High temporal resolution of glucosyltransferase dependent and independent effects of *Clostridium difficile* toxins across multiple cell types

Kevin M. D’Auria1, Meghan J. Bloom1,2, Yesenia Reyes2, Mary C. Gray2, Edward J. van Opstal2,3, Jason A. Papin1,4, Erik L. Hewlett2,4

1 Department of Biomedical Engineering, University of Virginia

PO Box 800759

Charlottesville, VA 22908

2 Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia

PO Box 801340

Charlottesville, VA 22908

3 Current address: Vanderbilt University School of Medicine.

340 Light Hall

Nashville, TN 27232

4 Corresponding author. Equal contribution.

KD: kd3jd@virginia.edu

MB: mjb8vv@virginia.edu

YR: yesenia.reyes@utdallas.edu

MG: mrc6r@virginia.edu

EO: edward.j.van.opstal@vanderbilt.edu

JP: papin@virginia.edu

EH: eh2v@virginia.edu

**ABSTRACT**

**Background**

*Clostridium difficile* toxins A and B (TcdA and TcdB), homologous proteins essential for *C. difficile* infection, affect the behavior and morphology of several cell types with different potency and timing. However, precise morphological changes over various time scales, which help explain the roles of cell types, are poorly characterized. The toxins’ glucosyltransferase domains are critical to their deleterious effects, and cell responses to glucosyltransferase-independent activities are incompletely understood. By tracking morphological changes of multiple cell types to *C. difficile* toxins with high temporal resolution, newly characterized cellular responses to TcdA, TcdB, and a mutant, glucosyltransferase-deficient TcdB (gdTcdB) are elucidated.

**Results**

HUVECs, J774 macrophages, and four epithelial cell lines were treated with TcdA, TcdB, and gdTcdB. Impedance changes across cell cultures were measured to track changes in cell morphology. Metrics from impedance data, developed to quantify rapid and long-lasting responses, were used to build standard curves with wide dynamic ranges that defined cell line-specific toxin sensitivities. Except for T84 epithelial cells, all cell lines were more sensitive to TcdB than TcdA. Macrophages rapidly stretched and arborized, and then increased in size in response to TcdA and TcdB but not gdTcdB. High concentrations of TcdB and gdTcdB (>10 ng/ml) resulted in loss of intact macrophages. In HCT8 epithelial cells, gdTcdB (1000 ng/ml) elicited a cytopathic effect only after several days, yet it was capable of delaying TcdA and TcdB’s rapid effects. gdTcdB did not delay TcdA’s stimulation of macrophages.

**Conclusions**

Epithelial and endothelial cells have similar responses to toxins yet differ in timing and degree. Relative potencies of TcdA and TcdB in mouse epithelial cells *in vitro* do not correlate with potencies *in vivo*. gdTcdB is not entirely benign in HCT8 cells. TcdB requires glucosyltransferase activity to stimulate macrophages, but cell death from high TcdB concentrations is glucosyltransferase-independent. Competition experiments with gdTcdB show TcdA or TcdB round HCT8 cells through common mechanisms, yet macrophages are stimulated through potentially different pathways. This first-time, precise quantification of multiple cell lines provides a comparative framework for contextualizing previous research and delineating the roles of different cell types and toxin-host interactions.

**Keywords**

*Clostridium difficile*, Toxin A, Toxin B, glucosyltransferase, Epithelial, Endothelial, Macrophage

