UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

PANEL DE GTPASAS GLICOSILADAS POR DIFERENTES TcdBs DE *Clostridium difficile* Y SU RELACIÓN CON EL POTENCIAL PATOGÉNICO DE DISTINTAS CEPAS

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Microbiología, Parasitología y Química Clínica para optar al grado y título de Maestría Académica en Microbiología con énfasis en Bacteriología

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RESUMEN

Estudios recientes han demostrado que TcdB es el principal factor de virulencia de *Clostridium difficile* y que las variaciones en virulencia que se presentan entre las distintas cepas, podrían estar relacionado con esta toxina, tal como en el caso de la cepa epidémica NAP1 y las cepas que presentan una TcdB variante. Sin embargo, no se conoce con exactitud cuál es el papel que tienen estas distintas TcdBs sobre el potencial patógeno de las cepas. Por tanto, en este trabajo se analizaron cuatro toxinas distintas ($TcdB_{NAP1}$, $TcdB_{NAP1v}$, $TcdB_{NAP9}$ y $TcdB_{VPI10463}$) con el fin de dilucidar el papel del panel de sustratos modificados por estas proteínas en la patogénesis de *C. difficile*. Por medio de ensayos de glicosilación se determinó el espectro de GTPasas modificadas y se determinaron los efectos celulares inducidas por las distintas TcdBs en términos de efecto citopático (CPE), muerte celular y activación inmune. Asimismo, se evaluó el potencial patogénico asociada a cada TcdB por medio del ensayo de asa ligada en ratón. Por medio de ensayos *in vitro* y *ex vivo* se determinó que $TcdB_{NAP1}$ es capaz de glicosilar un panel más amplio de sustratos. Esta toxina es capaz de glicosilar a las GTPasas Rho y Ras, mientras que $TcdB_{VPI10463}$ sólo modifica a RhoA, Rac1 y Cdcd42, y $TcdB_{NAP1v}$ y $TcdB_{NAP9}$ glicosilan a Rac1 y R-Ras. Una comparación de la cinética de estas proteínas en distintas líneas celulares reveló de manera indirecta, que la tasa de entrada a las células eucariotas es similar para todas las toxinas a pesar de que sólo $TcdB_{NAP1}$ y $TcdB_{NAP1v}$ comparten los dominios de unión al receptor y de autoprocesamiento. En cambio, el tipo de CPE causado por cada toxina se asocia a un dominio glicosiltransferasa (GTD) similar. Aunado a esto, los eventos biológicos asociados a la intoxicación parecen relacionarse con el panel de sustratos glicosilados por cada toxina. Los datos obtenidos sugieren que RhoA determina el tipo de CPE inducido ya que las toxinas que modifican esta GTPasa inducen un CPE arborizante, mientras $TcdB_{NAP1v}$ y $TcdB_{NAP9}$ inducen un efecto variante. Asimismo, la vía de muerte celular inducida como resultado de la glicosilación de Ras por $TcdB_{NAP1v}$ y $TcdB_{NAP9}$, parece ser distinta a la vía apoptótica inducida por las otras dos toxinas. Por otro lado, la inactivación de RhoA por $TcdB_{NAP1}$ y $TcdB_{VPI10463}$ parece estar relacionada con la activación de macrófagos y la liberación de TNF-α. Finalmente en un modelo *in vivo*, $TcdB_{NAP1}$ es capaz de inducir probablemente debido al mayor panel de GTPasas modificadas, una mayor activación inmune caracterizada por la liberación de Il-6 e Il-1β y disrupción del epitelio, los cuales son eventos asociados a un pronóstico negativo de la enfermedad. Por otro lado, la intoxicación con $TcdB_{NAP1v}$ resultó en mayor congestión vascular y fusión de vellosidades. En conclusión, nuestros resultados sugieren que la virulencia aumentada y el potencial patógenico asociado a TcdB, descrito para las cepas como NAP1 y cepas toxina A negativas que portan TcdB variante, podrían estar asociados a diferencias en la glicosilación de GTPasas por sus TcdBs.

ABSTRACT

Recent studies have shown that TcdB is the major virulence factor of *Clostridium difficile* and that variations in virulence seen among strains could be related to this toxin, such as in the case of the epidemic NAP1 isolates and the strains that harbor a variant TcdB. However, the relative contribution of distinct TcdBs on the pathogenic potential of different strains is not clearly understood. In this context, we used a panel of four *C. difficile* TcdBs to address the impact of variations in substrate modification on *C. difficile* pathogenesis: $TcdB_{NAP1}$, $TcdB_{NAP1v}$, $TcdB_{NAP9}$ and $TcdB_{VPI10463}$. Glucosylation assays were performed in order to determine the panel of modified GTPases and the cellular effects induced by each TcdB were assessed in terms of cytopathic effect (CPE), cell death and immune activation. Furthermore, the pathogenic potential associated to each TcdB was determined in the ileal loop mouse model. *In vitro* and *ex vivo* assays revealed that $TcdB_{NAP1}$ is able to glucosylate a broader panel of substrates as this toxin glucosylates both Rho and Ras GTPases, whereas $TcdB_{VPI10463}$ only modifies RhoA, Rac1 and Cdcd42, and $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ modify Rac1 and R-Ras. Through a comparison of the kinetics of intoxication on different cell line, we indirectly show that the rate of toxin uptake is similar for all toxins even if only $TcdB_{NAP1}$ and $TcdB_{NAP1v}$ share the receptor-binding and autoprocessing domains, whereas the kind of CPE caused by each toxin is associated to a similar glucosyltransferase domain (GTD). In line with this, the panel of substrates targeted by each toxin seems to correlate with the biological events induced upon intoxication. Our study indicates that RhoA seems to dictate the type of CPE induced as toxins that modify this GTPase induce an arborizing CPE, while $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ induce a variant effect. Furthermore, variant toxins appear to cause other mechanisms different from apoptotic events due to Ras glucosylation, while RhoA inactivation by $TcdB_{NAP1}$ and $TcdB_{VPI10463}$ seem to correlate with macrophage activation and TNF-α release. Finally, we show in an *in vivo* model that $TcdB_{NAP1}$ is able to induce, probably as result of the extended panel of GTPases glucosylated, a stronger immune activation with release of Il-6 and Il-1β and epithelial disruption, which are important hallmarks of fatal outcome of disease. On the other hand, intoxication with $TcdB_{NAP1v}$ resulted in a greater vascular congestion and villus fusion. Overall, our results suggest that the increased virulence and the pathogenic potential associated to TcdB, described for strains such as NAP1 and TcdA-negative strains, could be associated to differences in GTPase modification by these TcdBs.

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LIST OF ABBREVIATIONS

Arf: ADP-ribosylation factor

CDI: *Clostridium difficile* infection

CPE: Cytopathic effect

CROPs: Combined repetitive oligopeptides

GAP: GTPase activating proteins

GDI: Guanine nucleotide dissociation inhibitors

GEF: Guanine nucleotide exchange factor

GTD: Glucosyltransferase domain

IL: Interleukin

IP6: Inositlhexaisophosphate-6

LCTs: Large clostridial toxins

LPS: Lipopolysaccharide

MPO: Mieloperoxidase

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

PaLoc: Pathogenicity locus

PI: Phosphatidylinositol

PK: Proteinase K

Ras: Ras sarcoma proteins

Rab: Ras-like proteins in brain

Ran: Ras-like nuclear proteins

Rho: Ras homologues proteins

R-Ras: Ras related proteins

Rap: Ras proximal proteins

TNF: Tumor necrosis factor

1. INTRODUCTION

1.1 General characteristics of *Clostridium difficile* infection

Clostridium difficile, a Gram positive, spore forming anaerobe is the leading agent of antibiotic associated diarrhea in hospitalized patients (1, 2). Some strains of this bacterium harbors mobile genetic elements and point mutations that confer resistance to antibiotics such as clindamycin, cephalosporines and fluoroquinoles (3, 4). Antibiotic treatment modifies the balance of commensal microbiota, allowing the ingested *C. difficile* spores to extensively germinate, colonize the gut and secrete the large clostridial toxins (LCTs), causing the disease. The resulting *C. difficile* infection (CDI) can lead to a variety of clinical outcomes that range from mild diarrhea to potentially fatal pseudomembranous colitis (5).

In the last decade the prevalence and severity of CDI has increased in hospital settings and community-onset cases (6). This increase has been attributed to spore persistence in the environment and the emergence of multidrug resistance strains and more virulent isolates. The costs of healthcare and the burden associated to CDI have increased as well (7). Interestingly among the risks factors related to CDI, high economic income of patients and healthcare access seem to be important (8).

1.2 Main virulence factors of *C. difficile*

The main virulence factors associated to CDI are two large clostridial exotoxins, TcdA and TcdB. Virulent strains produce TcdA and TcdB or only TcdB(5). These toxins are encoded in a pathogenicity locus (PaLoc) of 19.6 kb, which also encodes for a holin-like structure (TcdE), and two regulators of toxin gene expression, TcdC (putative negative regulator) and TcdR (sigma factor) (Fig. 1) (9).

The regulation of toxin production involves several sigma factors and has been related to flagellar gene expression, sporulation and catabolic repression (10–13).

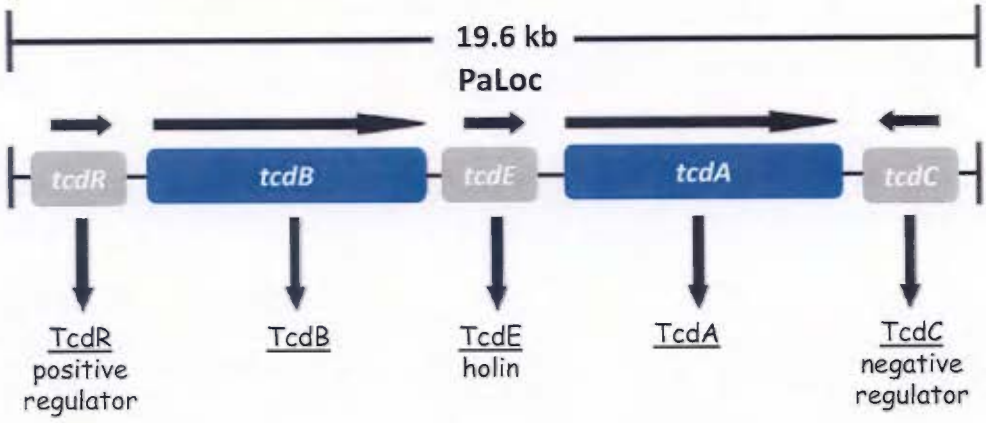


Figure 1. *C. difficile* PaLoc organization. Taken from Voth and Ballard, 2005 (5).

TcdA and TcdB are composed of various functional domains. The carboxyl terminal domain is responsible for delivering the N-terminal glucosyltransferase domain (GTD) inside the cell. The C-domain shows a number of short, homologous regions with repetitive oligopeptides (CROPs); this region binds to host cellular receptors. A translocation domain with autoprocessing cysteine protease function is found between a hydrophobic region and the GTD. The GTD is composed of an enzymatic catalytic domain and a substrate recognition domain (14).

Figure 2. Organizational structure of TcdB. Taken from Belyi and Aktories, 2010 (15).

C. difficile toxins enter the eukaryotic cell through clathrin-mediated endocytosis in a dynamin-dependent process (16). A dual receptor hypothesis for TcdB has been suggested as it binds to the cell through the CROPs domain and a second receptor binding domain (17, 18). Recently, the chondroitin sulfate proteoglycan 4 molecule and the poliovirus receptor-like 3 have been identified as receptors for TcdB and the sucrose-isomaltase and the p-glycoprotein have been reported as receptors for TcdA (19–21). Once in the acidic endosome, the hydrophobic region of the toxin forms a pore in the membrane and the autoproteolytic domain encounters its co-factor inositolhexaisophosphate (IP6) mediating the release of the GTD into the cytosol. In the cytosol the enzymatic domain irreversibly monoglucosylates small GTPases of the Rho and Ras families using UDP-glucose as a substrate, in a threonine residue located in the effector domain of these proteins (Fig.3) (15). Consequently, important signaling pathways in the cell are affected. Overall, the action of these toxins results in massive colonic fluid secretion, inflammation and colonic tissue necrosis (5).

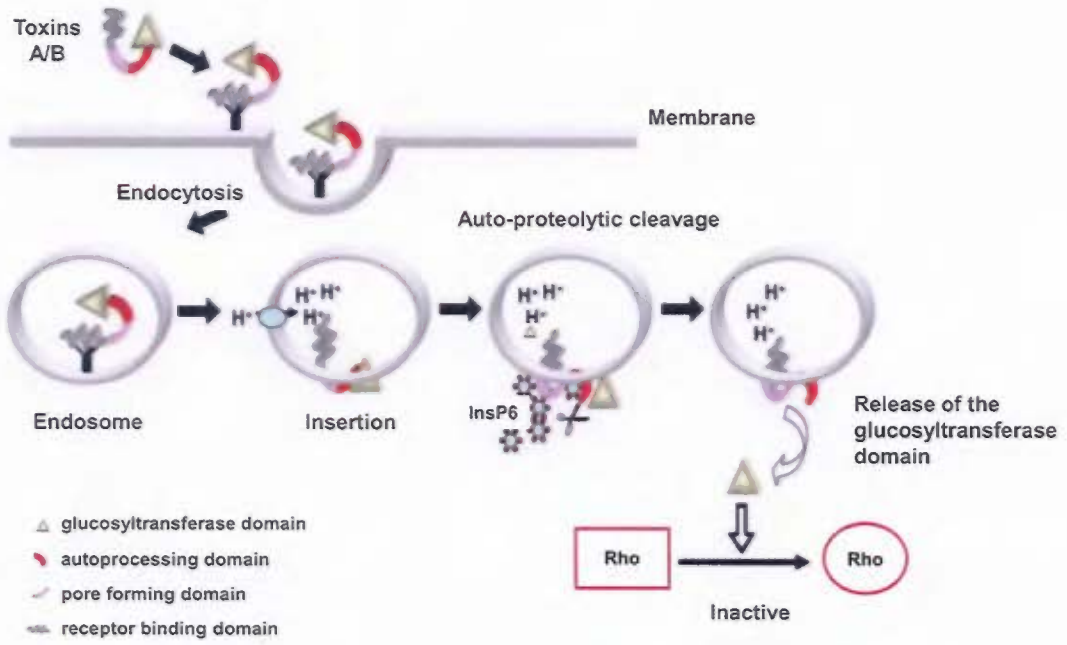


Figure 3. Toxin uptake and mechanism of action. Taken from Belyi and Aktories, 2010 (15).

1.3 Other virulence factors of *C. difficile*

A small group of toxigenic strains produce a third toxin known as binary toxin which is encoded by genes located in the CdtLoc locus. The binary toxin is an actin ADP-ribosylating toxin composed of an enzymatic ADP-ribosyltransferase (CDTa) and a CDTb that binds to the lipolysis-stimulated lipoprotein receptor and translocates the CDTa into the cytosol (22). CDTa ribosylates actin in an arginine residue, inducing its depolimerization and eventually the complete cytoskeleton is disassembled. Mutant strains that only encode for binary toxin can induce inflammation in the hamster model of CDI (23). Moreover, the binary toxin has been reported in strains associated with increased severity of CDI and studies suggest that this toxin favors adhesion through modification of host microtubules (24) .

Other virulence factors described for *C. difficile* include molecules involved in colonization and adhesion events, like the S-layer proteins (25), the fibronectin binding protein FBp68 (26), flagellar proteins (27), the heat shock protein GroEL (28) and various hydrolytic enzymes (29). The S-layers proteins have also been associated to immune activation contributing to the pathogenesis induced by the glucosylating toxins. Nevertheless, unlike for toxins, no variations between epidemic and non-epidemic strains have been reported for these virulence factors (30).

1.4 The Rho and Ras family of GTPases

The small GTPases are molecular switches involved in the regulation of numerous cellular events such as motility, organization of the actin cytoskeleton, apoptosis, cell cycle progression and membrane trafficking (15). These proteins are monomeric G proteins that switch from activated state when bound to GTP to inactivated state in the GDP-bound conformation. GDP/GTP exchange is regulated by the guanine nucleotide exchange factor (GEF) and GTP hydrolysis is activated

by GTPase activating proteins (GAP). In their activated state, the GTPases interact with multiple effector molecules and control diverse cellular activities (31).

