2A), TcdBVPI and TcdB NAP1 induced a decrease in the intensity of the bands, demonstrating the glucosylation of both Rac1 and RhoA. However, in PMNs, we did not observe a reduction in the intensity of the Rac1 or RhoA bands in PMNs treated with TcdB (Fig. 2B).

3.3. TcdB does not affect the phagocytic or bactericidal activity of PMNs

Given that PMNs could be affected by TcdB despite the absence of GTPase glucosylation, we evaluated the phagocytic and bactericidal activity of PMNs treated with TcdB. For this, we incubated PMNs with latex particles or *Staphylococcus aureus*, respectively.

The phagocytic activity showed no significant differences between the control (PMNs without toxin) and PMNs treated with $TcdA_{VPI}$, $TcdB_{VPI}$, $TcdA_{NAP1}$, or $TcdB_{NAP1}$ at the evaluated times (Fig. 3). In general, the percentage of phagocytosis with latex beads increased between one and 2 h of incubation in all conditions.

The control group (PMNs without toxin) and PMNs treated with $TcdA_{VPI}$, $TcdB_{VPI}$, showed a gradual decrease in the viability of *S. aureus* at 10 and 20 min of exposure. However, we did not observe significant differences between the treatments and the control group (Fig. 4). Thus, our results show that PMN's phagocytic and bactericidal activity was not affected by TcdB.

TcdB does not promote the production of IL-8 and TNF-α but decreases their amount upon LPS stimulation.

The treatment of total leukocytes (whole blood) with $TcdA_{VPI}$, $TcdB_{VPI}$, $TcdA_{NAP1}$, and $TcdB_{NAP1}$ alone induced low IL-8 and TNF-α (<50 pg/mL); like the untreated negative control. However, leukocytes pretreated with TcdB toxins and subsequently stimulated with LPS showed a significant decrease in the amount of IL-8 and TNF-α. In TcdA pretreatment and consequent LPS stimulation, the quantity of cytokines was similar to the LPS control (Fig. 5).

3.4. TcdB treated-PMNs do not increase CD11b marker on the cell surface but decrease its expression upon LPS stimulation compared to untreated PMNs

We evaluated the effect of $TcdB_{VPI}$ and $TcdB_{NAP1}$ on the expression level of different surface cell markers related to cell activation in isolated PMNs upon LPS stimulation. To select the best cell marker for evaluating the activation state of PMNs, we assessed the expression of CD35, CD66b, and CD11b before and after LPS stimulation. The cell marker that showed the most significant difference was the adhesion and migration marker CD11b, with a 42% increase in the PMN cell surface (Fig. S1).

The expression of CD11b was then evaluated in isolated PMNs pretreated with TcdB or TcdA toxins and subsequently stimulated with LPS. Analogous to what we observed with the amount of IL-8

