Early cell death induced by *Clostridium difficile* TcdB: Uptake and Rac1-glucosylation kinetics are decisive for cell fate.

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Abstract

Toxin A and Toxin B (TcdA/TcdB) are large glucosyltransferases produced by *Clostridium difficile*. TcdB but not TcdA induces reactive oxygen species (ROS)-mediated early cell death when applied at high concentrations. We found that non-glucosylated Rac1 is essential for induction of early cell death since inhibition of Rac1 impedes this effect. Early cell death only occurs when TcdB is rapidly endocytosed. This was shown by generation of chimeras using the trunk of TcdB from a hypervirulent strain. TcdB from hypervirulent strain has been described to translocate from endosomes at higher pH values and thus, meaning faster than reference type TcdB. Accordingly, intracellular delivery of the glucosyltransferase domain of reference TcdB by the trunk of TcdB from hypervirulent strain increased early cell death. Furthermore, proton transporters such as NHE sodium/proton exchanger or the ClC-5 anion/proton exchanger, both of which contribute to endosomal acidification, also affected cytotoxic potency of TcdB: Specific inhibition of NHE reduced cytotoxicity, whereas transfection of cells with the endosomal anion/proton exchanger ClC-5 increased cytotoxicity of TcdB. Our data suggest that both the uptake rate of TcdB into the cytosol and the status of non-glucosylated Rac1 are key determinants that are decisive for whether early cell death or delayed apoptosis is triggered.
Introduction

The main pathogenicity factors TcdA and TcdB of Clostridium difficile are large clostridial glucosyltransferases that mono-glucosylate Rho GTPases. The isolated toxins are sufficient to induce all clinical symptoms and to work fatally in an animal model (Just et al., 1995; Kuehne et al., 2010). These two protein toxins mediate their cell entry by receptor-based endocytosis which exploits clathrin- and/or dynamin-dependent pathways. (Papatheodorou et al., 2010; Gerhard et al., 2013; Chandrasekaran et al., 2016; Aktories et al., 2017). Both toxins, TcdA and TcdB, escape early endosomes by insertion into the vesicular membrane and by translocation of at least the N-terminal glucosyltransferase (GT) domain through a pore built in the vesicular membrane (Giesemann et al., 2006). Acidification of the endosomal compartment by the v-ATPase proton pump is essential for this process. A drop in the endosomal pH induces the required conformational changes of the toxins (Salnikova et al., 2008). Once the GT domain (GTD) has entered the cytosol, inositol hexakisphosphate and reducing conditions induce its cleavage by allosterical activation of the toxin-inherent cysteine protease domain adjacent to the GTD (Egerer et al., 2007). Release of the GTD from the trunk of the toxin prevents its lysosomal degradation and gives mobility to reach substrate GTPases for their UDP-glucose-dependent modification.

This cell entry process is identical for all large clostridial glucosyltransferases, albeit differences between TcdA and TcdB (and putatively TcsL and TcsH from C. sordellii) exist regarding the receptor for cell surface binding. The substrate specificity of TcdA and TcdB is almost identical. Main substrate GTPases are RhoA/B/C, Rac1/2, and Cdc42. It is assumed that the toxins mostly differ in their uptake kinetics and those of substrate glucosylation. Differences in substrate specificity of both toxins are known for e.g. TCL,Ral and Ras, which seem to be specific substrates for either TcdB
(TCL) or TcdA (RalA/B, H/K/N-Ras), respectively (Zeiser et al., 2013; Genth et al., 2014). All these systematic approaches to specify substrate GTPases, however, bear the disadvantage of either not representing intracellular conditions (recombinant in vitro system) or not being able to distinguish between homologous GTPases when peptides with identical sequences were detected (mass spectrometry analysis). Thus, it is not clear whether these comparably small differences in substrate specificity are responsible for the drastically, i.e. about 1,000-fold, increased cytotoxic potency of TcdB compared to TcdA. The specific cytotoxic effect of TcdB is independent of glucosyltransferase activity (Farrow et al., 2013; Wohlan et al., 2014) and is also independent of the intracellular release of the GTD (Chumbler et al., 2012). Neither TcdA nor variant TcdB (from strain 1470) triggers this kind of early cell death (ECD)/pyknosis (Farrow et al., 2013; Wohlan et al., 2014). It is induced only at high concentrations, about 1,000-fold higher than the minimal concentration required for cell rounding (cytopathic effect). Functional inactivation of the NADPH oxidase by siRNA-mediated knock down of NOX1 or NOX3 or by a pharmacological inhibitor as well as scavengers for reactive oxygen species (ROS) prove that the early cell death is triggered by ROS, although the mechanism by which TcdB induces ROS is not known (Fradrich et al., 2016). Our observations suggest that a rapid intracellular appearance of an adequate amount of the GTD from TcdB drives cells into early cell death in a Rac1-dependent manner.

For our study, we generated standardized chimeras of TcdB exploiting the reference translocation machinery, which is the trunk of toxin lacking only the GT domain, of TcdB VPI10463 (toxinotype 0) or of TcdB of the hypervirulent strain R9385 (toxinotype XIVb) according to classification by Rupnik and Janezic (Rupnik et al., 2016). It was previously described that TcdB from hypervirulent strain BI17 6493 (ribotype 027) translocates more rapidly into host cells due to essential
conformational changes performed at higher pH-levels than necessary for translocation of the reference TcdB (Lanis et al., 2010). We here used TcdB from strain R9385 for our studies, because the GT domain is almost identical to that of TcdB(F) from strain 1470 (toxinotype VIII), which is well characterized. The trunk of TcdB<sub>R9385</sub>, however, is identical to that of TcdB<sub>R20291</sub> except for 12 amino acids within the cysteine protease domain and can therefore be recognized as equivalent to TcdB<sub>R20291</sub>. By manipulating Na<sup>+</sup> and Cl<sup>-</sup> transport into endosomes, we were able to increase or decrease the cytotoxic effect of TcdB. Thus, intracellular flush of toxin and kinetics of Rac1 inhibition are the two determinants for cytotoxicity of TcdB.