The Ras superfamily of small GTPases is divided into five families: the Ras sarcoma proteins (Ras), the Ras homologues proteins (Rho), the Ras-like proteins in brain (Rab), the ADP-ribosylation factor (Arf) and the Ras-like nuclear proteins (Ran) (32). Alternative splicing events and post-translational modifications of these proteins dictate their cellular location and thus the signal transduction pathways regulated (32). Rho GTPases are also regulated by guanine nucleotide dissociation inhibitors (GDI) which maintain the inactive GTPase in the cytosol and transport the active form to the target compartments (15). (Fig. 4)

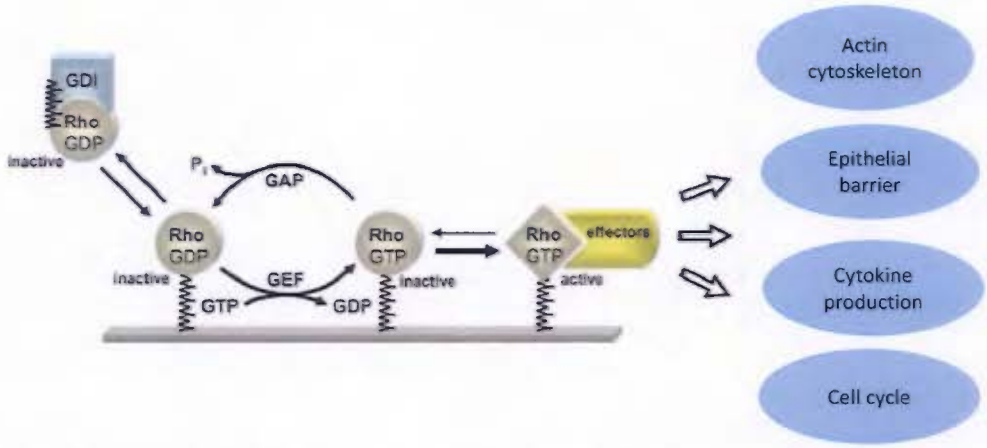


Figure 4. Rho GTPase regulation. Taken from Jank and Aktories, 2008 (14).

The Rho GTPases are mainly involved in actin cytoskeleton regulation. Rac and Cdc42 induce actin polymerization through the Arp2/3 complex and RhoA activates formins such as mDia, which interact with profilin/actin monomers promoting actin polymerization. Rac is involved in membrane ruffling (33), and Cdc42 is involved in filopodia formation (34). RhoA is associated to formation of focal adhesions and stress fibers through the effector protein Rho kinase (35). These proteins also regulate the assembly of tight junctions via actin cytoskeleton control (36).

The Ras GTPases control various signaling pathways when activated by extracellular stimuli. These pathways include extracellular signal-regulated kinases (Raf/MEK/ERK), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinases (MAPK) and the nuclear factor-kappa B (NF-κB) pathway (37). These pathways regulate cell proliferation, actin remodeling and apoptosis. The Ras related (R-Ras) GTPases include the R-Ras, R-Ras2 (TC21) and R-Ras3 (RasM) isoforms which are 70% identical, and the Ras proximal (Rap) group is composed of the Rap1 and Rap2 isoforms which are 60% identical (38). Both, R-Ras and Rap proteins are also involved in cytoskeleton control and mitogenesis (37).

1.5 Cellular effects induced by glucosylating toxins

Regulation of actin polymerization is orchestrated by Rho GTPases, hence the modification induced by *C. difficile* toxins causes a redistribution of the actin cytoskeleton structure. This disruption leads to morphological changes which result in cell rounding and formation of neutrite like retraction fibers as seen in cell cultures (cytopathic arborizing effect) (39). Apoptotic cell death events have also been associated to the glucosylation of small GTPases. Activation of caspase 3, 8 and 9 with chromatin condensation and nucleus fragmentation have been described in cells treated with TcdA and TcdB (5, 40). TcdA and TcdB also induce cytokine production by epithelial cells and monocytes in glucosyltransferase-dependent and –independent events (41–43).

Cell rounding and cell death are temporally distinct outcomes, while cell rounding is seen at earlier times of intoxication, cell death has been reported after 24 hours post intoxication (5). It has been suggested that the cytopathic effect might be more important than cell death since the consequences of actin reorganization relate to the pathogenesis of CDI (5). One of these main events is the disruption of epithelial tight junctions (44). TcdA reduces epithelial resistance within 8 hours of intoxication (45). TcdB induces cell rounding within 2 hours and modifications of apical and basal F-actin have been associated to the loss of occludin and ZO-1 in

tight junctions (44). These events contribute to the increase in epithelial permeability, fluid secretion, and inflammatory response in CDI. Intoxication then leads to the release of intelukin-8 (IL-8), IL-6 and tumor necrosis factor (TNF)-α (46) by epithelial cells and macrophages, inducing neutrophil chemiotaxis (47) and pseudomembranous colitis develops when the inflammatory process persists (5).

1.6 Role of TcdB in disease

Previous studies showed that TcdA alone could induce most of the effects elicited by *C. difficile* in the hamster model. On the contrary, and in spite of being one thousand times more potent than TcdA (48), TcdB alone could not induce these effects in animals unless it was administered with TcdA. These observations suggested that both toxins acted synergistically (49). However, recent work provides evidence that TcdB is necessary for *C. difficile* associated disease as mutant strains that only encode for TcdA are not virulent (50) and virulent circulating strains that do not produce TcdA (TcdA-negative strains) are fully capable of causing disease (51). Interestingly, native toxin B purified from the latter group of strains induces a different CPE on cells and consequently these proteins have been identified as variant TcdBs (51). Nosocomial outbreaks caused by TcdA-negative strains have increased in the last decade (52) as well as the severity of disease associated to this group of strains (53).

Overall, these facts suggest that differences in TcdB could have an important role in CDI and could be a possible factor that contributes to the pathogenic potential of different *C. difficile* strains.

1.7 Epidemic strains of *C. difficile*

The epidemic *C. difficile* strain NAP1/027 has rapidly spread and has been responsible for epidemic outbreaks worldwide (54–57). Furthermore, the increase in severity and recurrences of CDI has been related to the emergence of this group

of strains. Among the factors that have been proposed to contribute to the virulence potential of NAP1 strains are fluoroquinolone resistance, higher sporulation rates and increased toxin production (58–60). This group of strains encodes for the binary toxin and contains an 18–base pair deletion and a point mutation at position 117 in the gene *tcdC* which might be related to toxin overproduction (61). In addition, their TcdB has a higher cytotoxic potency on cell lines as CHO cells and 3T3 fibroblasts, suggesting that this form of TcdB could be an additional factor contributing to the increased pathogenesis induced by NAP1 strains (62, 63). The high toxicity displayed by this toxin is due to a more rapid cell entry and efficient autoprocessing (62). These characteristics have been linked to structural differences of TcdB, particularly in the CROPs domain (63, 64).

2. JUSTIFICATION

The increase in severity and recurrences of CDI has been related to the emergence of epidemic strains like the NAP1 strains. Furthermore, the prevalence and severity of disease associated to TcdA-negative strains that harbor variant TcdBs are on the rise. Evidence suggests that variations in TcdB could contribute to the differences in the virulence potential of different *C. difficile*. Nevertheless, the contribution of differences in the enzymatic activity of distinct TcdBs on the biological events induced upon intoxication has not been explored. Under this perspective, a parallel comparison of the modified substrates and cellular effects induced by the TcdBs secreted by various *C. difficile* strains such as NAP1 and TcdA-negative strains is necessary in order to outline the relationship between the pathogenic potential associated to TcdB and the panel of glucosylated GTPases.

3. HYPOTHESIS

The panel of small GTPases modified by different TcdBs plays a role in the pathogenicity of *C. difficile* strains.

4. OBJECTIVES

4.1 Main objective

To determine the relationship between the pathogenic potential associated to TcdB from different *C. difficile* strains and the panel of small GTPases glucosylated by this toxin.

4.2 Specific objectives

1. To compare the panel of GTPases glucosylated *in vitro* and *ex vivo* by TcdB from *C. difficile* strains NAP1, NAP1v, VPI 10463 and NAP9.
2. To compare the effects induced by TcdB from *C. difficile* strains NAP1, NAP1v, VPI 10463 and NAP9 on the cytoskeleton (cytopathic effect), cell death and immune activation.
3. To compare the pathogenic potential associated to TcdB from *C. difficile* strains NAP1, NAP1v and VPI 10463 in the ileal loop mouse model.

5. MATERIALS AND METHODS

5.1 *C. difficile* strains and TcdB sequence analysis

From a collection of *C. difficile* clinical isolates obtained at the Laboratorio de Investigación de Bacteriología Anaerobia (LIBA) from the University of Costa Rica, three distinct strains were chosen in order to analyze the impact of TcdB on the pathogenic potential. These included a NAP1 epidemic strain, a NAP1 variant strain (NAP1v) whose supernatant induced a different cytopathic effect in cell culture and a NAP9 strain (TcdA-negative strain) (54, 64). These strains had been typed by pulsed field gel electrophoresis and whole-genome sequences had been obtained using sequencing-by-synthesis HiSeq platform (Illumina) (65). The VPI 10463 strain, obtained from cryopreservation conditions at -80 °C at LIBA, was also included as a reference and historic strain of *C. difficile* toxin studies. For this study, TcdB sequences were extracted manually and aligned with MAFFT (66) or MUSCLE (67). Sequences were compared to TcdB from the reference strain VPI 10463 (Genbank number FN545816).

5.2 Quantitation of secreted toxins

Strains were grown in TYT broth (3% Bacto tryptose, 2% yeast extract, and 0.1% thioglycolate, pH 6.8) for 24 hours, as described previously (65). After this period, bacteria were removed by centrifugation at 20000 x g for 30 min. Proteins from bacterial free supernatants were concentrated by methanol/choloroform precipitation (23). Pellets were air dried and resuspended in loading buffer (50 mM Tris-HCl pH 6.8, 1 % SDS, 0.01 % Bromophenol Blue, 5 % β-mercaptoethanol and 10 % glycerol). Proteins were then separated in 7.5% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes and analyzed by Western blot with monoclonal anti-TcdA (TTC8) or anti-TcdB (2CV) antibodies (tgcBIOMICS). Chemiluminescence signals emitted after addition of a goat anti-mouse IgG-horseradish peroxidase conjugate (Invitrogen) and the Lumi-Light Plus Western

blotting substrate (Roche) were recorded with a Chemidoc XRS documentation system (Bio-Rad).

5.3 Native toxin B purification

TcdA and TcdB were obtained from supernatants of strains grown in a dialysis system culture for 72 hours in brain heart infusion broth and purified as described previously (68). After this time, bacteria were removed by centrifugation at 20000 x g for 30 min and proteins found in the supernatant were precipitated with ammonium sulfate (70 %). Proteins were loaded onto an anion exchange DEAE-sepharose column pre-equilibrated with 50 nM NaCl pH 7.4. Toxins were eluted with 1 M NaCl pH 7.4 at a flow rate of 4 mL/min. Toxins were then purified by gel filtration in a Superdex 200 10/300 column (GE Healthcare), equilibrated with Hepes buffer (20 nM Hepes, 50 nM NaCl, pH 6.9) at a flow rate of 0.5 mL/min. Toxin positive fractions were pooled and concentrated in Hepes buffer by ultrafiltration with a 100 kDa membrane. Proteins were quantified using the Bradford method (Bio-Rad DC Protein Assay, Bio-Rad) and purity was assessed by SDS-PAGE and Coomassie staining. Final toxin identification was determined through mass spectrometry (LC-MS-MS) and only TcdB derived peptides were identified (not shown).

5.4 Cytopathic effect (CPE) produced by TcdBs

Confluent HeLa cells and 3T3 fibroblasts (cultured in DMEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C) grown in 12-mm glass slides were treated with 0.2 nM of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ or $TcdB_{NAP9}$. After the CPE was achieved in 100% of the cells, HeLa cells were visualized by light microscope. Fibroblasts were fixed with 3.5 % of paraformaldehyde for 10 minutes according to previously described protocols (51). Then, cells were permeabilized with 0.5 % Triton X-100 in PBS and treated with 0.5 g/ml fluorescein

isothiocyanate-phalloidin (FITC) for 30 minutes. Cytoskeleton modifications were evaluated by fluorescence microscopy.

5.5 *In vitro* glucosyltranferase activity of TcdBs

The ability of TcdBs to glucosylate different monomeric GTPases was performed through a radioactive assay, as previously described (18, 48). For this, 24.2 µM of UDP-[^{14}C]glucose (250 mCi/mmol; PerkinElmer), 2 µM of previously purified recombinant small GTPases (64) and 10 nM of each TcdB were mixed in a reaction buffer (50 mM Hepes pH 7.5, 100 mM KCl, 1 mM $MnCl_2$, 2 mM $MgCl_2$, and 0.1 mg/ml BSA). After 1 hour of incubation at 37 °C, the reaction was stopped with loading buffer and the proteins were separated by 10 % SDS-PAGE. Glucosylation of small GTPases was analyzed by phosphorimaging. Band intensities were measured with Image J software and were normalized to the Rac1 signal.

5.6 *Ex vivo* small GTPase modification assays

The TcdB ability to inactivate small GTPases was determined on confluent HeLa cells and RAW 264.7 macrophages grown in 6-well plates (cultured in DMEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C). Cells were intoxicated with 0.9 nM of the corresponding TcdB for the indicated times. After this period, the cells were treated as previously described (69). Cells were washed with PBS and lysed with precipitation buffer (1% Triton X-100, 0.1% SDS, 0.3% Nonidet P-40, 500 mM NaCl, 10 mMm $MgCl_2$ y 50 mM Tris, pH 7.2). Lysates were centrifuged at 20000 x g for 1 minute and 20 µl were separated as control for total amount of GTPases. Lysates were incubated with previously purified GST fusion effector proteins coupled to glutathione- Sepharose beads (69). After 1 hour of incubation at room temperature, activated proteins were pulldown by centrifugation with the GST-RBD (Cdc42), GST-RalGDS-RBD (Rap2A) and GST-Raf-RBD (R-Ras) (18, 69). Proteins were then resolved by SDS-PAGE and transferred to PVDF

membranes. RhoA and Rac1 proteins were detected with monoclonal antibodies that do not recognized the glucosylated isoforms, anti-RhoA (ab54835, Abcam) and anti-Rac1 (clone 23A8, Millipore). The other activated GTPases were detected using anti-Cdc42 antibodies (ab41429, Abcam), anti-RRas2 (ab156270, Abcam) or anti-Rap2A+Rap2B+Rap2C (ab73296, Abcam) by Western blotting. Chemiluminescence signals emitted after addition of a goat anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate (Invitrogen) were recorded as previously described. For graphical representation, band densities of R-Ras2 and Rap2A modifications were measured with Image J software and band intensities were normalized to the untreated cells signal. In Raw cells, actin was included as a loading control (A2066, Sigma-Aldrich).

5.7 Kinetics of intoxication induced by TcdBs

Confluent HeLa cells, 3T3 fibroblasts and Chinese hamster ovarian (CHO) cells grown in 96-well plates (cultured in DMEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C) were intoxicated with the indicated concentrations of each toxin (Fig. 8, 9, 10). The percentage of round cells in each well was evaluated every hour for a period of 12 hours and then at 24 hours.

5.8 Cell death assay

Confluent HeLa cells grown in 24 well plates (cultured in DMEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C) were intoxicated with the indicated concentrations of each toxin (Fig. 11) and Annexin V was used as a marker of initial apoptotic events. Cytotoxicity was evaluated after 8 hours and 24 hours of treatment. Cells were then harvested, washed in 1 X PBS and resuspended in 100 µL 1 X annexin binding buffer (Molecular Probes, Invitrogen). Two µL of Alexa Fluor 488 annexin V and 1 µL of 100 mg/mL propidium iodide (PI) working solution (Molecular Probes, Invitrogen) were added to the resuspended cells. After 15

minutes, stained cells were analyzed by flow cytometry using a Guava easyCyte Mini (Millipore). The percentage of stained cells was determined with FLOWJO, LLC Data analysis software.

