Glucosylation Drives the Innate Inflammatory Response to *Clostridium difficile* Toxin A

Carrie A. Cowardin,^a Brianna M. Jackman,^b Zannatun Noor,^a Stacey L. Burgess,^a Andrew L. Feig,^b William A. Petri, Jr.^{a,c}

Department of Microbiology, Immunology and Cancer Biology, University of Virginia, Charlottesville, Virginia, USA^a; Department of Chemistry, Wayne State University, Detroit, Michigan, USA^b; Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, Virginia, USA^c

Clostridium difficile is a major, life-threatening hospital-acquired pathogen that causes mild to severe colitis in infected individuals. The tissue destruction and inflammation which characterize *C. difficile* infection (CDI) are primarily due to the Rho-glucosylating toxins A and B. These toxins cause epithelial cell death and induce robust inflammatory signaling by activating the transcription factor NF- κ B, leading to chemokine and cytokine secretion. The toxins also activate the inflammasome complex, which leads to secretion of the pyrogenic cytokine IL-1 β . In this study, we utilized glucosylation-deficient toxin A to show that activation of the inflammasome by this toxin is dependent on Rho glucosylation, confirming similar findings reported for toxin B. We also demonstrated that tissue destruction and *in vivo* inflammatory cytokine production are critically dependent on the enzymatic activity of toxin A, suggesting that inhibiting toxin glucosyltransferase activity may be effective in combating this refractory disease.

Clostridium difficile is now the number one hospital-acquired infectious agent in the United States, causing an estimated 453,000 infections and 29,000 deaths annually (1). Infection with this pathogen typically occurs following disruption of the gut microbiome due to antibiotic use, resulting in mild to severe diarrhea, toxic megacolon, sepsis, and death (2). Disease manifestations are primarily mediated by the Rho-glucosylating toxins A and B (TcdA and TcdB, respectively), and one or both of these toxins are required for symptomatic infection (3–6). Certain strains of *C. difficile* also encode a third toxin, known as *C. difficile* transferase (CDT) or binary toxin (7). Although the contribution of CDT to pathogenesis is still under investigation, both TcdA and TcdB are known to cause cell death and induce inflammation. TcdA and TcdB are structurally similar, but remarkable differences in their ability to intoxicate and kill cells have been noted. In a murine cecal injection model of intoxication, TcdA has been shown to induce a dramatic inflammatory response leading to fluid accumulation and cytokine production (8). In contrast, TcdB is less effective at inducing inflammation in this model, prompting the hypothesis that disruption of tissue by TcdA may be a prerequisite for TcdB activity. TcdA and TcdB activities also differ *in vitro*, where TcdB is more effective at eliciting cell death at low concentrations (9).

Although these toxins are thought to share similar mechanisms of intoxication, the role of each step of this process in eliciting cell death is not well defined. To intoxicate cells, the C-terminal CROP domain binds to host cell receptors which are then internalized via receptor-mediated endocytosis. Acidification of the endosomal compartment leads to insertion of the translocation domain into the endosomal membrane. The glucosyltransferase and cysteine protease domains are then translocated out of the endosome through a pore formed by the translocation domain. Finally, the cysteine protease domain is responsible for autoprocessing of the glucosyltransferase domain, which is released into the cytoplasm to modify Rho GTPases via the irreversible addition of a glucosyl moiety. This modification prevents the GTPase function of RhoA, Rac1, and Cdc42 (10).

Recent work has sought to define the role of each toxin domain

in eliciting cell death. Reports indicate that autoprocessing of the cysteine protease domain was dispensable for the induction of cell death. Recombinantly expressed toxin containing inactivating mutations in the cysteine protease domain were capable of killing cultured epithelial cells (11). The same study also found that glucosyltransferase-deficient TcdB (TcdB D270N) was able to cause caspase 3/7 activation and lactate dehydrogenase (LDH) release from epithelial cells, suggesting that glucosyltransferase activity is also not required for cell death to occur. Furthermore, evidence also suggested that TcdB was capable of inducing two distinct cellular phenotypes via separate mechanisms. Low concentrations of TcdB lead to cell rounding due to Rho glucosylation, while high concentrations lead to pyknotic cell death characterized by shrinkage of cells and chromatin condensation (12). Thus, cell rounding and cell death may be glucosyltransferase dependent and independent, respectively (13). However, this distinction is less well understood for TcdA. Although TcdA also causes cell death, this process seems to be primarily dependent on glucosyltransferase activity, as mutation of the glucosyltransferase domain resulted in diminished cellular apoptosis (14, 15). Therefore, it appears that TcdA and TcdB may activate distinct cell death pathways, with cell death due to TcdA requiring glucosylation, while TcdB elicits glucosylation-independent cell death at high concentrations.