**INTRODUCTION**

*Clostridium difficile* infections, with an annual occurrence in the US of over 300,000, cause potentially fatal diarrhea and colitis [1]. These pathologies arise from the release of two potent, homologous, protein toxins—TcdA and TcdB—into the host gut. The toxins’ interactions with many cell types lead to disease, yet the relative sensitivities and roles of different cell types remain poorly understood. Both toxins disrupt the epithelial barrier by causing epithelial cells to round and detach [2]. Neutrophil infiltration and activation of other immune cells, driven by inflammatory signals, are also key to toxin-induced enteritis [3]. Though several molecular mediators of disease have been identified, little is understood about the host cell dynamics and the role of each cell type involved [4, 5]. To explore the toxins’ effects on different cells, facets of the host response have been studied using cell lines treated with TcdA and/or TcdB (e.g., release of cytokines [4, 6, 7], changes in cell morphology [8, 9], gene expression [10, 11], and cell death [12, 13]). Most of these assays used in previous studies are limited to few time points, and since both toxins affect cells rapidly (in less than one hour), it is unknown if either toxin has additional effects on finer time scales and if any of these effects are consistent across cell lines at comparable concentrations.

We and others have tracked temporal changes in cell morphology and attachment in response to TcdA or TcdB by continuously measuring electrical impedance across the surface of a cell culture [14-16]. When cells grow or increase their footprint or adherence, impedance rises. In contrast, cell rounding, shrinking, and/or death correspond to decreased impedance. This assay has primarily been used as a sensitive diagnostic—as a more quantitative replacement of assays that are dependent on visualization of cell rounding. In this study, we recognize that this impedance data, in addition to detecting toxin, can further be analyzed to reveal previously unrecognized, dynamic responses of host cells. Our analyses and associated metrics also allow precise comparisons between the effects of TcdA and TcdB and between different cell types. Using epithelial and endothelial cells, these analyses identify characteristics such as the minimal effective toxin concentrations and the shortest time to measurable toxin effects; standard curves with wide dynamic ranges can also be derived. Impedance changes of other cells, such as macrophages, are not as easily linked to known cell functions, but the data reveal toxin effects that would not otherwise be observed at lower temporal resolution. This knowledge contextualizes the potential roles and relative abilities of different cell types to respond directly to toxin during an infection

Impedance curves that profile cell responses also provide insight into the toxins’ molecular functions. TcdA and TcdB have glucosyltransferase domains that inactivate small GTPases. With the use of engineered mutant toxins, glucosyltransferase activity has been found necessary for cell rounding [17]. However, evidence that some glucosyltransferase-deficient mutants of TcdB (gdTcdB) are cytotoxic has raised questions about whether there are other, previously unknown toxin activities [18]. In order to identify changes dependent and independent of glucosyltransferase activity, we use gdTcdB to evaluate the dynamics of the response of macrophage and epithelial cell lines to gdTcdB, elucidating changes dependent and independent of glucosyltransferase activity. We also leverage the unique response profiles to TcdA, TcdB, and gdTcdB in order to investigate synergy or antagonism between toxins.

The cell response profiles define the dynamics of basic changes in cell physiology (e.g., cell rounding) across multiple cell types in response to TcdA, TcdB, and gdTcdB. This understanding identifies those times most representative of the entire cell response, delineates the contribution of glucosyltransferase activity to overall toxin effects, and suggests the relative roles of various cell during toxin-mediated disease.

**METHODS**

**Cell culture**

HCT-8 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate. J774A.1 cells were cultured in DMEM high glucose media supplemented with 10% FBS, 1 mM, and MEM nonessential amino acids (Gibco 11140). HUVEC cells (passage 3) were cultured in endothelial growth medium (EGM-Bullet Kit CC-3124, Lonza group). T84 cells were grown in an equal mixture of Ham’s F12 and Dulbecco’s modified Eagle’s media supplemented with 2.5 mM L-glutamine and 5% FBS. All cells were incubated at 37°C/5% CO2. In our analyses, we include our previous data from immortalized, mouse, cecal epithelial cells (hereon referred to as IMCE cells) which were derived by Becker et al. and incubated at 33°C as described by Becker et al. [16, 19]. TcdA and TcdB, isolated and purified from strain VPI-10643, were a generous gift from David Lyerly (TECHLAB Inc., Blacksburg, VA). Recombinant gdTcdB and TcdB were a generous gift from the laboratory of Aimee Shen.