5.9 Determination of tumor necrosis factor alpha (TNF-α) induction

Confluent RAW 264.7 macrophages grown in 24-well plates (cultured in DMEM supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C) were intoxicated with different concentrations of the indicated TcdB. Cells were also treated with 0.5 nM of TcdBs that were either inactivated at 70°C for 10 minutes or treated with 1 mg/ml proteinase K (70). Lipopolysaccharide (LPS) (10 µg/mL) from *E. coli* was used as a positive control. The concentration of TNF-α in the supernatants after 6 hours and 24 hours was determined by commercial enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (eBioscience).

5.10 Murine ileal loop model

Animal experimental procedures were approved by the University of Costa Rica Animal Care and Use Committee through CICUA 01-12 and CICUA 07-13 according to Law 7451: Bienestar de los animales, 26668-MICIT. Male Swiss mice of 20 to 25 g were subjected to fasting overnight and anesthetized with ketamine (60 mg/kg of body weight) and xylazine (5 mg/kg) (Eremer Pharma). Through a midline laparotomy, an ileal loop of approximately 4 cm was ligated, and 10 µg of each toxin or Hepes control solution were injected. Mice were sacrificed 4 hours after intoxication. The neutrophil accumulation in ileal tissue was evaluated through determination of myeloperoxidase (MPO) activity with the odianisidine dihydrochloride (Sigma) and H_2O_2 assay (71). For this, 100 µg of tissue were macerated in HTAB (50 mM hexadecyltrimethylammonium bromide in KH_2PO_4 buffer, pH 6.0). MPO activity was determined at 450 nm, and calculated as the enzyme activity that catalysis the conversion of 1 µmol substrate into product in 1 minute. The concentrations of the proinflammatory cytokines IL-1β, IL-6, and TNF-

α in ileal tissue were also determined (65). Tissues were macerated in 1 X PBS (pH 7.0) and cytokine concentration in homogenates was determined by commercial ELISA according to the instructions of the manufacturer (eBioscience). Intestinal sections were also fixed in formalin and stained with hematoxylin and eosin for histopathological evaluation according to previous protocols (72). The samples were evaluated for the severity of epithelial damage, vascular congestion, and villus fusion using a histopathological score (HS) scale ranging from 0 (absence of alterations) to 3 (severe) (72). All samples were evaluated by an independent pathologist at the Pathology Laboratory of the Escuela de Medicina Veterinaria of the National University of Costa Rica.

6. RESULTS

6.1 The type of cytopathic effect induced by TcdBs is associated to a similar GTD.

Four distinct strains were chosen in order to analyze the impact of TcdB on the pathogenic potential: a NAP1 epidemic strain, a NAP1 variant strain (NAP1v) whose supernatant induced a different cytopathic effect in cell culture, a NAP9 strain (54, 64) and the VPI 10463 historic strain. Both NAP1 isolates and the VPI 10463 strain produce high amounts of TcdA and TcdB as observed when assessed by Western Blot (Fig. 5A). On the other hand, the NAP9 strain does not produce high levels of TcdB and no TcdA was detected. This result along with whole genome sequencing and PaLoc analysis (data not showed) (54, 64) confirms that the NAP9 strain is in fact a TcdA-negative strain.

To characterize the morphological effects induced by TcdB, native toxin from each strain was obtained. After purification, toxin identification and purity were evaluated by mass spectrometry (data not showed) and Coomassie blue staining (Fig. 5B). HeLa cells and 3T3 fibroblasts were then intoxicated and the cytopathic effects were recorded. The classic arborizing effect was observed in cells treated with $TcdB_{NAP1}$ and $TcdB_{VPI10463}$. Whereas cells intoxicated with $TcdB_{NAP9}$ and $TcdB_{NAP1v}$ showed a distinct cytopathic effect, characteristic of variant TcdBs. Cell rounding and detachment were induced by these toxins, but there was no evidence of protrusion formation (Fig. 5C). These results indicate that TcdB from the NAP1v strain was responsible for the variant cytopathic effect reported earlier in the supernatant-treated cells (64).

Previous phylogenetic analysis of the NAP1v strain revealed that its PaLoc is closely related to that of epidemic NAP1 (64). However, $TcdB_{NAP1v}$ clearly induces an effect similar to $TcdB_{NAP9}$. Thus, we decided to compare the toxins at sequence level using the sequence of $TcdB_{VPI10463}$ as reference. This comparison showed

that the primary sequence of the GTD is similar between $TcdB_{NAP1}$ and $TcdB_{VPI10463}$ (96%), but in $TcdB_{NAP1v}$ this region is more closely related to the corresponding region of $TcdB_{NAP9}$ (Fig. 5D). The identity in the first 546 amino acid residues between $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ is 100 %, whereas that between $TcdB_{NAP1v}$ and $TcdB_{NAP1}$ is 80 %. On the other hand, in the CROPs domain, which corresponds to amino acids 1645 to 2366, there is a 99 % identity between $TcdB_{NAP1v}$ and $TcdB_{NAP1}$. Overall, TcdBs that induce a similar CPE have a high identity at the GTD level. In addition, the NAP1 toxins share the CROPs region, which is different from the corresponding domain of $TcdB_{VPI10463}$ and $TcdB_{NAP9}$.

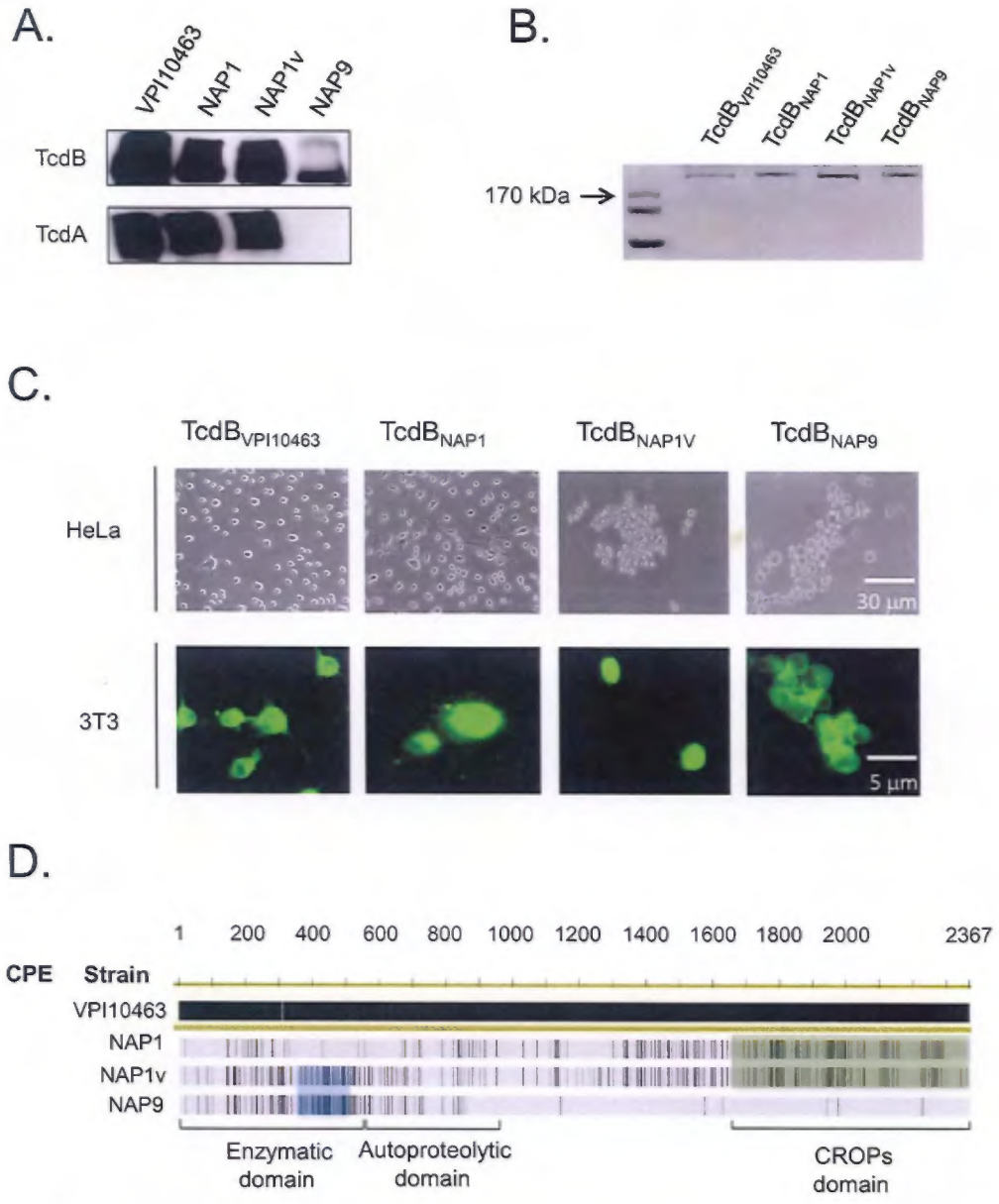


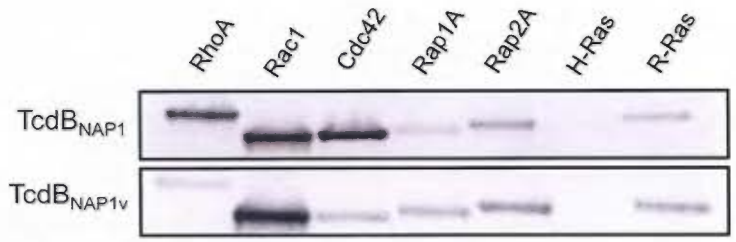
Figure 5. Characterization of TcdB secreted by different C. difficile strains. (A) Quantification of toxin production by different *C. difficile* strains in 24 hours bacterial-free supernatants. Proteins were precipitated with methanol-chloroform and probed with monoclonal antibodies against TcdA and TcdB. (B) TcdBs were obtained from supernatants of strains grown in dialysis system culture and purified by ionic exchange chromatography and gel filtration. Purity of toxins was assessed by Coomassie stained 7.5% SDS-PAGE, loaded with

1.5 µg of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{NAP9}$. (C) HeLa cells and 3T3 fibroblasts were treated with 0.2 nM of purified toxins. Cells were treated until a CPE was achieved in 100 % of the cells. Images of the morphological effects induced upon intoxication were obtained for HeLa cells by light microscopy and for fibroblasts by fluorescence microscopy. In order to see actin cytoskeleton modifications, fibroblasts were stained with fluorescein isothiocyanate-phalloidin. (D) Sequence alignment of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{NAP9}$. Black blocks represent disagreements to the sequence of $TcdB_{VPI10463}$, which was selected as a reference for the alignment. The blue box highlights a distinct glucosyltransferase region of $TcdB_{NAP9}$ and $TcdB_{NAP1v}$, absent in $TcdB_{NAP1}$. The green box shows sequence stretches in the repetitive CROPs domain shared by $TcdB_{NAP1}$ and $TcdB_{NAP1v}$, which are different in $TcdB_{VPI10463}$ and $TcdB_{NAP9}$.

6.2 $TcdB_{NAP1}$ glucosylates a broader spectrum of small GTPases

Since $TcdB_{NAP1}$ and $TcdB_{NAP1v}$ vary mainly in their GTD domain, we wanted to evaluate whether the differences in the cytopathic effect were related to the panel of glucosylated GTPases. The glucosylation pattern of $TcdB_{NAP1}$ and $TcdB_{NAP1v}$ was determined through a radioactive *in vitro* assay. $TcdB_{NAP1v}$ readily glucosylated Rac1 (Fig. 6A) and modified to a lesser extent Cdc42, Rap1A, Rap2A and R-Ras (Fig. 6B). $TcdB_{NAP1}$ targets proteins reported for toxins that induce an arborizing effect, RhoA, Rac1 and Cdc42 (Fig. 6A) (73). However, to our surprise, this toxin was also able to glucosylate Rap1A, Rap2A and R-Ras at levels similar to those of $TcdB_{NAP1v}$ (Fig. 6B), suggesting that TcdB from NAP1 could modify a broader group of small GTPases without inducing a variant cytopathic effect.

A.



B.

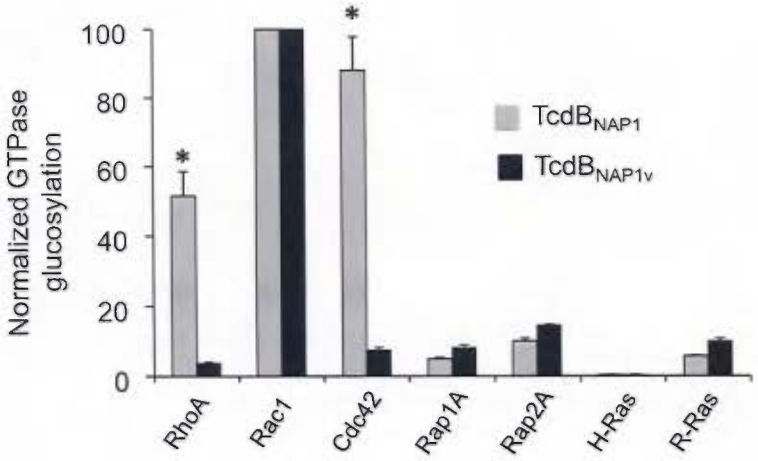


Figure 6. In vitro glucosyltransferase activity of TcdBs. (A) 10 nM of TcdB$_{NAP1}$ and TcdB$_{NAP1v}$ were tested for their ability to glycosylate a panel of recombinant GTPases using UDP-[^{14}C]glucose as a co-substrate. Labeled bands were detected by phosphorimaging analysis. (B) The band intensities quantified by densitometry. Each experiment was normalized to Rac1 signal. Means ± SEM of three independent experiments are showed. *P < 0.05 (One-way ANOVA with Bonferroni´s correction).

To confirm that in fact $TcdB_{NAP1v}$ was able to glucosylate the Ras family of GTPases, pull down assays in intoxicated HeLa cells were performed. For this purpose, cells were treated with $TcdB_{NAP1}$, $TcdB_{VPI10463}$, $TcdB_{NAP1v}$ or $TcdB_{NAP9}$. Rac1 completely disappeared in all intoxicated cells after 6 hours of treatment, whereas RhoA was only modified by toxins that induce an arborizing effect (Fig. 7A). In this case, the antibody to Rac and Rho used did not recognize the glucosylated form (64), therefore no loading control of total GTPase is shown. Cdc42 was only glucosylated by $TcdB_{NAP1}$ or $TcdB_{VPI10463}$ at 6 hours but after 24 hours all four toxins inactivated this protein (Fig. 7A). This difference indicates that even though $TcdB_{NAP1v}$ or $TcdB_{NAP9}$ could modify Cdc42, this GTPase might not be a preferred substrate for variant toxins.

In agreement with the *in vitro* experiments, $TcdB_{NAP1}$ and $TcdB_{NAP1v}$ were able to partially glucosylate Rap2A at early intoxication times (Fig. 7B). Interestingly, at late intoxication times $TcdB_{NAP1}$ treated cells, the levels of activated Rap2A increased. The antibody used is targeted against Rap2A-Rap2B-Rap2C, suggesting an activation compensatory mechanism of Rap2B and/or Rap2C. $TcdB_{NAP1}$ was the only toxin that could inactivate the R-Ras2 isoform after 6 hours of intoxication (Fig. 7B), confirming its ability to modify an extensive panel of GTPases. Furthermore, $TcdB_{NAP9}$ that did not glucosylate Rap2A was able to glucosylate R-Ras2; which could mean that even if these toxins have a high level of identity at the GTD domain, the targeted substrates may vary.