Interestingly, differences in the ability of TcdA and TcdB to cause an inflammatory response have also been documented. While both toxins are able to activate the inflammasome to secrete

Received 14 April 2016 Accepted 25 May 2016

Accepted manuscript posted online 6 June 2016

Citation Cowardin CA, Jackman BM, Noor Z, Burgess SL, Feig AL, Petri WA, Jr. 2016. Glucosylation drives the innate inflammatory response to *Clostridium difficile* toxin A. Infect Immun 84:2317–2323. doi:10.1128/IAI.00327-16.

Editor: V. B. Young, University of Michigan

Address correspondence to William A. Petri, Jr., wap3g@virginia.edu.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

interleukin 1 β (IL-1 β), TcdB is able to induce inflammasome activation at much lower concentrations than TcdA (16), mirroring the cell death phenotype. Conflicting results have been reported regarding the ability of TcdB to induce inflammasome activation. One group reported that glucosyltransferase-deficient TcdB (W102A/D288N) was able to induce IL-1 β secretion in the human monocytic cell line THP-1. On the other hand, a second group employed the same mutations and reported that glucosyltransferase-deficient TcdB was unable to induce IL-1 β secretion in murine primary bone marrow-derived macrophages (17), suggesting that there may be differences in the response to the toxins between murine and human cells. The role of glucosylation in inflammasome activation by TcdA is less well understood. Although it has been reported that TcdA glucosyltransferase activity is necessary for tumor necrosis factor alpha (TNF- α) production (18), inflammasome activation is a distinct process which requires two separate signaling events to occur. The first step, referred to as priming, involves recognition of a danger signal which activates the transcription factor NF- κ B and induces pro-IL-1 β gene expression (19). The second activation step leads to assembly of the inflammasome and activation of caspase 1. Caspase 1 is responsible for the processing of pro-IL-1 β into its mature, secreted form. Although multiple inflammasomes which recognize diverse stimuli have been identified, evidence suggests that the Pyrin inflammasome is primarily responsible for IL-1 β secretion in response to TcdA and TcdB (17). Indeed, this inflammasome is thought to be activated in response to Rho GTPase modification, lending support to the idea that glucosylation is required for inflammasome activation, particularly with regard to TcdB.

In order to clarify the role of Rho glucosylation by TcdA in inflammasome activation, as well as to further investigate glucosylation *in vivo*, we utilized purified wild-type TcdA (WT-TcdA) as well as an enzymatically inactive mutant of TcdA (NVN-TcdA). Originally developed by Teichert et al., NVN-TcdA possesses two mutations (D285/287N) which together reduce glucosylation of Rho family proteins 6,900-fold compared to the wild-type toxin (20). These mutations have been used extensively in previous studies to investigate glucosylation-dependent phenotypes, and the mutant toxin is capable of entering cells to an extent similar to that of wild-type TcdA (18). We used this mutant to show for the first time that inflammasome activation by TcdA is enhanced by Rho glucosylation and that glucosylation is absolutely required for tissue damage and an inflammatory response *in vivo*.

MATERIALS AND METHODS

rTcdA plasmid constructs. Initial amplification of the *tcdA* gene from *C. difficile* genomic DNA (strain ATCC 9689) was performed using forward and reverse primers, 5'-ATGCTCTTAATATCTAAAGAAG-3' and 5'-TTAGCCATATATCCCAGG-3', and the gene was cloned into the pCR-XL-TOPO vector (Invitrogen). A SalI restriction site was introduced to split the gene (8,130 bp). The 5' fragment was cloned into pUC19 using forward primer 5'-GACTGAGAATTCGCCCTTATGTC-3' and reverse primer 5'-GACTAGACTTCCTTCTGTGACCG-3', followed by site-directed mutagenesis (SDM) to correct any mutations (pANK-10405). The 3' end was reamplified from genomic DNA with respective forward and reverse primers, 5'-GTCGACAGAAGGAAGTG-3' and 5'-GGTACCATATATCCCAGG-3'. Both plasmids were then digested (Cfr 10I and SalI) and ligated together to give the holotoxin construct pANK-10408, which was then subcloned into the pWH1520 shuttle vector (*cat* gene removed; MoBiTec) to create the final wild-type recombinant TcdA (rTcdA) expression construct pANK-80406. Further, pANK-10408 underwent SDM

(D285N and D287N) using the QuikChange XL-SDM kit (Stratagene) with respective forward and reverse primers, 5'-GGCGGAGTATATTAAATGTGAATATGCTTCCAGGTATTCACCTCTG-3' and 5'-CAGAGTGAATACCTGGAAGCATATTAACATTTAAATATACTCCGCC-3', and was also placed in pWH1520 for expression of NVN mutant rTcdA (pANK-80407).