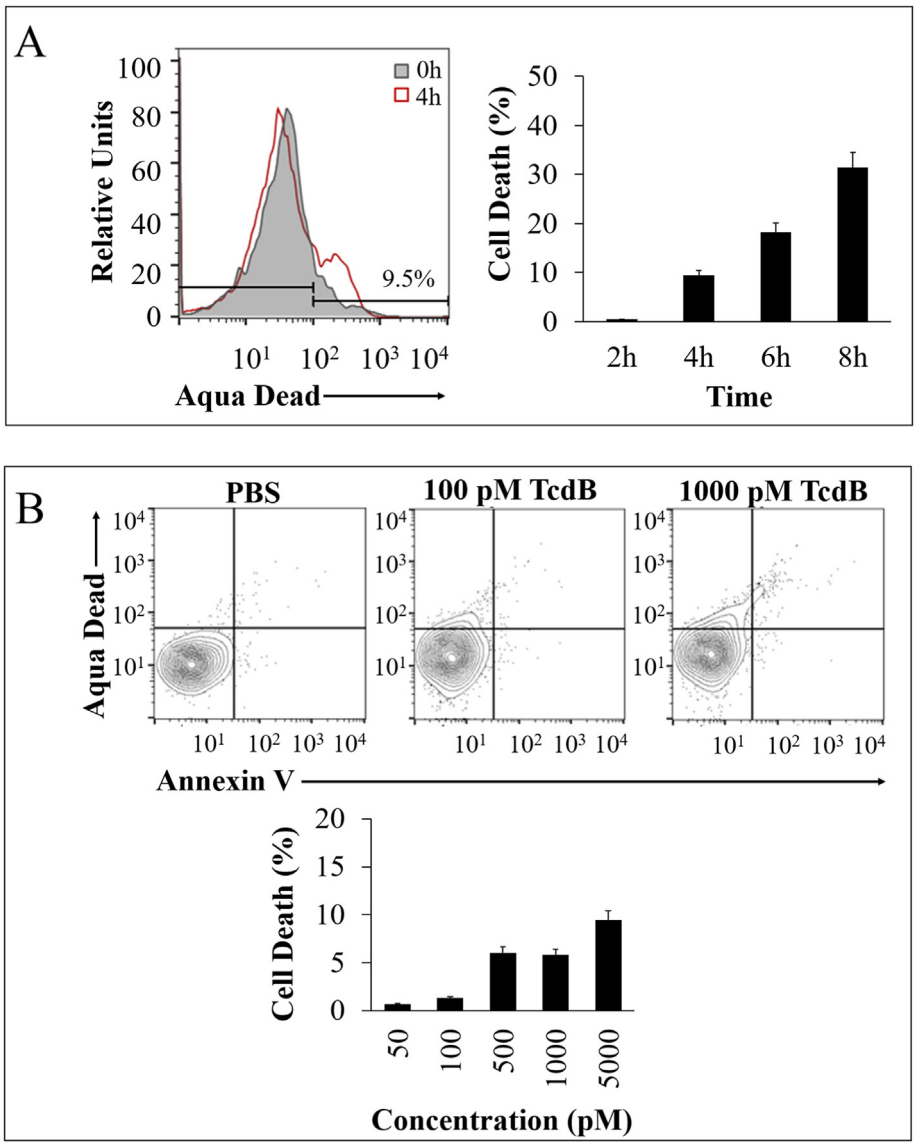

Fig. 1. Determination of the sub-lethal concentration of TcdB $_{NAP1}$ in PMNs in whole blood. (A) The percentage of death of PMNs treated with 5000 pM TcdB was determined using the AquaDead death marker at 2-h intervals by flow cytometry. (B) The percentage of death of PMNs was evaluated using increasing concentrations of TcdB $_{NAP1}$NAP1 at 4 h using the death markers AquaDead and Annexin V by flow cytometry. Error bars represent a 5% error rate. Experiments were repeated at least three times.

and TNF-α in total leukocytes, pretreatment with TcdB or TcdA toxins alone did not induce a significant expression of CD11b. Similarly, PMNs pretreated with TcdB toxins and stimulated with LPS showed a substantial reduction in the surface expression of CD11b compared to the LPS control. However, we did not observe such an inhibitory effect with TcdA toxins (Fig. 6).

3.5. Plasma does not affect TcdA or TcdB toxicity in HeLa cells

Given that some experiments on neutrophils were performed on whole blood, we evaluated whether plasma exerts an inhibitory effect on the action of toxins. Therefore, we treated HeLa cells with $TcdA_{VPI}$, $TcdB_{VPI}$, $TcdA_{NAP1}$, and $TcdB_{NAP1}$ and supplemented or not with 10% human plasma. A change in cell morphology (cell rounding) of more than 50% of the cells per well was considered positive. We did not observe a difference in the cytopathic effect induced by the different toxins between cells incubated in the presence or absence of plasma (Fig. 7).

4. Discussion

Here we show that using a sub-lethal concentration, PMNs were resistant to the cytotoxic activity of TcdB (mediated by the glucosylation of GTPases). Additionally, we show that TcdB induced no alteration of the phagocytic or bactericidal capacity of the PMNs and no cellular activation in total leukocytes in blood (measured by the production of IL-8 and TNF-α) and in PMNs (measured by the surface expression of CD11b). TcdB also showed an immunomodulatory effect that partially altered PMN's activation capacity against LPS.