**Results**

*TcdB-induced early cell death depends on ROS*

TcdB induces ROS-triggered early cell death (ECD). As previously shown, the predominant morphological signs are pyknotic characteristics such as chromatin condensation and membrane blistering (Fig. 1A) (Wohlan et al., 2014). To investigate the relationship of cell rounding, ECD, and ROS in more detail, we determined numbers of either round or pyknotic cells after treatment with 3 nM TcdB. Combined DAPI incorporation assays and microscopic analyses revealed the number and morphotype of cells that lacked viability due to toxin treatment. Overlay of phase contrast and fluorescence images in Figure 1B revealed that only pyknotic cells with condensed nucleus were DAPI-positive, whereas rounded cells are acknowledged as viable based on DAPI exclusion. Apocynin (1 mM), an inhibitor of the NADPH oxidase and a ROS scavenger, reduced the number of DAPI positive cells. Both morphotypes, rounded and pyknotic, were mutually exclusive. The concentration-dependent cell rounding and pyknosis induced by TcdB is shown in Fig. 1C (left panel). At a fixed concentration of 3 nM TcdB, apocynin reduced the number of
pyknotic, i.e. DAPI-positive cells, and increased the number of round cells in a concentration-dependent manner (right panel). Thus, ROS-induced pyknosis is dominant and is initiated before cell rounding. The kinetics of intracellular ROS production are shown in Fig. 1D. To exclude effects based on glucosylation, we used the mutant TcdB NXN which is glucosyltransferase-deficient due to two point mutations in the DXD motif (D286/288N) (Wohlan et al., 2014). HEp-2 cells treated with 3 nM TcdB NXN displayed no significant ROS production within the first hour of treatment as measured by DHE-positive nuclei. Instead, DHE-positive nuclei appeared only beyond 60 min of treatment. This nicely correlated with the protection assay using apocynin as shown in Fig. 1E. Apocynin (1 mM) significantly reduced TcdB-induced loss of cell viability only when applied within the first 60 min after toxin addition. When apocynin was added 90 min after the onset of TcdB treatment, no significant reduction of cell death was detected after 210 min. Thus, cell fate (cell rounding vs. ECD) is irreversibly determined already within the first 60 min of toxin application. In fact, the removal of toxin by medium exchange even 10 min after toxin addition only slightly reduced ECD detected after 4 h compared to cells incubated with TcdB for the whole period (Supplementary Fig. S1).

ROS production by NADPH oxidase (NOX1, NOX2 and possibly NOX3) is regulated by Rac1 (Ueno et al., 2005; Hordijk, 2006; Ueyama et al., 2006; Kao et al., 2008). As shown previously, knock down of NOX1 or NOX3 (depending on cell line) or knock down of Rac1 reduces ROS production and the cytotoxic effect of TcdB (Farrow et al., 2013). In pull down assays of cell lysates from HEp-2 cells, we estimated possible activation of Rac1 (Fig. 1F). Immunoblots show active and total Rac1 in HEp-2 lysates after 5, 20 and 210 min treatment with 0.03 nM TcdB, 3 nM TcdB and 6 nM TcdB NXN. The densitometric evaluation of immunoblots is shown in Fig. 1G. The experiments revealed no significant overall Rac1 activation by TcdB and TcdB
NXN 5 min after toxin addition. After 20 min, active Rac1 was reduced below control levels in cells treated with low or with high concentrations of TcdB. Only in TcdB NXN treated cells, unchanged levels of active Rac1 were observed over the whole period of treatment. However, the pull down assays did not provide evidence of significant overall Rac1 activation. Assuming full Rac1 activity for induction of ECD, the pull down experiments suggest a time frame of only a few minutes before noteworthy Rac1 inhibition occurs by TcdB-catalyzed glucosylation.

The cytotoxic effect is independent of glucosyltransferase activity

The pull down assays for total cellular Rac1 activity did not exclude local Rac1 activation at single signalosomes. To further define the role of Rac1 in ECD, we additionally tested TcdB in Rac1-deficient mouse embryonic fibroblasts (Rac1−/− MEFs). Whereas Rac1<sup>flox/flox</sup> control MEFs showed typical signs of ECD at high concentrations of TcdB, the number of pyknotic cells was drastically reduced in TcdB-treated Rac1<sup>−/−</sup> MEFs (Fig. 2A,B). To show that glucosylated Rac1 indeed prevents ECD, we pre-incubated cells with the cytopathic concentration of the chimera B(F)-B<sub>ref</sub>, which was built of the GTD of TcdB(F) strain 1470 and the trunk of TcdB strain VPI10463 (termed B(F)-B<sub>ref</sub>). The chimera has Rac1 as major substrate, but does not induce ECD (Wohlan <i>et al.</i>, 2014). Preincubation of HEp-2 cells with 0.03 nM B(F)-B<sub>ref</sub> completely prevented induction of ECD by the subsequently applied glucosyltransferase-deficient TcdB NXN (termed B<sup>NXN</sup>-B<sub>ref</sub>) at high concentration (Fig. 2C). Here we chose TcdB NXN for induction of ECD in order to avoid interference with glucosylation of Rho GTPases when applying both toxins. Different toxin preparations show various impurities and toxin fragments that putatively bias experiments. We therefore investigated ECD in competition experiments to exclude fragments block toxin effects (Supplementary Fig. S2). The lack of ECD did not
correlate with impurities or was due to competition with toxin fragments on receptor
level.
Since Rac1 was described to participate in endocytosis (Sanlioglu et al.,
2000; Soriano-Castell et al., 2017), we checked the effect of Rac1 inactivation on
uptake of TcdB. To this end, we performed sequential glucosylation-sensitive
[^32P]ADP-ribosylation of RhoA, since RhoA is a substrate for TcdB but not for
TcdB(F). Preincubation of cells with B(F)-Bref did not reduce subsequent
glucosylation of RhoA/B by TcdB (Fig. S3). We also found that ECD induced by high
concentrations of TcdB was also abrogated or reduced if cells were pre-incubated
with only cytopathic concentration of TcdB or TcdA, respectively (data not shown).
Although it is known that the glucosyltransferase-deficient TcdB mutant also exhibits
a cytotoxic effect, we cross checked this effect by using the UDP-glucose-deficient
cell line DonQ and its revertant G3. This cell line is devoid of UDP-glucose to the
largest extent and, thus, can be used to investigate the role of intracellular UDP-
glucose concentrations in ECD. As shown in Fig. 2D, DonQ cells show an over
1,000fold decreased level of UDP-glucose compared to the revertant G3 cells, which
was almost at the limit of detection. The UDP-glucose level in the revertant G3 is
comparable to the level that can be measured in HEp-2 cells. The morphotype of
control cells, round cells and pyknotic G3 cells is shown in Fig. 2E, and the bar chart
(Fig. 2F) shows the numerical analysis. TcdB induced cell rounding to an extent of
more than 90% in G3 cells at low concentration (3 pM), but not in DonQ cells, where
number of round cells did not differ from untreated control cells. However, a high
concentration of TcdB (3 nM) induced the pyknotic morphotype in more than 90% of
G3 and 70% of DonQ cells, again verifying that the glucosylation reaction is
dispensable for induction of ECD.
Translocation and glucosylation characteristics account for specific cytotoxic effects of TcdB.