***Bacillus megaterium* protoplast generation.** One milliliter of *B. megaterium* cells (grown overnight in 5 ml of Difco antibiotic medium 3 [AB3] at 37°C and 250 rpm) was used to inoculate 50 ml of fresh AB3, which was incubated (37°C and 250 rpm) until an A_{550} of 1.0 was reached, harvested, resuspended in 5 ml of SMMP (1:1 AB3-SMM [40 mM maleic acid, 80 mM NaOH, 40 mM MgCl₂, 1 M sucrose]), and transferred to a 100-ml flask. A 2-mg/ml concentration of lysozyme was added, and cells were incubated (37°C and 100 rpm for 45 to 60 min), harvested (room temperature [RT] and 2,250 rpm for 10 min), washed with 5 ml of SMMP, and harvested again as before. Finally, cells were suspended in 5 ml of SMMP (containing 10% [wt/vol] glycerol), aliquoted, and stored at -80°C (21).

***B. megaterium* plasmid transformation, cell growth, and rTcdA expression.** Two hundred microliters of *B. megaterium* protoplasts was thawed on ice, where 0.5 to 1 μ g of *E. coli*-miniprep plasmid DNA and 600 μ l of PEG-P (40% [wt/vol] polyethylene glycol 6000 [PEG 6000], 500 mM sucrose, 20 mM sodium maleinate, 20 mM MgCl₂ [pH 6.5]) were added. The solution was inverted gently to mix and then incubated on ice and then at RT (2 min each), followed by addition of 2 ml of SMMP, gentle inversion, and harvesting (ambient temperature and 3,000 rpm for 10 min). The supernatant was removed, and 500 μ l of fresh SMMP was added before outgrowing (37°C and 100 rpm for 90 min). Finally, transformed culture was plated (LB agar with 10 μ g/ml of tetracycline [Tet]), incubated (37°C for 16 h), and restreaked on Tet plates to ensure selection. Cells containing the rTcdA plasmid were grown overnight (in 10 ml of LB and 10 μ g/ml of Tet at 37°C and 250 rpm for 16 to 19 h), and the entire overnight culture was transferred to a 1-liter flask. Cells were then grown to an optical density at 600 nm (OD₆₀₀) of 0.4, induced by addition of 0.5% D-xylose, incubated for 6 to 12 h, harvested (8,500 $\times$ g and 4°C for 10 min), and stored at -80°C until use.

Purification of WT and NVN mutant rTcdA. Thawed cell pellets containing lysis buffer (50 mM Na₂HPO₄, 300 mM NaCl, 10 mM imidazole [pH 8]) and an EDTA-free cocktail of protease inhibitors (Roche) were lysed by sonication and centrifuged to clarify (15,000 rpm and 4°C for 45 min). The supernatant was filtered through 0.8- μ m and 0.22- μ m syringe filters. The crude lysate was first purified by a nickel-chelated HiTrap column (GE Healthcare) and eluted in 250 mM imidazole. Fractions were then subjected to size exclusion chromatography (HiLoad 16/600 Superdex 200 pg) and a second application to the HiTrap column for concentration purposes. Purified toxins were dialyzed into storage buffer (50 mM HEPES-K, 100 mM KCl, 1 mM MgCl₂ [pH 7.5]) and stored at 4°C.

Mice and cells. Eight- to 12-week-old male C57BL/6 mice were purchased from the Jackson Laboratory. All animals were housed under specific-pathogen-free conditions at the University of Virginia's animal facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. Bone marrow-derived dendritic cells (BMDCs) were generated as previously described (22). THP-1 Blue (catalog no. thp-nfkb) and Raw Blue (catalog no. raw-sp) cells were obtained from InvivoGen and grown according to the vendor's instructions.

