INVESTIGATING *CLOSTRIDIOIDES DIFFICILE* CYTOTOXICITY IN THE PHYSIOLOGICALLY RELEVANT HUMAN INTESTINAL ENTEROID MODEL

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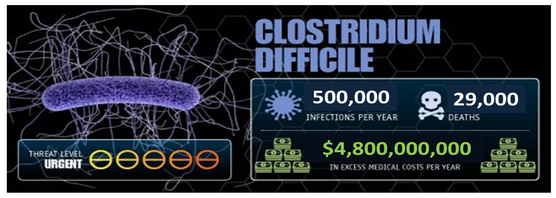
Abstract

Background: *Clostridioides difficile* is a nosocomial pathogen that produces toxins to cause life threatening diarrhea and colitis. Toxins bind cell surface receptors, are endocytosed, and inactivate GTP-binding proteins, leading to the collapse of the actin cytoskeleton. These cytoskeletal alterations produce a cell rounding phenotype that has been used as a measure of *C. difficile* toxin activity in a cancer-derived and immortalized cell lines. However, the biological relevance of these model systems is limited. To date, few studies have examined the distribution of *C. difficile* toxin receptors in the human small intestine or examined toxin sensitivity. We hypothesized that human intestinal enteroids (HIEs), as the most physiologically relevant in vitro model system available, expresses the native toxin receptors and provide a new model to dissect *C. difficile* cytotoxicity in the small intestine, providing insights into CDI-enteritis. **Methods & Results**: We generated biopsy-derived jejunal HIE and Vero cells which stably express LifeAct-Ruby, a fluorescently label of F-actin, to monitor actin cytoskeleton rearrangement by live-cell microscopy. Imaging analysis revealed that toxins from pathogenic *C. difficile* strains (R20291, 630, M68) elicited cell rounding in a strain-dependent manner. Interestingly, HIEs were tenfold more sensitive to toxin A (0.001 µg/ml) than toxin B (0.01 µg/ml). HIEs were also less sensitive to toxins derived from several *C. difficile* strains (R20291, 630, M68) or purified toxins when compared to Vero cells. By qPCR we paradoxically found that jejunal HIEs expressed greater quantities of toxin receptor mRNA compared with Vero cells, and yet exhibited decreased sensitivity to *C. difficile* toxins when compared to traditionally used cell lines. We reasoned that these differences may be explained by components, such as mucins, that are present in HIEs cultures that are absent in immortalized cell culture models. Addition of human-derived mucin MUC2 to Vero cells delayed cell rounding, indicating the mucus serves as a barrier to toxin-receptor binding. **Conclusions**: This work highlights that investigation of *C. difficile* infection in the human derived enteroid model can provide important insights into the intricate interactions between toxins and the human intestinal epithelium.

Background



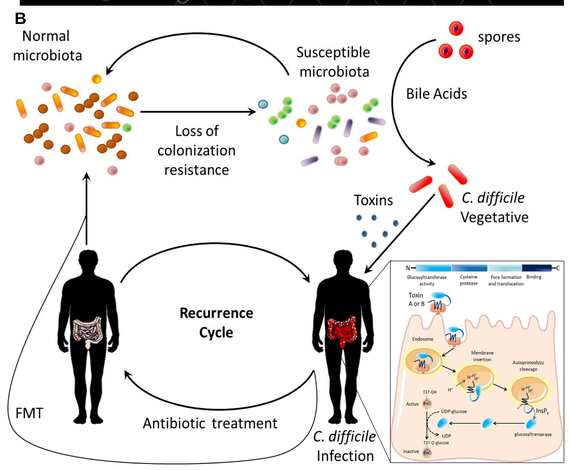


Fig 1. **A**. The Center for Disease Control has upgraded the threat level of *Clostridium difficile* infection (CDI) to urgent, reflecting the high likelihood of hospital and community acquired infection. In the United States, *C. difficile infection* is responsible for 500,000 infections and 29,000 deaths, which correlated with $4.8 billion in excess medical care costs. These staggering statistics highlight the need to better understand the mechanisms of CDI. **B**. The human gut is comprised a gut microbiota that is dominated by Bacteroidetes and Firmicutes. Exposure to select antibiotics alters the microbial population. In this setting, introduction of chemical and heat resistant *C. difficile* spores can result in vegetative cells which produce toxin and cause CDI. Current treatment for CDI includes antibiotics and fecal microbiota transplant (FMT) for recurrent CDI. Toxins are known to bind to select receptors and which become internalized into enterocytes. Inside the cell, the glycosylatransfer toxin domain inactivates Rho/Rac kinase and stimulates actin cytoskeleton rearrangement (Modified from Bitton & Young 2018).