Obviously, high concentrations of TcdB and signaling competent Rac1 are the two prerequisites for ECD, whereas inhibition of Rac1 counteracts ECD induced by TcdB. From this, it can be concluded that rapid uptake of sufficient toxin is required before a critical level of Rac1 is glucosylated by the toxin itself. Therefore, the uptake and glucosylation kinetics of TcdB are considered to constitute the two determinants for the occurrence of ECD. To test this, we generated specific toxin chimeras either for efficient Rac1-glucosylation (based on the GTD of variant TcdB(F)) or for rapid translocation into target cells (based on the delivery domain of TcdB from a hypervirulent strain). Fig. 3A shows pairwise alignment of four toxins: TcdB VPI10463 representing the reference/historical strain, TcdB R20291 representing a hypervirulent strain, TcdB 1470 as historical strain with a variant TcdB inducing sordellii-like phenotype, and TcdB R9385 representing a variant TcdB of a hypervirulent strain that induces a sordellii-like phenotype in cell culture. The sordellii-like phenotype provides rounding of cells with sometimes filopodia-like structures. In contrast, the cytopathic effect induced by TcdB VPI10463 is characterized by rounding with neurite-like protrusions (Chaves-Olarte et al., 1999;Chaves-Olarte et al., 2003). The alignment illustrates that 1) the GTD of TcdB 1470, and TcdB R9385 are almost identical, except three amino acid residues and 2) the trunk of TcdB R20291 and TcdB R9385 are also considered as identical except for 12 amino acid residues, all within the cysteine protease domain. Figure 3B shows comparison of the cytotoxic effect of the GTD and the glucosyltransferase-deficient GTD when translocated into HEp-2 cells by either the trunk of TcdB reference (B-B ref, B NxN-B ref) or the trunk of TcdB hypervirulent (B-B hyp, B NxN-B hyp). The data clearly show that the glucosyltransferase-deficient GTD is more potent in inducing ECD than the wild type GTD. Furthermore, the cytotoxic effect is
even more pronounced when the GTD is delivered into cells by the trunk of TcdB
from the hypervirulent strain. The same experiment was performed with the GTD of
TcdB_{1470}, which has Rac1 as major substrate. We previously observed that neither
wild type TcdB(F) nor the chimera of B(F)-B_{\text{ref}} induced pyknosis (Wohlan et al., 2014).
However, when the GTD of TcdB(F) was fused to the trunk of TcdB_{\text{hypervirulent}} (B(F)-
B_{\text{hyp}}), we observed a slight increase in LDH release assay as surrogate for cell death
(Fig. 3C). Even more, the cytotoxic potency was completely unmasked when the
glucosyltransferase-deficient chimeras B(F)^{NXN}-B_{\text{ref}} and B(F)^{NXN}-B_{\text{hyp}} were applied in
cytotoxicity assay, validating our hypothesis that rapid Rac1-glucosylation
counteracts ECD. Interestingly, the pyknotic effect was also achieved when
TcdB NXN was translocated by an acidic shift of medium to pH 4.8, indicating that
translocation independent of endocytosis is sufficient for induction of ECD (Fig. 3D).
This was not the case for wild type TcdB, stressing the importance of Rac1
glucosylation as critical factor in ECD. TcdA and TcdA NXN as well as TcdB(F) were
included into experiments. All toxins except their glucosyltransferase-deficient NXN
mutants induced cell rounding to different degrees, showing positive cytosolic
translocation by pH shift (data not shown).
These results strongly suggest that the translocation kinetics of TcdB is decisive for
induction of early cell death. Yuan and coworkers showed that deletion of TcdB
receptor CSPG4 in HeLa cells required higher toxin concentrations to achieve the
cytotoxic effect (Yuan et al., 2015). The observation that deletion of PVRL3/nectin-3,
another receptor for TcdB, prevented the cytotoxic effect as reported by LaFrance
and coworkers is therefore not in contradiction (LaFrance et al., 2015). If the most
abundant receptor in target cells is deleted, a concentration of intracellular toxin
sufficient to induce early cell death might not be achieved. As shown by Manse and
Baldwin, PVRL3 interacts with the region of amino acids 1372-1493 within TcdB
Manse et al., 2015). Recent data show that CSPG4 binding also happens within the region of amino acids 1810-1850 (Gupta et al., 2017). As reported earlier, the CROP-deleted TcdB (TcdB 1-1852) is less potent than full length TcdB with respect to cell rounding (Olling et al., 2011). We here re-investigated this with respect to induction of pyknosis and found that TcdB 1-1852 does not induce early cell death as measured by WST release assay (Fig. 4A). While TcdB (3 nM) reduced cell viability to 60% of control value, TcdB 1-1852 did not affect cell viability even at 6 nM, although complete cell rounding verified its full cytopathic potency. This was also proven for TcdB from strain R20291, where even the complete CROP domain except four amino acids was removed (TcdB_{R20291} 1-1836) (Supplementary Fig. S4). We recently observed that the ClC-5 anion/proton exchanger makes cells more susceptible to TcdA and TcdB (Ruhe et al., 2017) ClC-5 supports the v-ATPase in acidification of endosomes and by generating a beneficial electrochemical gradient, thereby accelerating uptake and translocation of toxins (Piwon et al., 2000;Wang et al., 2000;Hara-Chikuma et al., 2005;Novarino et al., 2010). We here used the HEK293 cell model for investigation of the cytotoxic effect of TcdB. Wildtype HEK293 cells do not show signs of pyknosis and ECD when treated with TcdB chimera B-B_{hyp} up to 6 nM (Fig. 4B). mCherry-CIC-5 expressing cells, however, were significantly more susceptible to ECD than mock transfected cells expressing only mCherry when treated with the most potent chimera B-B_{hyp}. It can be assumed that both cell lines have comparable receptor pattern and that only endocytotic rates and translocation process might be the causal relationship with ECD induced by TcdB. mCherry transfected cells and mCherry-CIC-5 cells showed no difference in cell surface binding of TcdB R20291 as investigated in immunoblot analysis (Fig. 4C).
Based on these findings, we further investigated the role of proton transporters in influencing endosomal pH and thus, translocation of TcdB. When HEp-2 cells were intoxicated in Iscove’s Modified Dulbecco’s medium (IMDM) instead of Minimum Essential Medium Eagle (MEM), TcdB-induced pyknosis and ECD were much less pronounced (Fig. 5A). Both media differ in their sodium chloride concentrations containing either 77 mM (IMDM) or 117 mM (MEM). Sodium as well as chloride gradients are utilized for proton transport by the sodium/proton exchanger NHE1-7 or by anion/proton transporter ClC3-7. As shown in Fig. 5A, TcdB-induced pyknosis in HEp-2 cells kept in IMDM is roughly 20% of that observed when treated in MEM. Adjustment of the NaCl concentration in IMDM from 77 mM to 117 mM significantly increased the number of pyknotic cells after treatment with TcdB. Involvement of the sodium/proton exchanger NHE was investigated by specific inhibition. EIPA (5-(N-ethyl-N-isopropyl)-Amiloride), an inhibitor of several NHE isoforms dose-dependently inhibited TcdB-induced pyknosis. Complete inhibition was achieved at 50 µM (Fig. 5B). The corresponding micrographs show that only pyknosis is inhibited but not cell rounding at that concentration. Additional experiments dissected between the specific sodium or chloride gradient: cells were treated with TcdB in IMDM supplemented either with 40 mM choline chloride or 40 mM sodium hydrogencarbonate (Fig. 5C). In both cases, pyknosis was significantly increased to levels observed in MEM, indicating that either ion gradient supports toxin uptake. Pyknosis did not result from increased osmolarity, since addition of 80 mM sorbitol did not sensitize cells in IMDM for TcdB-induced ECD as 40 mM NaCl did (Fig. 5D).