Cell stimulation. BMDCs were harvested, and 2×10^5 cells per well were seeded into 96-well tissue culture plates. Cells were stimulated and incubated for 24 h at 37°C with 5% CO₂. Cells were spun down at 300 $\times$ g for 5 min, and the supernatant was harvested and frozen down at -80°C. Raw Blue and THP-1 Blue cells were harvested and resuspended in complete media, and 1×10^5 cells per well were seeded into 96-well tissue culture plates. Cells were stimulated and incubated for 20 h at 37°C with 5% CO₂. Cells were spun down at 300 $\times$ g for 5 min and the supernatant was harvested, except for positive controls for cytotoxicity, which were

lysed with Triton X-100 according to the manufacturer's instructions prior to supernatant aspiration.

Cecal injection and histology. Mice were anesthetized with ketamine-xylazine before surgery, and midline laparotomy was performed. The cecum was located and injected with 15 μ g of purified WT-TcdA, NVN-TcdA, or control buffer (mock treatment) in 100 μ l of 0.9% normal saline as previously described (23). Incisions were sutured and mice were monitored during recovery. Animals were monitored throughout the course of the 8-h period and humanely euthanized if moribund. To generate histological sections, mice were sacrificed at 8 h, the ceca were removed, and a sample was placed in Bouin's solution (Sigma) for 24 h. Tissue samples were moved to 70% ethanol before paraffin embedding and sectioning. Sections were mounted on slides and stained with hematoxylin and eosin prior to microscopic examination. Slides were scored blinded, with a score from 0 to 3 assigned based on 5 parameters: epithelial disruption, submucosal edema, inflammatory infiltrate, mucosal thickening, and luminal exudate. Scores were added for each sample, and the total score was plotted for each animal.

Detection of cytokines, NF- κ B activation, and cell death. Cytotoxicity was measured by LDH release assay according to the manufacturer's instructions (Promega). NF- κ B activation was quantified by adding cell supernatant to Quanti-Blue reagent according to the manufacturer's instructions. IL-1 β , IL-6, and CXCL1 were detected in protein supernatants from BMDCs using the mouse IL-1 β and IL-6 Ready-Set-Go! enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) and the mouse CXCL1 DuoSet kit (R&D Systems) according to the manufacturers' instructions. To measure cecal cytokines following cecal injection, total cecal lysate was generated by removing the cecum and rinsing it gently with phosphate-buffered saline (PBS). Tissue was bead beaten for 1 min in 400 μ l of lysis buffer I (1 $\times$ HALT protease inhibitor [Pierce], 5 mM HEPES). Four hundred microliters of lysis buffer II was added (1 $\times$ HALT protease inhibitor [Pierce], 5 mM HEPES, 2% Triton X-100), and tubes were inverted gently. Tissue samples were incubated on ice for 30 min, followed by a 5-min spin at 13,000 $\times$ g and 4°C. The supernatant was removed to a fresh tube, and total protein concentration was assessed by the bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce). Cytokine concentration is shown relative to total protein concentration below.

RESULTS

To begin to assess the role of glucosylation in the response to TcdA, we first examined the ability of WT-TcdA and NVN-TcdA to cause cell death and activate the transcription factor NF- κ B. We utilized two distinct cell lines which both express secreted embryonic alkaline phosphatase (SEAP) under the control of a promoter containing multiple NF- κ B binding sites. Cell death was assessed by measuring lactate dehydrogenase (LDH) release into the culture media, which occurs following plasma membrane damage. We sought to test a physiologically relevant dose of TcdA, which was found in the serum of infected piglets at concentrations ranging from 1 pg/ml to 10 ng/ml (24). We also included several concentrations both higher and lower than this range to clarify any potential differences between WT- and NVN-TcdA. Upon treating the cells with WT-TcdA and NVN-TcdA, we noted distinct differences in cell death and NF- κ B activation between the two cell lines. In the human monocytic cell line THP-1, WT-TcdA induced significantly more cell death at the two highest concentrations than did NVN-TcdA (Fig. 1A). However, in the Raw murine macrophage cell line, no significant differences in cytotoxicity were noted (Fig. 1C). In contrast, no difference in the ability of WT-TcdA and NVN-TcdA to activate NF- κ B was observed in THP-1 cells, while in Raw cells, WT-TcdA induced significantly more NF- κ B activation at almost all concentrations tested (Fig. 1B

and D). These results reinforce previous findings which indicate that distinct cell types respond differently to intoxication with TcdA and TcdB (13), and they suggest that NF- κ B activation and cell death may involve distinct pathways.