Studies on the effects of toxins at the cellular level have been performed mainly in epithelial cells and cell cultures, few in immune cells, and to a lesser extent in PMNs. The differences in the cytotoxic potencies of toxins depend primarily on the cell line studied [28]. Additionally, the models of exposure to toxins (concentration, time, type of toxin used) vary from study to study, which complicates the data interpretation. For instance, unlike our conclusions, Goy et al. [24] reported that TcdB activates human

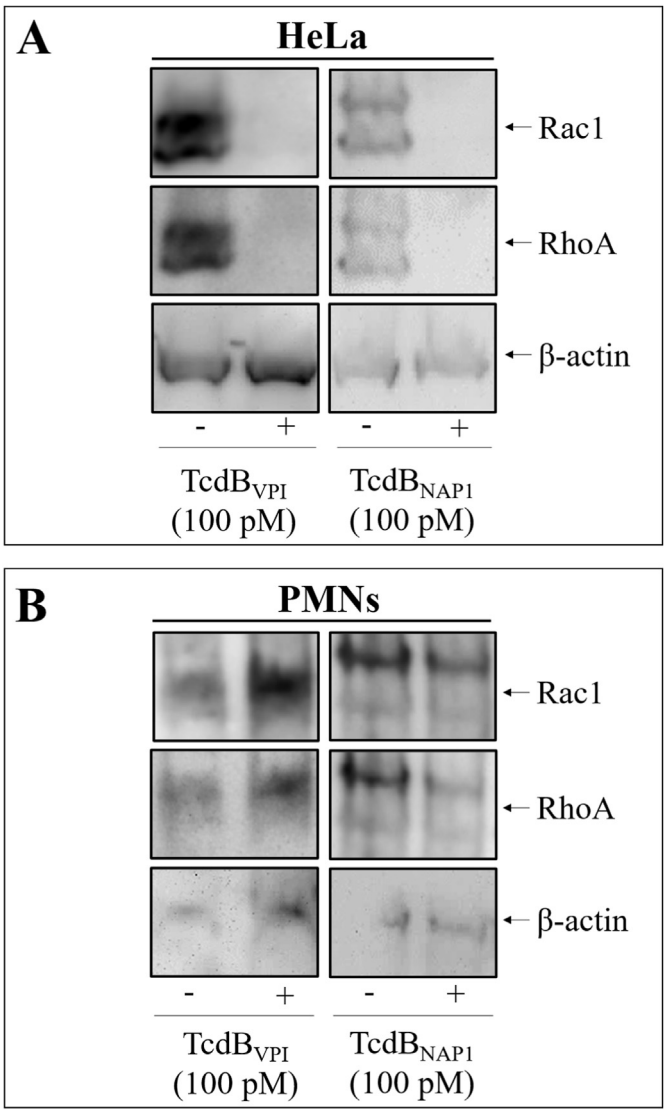

Fig. 2. Evaluation of Rac1 and RhoA glucosylation in HeLa and PMN cells. (A) HeLa cells and (B) purified PMNs were treated with 100 pM of TcdB$_{VPI}$ and TcdB $_{NAP1}$ for 4 h. Cells were lysed, and glucosylation of Rac1 and RhoA was determined by Western Blot. Specific anti-Rac1 and anti-RhoA antibodies recognizing the non-glucosylated form of these proteins were used for detection. Untreated (−) cells were included as negative controls of glucosylation β-actin immunodetection was used as a loading control. Glucosylation was evidenced as a decrease in the intensity of the bands. Experiments were repeated at least three times. One way ANOVA ($p < 0.05$) was performed.

PMNs. However, the authors use a different model, including (i) a different activation assay (influx of extracellular Ca2+), (ii) use of a recombinant TcdB or its fragments instead of the native toxin, (iii) use of different toxin concentrations (from 10 to 300 nM) and (iv) assay performed in a specific buffer used for measuring intracellular calcium (Fura-2 buffer). They attribute PMN activation to a non-cytotoxic recombinant fragment of TcdB.

Given that PMNs are short-living cells, we first validated a procedure to treat PMNs with TcdB at the maximum exposure time and highest toxin concentration inducing the least cell death. All experiments were performed under the same experimental conditions. Despite the high concentrations (5000 pM) used during some validation steps, the percentage of cell death did not exceed 10%. Likewise, it was previously shown that toxins from culture filtrates of *C. difficile* did not affect the viability of PMNs [22].