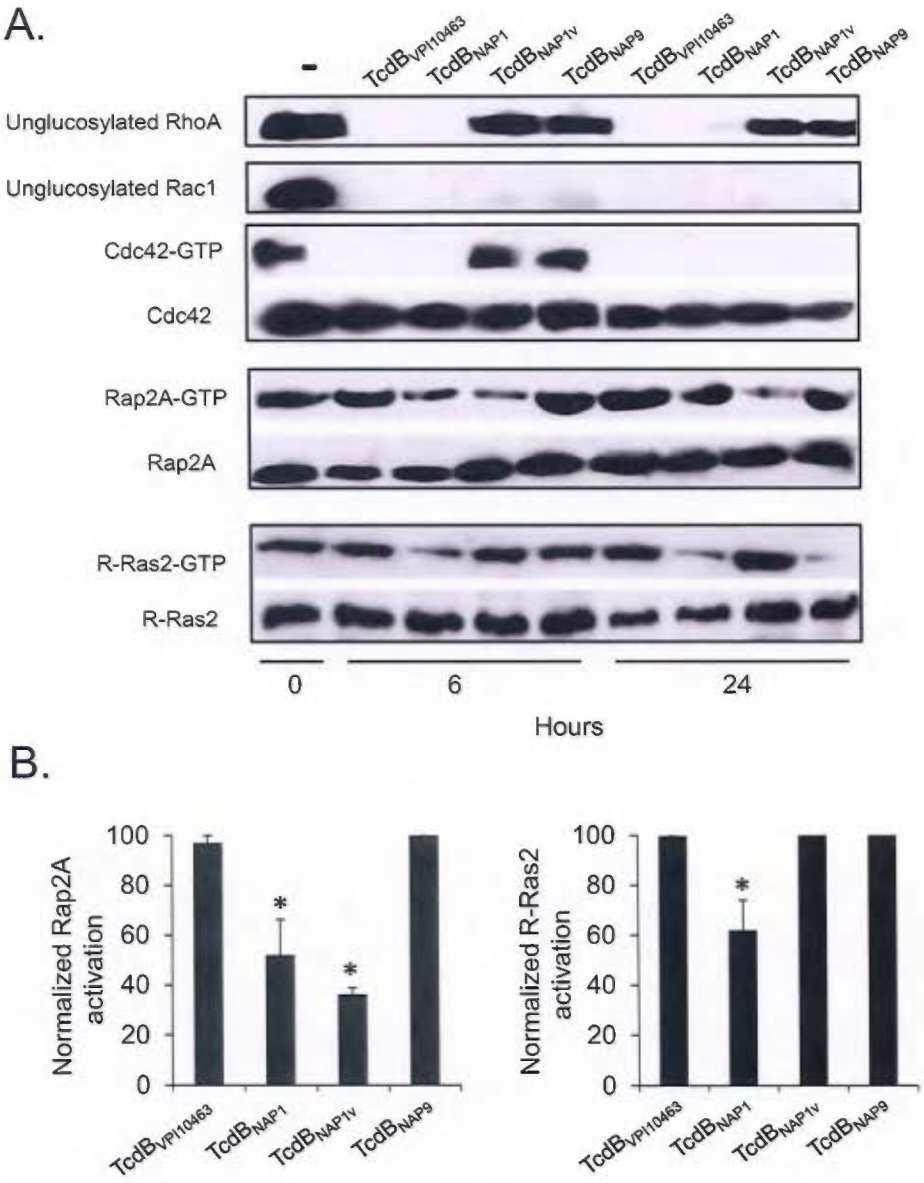


Figure 7. Ex vivo glucosyltransferase activity of TcdBs. (A) Effect of TcdBs on the activation state of small monomeric GTPases. HeLa cells were intoxicated with 0.9 nM of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ or $TcdB_{NAP9}$ for the indicated times. After treatment cells were lysed. One part of the lysates was used as control for total amount of GTPases for Cdc42, Rap2A and R-Ras, and the rest was incubated with RBD-GST (pull down of Cdc42-GTP), GST-RalGDS-RBD (pull down of Rap-GTP) or GST-Raf-RBD (pull down of R-Ras2-GTP)-sepharose beads. Precipitated GTP-loaded proteins and unglucosylated RhoA and Rac1 were detected by Western

blotting. GTPases were detected using anti-RhoA, anti-Rac1, anti-Cdc42, anti-Rap2A-Rap2B-Rap2C and anti-R-Ras2 respectively. Negative control cells were left untreated. (B) The band intensities of Rap2A and R-Ras2 activation at 6 hours post intoxication were quantified by densitometry. Each experiment was normalized to the untreated cells control. Error bars mark the SEM from the mean. $*P < 0.05$, compared to the groups without asterisk (One-way ANOVA with Bonferroni´s correction).

6.3 TcdBs have similar cytotoxic potencies

In order to address whether the cytotoxic potency is dependent of the panel of glucosylated GTPases, the kinetics of cell rounding induced by this group of toxins was determined in various cell lines. Cells were intoxicated with different concentrations of each toxin and the cytopathic effect was evaluated across time. In HeLa cells the cytotoxic potency of $TcdB_{NAP9}$ was lower than that observed for other toxins (Fig. 8); in 3T3 fibroblasts this observation was also true at low toxin concentrations (Fig. 9). These results initially lead to the idea that that the type of cytopathic effect could be related to this difference. However, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{VPI10463}$ present similar cytotoxic kinetic patterns in all cell lines (Fig.8, 9, 10). Furthermore, the sensitivity to all toxins varied within cell lines, in agreement with other studies that suggest that cellular receptor densities could account for cell line sensitivity (74).

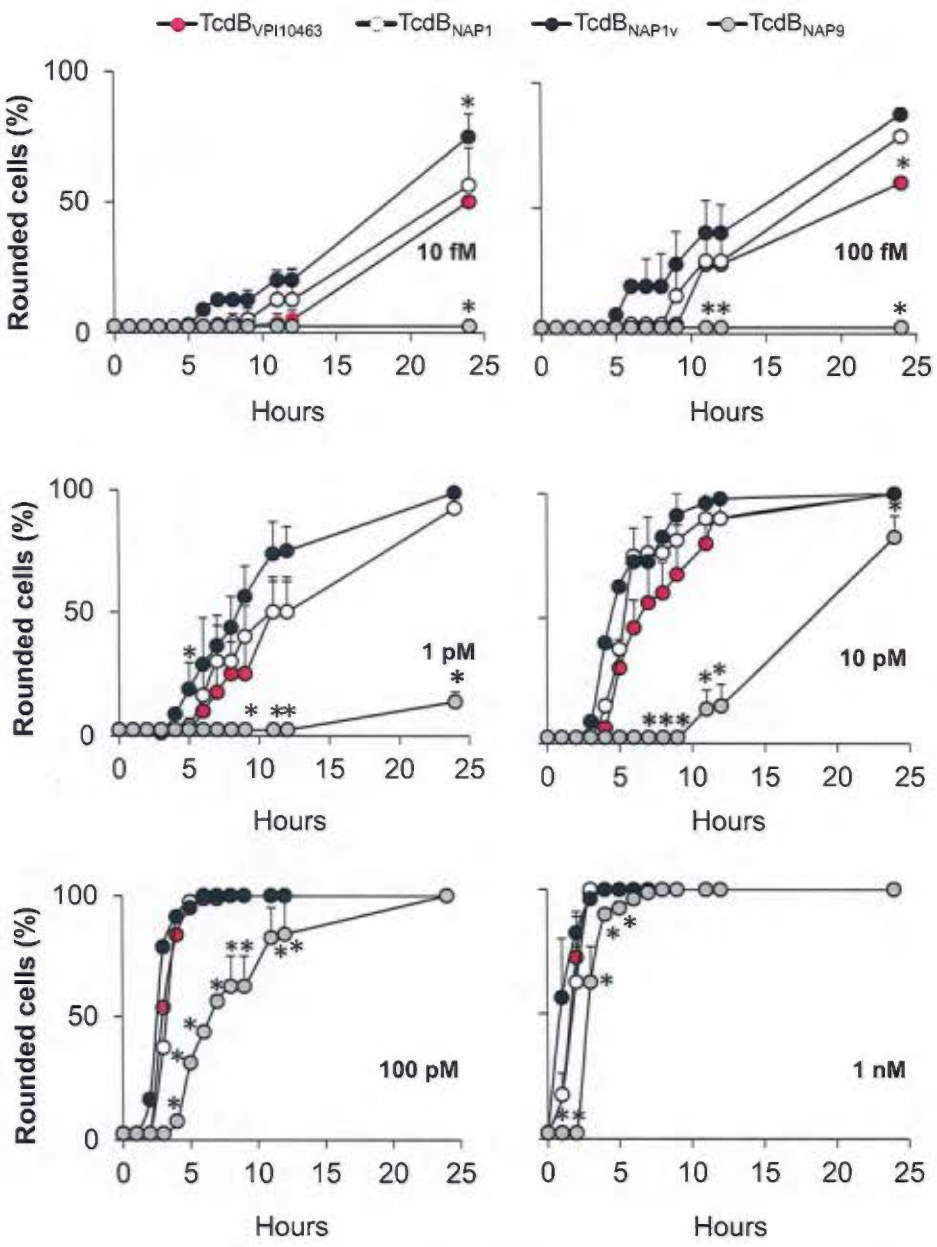


Figure 8. Kinetics of intoxication by different TcdBs on HeLa cells. HeLa cells were treated with the indicated concentrations of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{NAP9}$.The percentage of round cells in each well was evaluated at the indicated times. Means ± SEM of three independent experiments are showed. *$P < 0.05$ (One-way ANOVA with Bonferroni´s correction).

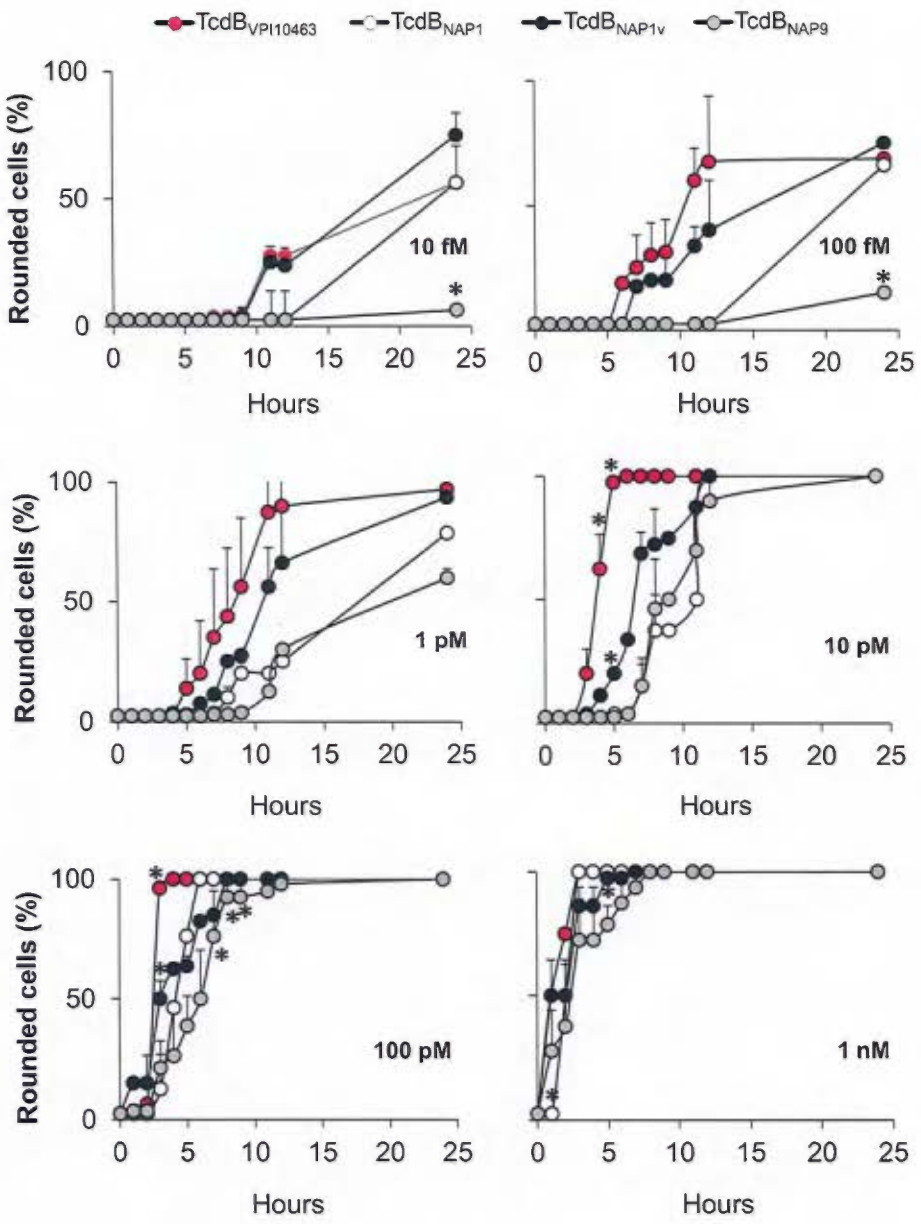


Figure 9. Kinetics of intoxication by different TcdBs on 3T3 fibroblasts. 3T3 fibroblasts were treated with the indicated concentrations of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{NAP9}$.The percentage of round cells in each well was evaluated at the indicated times. Means ± SEM of three independent experiments are showed. *$P < 0.05$ (One-way ANOVA with Bonferroni´s correction).

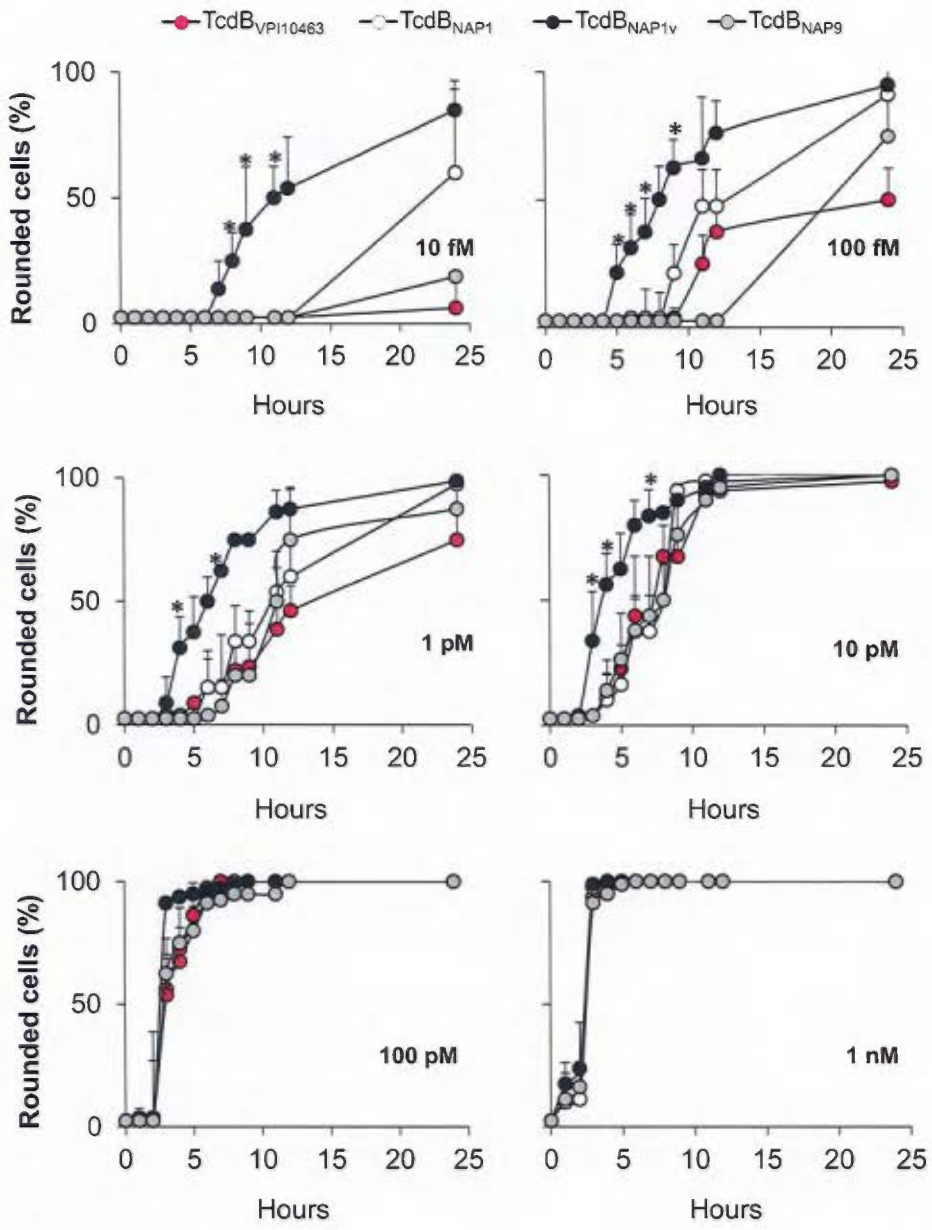


Figure 10. Kinetics of intoxication by different TcdBs on CHO cells. CHO cells were treated with the indicated concentrations of TcdBVPI$_{10463}$, TcdB$_{NAP1}$, TcdB$_{NAP1v}$ and TcdB$_{NAP9}$.The percentage of round cells in each well was evaluated at the indicated times. Means ± SEM of three independent experiments are showed. *P < 0.05 (One-way ANOVA with Bonferroni´s correction).