Next, we investigated the ability of NVN-TcdA to induce secretion of the cytokines IL-6 and CXCL1. IL-6 is a proinflammatory cytokine with numerous diverse functions and has been found to be highly upregulated during *C. difficile* infection (25, 26). CXCL1, a chemokine responsible for recruiting neutrophils in mice and a murine homolog of human IL-8, is also dramatically increased during infection (27). Using primary murine bone marrow-derived dendritic cells (BMDCs), we found that WT-TcdA was able to induce significantly higher levels of both IL-6 and CXCL1 than NVN-TcdA at two different concentrations (Fig. 2), suggesting that the glucosyltransferase activity of the toxin could impact secretion of downstream inflammatory mediators.

Because these results suggest that glucosylation contributes to cytokine production, we next investigated the ability of NVN-TcdA to induce IL-1 β secretion. We found that alone, neither WT-TcdA nor NVN-TcdA induced significant levels of IL-1 β at low concentrations (2 ng/ml) (Fig. 3A). This was expected, as TcdA is able to activate but not prime the inflammasome. However, when lipopolysaccharide (LPS) was added as a priming signal in combination with WT- or NVN-TcdA, significant IL-1 β release was detected. WT-TcdA induced higher levels of IL-1 β secretion than NVN-TcdA from the murine BMDCs, suggesting that glucosylation does contribute to inflammasome activation in response to TcdA. We verified this phenotype in human THP-1 cells by measuring caspase 1, which is responsible for the cleavage of pro-IL-1 β and is secreted upon inflammasome activation (28). In human monocytes, significantly more caspase 1 was detected in the cell supernatants in response to WT-TcdA than NVN-TcdA (Fig. 3B), supporting the observation that glucosylation contributes to inflammasome activation.

Because glucosylation appeared to be key to the induction of inflammation, we next tested whether this enzymatic activity contributes to inflammatory processes *in vivo*. To answer this question, we used a cecal injection model of intoxication. In this procedure, mice were anesthetized and underwent laparotomy, during which purified toxins were injected directly into the ceca. Eight hours following injection of the toxins, tissue damage was assessed by examining hematoxylin-and-eosin-stained sections of the cecal tissue. We found that WT-TcdA induced dramatic tissue disruption after injection of purified toxin, while NVN-TcdA-treated ceca appeared similar to mock-treated controls (Fig. 4A to C). Sections were assessed for epithelial disruption, inflammatory infiltrate, submucosal edema, mucosal thickening, and luminal exudate. Quantification of the histological scores (Fig. 4D) revealed a significantly higher damage score for WT-TcdA-treated mice than for NVN-TcdA-treated mice, demonstrating that glucosylation activity is critical for tissue disruption and inflammation *in vivo* (Fig. 4D).

To quantify inflammatory mediators following intoxication, we assessed cytokine levels within the cecal tissue of these mice. IL-6, CXCL1, and IL-1 β closely mimicked the trend observed with histological scores, with WT-TcdA inducing significantly more of these cytokines than in the mock-treated and NVN-TcdA-treated groups, which showed roughly equivalent levels (Fig. 5). Thus, glucosylation is necessary for both tissue damage and an inflammatory response during intoxication. These findings raise

FIG 1 Contribution of TcdA enzymatic activity to cell death and NF- κ B activation. (A and B) THP-1 monocytes were exposed to either WT-TcdA or NVN-TcdA at the indicated concentrations for 24 h. Lactate dehydrogenase release into the culture media was assessed by fluorometric assay (A). SEAP levels in the culture media were quantified at 24 h by the QUANTI-Blue assay (B). (C and D) Raw Blue NF- κ B reporter macrophages were exposed to WT-TcdA or NVN-TcdA at the indicated concentrations. LDH (C) and SEAP (D) levels in the culture media were quantified at 24 h. Data were combined from three independent experiments performed in triplicate. ***, $P < 0.001$ (by Student's t test).

the idea that disruption of glucosylation could provide a viable therapy for CDI.

