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C. difficile-associated antibiotics alter human mucosal barrier functions by microbiome-independent mechanisms.

Jemila C. Kester, Douglas K. Brubaker, Jason Velazquez, Charles Wright, Douglas A. Lauffenburger, and Linda G. Griffith*

Department of Biological Engineering
Massachusetts Institute of Technology
77 Massachusetts Avenue, Cambridge, MA, 02139

*corresponding author: griff@mit.edu
Abstract

A clinically relevant risk factor for *Clostridioides difficile*-associated disease (CDAD) is recent antibiotic treatment. Although broad-spectrum antibiotics have been shown to disrupt the structure of the gut microbiota, some antibiotics appear to increase CDAD risk without being highly active against intestinal anaerobes, suggesting direct non-antimicrobial effects. We examined cell biological effects of antibiotic exposure that may be involved in bacterial pathogenesis using an *in vitro* germ-free human colon epithelial culture model. We found a marked loss of mucosal barrier and immune function with exposure to the CDAD-associated antibiotics clindamycin and ciprofloxacin distinct from pretreatment with an antibiotic unassociated with CDAD, tigecycline, which did not reduce innate immune or mucosal barrier functions. Importantly, pretreatment with CDAD-associated antibiotics sensitized mucosal barriers to *C. difficile* toxin activity in primary cell-derived enteroid monolayers. These data implicate commensal-independent gut mucosal barrier changes in the increased risk of CDAD with specific antibiotics and warrant further studies in *in vivo* systems. We anticipate this work to suggest potential avenues of research for host-directed treatment and preventive therapies for CDAD.
Introduction

Clostridioides difficile-associated disease (CDAD) is a CDC urgent public health threat (1), with 453,000 incident cases in the U.S. in 2011 (2). CDAD is estimated to account for more than 44,500 deaths and over $5 billion in related healthcare costs in the United States each year (3). CDAD treatment failure is increasing due to rising levels of antibiotic resistant (4) and hypervirulent (5) strains of *C. difficile* and high rates of persistent and recurrent infections (6). New treatment and prevention strategies are needed. A promising strategy for treating some bacterial infections is host directed therapy (7). Translation to CDAD therapy requires a better understanding of disease susceptibility.

CDAD pathogenesis requires the outgrowth of the etiologic agent, *Clostridioides difficile* (*C. difficile*), in the gastrointestinal tract. While a functional gut microbiome is able to prevent the outgrowth of *C. difficile* (8), in large part due to bacterial-dependent production of secondary bile acids (9), loss of a functional gut microbiome allows for outgrowth of the pathogen. Once sufficient cell density is reached, the bacteria begin secreting toxins (10), specifically TcdB, which is primarily responsible for the disease’s symptoms and pathogenesis (11).

A clinically relevant risk factor for CDAD is recent antibiotic treatment (12). There is substantial evidence supporting a causal link between microbiome disruption by antibiotics and CDAD (reviewed in (13)); however, the antibiotic effect on the microbiome seems unlikely to be the sole causal mechanism for CDAD. Microbiome signatures of patients with and without CDAD (14) are more pronounced than those arising from antibiotic treatment alone, which produces widely varying changes to microbial community structure (15-17). Notwithstanding the frequency of proteobacteria blooms following antibiotic exposure (18), no shared taxonomic change has been identified in successful fecal microbial transplant donors (19) or recipients (20) in other diseases, and alterations to bacterial load do not correlate with risk of CDAD (21).
together, these data suggest one or more host-dependent contribution to antibiotic-associated risk of CDAD.

Recent work has shown that host-acting drugs have a significant effect on bacteria. The inverse has also been shown: a commonly prescribed antibiotic cocktail alters the mitochondrial function of enterocytes in germ-free mice, demonstrating the commensal-independent effect of anti-bacterials on the host gut mucosal barrier in this rodent model. Yet, the effects of CDAD-associated antibiotics on the host—especially the human host—and how these effects might contribute to CDAD is not known. Antibiotics are known to affect human host responses in other settings, such as the well-known alterations in airway inflammation and mucosal barrier behavior induced by macrolides. Extensive and lasting effects on gene expression and function in the immune and multiple organ systems, in association with generation of reactive oxygen species, were observed in mice exposed to ciprofloxacin, one CDAD-associated antibiotic. Though previous studies have explored the effects of antibiotics in general on the host in a variety of animal models and tissues—including the gut—isolating the effects of particular antibiotics on host-dependent mechanisms of antibiotic-associated CDAD requires a controlled study assessing these mechanisms for multiple antibiotics with varying degrees of CDAD-associated risk in the same experimental context, preferably with models that include elements of the human mucosal barrier response.
Results