**Electrical Impedance Assay**

Impedance was measured using the xCELLigence RTCA system (ACEA Biosciences), which consists of an RTCA DP Analyzer and 16-well E-plates. PBS was filled around all wells to prevent evaporation. In each well, 100 uL media was incubated at room temperature for 30 minutes, and one baseline reading was taken. Cells in 100 uL media were then added and allowed to settle at room temperature for 30 minutes. Plates were then moved inside the RTCA DP Analyzer inside a CO2 incubator at 37°C. Subsequent readings were taken at frequencies ranging between every 4 seconds to every 10 minutes, with higher frequency measurements reserved for times directly before toxin addition to at least 6 hours after addition (complete protocols and data files available in the Supplemental Material).

Since the impedance measurements are sensitive to slight movements or vibrations, the method by which toxin was added to cells was an important consideration. In our initial experiments, mechanical agitation and replacement of media sometimes caused small, sharp spikes in electrical impedance. To minimize disturbances, plates were not removed from the RTCA Analyzer once seated. Toxins prepared in media (10x) were gently pipetted using only one to two depressions. Media was not replaced after the addition of toxin.

**Analyses**

The protocols, data, computer code, and instructions for running the code that reproduce all results and figures are provided in the Supplemental Material.

**RESULTS**

**Quantification of the cytopathic effects elicited by TcdA and TcdB**

In order to assess the cytopathic effects of TcdA and TcdB, we measured changes in impedance across the surface of electrode-embedded wells (Methods). Impedance is dependent upon cell number, adherence, and morphology. It increases as cells proliferate or spread and decreases when toxin is added and cells round up (Figure 1). The rate at which impedance decreases is dependent on the toxin, toxin concentration, and cell type (Figures 2A and 2B). To summarize the data-rich “impedance curves”, we calculated simple metrics: the area between the curves of control and toxin-treated cells (ABC, gray area in inset of Figure 1), the maximum slope of a curve (MaxS), and time for a curve to decrease by 50% (TD50, Figure 1). A negative ABC indicates that the impedance curves of toxin-treated cells are below the curves of untreated cells. Blue, dashed lines in Figure 2C show the variability of the ABC of control cells from their average impedance curve. Standard curves relating TD50 to toxin concentration have been generated before [20], and we found that our metrics, ABC and MaxS, also produce log-linear calibration curves (Figure 2C). Among replicates, differences in timing translated to differences in TD50, as expected, whereas MaxS values were more similar. In simpler terms, for replicates within and between experiments, the time required to observe a change in impedance was more variable than the rate of the change. For this reason, we found that MaxS, instead of TD50, better quantified rapidity of the cell response to high toxin concentrations. The other metric, ABC, captures long-term effects by integrating readings over several hours. The minimal concentration to induce a change in impedance from control is denoted as the minimal cytopathic concentration (MCC; Figure 2C). When ABC and MaxS are considered together, toxin concentration can be determined with a dynamic range spanning six orders of magnitude or more (depending on toxin and cell type). Together, these metrics allow for millions of data points and hundreds of wells to be simultaneously visualized and summarized to dozens of numbers or fewer that can be easily interpreted (e.g., Figure 2D and Supplement).

**Epithelial and endothelial cells: similar characteristic responses but different sensitivities to TcdA and TcdB**

In the first set of comparisons, we chose four well-characterized cell types or cell lines—one endothelial (HUVECs) and three epithelial (CHO, HCT8, and T84)—and one immortalized, cecal, mouse epithelial cell line (IMCE, see Methods). For these five cell lines, the MCC for TcdA and TcdB varied over ranges of 0.1-1 ng/ml and 0.1-100 pg/ml, respectively (Figure 2D). We did not find a maximal effective concentration of either toxin (1 μg/ml was the highest concentration tested). TcdB was consistently 100-1000 times more potent than TcdA, except in T84 cells, which were equally sensitive to TcdA and TcdB (as measured by MCC). The curves were largely similar in that they all consisted of a short delay followed by a sharp decrease that then leveled off (Figure 2A); differences were primarily in scale. Determining the time to the onset of the first toxin effects was complicated slightly by the physical process of adding toxins to wells—a process which caused disturbances that temporarily affected impedance (note the early “bump” in Figure 2A). Nevertheless, differences between control and toxin-treated cells can be distinguished. Across all cell types, the time required for an impedance curve to diverge from control was more than ten minutes. Nothing clearly suggested an immediate response to toxin binding. Morphological changes might not occur until after toxins enter cells and glucosylate Rho proteins. We next examined early effects of toxins on macrophages and investigated the contribution of glucosyltransferase activity to the dynamics of cell responses.