Hypothesis

Human intestinal enteroids (HIEs), as the most physiologically relevant *in vitro* model system, expresses the important native toxin receptors and provide an ideal model to dissect *C. difficile* toxin activity

Methods

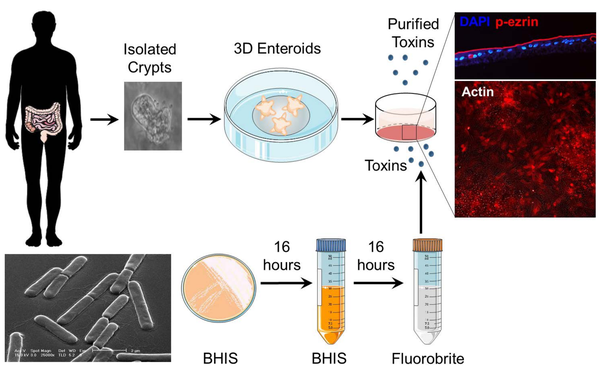


Fig 2. Methods for testing hypothesis. Human Intestinal Enteroids (HIEs) derived from jejunum were lentivirus transduced with LifeAct-Ruby labeling F-actin. HIEs were grown in 2D monolayers and treated with purified C. difficile toxins (List biologicals) or toxin-containing fluorobrite supernatant from *C. difficile* cultures (R20291, M68, 630 and non-toxin CD37). HIEs or cells lines were live imaged overnight (16 hr) to assess toxin effects on F-actin.

Results

C. difficile strains exhibit differences in their genome and toxin genes

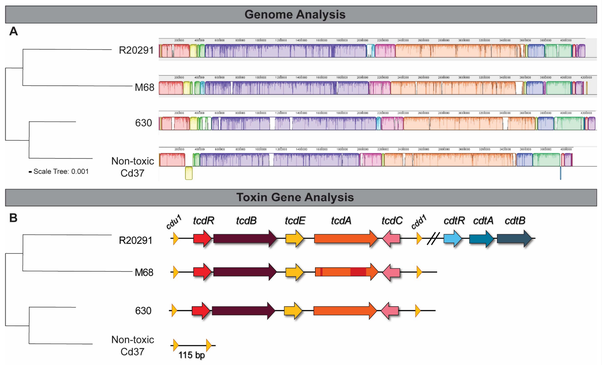


Fig 3: **A**. (Left) Phylogenetic tree generated from a concatenated nucleotide sequence alignment of *C. difficile* strains R20291 (ribotype 027), 630 (ribotype 012), M68 (ribotype 017), and non-toxic Cd37 core genes. The horizontal bar at the base (scale tree) represents 0.001 substitutions per nucleotide site. (Right) Genome alignment of *C. difficile* strains complete genomes (single assembled contig) was performed using progressiveMauve. **B**. (Left) Phylogenetic tree. (Right) Toxin genes were examined in each strain and graphically represented by arrows.