DISCUSSION

As essential virulence factors of *C. difficile*, toxins A and B are well understood to cause significant tissue damage and inflammation. Although the mechanisms are less clear, both toxins are able to kill cells and induce inflammatory signaling. The role of glucosylation

in activation of the inflammasome by TcdB has been investigated previously, with contradictory results reported. The contribution of glucosylation by TcdA is even less well characterized. In this study, we sought to determine whether Rho glucosylation activity following intoxication with TcdA was essential for cytotoxicity, activation of the inflammasome, and cytokine secretion. Treatment of cells with WT-TcdA and NVN-TcdA revealed dramatic differences in cytotoxicity and NF- κ B activation which were cell

FIG 2 Impact of TcdA enzymatic activity on cytokine secretion. Murine bone marrow-derived dendritic cells were incubated for 24 h with WT-TcdA or NVN-TcdA at the indicated concentrations. CXCL1 (A) and IL-6 (B) protein levels within the culture supernatants were assessed by ELISA. Data were combined from three independent experiments performed in triplicate. **, $P < 0.01$; ***, $P < 0.001$ (by Student's t test).

FIG 3 Role of TcdA enzymatic activity in inflammasome activation. (A) Murine bone marrow-derived dendritic cells were incubated with 2.5 ng/ml of WT-TcdA or NVN-TcdA for 24 h in the presence or absence of LPS as a priming signal. Inflammasome activation, indicated by IL-1 β secretion, was assessed by ELISA. (B) Human THP-1 monocytes were incubated for 24 h with WT-TcdA or NVN-TcdA, and caspase 1 release was evaluated by ELISA. Data were combined from three independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by Student's t test [A] and one-way analysis of variance [B]); NS, not significantly different from the value for the untreated sample.

type dependent. Intoxication of human monocytes with WT-TcdA induced dose-dependent cell death which was largely attenuated in the absence of glucosylation activity. However, WT-TcdA and NVN-TcdA displayed similar abilities to activate NF- κ B in these cells. Conversely, in murine macrophages, there was no difference between WT-TcdA and NVN-TcdA with regard to cell

death, while WT-TcdA was significantly more effective at activating NF- κ B.

These results suggest that not only are the consequences of intoxication different depending on the cell type in question, but also cell death and activation of cytokine production may follow different pathways. Both outcomes may be influenced by the magnitude of the signal generated, and this may also be dependent on cell type. Indeed, Raw macrophages are known to be deficient in an essential inflammasome adaptor protein, apoptosis-associated speck-like protein (ASC) (29). This differential expression of ASC could influence cytotoxicity following intoxication, as cells that cannot undergo inflammasome activation may exhibit decreased death by pyroptosis, although it is unclear whether pyroptotic cell death contributes to cytotoxicity in response to TcdA. Additionally, lack of ASC could influence NF- κ B activation by preventing IL-1 β release, as this cytokine can further activate NF- κ B signaling via the IL-1 receptor. Finally, differences in TcdA receptor expression could account for some of the dissimilarities observed. Although varied consequences of intoxication depending on cell type have been reported previously, our data emphasize the implications this may have for the immune response to *C. difficile* by suggesting that the type of immune cells recruited during an inflammatory response could profoundly shape the consequences of intoxication.

In our model of cecal injection, glucosylation by TcdA was required for tissue damage to occur, suggesting that disruption of Rho family GTPases plays a major role in the pathogenesis of *C. difficile* toxin A. Although epithelial intoxication and death likely play a major role, the extent to which pathogenesis is dependent on intoxication of immune cells remains to be determined. Future investigation in this area is essential to correctly interpreting the role of the host immune response during disease. Correspondingly, production of IL-6, CXCL1, and IL-1 β *in vivo* and *in vitro* also depended on glucosylation. Glucosylation could influence host cytokine production in multiple ways, perhaps by directly inducing inflammatory cytokine production by damaged epithelial cells, leading to recruitment of particular immune cell subsets, or by regulating inflammatory signaling within recruited immune cells via their intoxication. Although the contribution of these individual inflammatory cytokines to pathogenesis remains to be determined, we conclude that glucosylation by TcdA is a key step

FIG 4 Contribution of TcdA enzymatic activity to tissue damage *in vivo*. Fifteen micrograms of equivalent buffer (Mock) (A), recombinant WT-TcdA (B), or NVN-TcdA (C) was administered intracecally via laparotomy. The incision was closed and animals were monitored for 8 h before being humanely euthanized. Cecal sections were fixed in Bouin's solution for 18 h before being subjected to paraffin embedding, sectioning, and hematoxylin and eosin staining. Samples were scored blinded based on 5 parameters (submucosal edema, inflammatory infiltrate, epithelial disruption, luminal exudate, and mucosal thickening). Data represent the cumulative score for each section (D). Data are representative of two independent experiments ($n = 13$). *, $P < 0.05$; **, $P < 0.01$ (by Student's t test).