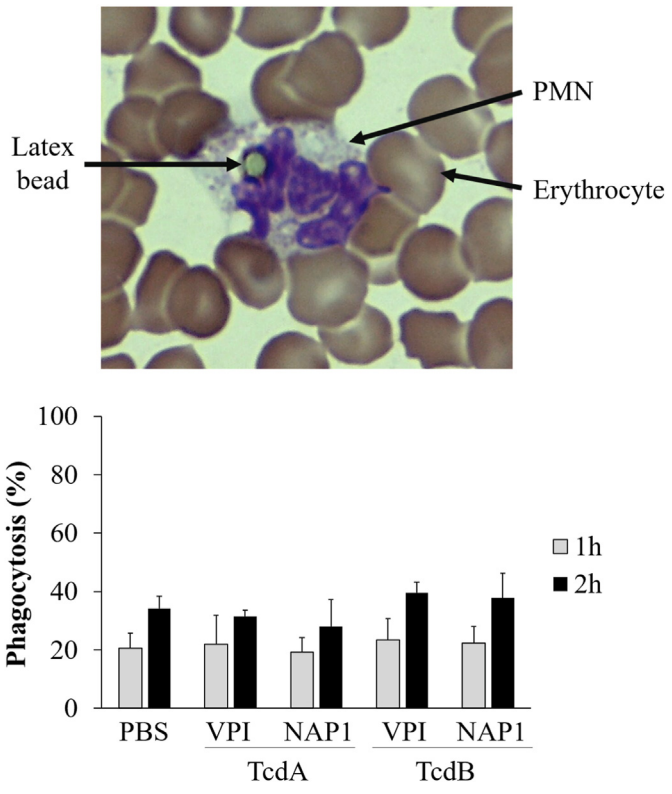

Fig. 3. Evaluation of the phagocytic activity of PMNs. Whole blood was treated with TcdA$_{VPI}$, TcdB$_{VPI}$, TcdA $_{NAP1}$, and TcdB $_{NAP1}$ for 4 h and subsequently incubated with latex particles for an additional one and 2 h to determine the phagocytic capacity of PMNs. A total count of 100 PMNs was made. The percentage of infection corresponds to the amount of PMNs that phagocytosed at least one latex particle. The bottom picture is a representative image of how latex particles were internalized. Error bars represent the standard deviation. Experiments were repeated at least three times. One way ANOVA ($p < 0.05$) was performed.

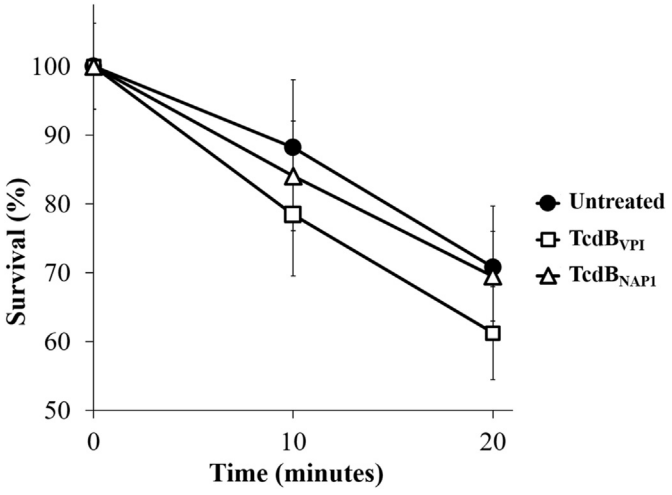

Fig. 4. Evaluation of the bactericidal activity of PMNs. Purified PMNs (1×10^7 cells/mL) were incubated with TcdB$_{VPI}$ or TcdB $_{NAP1}$ for 4 h. In the last 20 min of this incubation, *S. aureus* (1×10^8 bacteria/mL) was added. The survival percentage of the bacteria was determined by counting the CFU at 10 and 20 min of incubation. The mean values of three independent experiments are shown. Error bars represent the standard deviation. Experiments were repeated at least three times. One way ANOVA ($p < 0.05$) was performed.