The data described above indicate that a high uptake rate of TcdB triggers ECD. The HEp-2 cell line used in this study is predestinated for ECD since all known receptors as well as the endosomal NHE6 and ClC-5 are expressed, as shown by cDNA-microarray (Supplementary Fig. S5).
Discussion

Along with previous reports, our present data point out that the lack of functional Rac1 prohibits early cell death induced by high concentrations of TcdB. In the absence of (functional) Rac1 or in the presence of a predominant pool of glucosylated Rac1, TcdB fails to induce ROS-mediated early cell death. Sufficient and signaling-competent Rac1 is required within the initial phase of intoxication to allow activation of a dominant and irreversible process ending up in ECD. Pull down assays showed that 20 min after application of 3 nM TcdB, the amount of active Rac1 was decreased to more than 70%, indicating a time window of only a few minutes in which TcdB can induce fatal signaling by a so far unknown mechanism.

Induction of pyknosis does not prevent glucosylation of Rho GTPases. Glucosylation also occurs in those cells that are dedicated to early cell death, but without consecutive cell rounding. Obviously, the very initial phase of intoxication is decisive for whether the cytotoxic effect, i.e. Rac1-dependent ROS-production, or the cytopathic effect (cell rounding due to inhibition of Rho GTPases) is prevailing. Our data suggests that rapid uptake accelerates ECD. Uptake of toxin does not only mean endocytosis, but encompasses the whole process from cell surface binding to entering the cytosol. Thus, several parameters constitute the prerequisites for immediate cytotoxicity: 1) high concentration of TcdB, 2) specific toxinotypes from hypervirulent strains, 3) receptor abundancy, and 4) support of the vesicular ATPase to acidify endosomal lumen. The latter phenomenon can be achieved by increasing extracellular sodium or chloride concentration, which drives proton efflux. Our results presented here show that spontaneous change of culture medium can strongly reduce the cytotoxic effect of TcdB. This finding clearly speaks against a specific receptor being in charge of cytotoxic signaling, as shown for PVRL3 (LaFrance et al.,
2015). Nevertheless, receptor abundancy and endocytotic rates of receptors define uptake and intracellular flush of toxin, making any highly abundant receptor an according candidate for mediating cytotoxicity. This supposedly also accounts for the other known TcdB receptors Frizzled-2,1,7 and chondroitin sulfate proteoglycan-4 (CSPG4) (Yuan et al., 2015; Tao et al., 2016), as long as they are associated with high endocytotic rates. In this study we also used the CROP-truncated version of TcdB (aa 1-1852). This truncated toxin does still contain part of the CROPs that are responsible for binding to CSPG4 (Tao et al., 2016). This surely implicates that the CROPs contribute to toxin uptake in another way than mere CSPG4 binding. We nevertheless used a CROP-truncated version of TcdB from strain R20291, ending at position 1836, which is only four amino acids downstream of the beginning of the CROPs (Orth et al., 2014). TcdB R20291 (aa1-1832) which interacts with only non-CROP receptors, was as less potent in inducing ECD as TcdB VPI1043 (aa 1-1852). The most compelling reason speaking for translocation of protein instead of mere receptor binding is the striking difference between the two culture media MEM and IMDM, which turned out to significantly affect cytotoxicity of TcdB. To differentiate between sodium or chloride gradient, we reconstituted either concentration by applying sodium hydrogen carbonate or choline chloride to result in sodium/chloride concentrations found in MEM. Furthermore, we applied the amiloride derivate EIPA as inhibitor for sodium/proton exchanger NHE. The cytotoxic effect was strongly reduced by EIPA, without complete inhibition of toxin uptake. EIPA-treated cells completely rounded up, which indicated only reduced uptake instead of complete abolishment, as it can be achieved by bafilomycin A. The role in toxin uptake of the chloride/proton antiporter CIC-5, which directly acidifies endosomes, was reported before (Smith et al., 2010; Ruhe et al., 2017). All strategies gave results that were in line with our hypothesis of a Rac1-dependent effect mediated by intracellular flush of
toxin. We assume that the protective effect of EIPA on TcdB-induced ECD is based on pH-dependent translocation of TcdB, modulated by the endosomal NHE isoform 6 (Ohgaki et al., 2008; Fukura et al., 2010; Ohgaki et al., 2010). However, inhibition of an outward-directed proton efflux can affect activity of Rac1 as shown by Koivusalo and coworkers, since submembranous pH modulates activation of GTPases (Koivusalo et al., 2010). In that study, it was shown that Rac1 activity decreases in a local, acidified milieu, which could be achieved by inhibition of NHE. As shown in our present study, inactivation of Rac1, in turn, reduces the cytotoxic effect of TcdB. In the context of chloride-and ClC-5-dependent effects, we nevertheless assume a NHE-dependent intra-endosomal effect on toxin translocation instead of mediating local Rac1-activity.