Next, we decided to study the cytotoxic activity through a cell death assay. For this purpose, HeLa cells were treated with the different TcdBs and cell death was evaluated at an earlier time of intoxication and after 24 hours of treatment. Cells were treated with picomolar concentrations of toxins since nanomolar concentrations activate cell death pathways that are independent of the glucosyltransferase activity of these proteins (75). After 8 hours of treatment, the percent of cell death induced by all toxins was low, with less than 10% of the cells being stained (Fig. 11A and B). The number of cells stained with anexin V, was not statistically different from the percent of positive untreated control cells. Interestingly after 24 hours of intoxication, the number of positive cells was statistically higher when cells were treated with 10 pM of $TcdB_{NAP1v}$ and 100 pM of $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ (Fig. 11C and D). Furthermore, more than 20% of cells intoxicated for 24 hours with the variant toxins were positive for anexin V/PI, suggesting that membrane integrity could have been affected. On the other hand, $TcdB_{NAP1}$ did not induce more cell death that the other toxins in spite of glucosylating more GTPases (Fig. 11). Overall, these results indicate that TcdB does not induce high levels of cell death in HeLa cells; nevertheless, variant TcdBs seem to trigger cell death pathways that might be different from the ones induced by $TcdB_{NAP1}$ and $TcdB_{VPI10463}$.

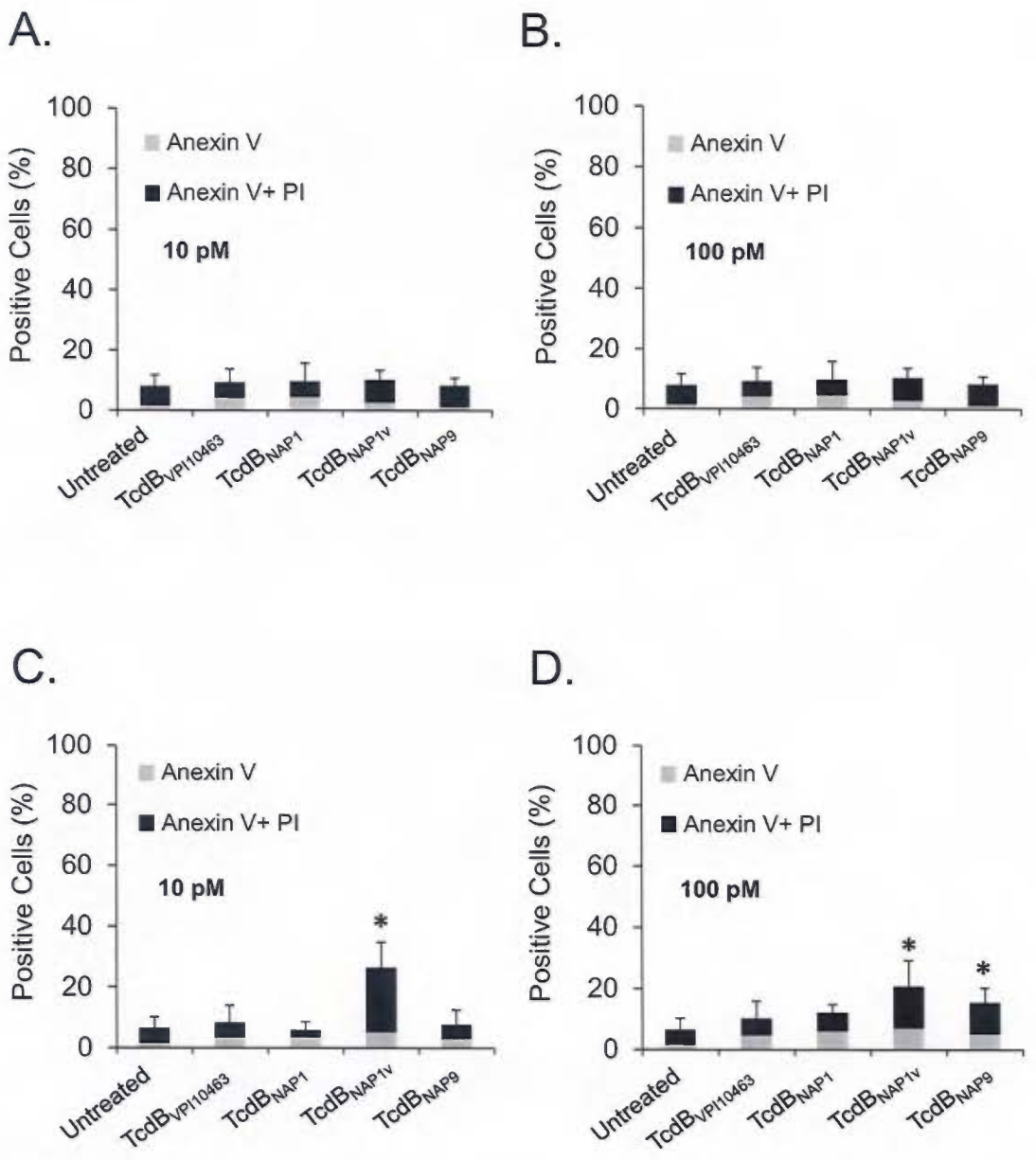


Figure 11. Cell death of HeLa cells induced by TcdBs. HeLa cells were treated with the indicated concentrations of $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ or $TcdB_{NAP9}$ for 8 hours (A and B) or 24 hours (C and D). Cell death was analyzed by flow cytometry using anexin/propidium iodide (PI) double staining. Means ± SEM of three independent experiments are showed. $*P < 0.05$ (One-way ANOVA with Bonferroni´s correction).

6.4 TNF-α release by macrophages is related to the panel of glucosylated GTPases

Studies by Sun et al. indicate that secretion of TNF-α by macrophages is associated with the glucosyltransferase activity of TcdA (42). In order to test whether this was also true for TcdB, Raw murine macrophages were treated with native toxin, heat-inactivated TcdB or TcdB treated with proteinase K. For all toxins, inactivation of their activity abolished their ability to induce TNF-α release (Fig. 12A), confirming that either the glucosyltransferase activity or at least the holotoxin conformation is needed for the release of TNF-α by TcdB. The production of this cytokine is dependent of toxin concentration as well. When cells were treated with different concentrations of each toxin, all toxins induced a higher level of secretion at 500 pM (Fig. 12B). Interestingly, TNF-α release was higher when macrophages were treated for 6 hours with $TcdB_{NAP1}$ or $TcdB_{VPI10463}$ (Fig. 13A). After 24 hours all toxins induced similar TNF-α concentrations (Fig. 13A). TNF-α production was not determined at higher concentrations of toxins since this condition induces *in vitro* cell lysis (data not shown), which has been reported as a glucosyltransferase-independent process in macrophages (74).

Since the enzymatic activity seems to be needed for the release of TNF-α by TcdB, we decided to evaluate the glucosylation of GTPases at 6 hours and 24 hours using the antibodies that do not recognize the glucosylated isoforms. Indeed, Rac1 was glucosylated at 6 hours by all toxins as no band was detected when assessed by Western blot. RhoA was modified as expected only by $TcdB_{NAP1}$ and $TcdB_{VPI10463}$, but in this case the signal corresponding to cells treated with $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ was greater than that of the untreated macrophages at 6 hours and then at 24 hours (Fig. 13B), indicating that RhoA is probably being activated or overexpressed across time as a result of intoxication. These observations suggest that the panel of glucosylated GTPases could be related to TNF- α release in this cell line.

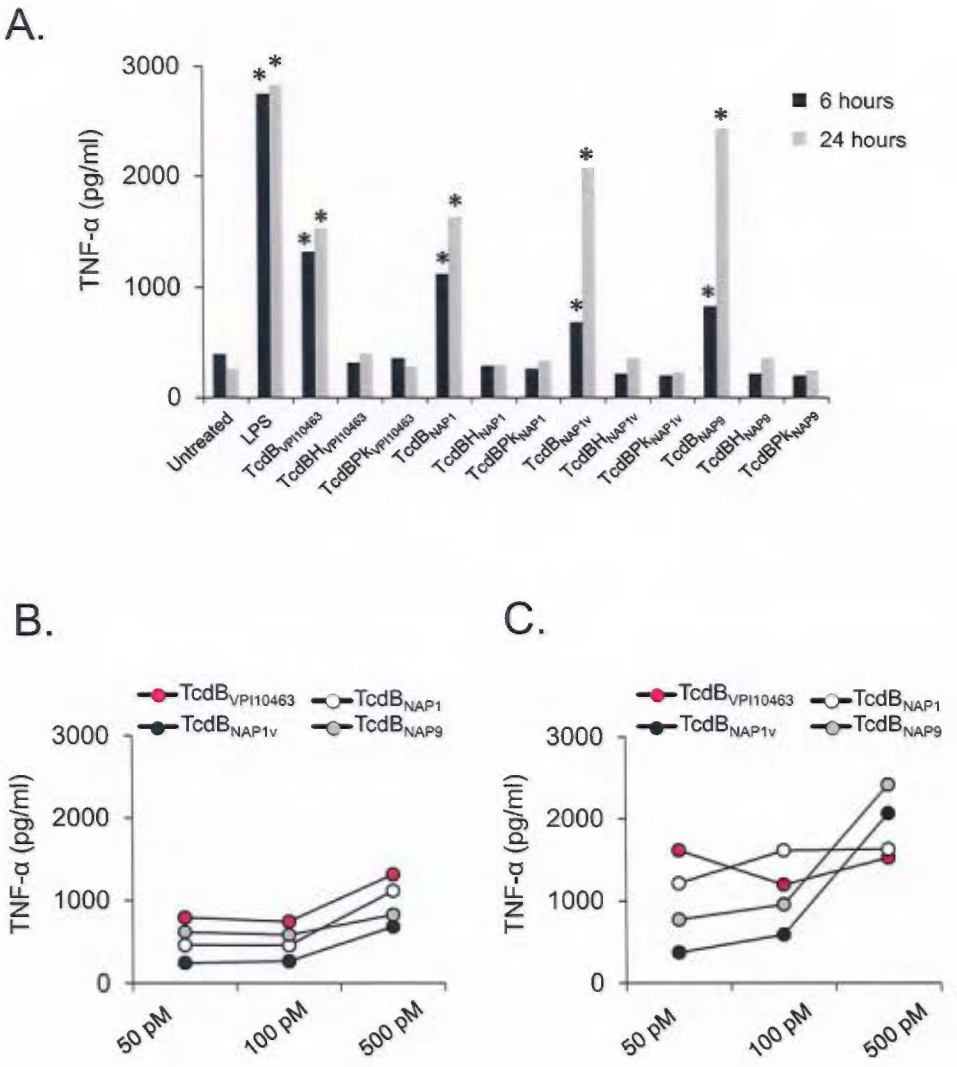
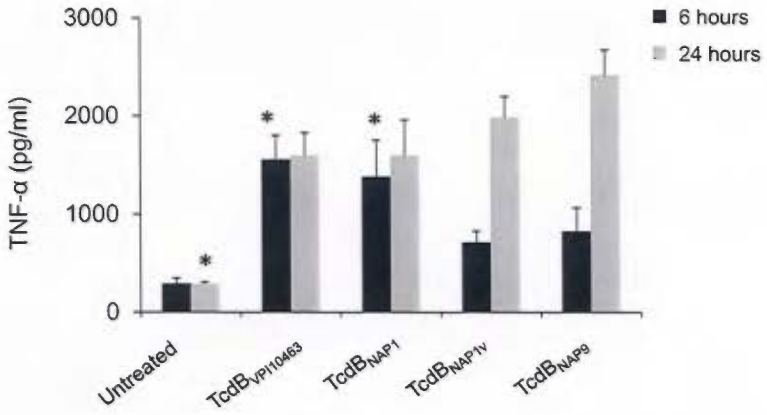


Figure 12. TNF-α production by RAW macrophages. (A) Cells were treated with 0.5 nM of TcdB$_{VPI10463}$, TcdB$_{NAP1}$, TcdB$_{NAP1v}$ and TcdB$_{NAP9}$ and cultured for 6 hours and 24 hours. Cells were also treated with toxins that were either inactivated at 70°C for 10 min (TcdB-H) or treated with proteinase K 1 mg/ml (TcdB-PK). LPS from *E. coli* was used as a positive control. (B) TcdBs were tested for their ability to induce TNF-α production by RAW cells at different concentrations. Cells were treated for 6 hours and (C) 24 hours. *$P < 0.05$ compared to the groups from the same treatment period without asterisk (One-way ANOVA with Bonferroni´s correction).

A.



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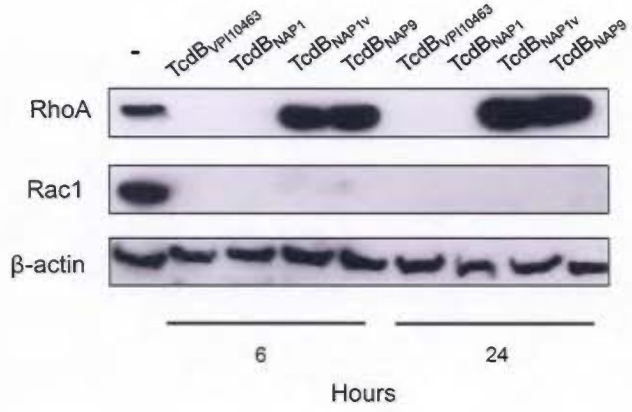

Figure 13. TNF-α release and glucosyltransferase activity of TcdBs in RAW macrophages. TcdBs were tested for their ability to induce TNF-α production by RAW cells and to glucosylate small GTPases. (A) Cells were treated with 0.5 nM of each TcdB. TNF-α production induced by TcdB$_{VPI10463}$, TcdB$_{NAP1}$, TcdB$_{NAP1v}$ and TcdB$_{NAP9}$ was determined after 6 hours of intoxication and 24 hours of intoxication. (B) After intoxication at the indicated times, cells were lysed and proteins were probed with antibodies against unglucosylated RhoA and Rac1. β-actin was included as a loading control. Means ± SEM of three independent experiments are showed. *$P < 0.05$ compared to the groups from the same treatment period without asterisk (One-way ANOVA with Bonferroni´s correction).

6.5 $TcdB_{NAP1}$ induces a stronger immune activation and ileal damage

The role of TcdB on the pathogenic potential was evaluated in the murine ileal loop model. In this case, $TcdB_{NAP9}$ was not evaluated as there were limitations in the amount of toxin concentration needed for this assay. The pro-inflammatory response was measured by MPO activity and IL-6 and IL-1β release after treatment with $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{VPI10463}$. Overall, $TcdB_{NAP1}$ induced a stronger immune activation. $TcdB_{NAP1}$ caused an increase in MPO activity compared to the other toxins and the control group (Fig. 14A), and the levels of IL-6 in ileal loops treated with $TcdB_{NAP1}$ were significantly higher than those of the control (Fig. 14B). The concentration of IL-1β also increased by three fold in $TcdB_{NAP1}$ treated tissues compared to $TcdB_{NAP1v}$ and the control (Fig. 14C). Disruption of ileal tissue was also evaluated, and once again, the epithelial damage was higher when loops were treated with $TcdB_{NAP1}$.