**Macrophages: rapid, sensitive, complex concentration-dependent responses to TcdA and TcdB**

J774 mouse macrophages were as sensitive and responsive to TcdA and TcdB as epithelial cells. The impedance of macrophages treated with TcdA (300 ng/ml) and TcdB (10 ng/ml) diverged from controls in 10 and 20 minutes, respectively (Supplement). In contrast to epithelial cells, however, the impedance of macrophages increased after toxin addition (Figure 3), and the responses of J774 cells to TcdA and TcdB differed in shape and scale. TcdA caused a rise in impedance at 0.1 ng/ml, and the magnitude and speed of this rise increased until TcdA concentration reached 100 ng/ml (Figure 3A). At higher concentrations, the slope of the rise continued to increase, yet the rise was inhibited, as if stopped prematurely before reaching its peak, and then impedance dropped below that of control cells (Figure 3A). Considering now TcdB, concentrations between 0.1 and 10 ng/ml caused impedance to rise and stabilize at more than double the initial value; only the slope of the rise (not the final height) was affected by toxin concentration (Figure 3A). These curves allowed us to resolve time points that would be of most interest for cell imaging. Increases in impedance, for TcdA and TcdB, correlated with rapid stretching and arborization of cells (appearance of many filopodia in Figure 3B) that suggest macrophage activation. Over the next 48 hours, cells increased in size and became more circular (Figure 3B). Subsequently for TcdA, decreased impedance correlated with a decrease in intact cells (Figure 3B). These results support complex dynamic responses of J774 cells to different toxin concentrations or a response driven by two or more cellular functions (e.g. activation and apoptosis are known to occur in monocytes and macrophages in response to TcdA and TcdB [12, 21-23]).

At concentrations of TcdB above 10 ng/ml, the impedance curves were entirely different than lower concentrations. Instead of rising, impedance fell because of a nearly complete cell death (see loss of intact cells in bottom row of images of Figure 3B). Hence, between 10 and 100 ng/ml, the response of macrophages to TcdB switches from a stimulatory response to death. Because this effect was so complete and rapid, we hypothesized that the glucosyltransferase activity of TcdB, when above 100 ng/ml, is not necessary to induce cell death. We used gdTcdB to investigate this. First, to better understand the effects of gdTcdB, we examined its ability to induce the well-known cytopathic effects of TcdA and TcdB in epithelial cells.

**Glucosyltransferase-deficient TcdB alters the effects of TcdA and TcdB on macrophages and epithelial cells**

Since the cytopathic effects of TcdA and TcdB have been attributed to their glucosyltransferase activities, we expected that gdTcdB would not cause cell rounding. Indeed, the impedances of gdTcdB-treated and untreated HCT8 cells were indiscernible in the first ten hours after toxin addition (Figure 4). However, gdTcdB caused an unexpected slow rise in impedance above that of control cells (Figure 4A). Imaging revealed continued growth, elongation, and close apposition of untreated cells, while gdTcdB-treated cells rounded slightly but remained attached (Figure 4A). The increased impedance elicited by gdTcdB was followed by a slow decrease towards that of TcdB-treated cells. This decrease, which took more than six days, was due to detachment of cells and disruption of cell morphology (Figure 4A). Hence, though gdTcdB does not round cells quickly as with TcdB, it still has unexplained, slow effects that alter the morphology of HCT8 epithelial cells.