C. difficile strains secrete toxins, maintain shape and viability after incubation in Fluorobrite DMEM

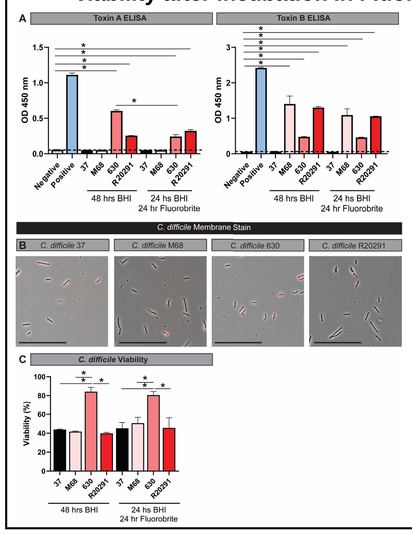


Fig 4: A. Toxin A and B ELISAs with *C. difficile* strains 37, M28, 630, and R20291 after 48 hrs in BHIS or 24 hr incubation in BHIS and an additional 24 hr incubation in Fluorobrite DMEM. **B.** FM 4-64 membrane staining in *C. difficile* 37, M28, 630, and R20291 after 24 hr incubation with BHIS and an additional 24 hr incubation in Fluorobrite DMEM. Scale bar = 50 µm. **C**. Cell viability as determined by BacLight Live/dead cell staining Cell staining was examined ex/em: 485/530 nm (live) and ex/em 485/630 nm (dead) on a Synergy H1 Microplate Reader and viabilities were calculated by dividing the fluorescence intensity of 485/530 by the fluorescence intensity at 485/630 x 100%.

HIEs exhibited delayed cell rounding in response to toxigenic *C. difficile* strains

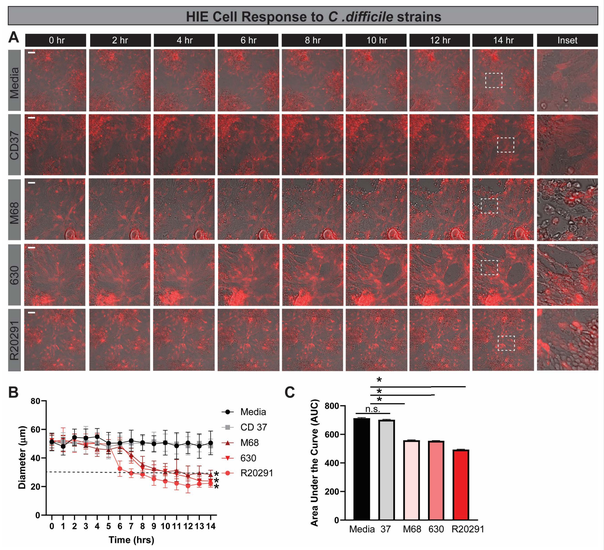


Figure 5: Human intestinal enteroids (HIEs) derived from the jejunum were transduced with the LifeAct-Ruby sensor, which labels F-actin with red fluorescent protein. Cell rounding was visualized by live cell microscopy on a Nikon TiE with 20x Plan Apo (NA 0.75) differential interference contrast objective, using a SPECTRA X LED light source and ORCA-Flash 4.0 sCMOS camera (scale bar =50 µm). **A.** Representative images of LifeAct-Ruby HIEs over time (1-16 hrs) after exposure to Fluorobrite DMEM medium alone or conditioned Fluorobrite from *C. difficile* strains CD37, M68, 630, or R20291. Insets indicate significant rounding occurs with toxigenic *C. difficile* strains (M68, 630, and R20291). **B**. FIJI (Formerly Image J) software was used to define cell membranes (as denoted by actin labeling) and cell diameter over time. **C.** Resulting curves were assessed for the area under the curve, which demonstrates decreased cell diameter with M68, 630, and R20291. *P<0.05, One Way ANOVA; n = 3 replicates/ 6 experiment.

Results

HIEs are less responsive than Vero cells to purified *C. difficile* toxin A and B