FIG 5 Quantification of the inflammatory response in mice challenged with glucosylation-deficient TcdA. Mice were challenged with 15 μ g of WT-TcdA or NVN-TcdA injected directly into the cecum via laparotomy. Cecal cytokine protein levels were assessed 8 h following injection by lysing tissue and subsequent ELISA for IL-6 (A), IL-1 β (B), and CXCL1 (C). Data are representative of two independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (by Student's t test).

leading to the profound tissue damage and inflammation observed during infection with *C. difficile*. This work also highlights the idea that disruption of glucosylation using therapeutic inhibitors could be a viable strategy for treatment of CDI. Current treatments involve use of the antibiotics which render patients susceptible, perpetuating a state of dysbiosis. Disruption of toxin activity is an attractive alternative therapy which would theoretically have minimal impact on protective host microbiota. Future work is needed to address the inhibition of glucosylation as a promising potential treatment for this increasingly common and poorly managed disease.

ACKNOWLEDGMENTS

We thank the UVA Research Histology and Flow Cytometry Cores for their assistance with sample preparation and analysis. We thank A. Criss, J. Casanova, U. Lorenz, and M. Kendall for helpful discussion. We thank A. Kerzmann for initial cloning work.

C.A.C. was supported by the Robert R. Wagner Fellowship from the University of Virginia School of Medicine and by NIH training grant 5T32AI07046-38. B.M.J. was supported by NSF grant 1306063 to Wayne State University.

FUNDING INFORMATION

This work was supported by NIH grants R01AI24214, R01AI026649, and R21AI114734 to W.A.P. and NSH grant 1306063 to A.L.F.

REFERENCES

1. 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. <http://dx.doi.org/10.1056/NEJMoa1408913>.
2. Ananthakrishnan AN. 2011. *Clostridium difficile* infection: epidemiology, risk factors and management. *Nat Rev Gastroenterol Hepatol* 8:17–26. <http://dx.doi.org/10.1038/nrgastro.2010.190>.
3. Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP. 2010. The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* 467:711–713. <http://dx.doi.org/10.1038/nature09397>.
4. Kuehne SA, Collier MM, Kelly ML, Cartman ST, Cockayne A, Minton NP. 2014. The importance of toxin A, toxin B and CDT in virulence of an epidemic *Clostridium difficile* strain. *J Infect Dis* 209:83–86. <http://dx.doi.org/10.1093/infdis/jit426>.
5. Carter GP, Chakravorty A, Nguyen TAP, 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. <http://dx.doi.org/10.1128/mBio.00551-15>.
6. Carter GP, Rood JI, Lyras D. 2010. The role of toxin A and toxin B in *Clostridium difficile*-associated disease. *Gut Microbes* 1:58–64. <http://dx.doi.org/10.4161/gmic.1.1.10768>.
7. Schwan C, Stecher B, Tzivelekis 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. <http://dx.doi.org/10.1371/journal.ppat.1000626>.
8. Lysterly DM, Saum KE, MacDonald DK, Wilkins TD. 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. *Infect Immun* 47:349–352.
9. Matarrese P, Falzano L, Fabbri A, Gambardella L, Frank C, Geny B, Popoff MR, Malorni W, Fiorentini C. 2007. *Clostridium difficile* toxin B causes apoptosis in epithelial cells by thrilling mitochondria. Involvement of ATP-sensitive mitochondrial potassium channels. *J Biol Chem* 282:9029–9041.