To investigate if TcdA and TcdB have overlapping activity, we performed experiments with gdTcdB plus TcdA or TcdB. We anticipated that gdTcdB would attenuate or delay the effects of TcdB and perhaps TcdA. Indeed, a tenfold excess gdTcdB delayed the onset of the effects of TcdA and TcdB (Figures 4B and 4C). Competition for shared substrates (Rho family proteins) of gdTcdB with TcdA and TcdB likely account for the delay, although other factors such as shared receptors may be responsible.

We next determined glucosyltransferase-dependent toxin effects on J774 macrophages. Low gdTcdB concentrations did not stimulate macrophages as did TcdB (Figure 5A). However, gdTcdB at or above 100 ng/ml killed macrophages as did TcdB at 100 ng/ml (Figure 5A). Hence, glucosyltransferase activity is required for macrophage stretching and arborization but not required for cell death at concentrations of TcdB at or above 100 ng/ml.

Since TcdA and TcdB caused different effects at high concentrations, we hypothesized that TcdA and TcdB have one or more distinct activities in macrophages. As expected, gdTcdB delayed the effects of TcdB on J774 cells (Figure 5B). However, gdTcdB did not clearly attenuate or delay the response of J774 cells to TcdA, suggesting that the prominent responses to TcdA and TcdB in these cells are due to distinct toxin activities or substrates (Figure 5C).

**DISCUSSION**

In this study we systematically profiled the dynamic responses of epithelial, endothelial, and macrophage cell lines to TcdA and TcdB, revealing relative sensitivities and complex concentration-dependent cell responses. While comparing results from different experimental systems is difficult, our data have allowed quantitative comparisons between cell types and between toxins under similar conditions, distinguishing which cell types may respond most quickly or most intensely when exposed directly to toxins. The impedance “response profiles” provide continuous readouts representing external changes in morphology and adherence that occur from several possible functions within the cell. We began to explore the mechanisms of these changes by using glucosyltransferase deficient TcdB (gdTcdB), revealing which molecular functions of the toxin contribute to different aspects of response profiles. The response profiles also raise many questions about the mechanisms for the novel differences we observed. Although addressing each of these in detail is beyond the scope of this study, we highlight, in the following text, the findings that bring about these questions, discuss their relevance to previous studies, and so explain how they improve our current understanding of host cell responses to TcdA and TcdB.

The cytopathic effects of TcdA and TcdB that led to their discovery are still used as the gold standard diagnostic for infection [24, 25]. Since most cytotoxicity assays are endpoint assays, the kinetics of these effects that are key to scientific research and clinical practice have not been characterized. With a continuous assay, we were better able to observe immediate effects of toxin. Although toxins may interact immediately with the toxin surface, the morphological differences (represented by impedance) occurred after a delay of ten minutes or more. Since TcdA (2.65 μg/ml) has been found to enter HT29 cells in 5-10 minutes, the delay we observed is likely because toxins must enter HCT8 cells to alter their morphology [26].

Epithelial and endothelial cell lines had the same characteristic changes in morphology, yet the rapidity of the changes distinguished different cell types, toxin concentrations, and TcdA versus TcdB. These differences could be summarized by condensing the data into metrics that represented the greatest rate of the change (MaxS) and the cumulative amount of change over several hours (ABC). When these metrics are considered together, standard curves over many orders of magnitude can be used to measure toxin concentration and determine the minimal amount of toxin necessary to induce an effect (MCC, Figure 2D). The CHO cell line was second-most sensitive to TcdB, making CHO cells a good choice for toxin detection. Indeed, a modified CHO cell line was used in the development of an ultrasensitive assay of toxin activity [14]. T84 cells, the least sensitive to TcdB, were similarly sensitive to TcdA and TcdB, as has been found previously [27]. For TcdB, the two rodent cell lines (CHO and IMCE) were more sensitive than the three human cell lines (HCT8, HUVEC, and T84), although more cell lines would be needed to confirm any species-specific sensitivity. For TcdA, cell line sensitivities were less variable than for TcdB, indicating that factors that make cells vulnerable to TcdA may be more consistent between cell lines.