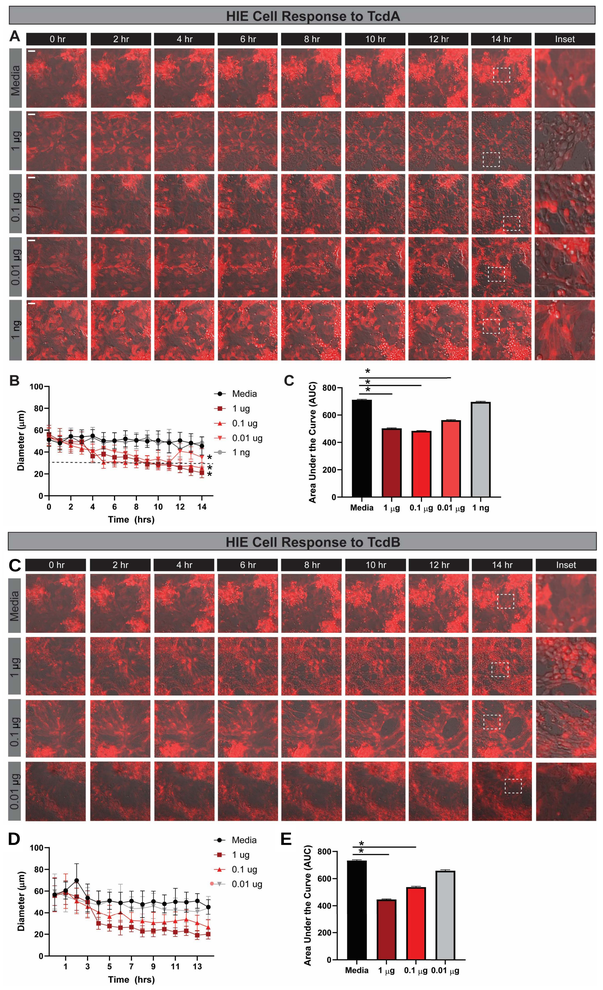


Fig 6: Representative images of LifeAct-Ruby HIEs cell over time (1-16 hrs) after exposure to purified TcdA (**A**) or TcdB (**C**) (List Biologicals) in Fluorobrite. FIJI analysis of cell diameter over time with TcdA (**B**) or TcdB (**D**). Area under the curve analysis for TcdA (**C**) or TcDB (**E**). *P<0.05, One Way ANOVA; n= 3 replicates/ 6 experiment.

Toxigenic *C. difficile* strains cause rapid cell rounding in Monkey Kidney Fibroblasts Vero cells

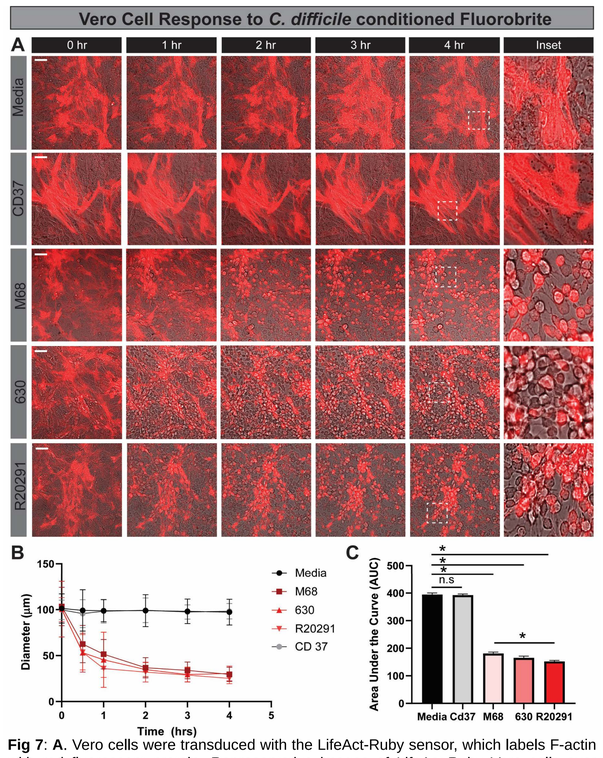


Fig 7: **A**. Vero cells were transduced with the LifeAct-Ruby sensor, which labels F-actin with red fluorescent protein. Representative images of LifeAct-Ruby Vero cells over time (1-4 hrs) after exposure to Fluorobrite DMEM medium alone or conditioned Fluorobrite from *C. difficile* strains CD37, M68, 630, or R20291. **B**. Cell diameter analysis. **C**. Area under the curve analysis. *P<0.05, One Way ANOVA.

Results

Purified toxin A and B cause rapid cell rounding in Monkey Kidney Fibroblasts Vero cells