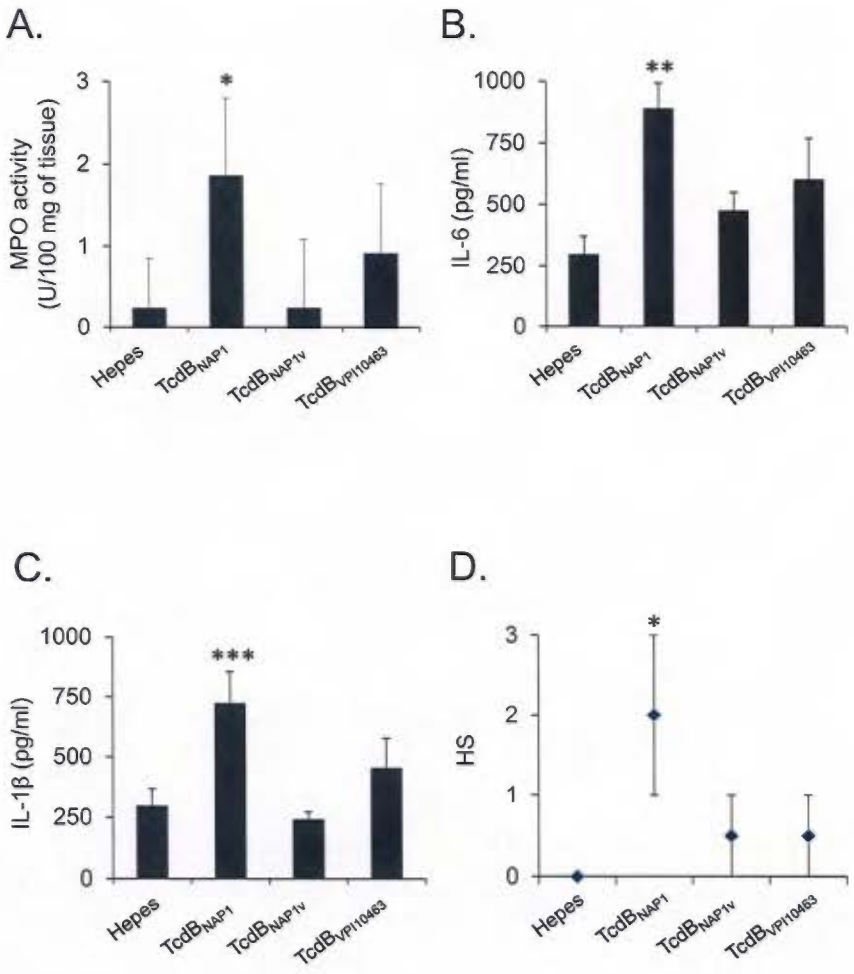

Figure 14. Murine ileal loop model. The pathogenic potential of TcdBs was assayed in the murine ligated ileal loop model. Ileal loops were inoculated with 10 μg of $TcdB_{VPI10463}$, $TcdB_{NAP1}$ and $TcdB_{NAP1v}$. The effect of the toxins on MPO activity (A) and induction of inflammatory cytokines (B and C) was measured. (D) Epithelial damage induced by the toxins was also determined using a histopathological score (HS) scale ranging from 1 (mild) to 3 (severe). Hepes was used as a negative control. Means ± SD, n ≥ 5. $^{*}P < 0.05$, $^{**}P < 0.05$ compared to Hepes, $^{***}P < 0.05$ compared to Hepes and $TcdB_{NAP1v}$ (One-way ANOVA with Bonferroni´s correction, Kruskal-Wallis test and Dunn's multiple-comparison test).

Interestingly, even if cytokine levels were not elevated in tissues treated with $TcdB_{NAP1v}$, this toxin induced blood accumulation in the blood vessels, leading to significant vascular congestion (Fig. 15A). Furthermore, villus fusion resulted in the loss of the epithelium architecture (Fig. 15B). These observations reaffirm the idea that differences in GTPase glucosylation could be related to distinct outcomes of intoxication.

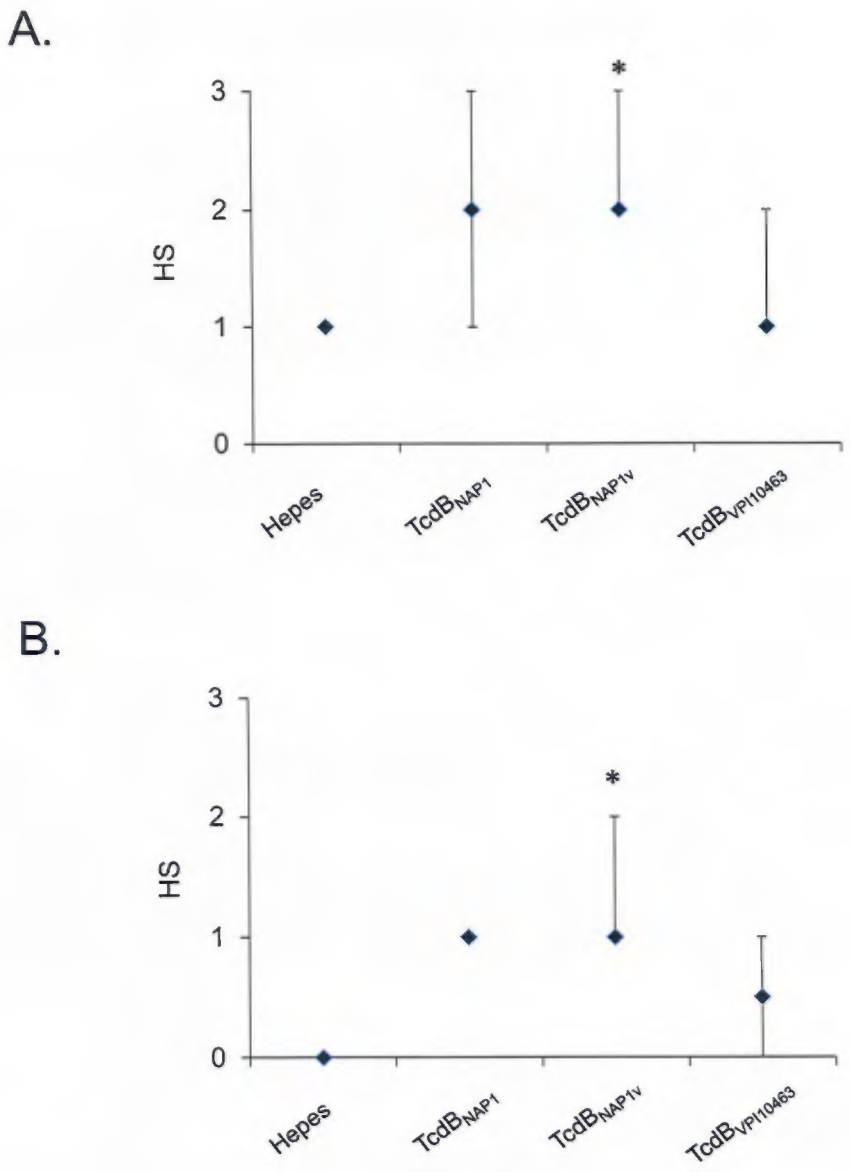

Figure 15. Histopathological scores of congestion and villus fusion in ileal tissue. Histopathological alterations of ileal loops treated with $TcdB_{VPI10463}$, $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and Hepes were determined, using a histopathological score (HS) scale of 1 (mild) to 3 (severe) for (A) congestion and (B) villus fusion. Means ± SD, $n \geq 5$. *$P < 0.05$ (Kruskal-Wallis test and Dunn's multiple-comparison test).

7. DISCUSSION

It has been shown that TcdB is essential for *C. difficile* virulence and its glucosyltransferase activity is required for purified toxin to cause symptoms that resemble CDI (50, 76, 77). In addition, previous reports have concluded that variations in TcdB could be an important factor for the increased virulence of the NAP1 strains (78, 79) and that differences not only in sequence, but also in substrate glucosylation are responsible for the variations of the CPE induced by TcdA-negative strains (69). In line with these studies, we decided to compare the activities of four distinct TcdBs that vary throughout their domains and possess a different panel of modified substrates. Through this panel of purified toxins, we evaluated the role of structural differences on the biological activities induced and assessed the relevance of the modification of different GTPases on the pathogenic potential associated to TcdB from various *C. difficile* strains.

Our sequence analysis and glucosyltransferase assays indicate that toxins that possess a similar GTD induce the same cytopathic effect, but do not share the same panel of modified GTPases. In this work we show that $TcdB_{NAP1}$ is able to glucosylate a broader panel of GTPases that includes the Rho and Ras family of proteins and we demonstrate by *ex vivo* activation assays that this toxin can even modify different Ras isoforms like R-Ras2; on the contrary $TcdB_{VPI10463}$ only modifies RhoA, Rac1 and Cdcd42. In the same context, $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ glucosylate Rac1 and R-Ras like other variant TcdBs (69), but $TcdB_{NAP1v}$ also modifies Rap2A and $TcdB_{NAP9}$ glucosylates R-Ras2.

Based on our results, it is tempting to hypothesize that even though 96% of the GTD is similar between $TcdB_{NAP1}$ and $TcdB_{VPI10463}$, and the surface residues which have been associated to substrate specificity are identical (64), additional divergent GTD residues could be contributing to the differences in the panel of glucosylated GTPases by these two toxins. Other comparative analysis using recombinant GTD have shown that TcdB from a non-epidemic NAP1 strain does not modify Ras

family of GTPases, and even if it glucosylates RhoA, this GTPase was described as a non-preferred substrate (80). Hence, conformational states present in native toxins that favor GTPase affinity, could account not only for the differences in substrates modified by similar GTDs but also for the varying results obtained with recombinant toxins. Studies have reported that recombinant TcdA has failed to show cell type specific effects that are induced by native TcdA (81), suggesting that the use of recombinant versus native toxins must be reconsidered in *C. difficile*.

The higher cytotoxicity of $TcdB_{NAP1}$ in the systemic mouse intoxication model has been attributed to a more efficient intracellular processing (82, 83). This would mean that the GTD of $TcdB_{NAP1}$ would reach the cytosol before other toxins and readily access intramolecular substrates (82). Considering that cell rounding is one of the initial outcomes of GTPase glucosylation, we expected for both $TcdB_{NAP1}$ and $TcdB_{NAP1v}$ would present higher cytotoxic potency compared to $TcdB_{VPI10463}$, as only the first two toxins share the regions determining receptor binding and entrance to the cell. Nevertheless, contrary to reports by Stabler et al. (63), our results show that the kinetics of cell rounding induced by all toxins is similar in the cell lines evaluated. Remarkably, recent unpublished data from our laboratory shows that the cytotoxic potencies in Caco-2 cells are different between $TcdB_{NAP1}$ and $TcdB_{NAP1v}$, as the variant toxin does not induce complete cell rounding after 24 hours of intoxication even at high toxin concentrations. Overall, these results suggest that the autoprocessing rate might not necessarily dictate the cytotoxic potency as had been previously reported (84). Instead, we postulate that a differential panel of GTPases glucosylated could account for the difference detected in our experiments.

In the case of $TcdB_{NAP9}$, the slower kinetics might be due to limitations faced in the toxin purification process, as NAP9 is not a toxin overproducer strain. Although equal amounts of toxin were used to assess this activity, degradation events during its purification might have led to the loss of activity. This could also be the situation

for previous results as Stabler et al. compared the cytotoxic potency of $TcdB_{NAP1}$ to that of a *C. difficile* 630 strain (63) which does not produce high amounts of toxin.

In agreement with the proposed hypothesis, variations in GTPase modification by similar GTD could also lead to different cellular responses. It seems like $TcdB_{NAP1}$ induces Rap2B and/or Rap2C activation after 24 hours of intoxication and this activation could be a result of Rap2A glucosylation. Indeed, Rap2A is found in its GTP bounded state due to its low sensitivity to GAPs (85), thus, modifications of this GTPase could activate compensatory mechanism via Rap2B and Rap2C. Another possibility for the activation of these proteins could be the R-Ras modification, as Ras and Rap proteins are antagonists (37). Furthermore, $TcdB_{NAP1v}$ possibly modifies all Rap2 isoforms as no signal was detected after 6 hours or 24 hours, whereas $TcdB_{NAP9}$ does not seem to glucosylate Rap2A. Additional studies have shown that A-negative strains do glucosylate Rap2A (69), so Rap2A might actually be glucosylated by $TcdB_{NAP9}$ and like in the case of $TcdB_{NAP1}$, this inactivation could be leading to the activation of Rap2B and/or Rap2C even at early times of intoxication. A detailed assessment with monoclonal antibodies against each isoform would allow us to confirm whether these GTPases are activated due to intoxication events or if Rap2A simply remains activated because it is not modified by $TcdB_{NAP9}$.

As expected, the CPE induced correlates with the GTPases that are modified. Previous results indicate that the variant CPE attributed to TcdB, which resembles the effect of *C. sordelli* lethal toxin TcsL (51), is associated to transient RhoA activation and R-Ras glucosylation (69). R-Ras glucosylation leads to integrin inactivation and focal adhesions disassembly causing detachment of the rounded cells (69). Interestingly, our study suggests that R-Ras modification might not be involved in the variant CPE since $TcdB_{NAP1}$ is able to glucosylate R-Ras at similar levels as $TcdB_{NAP1v}$ and after 24 hours all toxins glucosylate Cdc42, whereas only the arborizing inducing toxins glucosylate RhoA. Hence, RhoA modification seems to have an important role in the type of CPE induced, in concordance with reports

that describe that glucosylation of this protein is essential for classical CPE (86) and none of the variant toxins, including TcsL, modify this GTPase (73). Clearly, more studies that include other bacterial toxins that modify RhoA, are necessary in order to elucidate the role of RhoA inactivation on the CPE induced by large clostridial toxins.

Non-synchronized cultured cell lines are highly sensitive to the cytopathic effect induced by *C. difficile* toxins, but less sensitive to the activation of apoptosis (40, 87, 88), as seen in our results. Nevertheless, we show that the percent of cell death induced is higher in cells treated for 24 hours with 100 pM of each variant toxin, suggesting that variations in cytotoxicity events could also be related to the modified substrates, mainly Ras-Rho modifications. It has been reported that RhoA glucosylation leads to RhoB activation which results in activation of apoptotic cell death (87, 89). On the other hand, R-Ras inactivation can result in inhibition of ERK signaling pathways that regulate cell survival and proliferation (37) and Ras glucosylation can lead to non-apoptotic mitochondrial cell death pathways (37, 90). These events could explain why variant toxins seem to cause other mechanisms different from apoptotic events, such as necrosis. Under this perspective, the histopathological alterations induced by $TcdB_{NAP1v}$ in the ileal tissue reflect distinct cellular events, in which cell death pathways could account for the severity of CDI associated to strains that harbor variant TcdBs (76, 91) (Fig. 16). Alternatively, the increase in the percent of anexin V/PI positive cells induced by $TcdB_{NAP1v}$ and $TcdB_{NAP9}$ could also just be a resulting event of the cytopathic effect. Cell detachment could affect membrane permeability and thus allow PI entrance. However, studies in cells treated with TcdA and EDTA show that cell detachment leads to apoptosis (92), which does not seem to be the event induced by our variant toxins, meaning that probably the resulting effect is associated to direct pathways regulated by Ras and Rho. Additional cell death assays will allow us to confirm this observation.

Moreover, in HeLa cells $TcdB_{NAP1}$ did not display a higher cytotoxicity even if it glucosylates numerous GTPases. RhoB could be another substrate readily modified by this toxin as has been reported for other large clostridial toxins (73, 80), and its glucosylation could result at least in the first 24 hours, in inhibition of cell death, indicating that earlier biological effects such as inflammation and not apoptotic death are probably more important in NAP1 induced cytotoxicity.