CDAD-associated antibiotics induce distinct changes to host gene expression

Based on reported meta-analyses of community-associated *C. difficile* infection and antibiotics, we selected 3 antibiotics representing the odds ratio spectrum from less than 1 (tigecycline, no risk) to 6 (ciprofloxacin, medium risk) to 20 (clindamycin, highest risk) in order to achieve complete coverage of the CDAD risk landscape. Tigecycline, a derivative of tetracycline delivered intravenously, is currently used to treat CDAD and hence is an attractive antibiotic for this study. Although there are currently no epidemiological studies giving an odds ratio to tigecycline, we assigned it low risk based on its similarity to tetracycline, an antibiotic which showed no increased risk of CDAD in the meta-analyses, and its strong safety profile in clinical use, including for CDAD treatment.

We used clindamycin and ciprofloxacin for CDAD-associated antibiotics as they have the highest risk of CDAD among commonly-used antibiotics. Although all three are considered broad-spectrum antibiotics, they differ in their mechanism of action and species specificity. We considered mechanism of action as part of the study design: tigecycline (low risk) and clindamycin (high risk) share a similar mechanism of action, both targeting bacterial translation machinery. Conversely, ciprofloxacin (intermediate risk) inhibits bacterial DNA replication. Notably, ciprofloxacin has an FDA black-box label arising from adverse human side effects on the nervous and musculoskeletal systems, yet has been used to treat Crohn’s disease, a chronic gastrointestinal disorder, with a reasonable safety profile on the gastrointestinal tract though unclear efficacy. The mechanisms for effects of ciprofloxacin on human cells have implicated damage to mitochondrial DNA and alterations in DNA-modifying enzymes, but a clear picture has not emerged. Gene expression and phenotypic effects of...
these antibiotics on human colon mucosal barrier cells will thus help illuminate whether additional off-target effects of these drugs exist and should be further studied.

To test the commensal-independent effects of antibiotics on human tissue, we used a transwell-based \textit{in vitro} epithelial barrier without bacteria to model a germ-free human gut (34, 35). We treated mature mucosal barriers with antibiotics dosed from the basal side, using clinically-relevant dose ranges estimating $C_{\text{max}}$ in each case from a combination of the FDA inserts (Table 1) and published literature on pharmacokinetics for each drug, which provide $C_{\text{max}}$ values comparable to those in Table 1 (29, 36-41). The standard dosing for intravenous tigecycline includes a 100 mg loading dose followed by maintenance doses, resulting in $C_{\text{max}}$ values comparable to those shown in Table 1(29, 40); however, protocols involving daily higher doses (400 mg q 24 hr) are also used (42), motivating exploration of high-end dosing ranges.

Basal dosing of colonic epithelial monolayers at low doses near reported plasma $C_{\text{max}}$ is expected to replicate some but not all features of colonic epithelial exposure. The lack of significant metabolism \textit{in vitro} results in exposure to $C_{\text{max}}$ throughout the experiment, rather than episodically as \textit{in vivo}. Further, although the orally-dosed antibiotics are absorbed in the intestine with subsequent strong partitioning into tissue compartments, all 3 antibiotics considered in this study are also known to be partially excreted in feces in humans (36, 37, 43), resulting in apical exposure \textit{in vivo}. Tigecycline is given intravenously and is partly eliminated via biliary export into the intestine and passage to feces (41). Tigecycline also strongly partitions into tissue compartments (41, 43) with concentrations in colon tissue exceeding that in plasma (37, 43); whether it exhibits basal-to-apical transport in the colon is not reported in the literature, but the relatively high \textit{in vivo} basal concentrations suggest that our basal dosing scheme is reasonable for tigecycline. Orally-administered ciprofloxacin has variable absorption in the intestine (44) and with a relatively long half-life, builds up substantial concentrations in blood.
over several days of treatment. Ciprafloxacin is partly excreted in urine, but also in feces where
its presence may arise in part from systemic circulation via a known basal-to-apical transport
route in colon epithelia (36, 39). Thus, for ciprafloxacin, over the course of the 24-48hr
experiment, we likely exposed the cells in the ciprofloxacin case to both basal and apical
antibiotic due to basal-apical transport. Clindamycin is administered orally or systemically and in
either case partitions strongly into tissues and is also excreted into the intestine via biliary
transport (45). Whether it is transported basal-apically in colonic epithelia is unknown, but the
basal dosing used here is reasonable given the strong tissue partitioning.