Comparisons between TcdA and TcdB have often been a prominent research focus. TcdB is more cytotoxic in cell culture; TcdA is more enterotoxic in animal intoxication models [16, 28]; and there are varying results about which toxin is essential for *C. difficile* infection [29, 30]. Identifying which toxin contributes most to disease helps prioritize therapeutics. Comparisons between toxins are also valuable scientific tools. Differences in the toxins’ effects provide clues about their molecular activities. Also, by correlating differences in host cell responses to differences in disease severity, particular cell types or toxin activities can be prioritized. For instance, TcdA is more enterotoxic than TcdB in mice and hamster ceca, damaging the epithelial barrier [16, 31]. This agrees with findings that TcdB binds weakly in the hamster intestine, and TcdA binds epithelial cells [32, 33]. One might then expect that cecal epithelial cells from mice of the same genetic background as those used in the aforementioned *in vivo* studies (IMCE cells) would be more sensitive to TcdA than TcdB. Instead, IMCE cells were over 100 times more sensitive to TcdB than TcdA, suggesting that factors in addition to the cytopathic effects on epithelial cells are important in explaining the pathologies of toxins *in vivo*. The extracellular environment or other cell types may be the key mediators determining disease severity.

Macrophages are likely exposed to toxin after epithelial damage and play an important part in disease, changing morphology and releasing molecules that exacerbate inflammation [22, 34]. Previous studies have quantified the viability either TcdA- or TcdB-treated macrophages at one or two time points [21, 23, 35]. We characterized the effects of both toxins on macrophages, and the other cell types already presented, over many more concentrations and time points. J774 macrophages, HUVECs, and epithelial cells had similar sensitivity to toxins, indicating that all may be affected directly by toxins during disease. TcdA or TcdB rapidly stretched and arborized macrophages, which was reflected in increased macrophage impedance. However, the timing and concentration-dependent effects of TcdA and TcdB were different, as discussed below.

TcdA increased macrophage impedance, and a subsequent decrease in impedance correlated with a loss of intact cells. This agrees in part with Melo Filo et al. who reported that TcdA and TcdB killed 30% and 60%, respectively, of primary mouse macrophages (1 ug/ml at 24h) [23]. The balance of activation and death may therefore account for the rise and fall of impedance of TcdA-treated macrophages. At 100 ng/ml (the concentration at which the rise in impedance was greatest), two effects appear to be balanced. At higher concentrations, the stimulatory effect that raised the impedance occurred more rapidly but did not reach the same height, indicating that higher concentrations move the balance away from stimulation towards death and decreased adherence.

TcdB caused two distinct responses in J774 macrophages: stimulation (with “low” concentrations at or below 10 ng/ml) or death (with “high” concentrations above 10 ng/ml). Siffert et al. showed TcdB-treated, human macrophages arborize with little loss of viability (1ug/ml at 3h and 24h) [21]. This arborization corresponds with the morphological responses of J774 macrophages to low TcdB concentrations. It is possible that TcdB also causes two distinct response in human macrophages, but Siffert et al. only reported results at one concentration. Although much remains to be determined about the mechanisms of these effects, we have identified new characteristics of the dynamic responses of macrophages and these effects help to explain the role of macrophages during disease. In the intestine, macrophages likely respond to several signals begun during intoxication, and given their high sensitivity, may also respond directly to toxin in the intestine. Early stimulation of macrophages may contribute to acute inflammation, while eventual death correlates with macrophage depletion and neutrophil accumulation in *C. difficile* associated diarrhea [36].