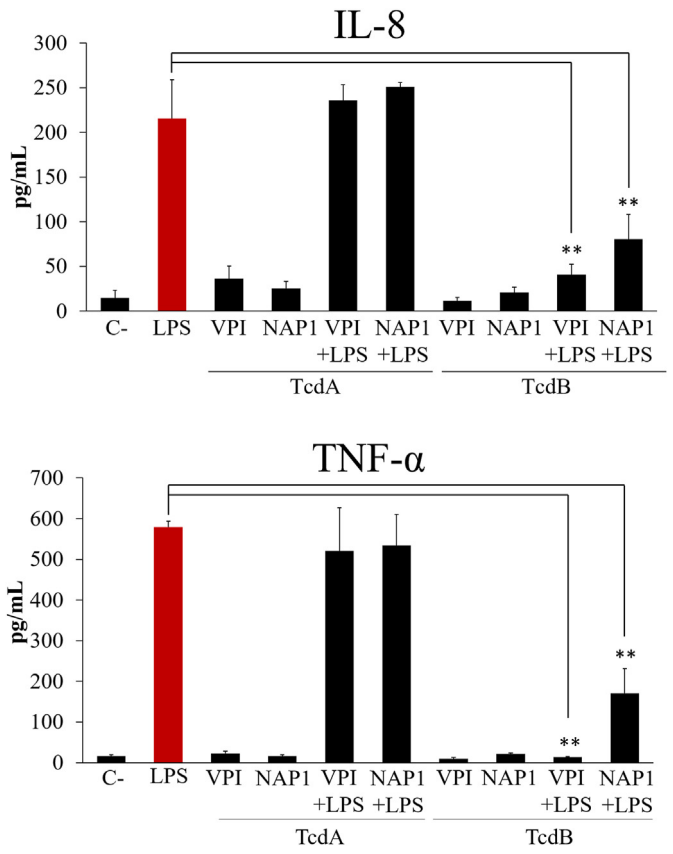

Fig. 5. Quantification of TNF-α and IL-8 in whole blood treated with TcdA and TcdB and stimulated with LPS. The production of IL-8 and TNF-α was determined by ELISA in whole blood plasma treated with 100 pM of $TcdA_{VPI}$, $TcdB_{VPI}$, $TcdA_{NAP1}$, and $TcdB_{NAP1}$ for 4 h in whole blood. LPS (10 μg/mL) was added as an activation control in some cases. The average results of three independent experiments are shown. Error bars represent the standard deviation. The values of $p < 0.01$ (**) are indicated.

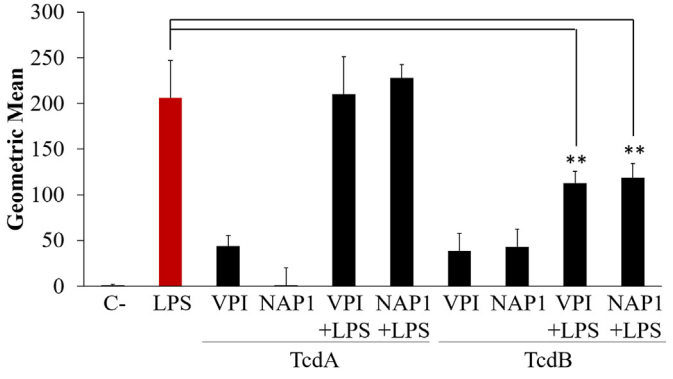

Fig. 6. Expression of CD11b in PMNs treated with toxins or stimulated with *E. coli* LPS. The increase in CD11b expression was quantified in PMNs incubated with 100 pM of $TcdA_{VPI}$, $TcdB_{VPI}$, $TcdA_{NAP1}$, and $TcdB_{NAP1}$ for 4 h in whole blood. *E. coli* LPS (10 μg/mL) was added as an activation control in some cases. As a negative control (C-), a no stimulation condition was left. Samples were analyzed by flow cytometry. The mean values of three independent experiments are shown. Error bars represent the standard deviation. The values of $p < 0.01$ (**) are indicated.

Besides the critical role of TcdA and TcdB in *C. difficile* pathogenicity [7], the severity of *C. difficile* disease is also determined by the intestinal inflammatory response and the recruitment of PMNs [16]. Therefore, the study of the physiological responses of PMNs in the presence of *C. difficile* toxins is essential to understand how these toxins modulate the activity of these inflammatory cells during *C. difficile* infection.

Rac GTPases play an essential role in coordinating chemotaxis and NADPH oxidase activation during the PMN response to invading microbes. RhoA GTPases function as regulators of PMN recruitment [29,30]. The Rho subfamily's GTPase inhibition induces early or immediate proinflammatory responses, such as IL-8 secretion, and eventually apoptosis of epithelial affected cells [31,32].

The pathogenesis induced by TcdA and TcdB is attributed to their glucosyltransferase activity and glucosyltransferase-independent effects, which have been reported to cause cell death due to high toxin concentrations [24,33–35]. In addition, glucosylation of Rho GTPases by *C. difficile* causes the reorganization of the actin cytoskeleton and may lead to apoptosis in many cell types [31]. However, there are few studies on the glucosylation activity on PMNs [24].