Aside from cell specific features, such as receptor abundancy, endocytic rate, endosomal acidification kinetics or Rac1 activity status, the toxin itself also determines early cytotoxicity. In this study, we also used chimeras of four different TcdB toxinotypes, which are representative of a historical reference strain (VPI10463), hypervirulent strains (R20291) and variants of TcdB from either a historical strain (1470) or a hypervirulent strain (R9385). For a direct comparison of either GTD-specific or the delivery domain (trunk)-specific effects, we generated chimeras. By applying these chimeras, we were able to differentiate between GTD-dependent and -independent effects of various GTDs, as well as between different translocation systems under standardized conditions. The results are complementary to what we found by manipulation of target cells: ECD depends on the translocation characteristics and on the kinetics of Rac1 glucosylation. This was most convincingly shown by variant TcdB(F). This toxin primarily has Rac1 and Ras, but not RhoA/B/C as substrate. We assume that the preferential Rac1 glucosylation abolishes the induction of ECD, since the glucosyltransferase-deficient mutant TcdB(F) clearly
induces ECD. Rac1-glucosylation obviously reduces TcdB-induced ECD, which is maximal when the respective NXN mutant is applied. The chimeras also revealed that different GTDs induce ECD to different extents when delivered by the same trunk of TcdB. Yet, we do not know whether the amino acid sequence, substrate specificity or different membrane crossing abilities account for these observations. All these findings together add up to a scenario where effective uptake of TcdB through the endosomal membrane triggers ECD. This process is Rac1-dependent and is executed by reactive oxygen species. Our present data do not unravel the mechanism by which ECD is induced, but help to understand the prerequisites and why this effect is not observed in every cell type. It is still unclear whether the early cytotoxic effect contributes to the pathology in C. difficile infections. If so, it can be assumed that only a minority of cells that is 1) most sensitive to TcdB and 2) is also exposed to relatively high concentrations of TcdB suffers from ECD. The fact that vaccination of rabbits and mice by using the genetically engineered toxoid TcdB NXN can be done without induction of local tissue damage argues for ECD being a cell culture phenomenon rather than significantly contributing to pathogenesis.

Materials and Methods

Materials. Glucosylation-sensitive monoclonal anti-Rac1/Cdc42 (clone 102) was from BD transduction Laboratories, monoclonal anti-GAPDH was from Zytomed Systems GmbH, Germany. Clostridium difficile strains: reference strain VPI10463 and strain 1470, serotype F were from the Pasteur Institute, Paris, France. Strain R20291 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Strain R9385 was generously provided by Nigel P. Minton, University of Nottingham.
Cloning of \textit{tcdB} genes and chimeras

All \textit{tcdB} genes were amplified from genomic DNA isolated by using the peqGold Bacterial DNA Kit from peqlab, Germany. \textit{C. difficile} TcdB from strain VPI10463 and the chimeric toxin TcdBF\textsubscript{1470}\textsuperscript{1-543} - TcdB\textsubscript{VPI10463}\textsuperscript{544-2366} (termed B(F)-B\textsubscript{ref}) were cloned in expression vector pHIS1522 and 6 x His tagged as described earlier (Wohlan \textit{et al.}, 2014). Following primers were used for PCR-based amplification of genes flanked by BsrGI and KpnI: 5'-TCATGTACAATGAGTTAGTTAATAGAAAACAG-3' was used as sense primer for all TcdB variants (VPI10463: Acc.-No. KC292162; 1470: Acc.-No. AF217292; R20291: Acc.-No. FN545816; R9385: Acc.-No. HM062502). The panTcdB sense primer 5'-GATCGGTACCCTTCACTAATGAGCTG-3' was used as antisense for TcdB VPI10463 and TcdB 1470. The primer 5'-GATCGGTACCCTTCACTAATGAGCTG-3' was used as antisense for TcdB variants from hypervirulent strains R20291 and R9385. The amplified genes were cloned with BsrGI/KpnI into modified pHIS1522 lacking a BamHI restriction site. The chimeras of TcdB variants were generated by site-directed, silent mutagenesis (Quickchange site directed mutagenesis kit, Stratagen, Germany) at position bp 1623 T $\rightarrow$ A and bp 1626 T $\rightarrow$ C to generate a BamHI restriction site at bp 1621-1626 (bp 1624-1629 for TcdB R9385), using mutagenesis primer 5'-GGATTATTTTGAGGATCCCTTGGAGAGATGATACTTG-3' and 5'-GATTATCATCTTCACCAGGGATCC TTCAAAAATAATTTTTC TTG-3' as sense and antisense primers, respectively in TcdB VPI10463 (TcdB GTX). In analogy, the BamHI restriction site was inserted into the TcdB R9385 expression vector by using mutagenesis primers 5'-CAAGAAAATTAT TTGGAAGGATCCCTTGGAGAGATGATACTTG-3' and 5'-CAAGTTTATCATCTTCTC TCCAAGGATCCCTTAAATTAATTTTTC TTG-3' as sense and antisense primers, respectively.
The chimeras of TcdB were generated by exchange of the glucosyltransferase domain (GTD, bp 1-1623) from TcdB GTX VPI10463 or from TcdB GTX R9385 with the amplified GTD from other TcdB variants. Therefore, the GTDs were excised by BsrGI and BamHI, and the cleaved expression construct complemented by ligation with PCR amplicons of the GTDs from indicated TcdB genes. For amplification, we used the panTcdB sense primer for all GTDs, and as anti-sense primer we used

5'-ACTGGA TCCTCAAAAATAATTTTTCTT GTATTCTTC-3' for TcdB VPI10463 GTD
or 5'-ACTGGAT CCTTCAAAAATAATTCCTTT TATATTCTTC-3' for TcdB1470 GTD.