The overall inflammatory reaction induced by large clostridial toxins indicates that a common mechanism such as the glucosyltransferase activity may be responsible for the outcomes in disease (93). Warny et al. have discussed that it is likely that early glucosylation of Rho proteins can induce MAP kinase activation resulting in the release of IL-8 by monocytes and epithelial cells (43). In this work, we determined that the glucosyltransferase activity is possibly needed for the release of TNF-α by TcdB. Another possibility could be that the holotoxin conformation is needed for the release of TNF-α but not the catalytic activity as has been debated for IL-1β secretion (41). However, our results show that differences in the enzymatic activity also determine variations in immune activation as RhoA glucosylating toxins induced higher amounts of TNF-α than variant toxins at earlier times of intoxication. In fact, RhoB activation due to RhoA inhibition could lead to NF-κB activation and thus TNF-α production (89, 94). Interestingly, variant toxins induced similar levels of TNF-α after 24 hours of treatment, possibly as a consequence of an increase in RhoA expression or activation due to modification of other GTPases. RhoA activation has also been reported to increase TNF-α as NF-κB translocation can be a RhoA dependent process (95).

It is believed that toxins can reach the subepithelial milieu after tight juntion disruption in CDI and thus encounter immune cells (36). Since monocytes are the initial cells recruited (36), activation of these cells by toxins in a glucosyltransferase -dependent manner could imply that the enzymatic activity has a direct effect on neutrophil infiltration, which is one of the main characteristics of pseudomembranous colitis (5) (Fig. 16). To further explore this concept, TNF-α

production and glucosylation induced by different concentrations of toxin could be evaluated at earlier times of intoxication, especially since the production of TNF-α seems to correlate with toxin concentration. In this context, it is likely that strains that produce high amounts of toxin and glucosylate RhoA could induce a higher immune response. In addition, studies with TcdBs that lack the glucosyltransferase activity and with other bacterial toxins that modify RhoA, can provide insight into the importance of RhoA-modification in cytokine production and in *C. difficile* induced inflammation.

Undoubtedly, persistent diarrhea in CDI and fatal outcome of disease correlates with intestinal inflammation (96). When we assessed the pathogenic potential of $TcdB_{NAP1}$, $TcdB_{NAP1v}$ and $TcdB_{VPI10463}$ in the ileal loop model, we observed that $TcdB_{NAP1}$ induced a higher immune activation. It has been shown that TcdB is in fact a potent inflammatory enterotoxin (46) and recent transcriptomic analysis in the mouse model of *C. difficile* revealed that TcdB from NAP1 is the major factor inducing host innate immune and proinflammatory responses (76). Additionally, low levels of cytokines involved in suppression of inflammation have been reported in NAP1-infected mice (97). Hence, in agreement with our results it is reasonable to suggest that the inflammation processes induced by $TcdB_{NAP1}$ could contribute to the high pathogenic potential described for NAP1 strains, and these events could be related to (i) an extended panel of GTPases being glucosylated and the overall inactivation/activation mechanisms induced by this toxin and (ii) the high amounts of toxin production that could result in RhoA glucosylation and thus in high levels TNF-α release by macrophages. Furthermore, this increased inflammatory event could also have an impact on the epithelial damage induced by $TcdB_{NAP1}$ as seen in our model and in other mouse models that describe epithelial disruption and inflammatory cell infiltration in cecal and colonic tissue (97) (Fig. 16). Antibody response to TcdB has been associated with protection from recurrent CDI (98) which highlights along with our results, the role of TcdB in NAP1 induced disease.

In summary, our results suggest that the biological events and even the overall outcome of CDI could be associated to differences in GTPase modification by TcdBs and this study contributes to the understanding of the variations in virulence seen between different *C. difficile* strains. Further experiments that address the role of harboring a variant toxin in *C. difficile* pathogenesis must be considered and the glucosyltransferase activity of these toxins must be evaluated in colonic cell lines in order to determine whether the reduced cytotoxic potency is due to a differential panel of glucosylated substrates. Finally, the analysis of the consequences of an augmented inflammatory process and the inactivation/activation mechanisms of GTPases induced by $TcdB_{NAP1}$ are a priority for our future research. For this purpose, *in vivo* glucoyltransferase assays in ileal and colonic tissues are already being assessed.

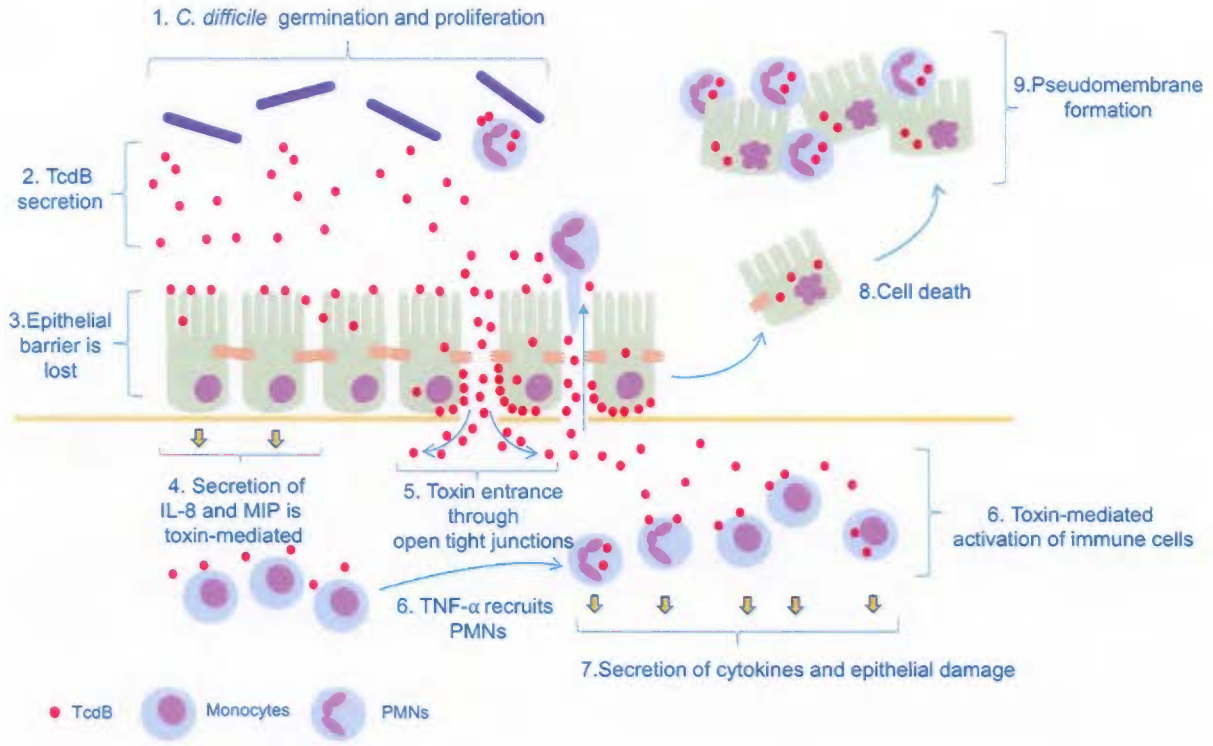

Figure 16. Role of TcdB in the pathogenesis of CDI. Model proposed for the pathogenic events associated to GTPase glucosylation by TcdB, leading to the induction of antibiotic-associated diarrhea and pseudomembranous colitis. Glucosylation of more GTPases and overproduction of $TcdB_{NAP1}$ could lead to an augmented inflammatory process, while $TcdB_{NAP1v}$ could cause a distinct cell death process leading to the loss of the epithelium architecture. MIP: Macrophage inflammatory protein. PMNs: Polymorphonuclear granulocytes.

8. CONCLUSIONS

- $TcdB_{NAP1}$ glucosylates a broader spectrum of small GTPases: Rho and Ras family of proteins.
- Even if these toxins have a high level of identity at the GTD domain, the targeted substrates may vary.
- The type of CPE induced by the different TcdBs is associated to a common GTD and RhoA seems to dictate the type of CPE induced.
- The kinetics of intoxication suggests that the rate of toxin uptake is similar for all toxins even if only $TcdB_{NAP1}$ and $TcdB_{NAP1v}$ share the receptor-binding and autoprocessing domains.
- The percent of cell death induced by variant TcdBs is higher and these toxins seem to trigger cell death pathways regulated by Ras and Rho that might be different from the ones induced by the other toxins.
- TNF-α release by macrophages is dependent of toxin concentration and is related to the panel of glucosylated GTPases. RhoA inactivation seems to correlate with macrophage activation and TNF-α release at earlier times of intoxication.
- $TcdB_{NAP1}$ triggers a stronger pro-inflammatory response, induces high levels of IL-6 and IL-1β at ileal level and induces significant ileal damage.
- $TcdB_{NAP1v}$ does not induce a strong inflammatory response in ileal tissue, but induces vascular congestion and villus fusion.
- Our presented data supports the hypothesis that the panel of substrates targeted by each TcdB seems to correlate with the biological events induced upon intoxication.

9. REFERENCES

1. **Kelly CP, LaMont JT**. 2008. *Clostridium difficile* — More difficult than ever. N Engl J Med **359**:1932–1940.
2. **Bartlett JG**. 2002. Antibiotic-associated diarrhea. N Engl J Med **346**:334–339.
3. **Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeño-Tárraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Dougan G, Barrell B, Parkhill J**. 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat Genet **38**:779–786.
4. **He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris SR, Fairley D, Bamford KB, D'Arc S, Brazier J, Brown D, Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox M, Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill J, Lawley TD**. 2012. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat Genet **45**:109–113.
5. **Voth DE, Ballard JD**. 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev **18**:247–263.
6. **Kurti Z**. 2015. Burden of *Clostridium difficile* infection between 2010 and 2013: trends and outcomes from an academic center in Eastern Europe. World J Gastroenterol **21**:6728.
7. **Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC**. 2015. Burden of *Clostridium difficile* infection in the United States. N Engl J Med **372**:825–834.

8. **Mao EJ, Kelly CR, Machan JT**. 2015. Racial differences in *Clostridium difficile* infection rates are attributable to disparities in health care access. Antimicrob Agents Chemother **59**:6283–6287.
9. **Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M, Eichel-Streiber C**. 1997. Transcription analysis of the genes *tcdA-E* of the pathogenicity locus of *Clostridium difficile*. Eur J Biochem **244**:735–742.
10. **Dineen SS, McBride SM, Sonenshein AL**. 2010. Integration of metabolism and virulence by *Clostridium difficile* CodY. J Bacteriol **192**:5350–5362.
11. **Antunes A, Camiade E, Monot M, Courtois E, Barbut F, Sernova NV, Rodionov DA, Martin-Verstraete I, Dupuy B**. 2012. Global transcriptional control by glucose and carbon regulator CcpA in *Clostridium difficile*. Nucleic Acids Res **40**:10701–10718.
12. **McKee RW, Mangalea MR, Purcell EB, Borchardt EK, Tamayo R**. 2013. The second messenger cyclic di-GMP regulates *Clostridium difficile* toxin production by controlling expression of *sigD*. J Bacteriol **195**:5174–5185.
13. **Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, Fairweather NF, Dougan G, Lawley TD**. 2012. The *Clostridium difficile spo0A* gene is a persistence and transmission factor. Infect Immun **80**:2704–2711.
14. **Jank T, Aktories K**. 2008. Structure and mode of action of clostridial glucosylating toxins: the ABCD model. Trends Microbiol **16**:222–229.
15. **Belyi Y, Aktories K**. 2010. Bacterial toxin and effector glycosyltransferases. Biochim Biophys Acta BBA - Gen Subj **1800**:134–143.
16. **Papatheodorou P, Zamboglou C, Genisyuerek S, Guttenberg G, Aktories K**. 2010. Clostridial glucosylating toxins enter cells via clathrin-mediated endocytosis. PLoS ONE **5**:e10673.
17. **Schorch B, Song S, van Diemen FR, Bock HH, May P, Herz J, Brummelkamp TR, Papatheodorou P, Aktories K**. 2014. LRP1 is a receptor for *Clostridium perfringens* TpeL toxin indicating a two-receptor model of clostridial glycosylating toxins. Proc Natl Acad Sci **111**:6431–6436.

18. **Pruitt RN, Chumbler NM, Rutherford SA, Farrow MA, Friedman DB, Spiller B, Lacy DB**. 2012. Structural determinants of *Clostridium difficile* toxin A glucosyltransferase activity. J Biol Chem **287**:8013–8020.
19. **Yuan P, Zhang H, Cai C, Zhu S, Zhou Y, Yang X, He R, Li C, Guo S, Li S, Huang T, Perez-Cordon G, Feng H, Wei W**. 2015. Chondroitin sulfate proteoglycan 4 functions as the cellular receptor for *Clostridium difficile* toxin B. Cell Res **25**:157–168.
20. **LaFrance ME, Farrow MA, Chandrasekaran R, Sheng J, Rubin DH, Lacy DB**. 2015. Identification of an epithelial cell receptor responsible for *Clostridium difficile* TcdB-induced cytotoxicity. Proc Natl Acad Sci **112**:7073–7078.
21. **Na X, Kim H, Moyer MP, Pothoulakis C, LaMont JT**. 2008. gp96 Is a Human Colonocyte plasma membrane binding protein for *Clostridium difficile* toxin A. Infect Immun **76**:2862–2871.
22. **Gerding DN, Johnson S, Rupnik M, Aktories K**. 2014. *Clostridium difficile* binary toxin CDT: mechanism, epidemiology, and potential clinical importance. Gut Microbes **5**:15–27.
23. **Kuehne SA, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP**. 2014. Importance of toxin A, toxin B, and CDT in virulence of an epidemic *Clostridium difficile* strain. J Infect Dis **209**:83–86.
24. **Schwan C, Stecher B, Tzivelekidis T, van Ham M, Rohde M, Hardt W-D, Wehland J, Aktories K**. 2009. *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. PLoS Pathog **5**:e1000626.
25. **Calabi E, Ward S, Wren B, Paxton T, Panico M, Morris H, Dell A, Dougan G, Fairweather N**. 2001. Molecular characterization of the surface layer proteins from *Clostridium difficile*. Mol Microbiol **40**:1187–1199.
26. **Hennequin C**. 2003. Identification and characterization of a fibronectin-binding protein from *Clostridium difficile*. Microbiology **149**:2779–2787.

27. **Tasteyre A, Barc M-C, Collignon A, Boureau H, Karjalainen T**. 2001. Role of FliC and FliD flagellar proteins of *Clostridium difficile* in adherence and gut colonization. Infect Immun **69**:7937–7940.
28. **Collignon A, Bourlioux P, Waligora-Dupriet A-J, Karjalainen T, Barc M-C, Hennequin C, Porcheray F**. 2001. GroEL (Hsp60) of *Clostridium difficile* is involved in cell adherence. Microbiology **147**:87–96.
29. **Poilane I, Karjalainen T, Barc M-C, Bourlioux P, Collignon A**. 1998. Protease activity of *Clostridium difficile* strains. Can J Microbiol **44**:157–161.
30. **Janoir C**. 2016. Virulence factors of *Clostridium difficile* and their role during infection. Anaerobe **37**:13–24.
31. **Vetter IR**. 2001. The guanine nucleotide-binding switch in three dimensions. Science **294**:1299–1304.
32. **Wennerberg K**. 2005. The Ras superfamily at a glance. J Cell Sci **118**:843–846.
33. **Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A**. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70**:401–410.
34. **Kozma R, Ahmed S, Best A, Lim L**. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol **15**:1942–1952.
35. **Jaffe AB, Hall A**. 2005. Rho GTPases: Biochemistry and Biology. Annu Rev Cell Dev Biol **21**:247–269.
36. **Aktories K, Barbieri JT**. 2005. Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol **3**:397–410.
37. **Ehrhardt A, Ehrhardt G, Guo X, Schrader J**. 2002. Ras and relatives—job sharing and networking keep an old family together. Exp Hematol **30**:1089–1106.
38. **Rosario M, Paterson HF, Marshall CJ**. 2001. Activation of the ral and phosphatidylinositol 3' kinase signaling pathways by the ras-related protein TC21. Mol Cell Biol **21**:3750–3762.