Here we showed that TcdB failed to glucosylate RhoA and Rac1 GTPases in PMNs. Thus, we conclude that PMNs have resistance to TcdB glucosyltransferase activity of *C. difficile*; therefore, they are refractory to the activities typically generated by this enzymatic activity in cells.

Different receptors for TcdB, such as FZD, PVRL3, and CSPG4 have been proposed [36–38]. TcdB receptors were studied in epithelial cells and other cells showing that FZD2/7, CSPG4, and PVRL3 seem to serve as cell surface binding receptors [39]; however, this has not been explored in PMNs yet. In addition, the activity of the sterol regulatory element-binding protein (SREBP), which regulates the content of cholesterol in membranes and is necessary for the pore formation by TcdB, has also been studied [40] but not in PMNs [15]. The alteration or absence of these elements in PMNs could eventually explain the low cytotoxic activity of TcdB in cells. This hypothesis remains to be explored.

Another possible mechanism of resistance to PMNs is myeloperoxidase (MPO) activity, which has been shown to inhibit the cytotoxic activity of TcdB [41]. In addition, the α-defensins contained in the granules of PMNs are another component that inhibits the glucosyltransferase activity of TcdB [42].

Despite the resistance of PMNs against TcdB, we evaluated the phagocytic and bactericidal activity. Similar to our results, it was previously shown that toxins from culture filtrates of *C. difficile* did not alter the phagocytic activity of PMNs [22]. We also showed that the bactericidal activity of PMNs treated with TcdB was not altered by using a standardized bacterial killing model [26]. This result agrees with the absence of the effect of small GTPases since phagocytosis and bactericidal activity depend on Rho and Rac. As previously mentioned, the disease caused by *C. difficile* depends on the effects of its toxins [35] and the proinflammatory response mediated by inflammatory molecules with the recruitment of PMNs [17]. We conclude from these results that TcdB does not affect the phagocytic and bactericidal activity of PMNs.

The glucosylation of small GTPases by *C. difficile* toxins has been linked to the induction of cytokines [43]. We show that total leukocytes treated with TcdA or TcdB did not produce higher amounts of IL-8 or TNF-α than the control group. This result suggests that leukocytes do not play an essential role in cytokine production in the presence of TcdA and TcdB. Similarly, TcdA or TcdB did not increase the expression of CD11b (adhesion and activation marker). These data contrast previous results showing that CD11b expression increased in the surface of PMNs when exposed to a culture medium of monocytes previously exposed to TcdA. However, when PMNs were treated directly with TcdA, there was no direct effect on CD11b surface expression [18].

We also evaluated if TcdB or TcdA somehow affected the capacity of activation of leukocytes in the presence of TcdA and TcdB