In order to capture extremes of response, Caco2/HT29 MTX monolayers were exposed for 24
hr at either standard dose concentrations (equivalent to C_{max}, Table 1) or higher doses, as listed in
Table 1, then harvested and lysed. RNA-seq identified gene expression changes under high and
low exposure conditions, with the largest number of transcriptional changes being driven by
ciprofloxacin exposure (Fig 1B-E). Tigecycline was insoluble in water, and therefore dosed in
DMSO. All other antibiotics were dosed in water and compared back to water vehicle. We found
DMSO exposure at high concentration had a significant effect on gene expression, and therefore
performed subsequent analysis using the lower concentrations of both DMSO and tigecycline
listed under “standard concentration” in Supplemental Fig S1.

Unsupervised hierarchical clustering of the 606 genes with statistically significant gene
expression changes in at least two exposure groups, using the high concentrations for
ciprofloxacin and clindamycin but the low concentration for tigecycline, revealed antibiotic-
specific alterations of the gut transcriptome (Supplemental Fig S2). This clustering showed
varying patterns of transcriptional response to exposure: several transcripts shared similar
expression configurations across experimental conditions, some had dose-dependent effects
correlating to increasing or decreasing CDAD risk, still others exhibited more complex behaviors.
not apparent from the initial clustering. However, the ciprofloxacin-driven expression changes dominated the clustering, highlighting the need for more nuanced computational analysis.

In order to identify genes with transcriptional changes shared between both CDAD-associated antibiotics, we used a self-organizing map (SOM). A SOM is a neural network-based unsupervised clustering technique that groups similar observations together on the SOM neurons. Here, we used the SOM to cluster gene transcript fold changes across antibiotic exposures to identify genes with expression changes associated with CDAD risk. Similar to other dimensionality reduction techniques, such as principal components analysis (PCA), SOMs produce a low-dimensional projection of high-dimensional data that facilitates visualization of patterns. However, unlike PCA or our previous hierarchical clustering, the SOM analysis merges two important features simultaneously: (i) it incorporates information about the expected number of clusters in the data by defining the number of SOM neurons based on experimental design (number of conditions); and (ii) it allows the data to drive identification of the most informative groups among those clusters (i.e., SOM neurons).

The architecture of the SOM employed here to map the 606 significant genes is based on increased or decreased gene expression (2 directions) in each of three (3) experimental conditions (i.e., clindamycin, ciprofloxacin, or tigecycline compared to its respective control), with an extra neuron for noisy profiles ($2^3+1=9$ neurons). Genes with similar expression patterns cluster in a node, with the number of genes per node indicated (Fig. 2A). Plotting neighbor weight distances allows for the visualization of similarities between nodes (Fig. 2B).

Each neuron of the SOM captured gene expression responses to antibiotic exposure that grouped according to changing CDAD risk ratios. These patterns could then be investigated by plotting line graphs of the gene fold changes across increasing CDAD risk for each node (Fig. 2C). Two nodes identified gene expression responses that were specifically elevated (Node 3) or...
repressed (Node 7) in response to CDAD-associated antibiotic exposure. Another two nodes (4 and 6) captured risk ratio-dependent changes in gene expression responses to CDAD-associated antibiotic exposure, with genes on Node 4 being more downregulated and genes on Node 6 being more upregulated in antibiotics with higher CDAD risk ratios. Altogether, nodes 3, 4, 6, and 7 capture a set of 261 genes with expression patterns common among ciprofloxacin and clindamycin that indicated a shared pattern of expression unique to the CDAD-associated antibiotics (Fig. 2C), despite different mechanisms of action between ciprofloxacin and clindamycin, and tigecycline and clindamycin being similar.

In order to identify the biological functions associated with CDAD-associated antibiotic exposure, we performed Gene Ontology Enrichment Analysis (GOEA) of each node (Fig. 2D, Supplemental Table S1). We would expect nodes that cluster by mechanism of action to be enriched in related GO terms. For instance, the gene expression responses common to tigecycline and clindamycin (Nodes 2 and 8) were enriched for the cellular targets of those drugs, translation machinery and chromosome maintenance (Fig. 2D). It is important to note that these targets are considered bacterial cellular components, yet we found they impacted mammalian cells. This finding from the SOM clustering that grouped known target-associated gene expression responses to tigecycline and clindamycin provided an important positive control for interpreting the biological functions associated with the other SOM neurons.