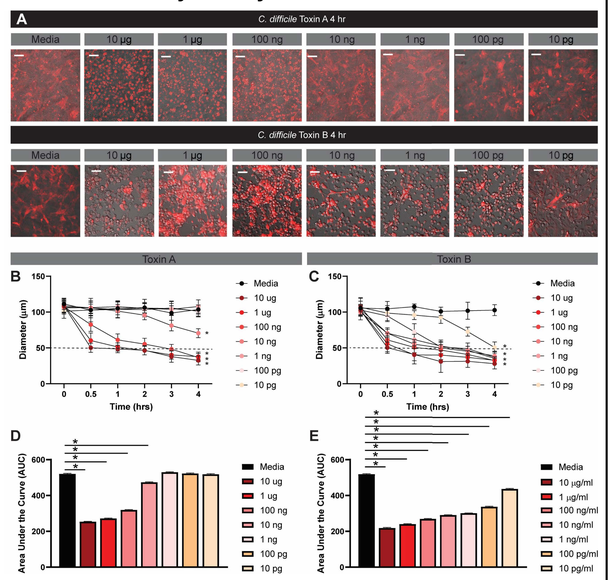


Fig 8: LifeAct-Ruby-transduced Vero cells incubated with purified TcdA or TcdB (List Biologicals). **A**. Representative images of LifeAct-Ruby Vero cells 4 hrs after exposure to Fluorobrite DMEM medium alone or purified toxins in Fluorobrite DMEM. Cell diameter over time for TcdA (**B**) and TcdB (**C**). Area under the curve analysis of TcdA (**D**) and TcdB (**E**). One Way ANOVA; n = 3 replicates/ 6 experiments.

HIEs express higher levels of toxin receptor mRNA than Vero cells

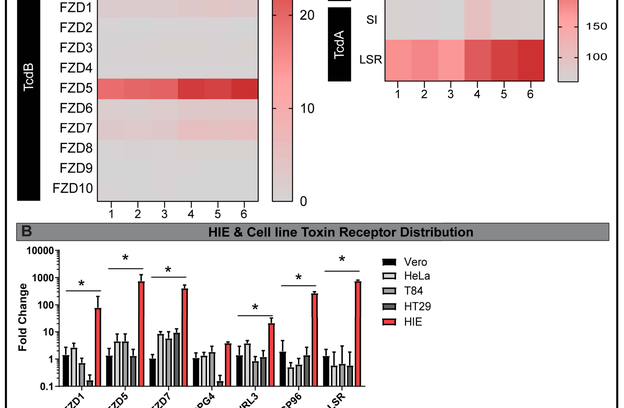


Fig 9: **A**. RNAseq of HIEs (untreated) revealed a unique profile of toxin receptors. We examined binary toxin (CDT) receptor LSR, TcdA receptorGp96, sucrase isomaltase (SI), and TcdB receptors [CSPG4, PVRL3, and Frizzled genes FZD]. Heat maps were generated from FPKM (Fragments Per Kilobase of transcript per Million mapped reads). **B**. qPCR analysis of HIEs, Vero, and cancer-derived HeLa, T84, and HT29 cells revealed high expression of toxin receptors in HIEs. Data represented as ΔΔCT normalized to 18S. *P<0.05, One Way ANOVA; n = 3 replicates, 6/experiment.

HIEs secrete MUC2 which acts as a decoy for TcdA and TcdB

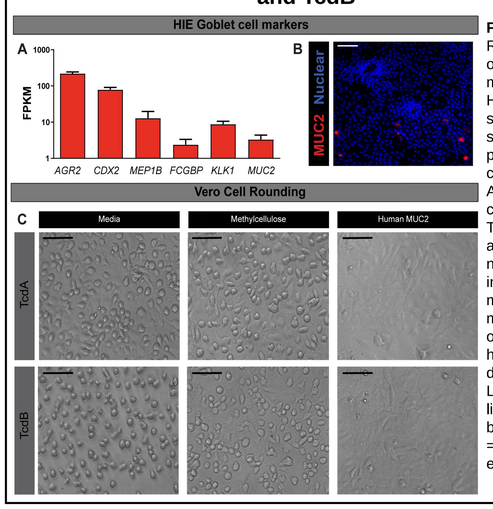


Fig 10: **A.** RNAseq analysis of goblet cell mark in untreated HIEs. **B.** MUC2 staining in HIE show MUC2 positive goblet cells. **C**. Decoy Assays in Vero cells. Purified TcdA (10 ng/ml) and TcdB (1 ng/ml) were pre-incubated with 1 mg/ml methylcellulose or 1 mg/ml human MUC2 derived from LS174T goblet-like cells. Scale bar = 100 µm. n = 3 replicates/3 experiments.