The cell responses described above prompted questions about toxin mechanisms. For instance, microinjection of TcdB’s glucosyltransferase domain is sufficient to induce cytopathic effects, yet are there changes in cell structure independent of glucosyltransferase activity [37]? We found that gdTcdB raised the impedance of epithelial cells above controls, and this difference was observed visually by tightly packed, visibly distinct control cells versus a smoother monolayer and slightly rounded gdTcdB-treated cells. The mechanisms for these differences are unclear. It is possible another toxin activity is unmasked when the strong cytopathic activity is removed or that the mutant glucosyltransferase affects substrates differently. After several days, gdTcdB causes cytotoxic or cytopathic effects. Chumbler et al. found that glucosyltransferase mutants were cytotoxic to HeLa cells after only 2.5h [18]. The different cell types (HCT8 versus HeLa) and different glucosyltransferase mutants may account for the differences in timing. It is also possible that any residual glucosyltransferase activity of the mutant toxin is not revealed until several days after treatment. The effects of mutant toxins have never been assessed over such long time scales with such great sensitivity.

The relatively benign effects of gdTcdB in the first hours after addition to HCT8 cells allowed us to investigate the effects of gdTcdB in combination with TcdA and TcdB. Since TcdA and TcdB are homologous, one might expect that gdTcdB should interfere with TcdA. Indeed, gdTcdB delayed the cytopathic effects of TcdA and TcdB. A first interpretation of this result is that TcdA and TcdB compete for cell entry. However, two studies using truncated toxins found that (1) the C-terminal domain (which is believed to be necessary for toxin internalization) of TcdA does not inhibit the effects of TcdB and (2) the TcdB C-terminus inhibits neither TcdA or TcdB-induced cell rounding [38, 39]. Hence, at the point of cell entry, TcdA and TcdB likely do not interfere with one another. Since the glucosyltransferase domains of TcdA and TcdB have many of the same Rho-family proteins as substrates, another interpretation is that the toxins compete after internalization. In this scenario, our results would indicate that gdTcdB is processed by the host cell, and its glucosyltransferase domain is still capable of binding Rho proteins or is at least close enough to interfere with TcdA. Although gdTcdB-mediated changes have the potential to reveal interesting mechanisms independent of glucosyltransferase activity, the results overall confirm the central role of the glucosyltransferase domains in eliciting the rapid, full effects of TcdA and TcdB in epithelial cells. However, as described later, glucosyltransferase activity may not be required for all toxin effects in all cell types.

gdTcdB often delayed the onset of cytopathic effects by one hour or less. Without high temporal resolution, we would have likely missed the time window in which TcdB+gTcdB was different than TcdB alone. This has implications in other studies that wish to identify other host factors that enhance or attenuate toxin effects. Without precisely tracking changes in cell structure, several potential inhibitors of toxin effects could be missed.

Since macrophages detect a variety of antigens, one might expect that the responses to toxin might not be entirely dependent on glucosyltransferase activity. J774 macrophages were stimulated and/or died after TcdA and TcdB exposure, and these two effects could be separated into glucosyltransferase-dependent and -independent effects. The killing action of TcdB at high concentrations was not glucosyltranseferase-dependent, but the stimulation of macrophages required glucosyltransferase activity. The concentration of gdTcdB and TcdB necessary to kill macrophages was similar, 100 ng/ml. Below this concentration, TcdB, but not gdTcdB, stimulated macrophages and caused them to spread. These effects imply that TcdB, and perhaps TcdA, are able to interact with host macrophages to induce cell death by a new, unknown mechanism.