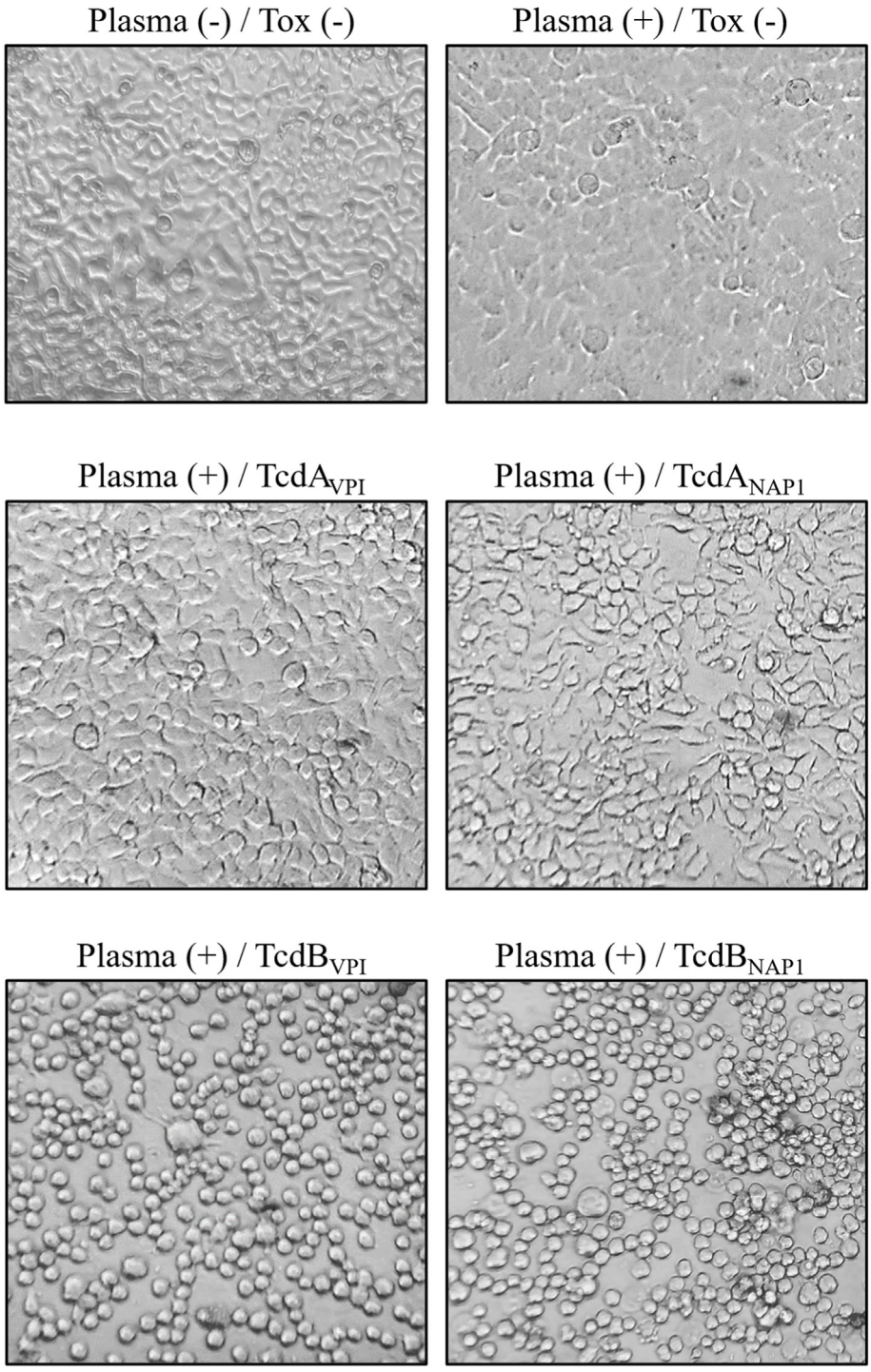

Fig. 7. Evaluation of the cytopathic effect of TcdA and TcdB in HeLa cells. HeLa cells were incubated in the presence of plasma with 100 pM of TcdA$_{VPI}$, TcdB$_{VPI}$, TcdA$_{NAP1}$, and TcdB $_{NAP1}$ for 24 h. The cytopathic effect was evaluated by direct observation under an inverted light microscope (10X). A change in cell morphology (cell rounding) in more than 50% of the cells per well was observed was considered a positive cytopathic effect.

using LPS as cell activator. We observed that TcdB but not TcdA inhibited the LPS-induced secretion of IL-8 and TNF-α in total leukocytes. Comparably, surface integrin receptor CD11b expression was inhibited in PMNs treated with TcdB and activated with LPS.

In vitro assays do not represent the natural physiological/pathological environment where multiple signals interact in the complex inflammatory response elicited by *C. difficile*. In addition, a possible weakness of this model is the use of a single PMNs donor. The use of single donors improves protocol standardization and reproducibility of the assays. However, data may variate (not the overall result) among donors. Despite these model limitations, our results show some insights into how *C. difficile* toxins may modulate PMNs activity during infection.

5. Conclusion

Our results show that under our experimental conditions, human PMNs are resistant to TcdB GTPase glucosyltransferase activity against RhoA and Rac1. We propose that *C. difficile* toxins induce the recruitment of PMNs to the site of infection (upon reaching the site of infection) and remain functional with their phagocytic and bactericidal capacities. Functional PMNs probably have a role in controlling *C. difficile*.

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CRediT authorship contribution statement

Catalina Chaves-Cordero: Investigation, Writing – review & editing. **Carlos Quesada-Gómez:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing. **Esteban Chaves-Olarte:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing. **Elías Barquero-Calvo:** Conceptualization, Methodology, Validation, Resources, Data curation, Visualization, Project administration.

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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Appendix A. Supplementary data

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