39. **Ottlinger ME, Lin S**. 1988. *Clostridium difficile* toxin B induces reorganization of actin, vinculin, and talin in cultured cells. Exp Cell Res **174**:215–229.
40. **Brito GAC, Fujji J, Carneiro-Filho BA, Lima AAM, Obrig T, Guerrant RL**. 2002. Mechanism of *Clostridium difficile* toxin a–induced apoptosis in T84 cells. J Infect Dis **186**:1438–1447.
41. **Ng J, Hirota SA, Gross O, Li Y, Ulke–Lemee A, Potentier MS, Schenck LP, Vilaysane A, Seamone ME, Feng H, Armstrong GD, Tschopp J, MacDonald JA, Muruve DA, Beck PL**. 2010. Clostridium difficile toxin–induced inflammation and intestinal injury are mediated by the inflammasome. Gastroenterology **139**:542–552.e3.
42. **Sun X, He X, Tzipori S, Gerhard R, Feng H**. 2009. Essential role of the glucosyltransferase activity in *Clostridium difficile* toxin-induced secretion of TNF-α by macrophages. Microb Pathog **46**:298–305.
43. **Warny M, Keates AC, Keates S, Castagliuolo I, Zacks JK, Aboudola S, Qamar A, Pothoulakis C, LaMont JT, Kelly CP**. 2000. p38 MAP kinase activation by *Clostridium difficile* toxin A mediates monocyte necrosis, IL-8 production, and enteritis. J Clin Invest **105**:1147–1156.
44. **Nusrat A, von Eichel-Streiber C, Turner JR, Verkade P, Madara JL, Parkos CA**. 2001. *Clostridium difficile* toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. Infect Immun **69**:1329–1336.
45. **Chen ML, Pothoulakis C, LaMont JT**. 2002. Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of t84 colonocytes exposed to *Clostridium difficile* toxin A. J Biol Chem **277**:4247–4254.
46. **Savidge TC, Pan W, Newman P, O'Brien M, Anton PM, Pothoulakis C**. 2003. *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. Gastroenterology **125**:413–420.
47. **Popoff MR, Bouvet P**. 2009. Clostridial toxins. Future Microbiol **4**:1021–1064.
48. **Chaves-Olarte E, Weidmann M, Eichel-Streiber C, Thelestam M**. 1997. Toxins A and B from *Clostridium difficile* differ with respect to enzymatic

potencies, cellular substrate specificities, and surface binding to cultured cells. J Clin Invest **100**:1734–1741.

49. **Kim PH, Iaconis JP, Rolfe RD**. 1987. Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. Infect Immun **55**:2984–2992.
50. **Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S, Gerding DN, Rood JI**. 2009. Toxin B is essential for virulence of *Clostridium difficile*. Nature **458**:1176–1179.
51. **Chaves-Olarte E, Low P, Freer E, Norlin T, Weidmann M, von Eichel-Streiber C, Thelestam M**. 1999. A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. J Biol Chem **274**:11046–11052.
52. **Elliott B, Squire MM, Thean S, Chang BJ, Brazier JS, Rupnik M, Riley TV**. 2011. New types of toxin A-negative, toxin B-positive strains among clinical isolates of *Clostridium difficile* in Australia. J Med Microbiol **60**:1108–1111.
53. **Du P, Cao B, Wang J, Li W, Jia H, Zhang W, Lu J, Li Z, Yu H, Chen C, Cheng Y**. 2014. Sequence variation in *tcdA* and *tcdB* of *Clostridium difficile*: ST37 with truncated *tcdA* is a potential epidemic strain in China. J Clin Microbiol **52**:3264–3270.
54. **Quesada-Gomez C, Rodriguez C, Gamboa-Coronado M d. M, Rodriguez-Cavallini E, Du T, Mulvey MR, Villalobos-Zuniga M, Boza-Cordero R**. 2010. Emergence of *Clostridium difficile* NAP1 in Latin America. J Clin Microbiol **48**:669–670.
55. **Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault A-M, Nguyen T, Frenette C, Kelly M, Vibien A, Brassard P, Fenn S, Dewar K, Hudson TJ, Horn R, René P, Monczak Y, Dascal A**. 2005. A predominantly clonal multi-institutional outbreak of *Clostridium difficile* –associated diarrhea with high morbidity and mortality. N Engl J Med **353**:2442–2449.

56. **Goorhuis A, Van der Kooi T, Vaessen N, Dekker FW, Van den Berg R, Harmanus C, van den Hof S, Notermans DW, Kuijper EJ**. 2007. Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in the Netherlands. Clin Infect Dis **45**:695–703.
57. **Smith A**. 2005. Outbreak of *Clostridium difficile* infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull **10**:E050630.2.
58. **Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC**. 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. The Lancet **366**:1079–1084.
59. **Akerlund T, Persson I, Unemo M, Noren T, Svenungsson B, Wullt M, Burman LG**. 2008. Increased sporulation rate of epidemic *Clostridium difficile* ype 027/NAP1. J Clin Microbiol **46**:1530–1533.
60. **Drudy D, Quinn T, O'Mahony R, Kyne L, O'Gaora P, Fanning S**. 2006. High-level resistance to moxifloxacin and gatifloxacin associated with a novel mutation in gyrB in toxin-A-negative, toxin-B-positive *Clostridium difficile*. J Antimicrob Chemother **58**:1264–1267.
61. **Matamouros S, England P, Dupuy B**. 2007. *Clostridium difficile* toxin expression is inhibited by the novel regulator TcdC: TcdC inhibits *C. difficile* toxin expression. Mol Microbiol **64**:1274–1288.
62. **Karas JA, Enoch DA, Aliyu SH**. 2010. A review of mortality due to *Clostridium difficile* infection. J Infect **61**:1–8.
63. **Stabler RA, He M, Dawson L, Martin M, Valiente E, Corton C, Lawley TD, Sebaihia M, Quail MA, Rose G, Gerding DN, Gibert M, Popoff MR, Parkhill J, Dougan G, Wren BW**. 2009. Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. Genome Biol **10**:R102.
64. **Quesada-Gómez C, López-Ureña D, Chumbler N, Kroh HK, Castro-Peña C, Rodríguez C, Orozco-Aguilar J, González-Camacho S, Rucavado A, Guzmán-Verri C, Lawley TD, Lacy DB, Chaves-Olarte E**. 2016. Analysis of

TcdB proteins within the hypervirulent clade 2 reveals an impact of RhoA glucosylation on *Clostridium difficile* proinflammatory activities. Infect Immun **84**:856–865.

65. **Quesada-Gómez C, López-Ureña D, Acuña-Amador L, Villalobos-Zúñiga M, Du T, Freire R, Guzmán-Verri C, Gamboa-Coronado M del M, Lawley TD, Moreno E, Mulvey MR, Brito GA de C, Rodríguez-Cavallini E, Rodríguez C, Chaves-Olarte E**. 2015. Emergence of an outbreak-associated *Clostridium difficile* variant with increased virulence. J Clin Microbiol **53**:1216–1226.
66. **Katoh K, Standley DM**. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol **30**:772–780.
67. **Edgar RC**. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32**:1792–1797.
68. **Pruitt RN, Chambers MG, Ng KK-S, Ohi MD, Lacy DB**. 2010. Structural organization of the functional domains of *Clostridium difficile* toxins A and B. Proc Natl Acad Sci **107**:13467–13472.
69. **Chaves-Olarte E**. 2003. R-Ras glucosylation and transient rhoa activation determine the cytopathic effect produced by toxin B variants from toxin A-negative strains of *Clostridium difficile*. J Biol Chem **278**:7956–7963.
70. **Goy SD, Olling A, Neumann D, Pich A, Gerhard R**. 2015. Human neutrophils are activated by a peptide fragment of *Clostridium difficile* toxin B presumably via formyl peptide receptor: TcdB activates neutrophils. Cell Microbiol **17**:893–909.
71. **De Araujo Junqueira AFT, Dias AAM, Vale ML, Spilborghs GMGT, Bossa AS, Lima BB, Carvalho AF, Guerrant RL, Ribeiro RA, Brito GA**. 2011. Adenosine deaminase inhibition prevents *Clostridium difficile* toxin A-induced enteritis in mice. Infect Immun **79**:653–662.
72. **Barreto ARF, Cavalcante IC, Castro MV, Junqueira AFTA, Vale MR, Ribeiro RA, Souza MHLP, Brito GAC**. 2008. Fucoidin prevents clostridium difficile toxin-A-induced ileal enteritis in mice. Dig Dis Sci **53**:990–996.

73. **Busch C, Aktories K**. 2000. Microbial toxins and the glycosylation of Rho family GTPases. Curr Opin Struct Biol **10**:528–535.

74. **D'Auria KM, Bloom MJ, Reyes Y, Gray MC, van Opstal EJ, Papin JA, Hewlett EL**. 2015. High temporal resolution of glucosyltransferase dependent and independent effects of *Clostridium difficile* toxins across multiple cell types. BMC Microbiol **15**:7.

75. **Chumbler NM, Farrow MA, Lapierre LA, Franklin JL, Haslam D, Goldenring JR, Lacy DB**. 2012. *Clostridium difficile* toxin B causes epithelial cell necrosis through an autoprocessing-independent mechanism. PLoS Pathog **8**:e1003072.

76. **Carter GP, Chakravorty A, Pham Nguyen TA, Mileto S, Schreiber F, Li L, Howarth P, Clare S, Cunningham B, Sambol SP, Cheknis A, Figueroa I, Johnson S, Gerding D, Rood JI, Dougan G, Lawley TD, Lyras D**. 2015. Defining the roles of TcdA and TcdB in localized gastrointestinal disease, systemic organ damage, and the host response during *Clostridium difficile* Infections. mBio **6**:e00551–15.

77. **Yang G, Zhou B, Wang J, Sun X, He X, Nie W, Tzipori S, Feng H**. 2008. Expression of recombinant *Clostridium difficile* toxin A and B in *Bacillus megaterium*. BMC Microbiol **8**:192.

78. **Stabler RA, Dawson LF, Phua LTH, Wren BW**. 2008. Comparative analysis of BI/NAP1/027 hypervirulent strains reveals novel toxin B-encoding gene (tcdB) sequences. J Med Microbiol **57**:771–775.

79. **Lanis JM, Barua S, Ballard JD**. 2010. Variations in TcdB activity and the hypervirulence of emerging strains of *Clostridium difficile*. PLoS Pathog **6**:e1001061.

80. **Genth H, Pauillac S, Schelle I, Bouvet P, Bouchier C, Varela-Chavez C, Just I, Popoff MR**. 2014. Haemorrhagic toxin and lethal toxin from *C lostridium sordellii* strain vVPI9048: molecular characterization and comparative analysis of substrate specificity of the large clostridial glucosylating toxins: Large clostridial glucosylating toxins substrate specifcity. Cell Microbiol **16**:1706–1721.

81. **Gerhard R, Burger S, Tatge H, Genth H, Just I, Hofmann F**. 2005. Comparison of wild type with recombinant *Clostridium difficile* toxin A. Microb Pathog **38**:77–83.

82. **Lanis JM, Hightower LD, Shen A, Ballard JD**. 2012. TcdB from hypervirulent *Clostridium difficile* exhibits increased efficiency of autoprocessing: Autoproteolysis of $TcdB_{HIST}$ and $TcdB_{HV}$. Mol Microbiol **84**:66–76.

83. **Lanis JM, Heinlen LD, James JA, Ballard JD**. 2013. *Clostridium difficile* 027/BI/NAP1 encodes a hypertoxic and antigenically variable form of TcdB. PLoS Pathog **9**:e1003523.

84. **Li S, Shi L, Yang Z, Feng H**. 2013. Cytotoxicity of *Clostridium difficile* toxin B does not require cysteine protease-mediated autocleavage and release of the glucosyltransferase domain into the host cell cytosol. Pathog Dis **67**:11–18.

85. **Ohba Y, Mochizuki N, Matsuo K, Yamashita S, Nakaya M, Hashimoto Y, Hamaguchi M, Kurata T, Nagashima K, Matsuda M**. 2000. Rap2 as a slowly responding molecular switch in the Rap1 signaling cascade. Mol Cell Biol **20**:6074–6083.

86. **Just I, Selzer J, Wilm M, Eichel-Streiber C von, Mann M, Aktories K**. 1995. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature **375**:500–503.

87. **Huelsenbeck J, Dreger S, Gerhard R, Barth H, Just I, Genth H**. 2007. Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI 10463 and toxin B from variant *Clostridium difficile* strain 1470. Infect Immun **75**:801–809.

88. **Genth H, Huelsenbeck J, Hartmann B, Hofmann F, Just I, Gerhard R**. 2006. Cellular stability of Rho-GTPases glucosylated by *Clostridium difficile* toxin B. FEBS Lett **580**:3565–3569.

89. **Huelsenbeck J, Dreger SC, Gerhard R, Fritz G, Just I, Genth H**. 2007. Upregulation of the immediate early gene product RhoB by exoenzyme C3 from *Clostridium limosum* and toxin B from *Clostridium difficile*. Biochemistry (Mosc) **46**:4923–4931.

90. **Feig LA, Buchsbaum RJ**. 2002. Cell signaling: life or death decisions of Ras proteins. Curr Biol **12**:R259–R261.

91. **Drudy D, Fanning S, Kyne L**. 2007. Toxin A-negative, toxin B-positive *Clostridium difficile*. Int J Infect Dis **11**:5–10.

92. **Mahida YR, Makh S, Hyde S, Gray T, Borriello SP**. 1996. Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: induction of interleukin 8 production and apoptosis after cell detachment. Gut **38**:337–347.

93. **Aldape MJ, Bryant AE, Stevens DL**. 2006. Clostridium sordellii infection: epidemiology, clinical findings, and current perspectives on diagnosis and treatment. Clin Infect Dis **43**:1436–1446.

94. **Prendergast GC**. 2001. Actin' up: RhoB in cancer and apoptosis. Nat Rev Cancer **1**:162–168.

95. **Hippenstiel S, Schmeck B, Seybold J, Krüll M, Eichel-Streiber C v, Suttorp N**. 2002. Reduction of tumor necrosis factor-alpha (TNF-α) related nuclear factor-kappaB (NF-κB) translocation but not inhibitor kappa-B (Iκ-B)-degradation by Rho protein inhibition in human endothelial cells. Biochem Pharmacol **64**:971–977.

96. **El Feghaly RE, Stauber JL, Deych E, Gonzalez C, Tarr PI, Haslam DB**. 2013. Markers of intestinal inflammation, not bacterial burden, correlate with clinical outcomes in *Clostridium difficile* infection. Clin Infect Dis **56**:1713–1721.

97. **Pawlowski SW, Calabrese G, Kolling GL, Freire R, AlcantaraWarren C, Liu B, Sartor RB, Guerrant RL**. 2010. Murine Model of *Clostridium difficile* Infection with Aged Gnotobiotic C57BL/6 Mice and a BI/NAP1 Strain. J Infect Dis **202**:1708–1712.

98. **Leav BA, Blair B, Leney M, Knauber M, Reilly C, Lowy I, Gerding DN, Kelly CP, Katchar K, Baxter R, Ambrosino D, Molrine D**. 2010. Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI). Vaccine **28**:965–969.

10. APPENDIX

UNIVERSIDAD DE COSTA RICA
VICERRECTORIA DE INVESTIGACION
COMITÉ INSTITUCIONAL DEL CUIDO Y USO DE ANIMALES

Lunes 25 de agosto de 2014
CICUA-38-14

Master
Carlos Quesada Gómez
Centro de Investigación en Enfermedades Tropicales

Estimado M.Sc. Quesada:

En la sesión número 140 del Comité Institucional del Cuido y Uso de Animales (CICUA), del jueves 14 de agosto del 2014, se evaluaron las propuestas en hamster y ratones del proyecto de investigación "Determinación del potencial patogénico y la virulencia de cepas epidémicas NAP1 y endémicas "NAPCR1"de *Clostridium difficile*, aisladas a partir de heces de pacientes con diarrea de hospitales costarricenses".

El Comité aprueba las propuestas y se determina otorgar un mismo código de aprobación para ambas.

Atentamente,

Dra. Sara González Camacho
Coordinadora
CICUA

Cc: Dr. Domingo Campos, Dirección de Gestión de la Investigación.
Archivo