The glucosyltransferase-deficient mutants D286/288N were also generated by site-directed mutagenesis as described earlier (Wohlan et al., 2014). For mutagenesis of TcdB (1470), the following sense and antisense primers were used: Sense:

5'-GGTGAGTCTATCTAAATGTTAATATGTTACCAGGAATACCC-3' and anti-sense: 5'-GGGT GTATTCCTGGTAACATATTAACATTTAGACTCCACC-3'. All constructs were sequenced.

Expression of recombinant toxins
All toxins were expressed in B. megaterium system (MoBiTec, Germany) according to standard protocols from supplier, as previously published (Burger et al., 2003).

The 6 x His-tagged toxins were purified on Ni²⁺-TED columns (Macherey-Nagel, Germany) by gravity flow. Buffer of eluted proteins was exchanged to storage buffer (50 mM NaCl, 20 mM Tris HCl, pH 7.2) by Zeba desalting columns (ThermoFisher, Germany).

The PAK-CRIB domain used for pull down experiments was generously provided by John Collard, Amsterdam. PAK-CRIB domain was expressed as GST fusion protein in E. coli TG1 cells using standard conditions.
Cell culture and generation of stably transfected HEK293 cells

HEp-2 cells and HEK293 cells were kept with standard culture conditions (37°C, 5% CO₂, humidified atmosphere) in Minimum Eagle’s Medium (MEM) or Dulbecco’s Modified Eagles Medium (DMEM), respectively, each supplemented with 10% fetal bovine serum, 100 µM penicillin, and 100 µg/ml streptomycin. Stably transfected HEK293 cells were cultured with additional 2 mM L-glutamin and 100 µg/ml G418.

Mouse embryonic Rac1^{fl/fl} and Rac1^{-/-} fibroblasts were maintained in DMEM, 4.5 g/l glucose supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate as described (Steffen et al., 2013). Chinese hamster lung fibroblasts “Don Q” cells and the revertants “G3” were generously provided by Monica Thelestam, Karolinska Institute, Stockholm, Sweden (Chaves-Olarte et al., 1996). Don Q and G3 were kept in Minimal Essential Medium with Earle’s salts, 5 mM L-glutamin, 10% fetal bovine serum, 100 µM penicillin, and 100 µg/ml streptomycin. All cell lines were subcultured three times a week. All experiments were performed using cells within the logarhythmic growth phase, if not stated otherwise.

Endpoints for cytopathic and cytotoxic effects of TcdB

Cell rounding and pyknosis

Cell rounding is recognized as the cytopathic effect, since even in completely rounded cells, viability is not necessarily decreased. In contrast to rounding up of cells, a second phenotype termed as pyknosis is associated with early cell death (cytotoxic effect). This phenotype is characterized by condensed chromatin and blistering and excludes cell rounding (Wohlan et al., 2014). These cells integrate DAPI into their nucleus as a sign of disintegration of the plasma membrane accompanied by loss of viability. Thus, DAPI incorporation can be used for detection
of early cell death (pyknotic effect) within the first hours of toxin treatment before apoptosis is executed. To determine percentages of rounded versus pyknotic cells, we either counted the total number of cells and the number of rounded/pyknotic cells in phase contrast images, or performed DAPI incorporation assays. Therefore, we added DAPI at a final concentration of 200 nM to cells for 10 min. After this, cells were subjected to microscopy (Leica Inverted-2). Phase contrast and subsequent fluorescence microscopy were performed in identical field of views, and rounded cells as well as DAPI-positive nuclei were counted and expressed as percentage of total cells or number of cells per area. DAPI incorporation assay was performed when large numbers of cells have to be counted for quantitative analyses. Pyknotic cells determined by morphotype were counted when qualitative analyses were preferred.

**pH-shift assay**

HEp-2 cells were seeded in 96 well plate and grown over night to 80 % confluency. For pH-shift assay cells were cooled on ice and medium was changed to precooled medium supplemented with 500 nM bafilomycin A1 containing the indicated toxin at given concentration. Cells were incubated for 15 min on ice to allow toxin binding. Then, medium was aspirated and pre-warmed HEPES solution (20 mM HEPES, pH 4.8, 110 mM NaCl, 10 mM KCl, 37°C) was added to cells. Cells were incubated at 37°C for three min. HEPES solution was removed and pre-warmed cell culture medium supplemented with 500 nM bafilomycin A1 was added to cells. After 4h incubation at 37°C pyknotic cells were detected by DAPI incorporation. Cell rounding was controlled by phase contrast microscopy. For control, a pH-shift assay with HEPES solution pH 7.4 was done in parallel to show complete inhibitory effect of bafilomycin A1.
**Cell viability determined by LDH-release and WST-assay**

To measure cell viability (either LDH-release or WST-assay), especially loss of cell viability by ECD, HEp-2/HEK293 cells were seeded into 96-well plates at a density of $10^4$ cells per well and cultured overnight. Cells were treated as indicated in results and cultured for four hours or as indicated otherwise. Extracellular LDH as indicator of cell lysis or membrane disintegration was determined by using the LDH kit, according to the protocol supplied. The metabolic activity of cells as marker for viability was also determined by performing WST-assay. Cell viability as measured by LDH release is expressed as percentage of total LDH determined after release induced by triton X-100. WST-1 and LDH assays were performed for complex sets of experiments with high numbers of replicates for simultaneous end point analysis.