10. Pruitt RN, Lacy DB. 2012. Toward a structural understanding of *Clostridium difficile* toxins A and B. *Front Cell Infect Microbiol* 2:28.
11. 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 autoproduct-independent mechanism. *PLoS Pathog* 8:e1003072. <http://dx.doi.org/10.1371/journal.ppat.1003072>.
12. Wohlan K, Goy S, Olling A, Srivatharajan S, Tatge H, Genth H, Gerhard R. 2014. Pyknotic cell death induced by *Clostridium difficile* TcdB: chromatin condensation and nuclear blister are induced independently of the glucosyltransferase activity. *Cell Microbiol* 16:1678–1692. <http://dx.doi.org/10.1111/cmi.12317>.
13. Auria KM, Bloom DMJ, 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. <http://dx.doi.org/10.1186/s12866-015-0361-4>.
14. Gerhard R, Nottrott S, Schoentaube J, Tatge H, Olling A, Just I. 2008. Glucosylation of Rho GTPases by *Clostridium difficile* toxin A triggers apoptosis in intestinal epithelial cells. *J Med Microbiol* 57:765–770. <http://dx.doi.org/10.1099/jmm.0.47769-0>.
15. Nottrott S, Schoentaube J, Genth H, Just I, Gerhard R. 2007. *Clostridium difficile* toxin A-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. *Apoptosis* 12:1443–1453. <http://dx.doi.org/10.1007/s10495-007-0074-8>.
16. 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, 552.e1–552.e3. <http://dx.doi.org/10.1053/j.gastro.2010.04.005>.
17. Xu H, Yang J, Gao W, Li L, Li P, Zhang L, Gong Y-N, Peng X, Xi JJ, Chen S, Wang F, Shao F. 2014. Innate immune sensing of bacterial modifications of Rho GTPases by the P2Y₁ inflammasome. *Nature* 513:237–241. <http://dx.doi.org/10.1038/nature13449>.
18. 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. <http://dx.doi.org/10.1016/j.micpath.2009.03.002>.
19. von Moltke J, Ayres JS, Kofoed EM, Chavarría-Smith J, Vance RE. 2013. Recognition of bacteria by inflammasomes. *Annu Rev Immunol* 31:73–106. <http://dx.doi.org/10.1146/annurev-immunol-032712-095944>.
20. Teichert M, Tatge H, Schoentaube J, Just I, Gerhard R. 2006. Application of mutated *Clostridium difficile* toxin A for determination of glucosyltransferase-dependent effects. *Infect Immun* 74:6006–6010. <http://dx.doi.org/10.1128/IAI.00545-06>.
21. Yang G, Zhou B, Wang J, He X, Sun X, Nie W, Tzipori S, Feng H. 2008. Expression of recombinant *Clostridium difficile* toxin A and B in *Bacillus megaterium*. *BMC Microbiol* 8:192. <http://dx.doi.org/10.1186/1471-2180-8-192>.
22. Bai L, Feuerer M, Beckhove P, Umansky V, Schirmacher V. 2002. Generation of dendritic cells from human bone marrow mononuclear cells: advantages for clinical application in comparison to peripheral blood monocyte derived cells. *Int J Oncol* 20:247–253.
23. D'Auria KM, Kolling GL, Donato GM, Warren CA, Gray MC, Hewlett EL, Papin JA. 2013. In vivo physiological and transcriptional profiling reveals host responses to *Clostridium difficile* toxin A and toxin B. *Infect Immun* 81:3814–3824. <http://dx.doi.org/10.1128/IAI.00869-13>.
24. Steele J, Chen K, Sun X, Zhang Y, Wang H, Tzipori S, Feng H. 2012. Systemic dissemination of *Clostridium difficile* toxins A and B is associated with severe, fatal disease in animal models. *J Infect Dis* 205:384–391. <http://dx.doi.org/10.1093/infdis/jir748>.
25. Hunter CA, Jones SA. 2015. IL-6 as a keystone cytokine in health and disease. *Nat Immunol* 16:448–457. <http://dx.doi.org/10.1038/ni.3153>.
26. McDermott AJ, Frank CR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB. 2014. Role of GM-CSF in the inflammatory cytokine network that regulates neutrophil influx into the colonic mucosa during *Clostridium difficile* infection in mice. *Gut Microbes* 5:1–9. <http://dx.doi.org/10.4161/gmic.28028>.
27. Song F, Ito K, Denning TL, Kuninger D, Papaconstantinou J, Gourley W, Klimpel G, Balish E, Hokanson J, Ernst PB. 1999. Expression of the neutrophil chemokine KC in the colon of mice with enterocolitis and by intestinal epithelial cell lines: effects of flora and proinflammatory cytokines. *J Immunol* 162:2275–2280.
28. Andrei C, Margiocco P, Poggi A, Lotti LV, Torrisi MR, Rubartelli A. 2004. Phospholipases C and A2 control lysosome-mediated IL-1 β secretion: implications for inflammatory processes. *Proc Natl Acad Sci U S A* 101:9745–9750. <http://dx.doi.org/10.1073/pnas.0308558101>.
29. Pelegrin P, Barroso-Gutierrez C, Surprenant A. 2008. P2X₇ receptor differentially couples to distinct release pathways for IL-1 β in mouse macrophage. *J Immunol* 180:7147–7157. <http://dx.doi.org/10.4049/jimmunol.180.11.7147>.