The high sensitivity of epithelial cells, endothelial cells, and macrophages to TcdA and TcdB suggests that all of these cells could be damaged by direct toxin interaction in the host. However, the amount and location of toxin during infection is very poorly understood. With sensitivities of cells reaching as low as 100 pg/ml, tracking toxins by immunohistochemistry is technically challenging. Antibody labeling has only detected toxin on fixed, toxin-treated tissues with concentrations greater than 1 ug/ml [33]. Assessing sensitivities *in vitro* provides an indirect measure of the roles of different cell types in isolation. In addition to their direct effects, TcdA and TcdB initiate a cascade of deleterious events involving multiple cells. Neuronal signals have been implicated in beginning the disease process, stimulating mast cells or macrophages that may then recruit other cells [40-43]. Neutrophil infiltration is a hallmark of intoxication, yet neutrophils *in vitro* require much higher toxin concentrations than all other cell types to be affected or recruited (>1 μg/ml) [3, 9, 44-46]. Hence, it is thought that neutrophils are recruited by signals secondary to toxin damage [3, 4, 47]. To confirm the low toxin-sensitivity of neutrophils, we did attempt to measure impedance changes of neutrophils in response to toxins, yet the variability in these primarily non-adherent cells (impedance largely measures adherence) was too high to identify differences (Supplement). Elements of the toxin responses of other cell types (e.g., mast cells [48-50], dendritic cells[51, 52], neurons [53, 54], fibroblasts [55, 56], etc.) have been studied, yet the dynamics of their responses—and in many cases concentration-dependent effects—are unknown. In the future, precisely capturing the time and concentration-dependent responses to TcdA and TcdB will better contextualize their potential roles in the host. Our analyses of endothelial cells, epithelial cells, and macrophages in the same experimental framework set a precedent for such comparisons. Furthermore, we show how data from sensitive, continuous assays, could be used to gain insight into cell function and molecular mechanisms and generate new hypotheses. The framework and simple analyses may also be used to investigate synergy, antagonism, or interactions between bacterial toxins and other host factors that affect cells over a wide range of time scales.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

**AUTHOR’S CONTRIBUTIONS**

KD and MB performed computational and statistical analyses and drafted the manuscript. KD made all figures and wrote the supplement. KD, MB, YR, MG, and EO performed experiments. KD, JP, and EH conceived of the study. KD, MB, YR, MG, JP, and EH participated in the study design and coordination. All authors edited drafts and approved the final manuscript.

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**FIGURE CAPTIONS**

**Figure 1.** **Measurement of toxins’ cytopathic effects by tracking electrical impedance across the surface of a cell culture.** All impedance readings were normalized to the impedance at the time toxin was added. Shaded regions above and below lines represent the standard deviation of technical replicates (n=2). Readings were taken as quickly as every four seconds (Methods). The brightness of each photograph was adjusted digitally (uniformly across an entire photograph) to make the overall brightness across all photographs similar.

**Figure 2. Quantification of cytopathic effects.** **(A and B)** The cytopathic effects between cell types and toxins can easily be distinguished. **(C)** The impedance curves can be analyzed to produce two metrics, ABC and MaxS, which can then be used to define the minimal cytopathic concentration (MCC). **(D)** The MCC of TcdA and TcdB for five cell lines define cell line specific sensitivities.

**Figure 3. Macrophage responses to TcdA and TcdB. (A)** Impedance curves from a selection of toxin concentrations for TcdA and TcdB. Both graphs represents one multi-well experiment where confluent cells were treated with toxin. **(B)** Pairing of impedance data with photographs to show the morphological changes represented in the impedance data. Since wells with electrodes are opaque, technical replicates in transparent wells were used for microscopy. Sub-confluent cultures were used so that structural changes in individual cells could more easily be observed.

**Figure 4. Response of HCT8 epithelial cells to gdTcdB, TcdA+gdTcdB, and TcdB+gdTcdB.** **(A)** Impedance curves of HCT8 cells treated with gdTcdB and corresponding photographs. **(B)** HCT8 cells treated with TcdB or gdTcdB and TcdB in combination. **(C)** HCT8 cells treated with TcdA or gdTcdB and TcdA in combination. The three graphs are from the same multi-well experiment but are representative of three independent experiments.

**Figure 5. Response of J774 macrophages to gdTcdB, TcdA+gdTcdB, and TcdB+gdTcdB. (A)** Impedance curves of J774 cells treated with gdTcdB and corresponding photographs. Concentrations at or below 10 ng/ml are denoted as “low”, and other concentrations are denoted as “high”. This data is derived from the same experiment shown in Figure 3B. **(B and C)** J774 cells treated with TcdB; gdTcdB and TcdB in combination; or gdTcdB and TcdA in combination. The data in the three graphs are derived from three independent experiments.