**DHE staining for $O_2^{-}$**

Determination of reactive oxygen species was done indirectly by staining of cells with dihydroethidium (DHE). DHE, oxidized by ROS, binds to DNA and changes fluorescence to red. To detect ROS production, cells were treated with TcdB NXN and additionally incubated with DHE (10 µM) for 60 min intervals during the time course of toxin treatment starting at time point 0, 1, 2, or 3 hours after addition of toxin. After 60 min incubation with DHE, fluorescent nuclei and pyknotic cells were counted. To do this, medium was exchanged with PBS and fluorescent nuclei were documented by fluorescence microscopy at an emission wavelength of 610 nm after excitation at 535 nm. Phase contrast microscopy was performed in identical field of views to determine pyknotic and total number of cells. Microscope and camera exposure settings were kept constant between experiments to enable comparability.
of samples. Cells with typical pyknotic morphology (blisters, condensed nuclei) were considered as pyknotic positive.

**Western blot analyses**

Binding assay of toxins was done with cells seeded in 6-wells. After treatment of cells with 6 nM toxin at 4°C for 30 min cells were washed three times with PBS and lysed in 100 µl Laemmli buffer. After brief sonication, lysates were boiled for 3 min and subjected to SDS-PAGE. Proteins were transferred from gels onto nitrocellulose membranes by semi-dry blotting, and nitrocellulose membranes blocked with 5% [w/v] milk powder in TBS-Tween afterwards. Nitrocellulose membranes were incubated with primary antibodies in TBS-Tween at 4 °C overnight. The appropriate horseradish-conjugated, secondary antibodies were employed by incubating for 45 min at room temperature. Proteins were detected by ECL reaction and documented by Kodak Image Station. Densitometrical evaluation of chemiluminescence signals was performed using Kodak 1D software.

**Pull down assay**

Active Rac1 was measured by pull down assay using immobilized GST-PAK-CRIB domain, which binds active, GTP bound Rac1. HEp-2 cell lysate was obtained by lysis of ~50% confluent cells from petri dishes (6 cm diameter) in 0.75 ml fishing buffer (Schoentaube *et al.*, 2009). Lysates were centrifuged at 10,000 x g for 5 min, and the supernatant was used for pull down by addition of approximately 10 µg of immobilized GST-PAK-CRIB domain, which were incubated for 45 min on a rotary wheel. GSH-sepharose with immobilized GST-PAK-CRIB domain was harvested by
centrifugation at 8,000 x g for 1 min and washed with fish buffer. 15 µl of Laemmli-
buffer concentrated 2-fold were added to the remaining precipitate, and proteins
denatured by incubation at 95°C for three minutes. The complete sample as well as
lysate samples for total Rac1 was loaded onto SDS gel and subjected to immunoblot
analysis.

Mass spectrometry analysis of nucleotide sugars

Cells were seeded onto petri dishes (3.5 cm diameter) and grown over night to reach
50-80% confluency. Cells were kept in their respective culture medium and treated
as indicated. Incubation was stopped by washing cells once with ice cold PBS. PBS
was removed and cell content extracted by addition of 300 µl extraction solution
(acetonitrile/methanol/H2O: 2:2:1 v/v/v) containing 250 ng/ml of internal standard.
Cells were scraped off from well bottom, and wells rinsed with additional 400 µl
extraction solution twice. Suspensions were pooled, heated for 15 min at 95°C and
kept at -20°C to precipitate proteins. Precipitates were pelleted by centrifugation at
16,000 x g, followed by removal of 80 µl of the supernatants for mass spectrometric
analyses. For this, supernatants were dried at 40°C under constant flow of nitrogen.
Dried pellets were resuspended in 100 µl H2O and centrifuged at 16,000 x g.
Subsequently, 50 µl of each supernatant were subjected to liquid chromatography for
quantification by analysis with coupled MS/MS (5500QTRAP). Precipitated protein
pellets from acetonitrile/methanol/H2O extraction were re-suspended in 500 µl 0.1 M
sodium hydroxide solution, heated for 15 min at 95°C, and protein concentrations
measured using Bradford assay. The level of UDP-glucose was calculated by the
ratio of area under the curve and protein amount of sample (AUC*mg⁻¹ protein).
Statistics
All statistics were performed using GraphPad Prism software 5.02 (GraphPad Software, San Diego, CA, USA, 2008). For statistical significance, a two-tailed *t*-test was performed and significance was set at a *p*-value of <0.05 (indicated by *), *p*-Value of <0.01 is indicated by **. Data are presented as arithmetic means ± standard deviations.

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Contributions
LAB evolved study concept, performed experiments, worked on the manuscript; HT cloned constructs, performed experiments; NR, AO and SG cloned constructs and validated toxin mutants; MT performed experiments; AKA and KR provided essential materials and worked on the manuscript; RG designed the study, performed experiments, and wrote the manuscript.

Legends to figures

**Fig. 1:** TcdB-induced early cell death depends on ROS. A) Micrographs show HEp-2 cells either untreated or treated for 4h with cytopathic concentration of TcdB
leading to cell rounding or treated with cytotoxic concentration (3 nM), leading to pyknosis, characterized by blistering and chromatin condensation. B) DAPI exclusion assay indicates loss of cell viability after 4h treatment in pyknotic cells but not in rounded cells. Shown are representative overlays of phase contrast and fluorescence micrographs of DAPI-positive nuclei. Apocynin, an inhibitor of the NADPH oxidase, reduces numbers of pyknotic cells when treated with TcdB. C) Cell rounding (grey circles) and pyknotic/DAPI positive cells (blue circles) induced by incubation for 4h with TcdB inversely correlate (left panel). Inhibition of ROS production by apocynin reduces number of pyknotic cells in favor of cell rounding (right panel) (means±SD, n=4). D) Kinetics of ROS production after application of 3 nM TcdB NXN measured in 60 min intervals. E) Inhibition of TcdB-induced early cell death by delayed application of apocynin. Asterisks indicate significant reduction of DAPI-positive cells compared to the 210-min value. F) Pull down assay showing active and total Rac1 in HEp-2 cells after 5-min, 20-min, and 210-min treatment with indicated concentrations of TcdB and TcdB NXN. Concentration of TcdB NXN was adjusted to obtain a pyknotic effect equivalent to TcdB. G) Graphical evaluation of active Rac1 relative to mean values of controls as determined by pull down assay. Shown are means±SD, n=3-7.

Fig. 2: Rac1 is required for induction of early cell death. A) TcdB (0.03 nM) induced cell rounding in Rac1^{fl/fl} mouse embryonic fibroblasts and also induced pyknotic phenotype in Rac1^{fl/fl} at 3 nM. TcdB at 0.03 nM and 3 nM only induced cell rounding in Rac1^{-/-} mouse embryonic fibroblasts. B) Statistical evaluation of pyknotic cells in Rac1^{fl/fl} (black bars) and Rac1^{-/-} cells (grey bars) treated with 3 nM TcdB (means±SD, n=4, p<0.01). C) Inhibition of Rac1 by pre-incubation of HEp-2 cells with 0.03 nM chimera B(F)-B_{ref} completely protects from 3 nM TcdB NXN-induced early cell death,
as measured by DAPI incorporation assay. D) UDP-glucose levels in HEp-2 cells.
DonQ cells (CHO strain lacking functional UGT1) and the revertant G3 of DonQ were
used as controls for different UDP-glucose levels. E) TcdB concentration-
dependently induced rounding up and pyknosis in the revertant G3 cells compared to
unchanged morphology. F) Quantification of normal, rounded and pyknotic DonQ and
G3 cells after treatment with TcdB. In contrast to revertant G3 cells, DonQ cells
showed less than 20% TcdB-induced cell rounding. The glucosylation-independent
pyknosis, however, was comparable to that observed in G3 cells (means±SD, n=6).

Fig. 3: Translocation and Rac1 glucosylation determine cytotoxic effect of TcdB. A)
Pairwise alignment of TcdB from different *C. difficile* strains with differences in amino
acids indicated by red lines. TcdB VPI10463: reference strain for historical toxin,
toxinotype 0. TcdB 1470: historical strain for variant toxin with different substrate
specificity (Rac1, Cdc42 and R-Ras), toxinotype VIII. TcdB R20291: toxin from
hypervirulent strain, RT027, toxinotype III. TcdB R9385: variant toxin with different
substrate specificity (Rac1 and H,K,N-Ras) from hypervirulent strain, toxinotype
XIVb. Note that the trunks of TcdB from hypervirulent strains R20291 and R9385 as
well as the glucosyltransferase domain (GTD) of variant TcdB from strains 1470 and
R9385 were considered as identical. Abbreviations: CPD: cysteine protease domain;
DD: delivery domain; RBD: receptor binding domain; CROP: combined repetitive
oligopeptides. B) Cytotoxic effect of chimeras (3 nM) on HEp-2 cells measured by
LDH release. The glucosyltransferase-deficient chimeras (B^{NXN-B}_{ref}, B^{NXN-B}_{hyp}) were
more cytotoxic than their glucosyltransferase-proficient counterparts. The chimeras
with trunk of toxin from hypervirulent strains were more cytotoxic than their
counterparts from reference strain (mean±SD, n = 8; p<0.01). C) Only the
glucosyltransferase-deficient chimeras (3 nM) of variant TcdB provoked significant
LDH release. D) pH-shift assay for translocation of toxins through plasma membrane.

Glucosyltransferase-deficient TcdB NXN but not TcdA NXN induced pyknosis after pH 4.8 shift-mediated entry into cells. Wild type TcdB induced pyknosis only when endocytosed but not by pH shift mediated translocation. All toxins except their glucosyltransferase-deficient NXN-mutant induced cell rounding, independent of their mode of cell entry.

Fig. 4: Facilitated endocytic uptake enhances pyknosis. A) TcdB (3 nM) induced cell rounding and pyknosis in HEp-2 cells whereas TcdB 1-1852 (6 nM) lacking the CROPs only induced cell rounding. Only full length TcdB significantly reduced cell viability, as measured by WST-assay. Statistical evaluation of eight separate experiments is shown in the right bar chart. B) Effect of chimeric B-B<sub>hyp</sub> on mock (mCherry) and mCherry-ClC-5 transfected HEK293 cells. ClC-5 supports vesicular H-ATPase in endosomal acidification. Pyknotic cells were detected in ClC-5 transfected cells treated with 6 nM of the chimera TcdB<sub>VPI10463</sub> 1-542-TcdB<sub>R9385</sub> 543-2366 (B-B<sub>hyp</sub>) but not in mock transfected cells. The right panel shows the statistical analysis of WST-assay, displaying cell viability of mock and ClC-5 transfected HEK293 cells treated with chimera B-B<sub>hyp</sub> (6 nM). Shown are means±SD, n=8. C) Binding assay revealed no difference in TcdB R20291 binding to the surface of mCherry transfected cells and mCherry-ClC5-transfected cells in immunoblot analysis with polyclonal anti-TcdB rabbit serum (left panel). Shown is one representative blot of three independent experiments. Densitometrical evaluation of bound TcdB to β-actin as protein load is shown in bar chart (means±SD, n=3).

Fig. 5: Sodium and chloride gradients modulate cytotoxic effect of TcdB in HEp-2 cells. A) Effect of NaCl concentration on cytotoxic effect of TcdB. B) Inhibition of
sodium/proton exchanger NHE reduced cytotoxic effect in high NaCl medium (MEM).

C) Specific supplementation of Na\(^+\) (NaHCO\(_3\)) or of Cl\(^-\) (choline chloride) significantly increased cytotoxic effect in low NaCl medium (IMDM). D) Increased NaCl concentration but not equi-osmolar sorbitol increased cytotoxic effect in low NaCl medium (IMDM).

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Table 1: Table 1 shows the composition of chimeras with the amino acids of fragments of TcdB from different *C. difficile* strains.

| Short name | GTD (amino acids) | Trunk (amino acids) | equivalent to |
|------------|------------------|---------------------|---------------|
| B - B<sub>ref</sub> | 1-540 (VPI 10463) | 541-2366 (VPI 10463) | historical TcdB VPI10463 |
| B(F) - B<sub>ref</sub> | 1-541 (1470) | 541-2366 (VPI 10463) | historical TcdB(F) 1470 |
| B(F) - B<sub>hyp</sub> | 1-541 (R9385) | 541-2366 (R9385) | hypervirulent strain TcdB(F) R9385 |
| B - B<sub>hyp</sub> | 1-540 (VPI 10463) | 541-2366 (R9385) | hypervirulent strain TcdB R20291 |