We then analyzed the SOM clusters that captured genes with shared expression response patterns to CDAD-associated antibiotics (Nodes 3, 4, 6, and 7), and distinct from low risk, to generate mechanistic hypotheses of host-dependent mechanisms of CDAD. The GOEA functional annotations of CDAD-associated antibiotic exposure showed an accumulation of cellular toxins in the cell via retrograde secretion (Node 3: toxin transport) coupled with a decrease in secretion out of the cell (Node 7). We found that as antibiotic CDAD risk ratios
increased, genes associated with immune signaling GO terms were suppressed (Node 4) and genes associated with cell-cell and cell-ECM connections were increased (Nodes 6 and 7) in a dose-dependent manner. Node 6 captured genes related to focal adhesion and anchoring junctions, pathways reported to be enhanced in gut wound-healing responses (46), suggesting CDAD-associated antibiotics might result in greater cell stress or death, a hypothesis that can be tested experimentally. The suppression in immune signaling may also alter important barrier function critical in host-pathogen response (reviewed in (47)), and likewise can be probed in subsequent experiments. Overall, GOEA of these SOM suggested that exposure to CDAD-associated antibiotics resulted in alterations to transport of extracellular components out of the cell, toxins into the cell, and a reduced immune capacity after only 24hrs of exposure.

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CDAD-associated antibiotics reduce mucosal barrier and immune functions

Based on the results of the SOM analysis, we hypothesized that CDAD-associated antibiotic exposure would result in acute and possibly persisting effects of impaired epithelial barrier and innate immune cell function. We tested these SOM predictions experimentally using three complementary levels of in vitro models: (i) acute effects on epithelial barrier survival and function exposed for 24 hr to standard doses of antibiotics listed in Table 1; (ii) chronic (3 days) effects on epithelial exposure to antibiotics and toxin; and (iii) acute effects on innate immune cell function exposed to standard concentrations of antibiotics listed in Table 1.

We assessed the barrier function of Caco2/HT29 MTX mucosal barriers following 24 hours exposure to standard concentrations of antibiotics (Table 1) dosed in the basal compartment. We find a small but significant increase in cell death in monolayers with ciprofloxacin exposure, which agrees with previous work using significantly higher concentrations (48), and clindamycin, which has not previously been demonstrated (Supplemental Fig S3). It is unclear whether this
increase is biologically significant, but the upregulation in adhesion-related genes is consistent with a wound-healing response (46). Despite the increase in cell death, none of the antibiotics used affected the physical integrity of the barrier as determined by transepithelial electrical resistance 24 hours post exposure (Supplemental Fig S4).

To better mimic the 3-7 day course of antibiotics in routine *in vivo* human treatment patterns, we extended the exposure period to a 3 day basal dose, again using the standard concentrations listed in Table 1. We quantified both cell-associated and secreted mucin production as this strongly influence microbial interactions with the mucosal barrier. Total cell-bound (Fig. 3A) mucin of Caco2/HT29 MTX monolayers was reduced robustly following exposure to both CDAD-associated antibiotics, while mucins in low risk CDAD exposure groups remain unchanged (Fig. 3A, B). These cell-bound mucins, including MUC1 and MUC4, are critical for protection from intestinal pathogens, as they alert the cell to the presence of invading pathogens through intracellular signal transduction (reviewed in (49)). Secreted mucins, which provide both a niche for beneficial microbes and a physical obstacle preventing pathogens from accessing the epithelial surface, were reduced with both CDAD-associate antibiotics, though the clindamycin exposure group does not reach statistical significance (Fig 3B). Together, this reduction in secreted and cell-bound mucins with CDAD-associated antibiotics could provide increased access of *C. difficile* and its toxins to the epithelia.

To assess the effect of our antibiotic panel on primary tissue, we repeated this experiment using primary cell-derived 2D enteroids. We assayed for one of the main transmembrane mucins in the colon (50), *muc17*, as it was highly expressed in this donor and was one of the mucins downregulated significantly in our RNAseq analysis. We found *muc17* was reduced with ciprofloxacin but not clindamycin or tigecycline exposure (Fig. 3C); however, we did not assay for other mucin genes in this donor. The inconsistency between cell lines and primary tissue...
could suggest a reduced role for mucin changes in clindamycin exposure, or a donor-specific reduced effect of clindamycin. Other cell-associated mucins (e.g., Muc1 or Muc4) may be contributing to the changes seen in Fig. 3A in this primary cell donor.

To assess the effect of extended, low-dose antibiotic exposure on immune function, we treated an immune-competent mucosal barrier (Caco2/HT29 MTX monolayers with monocyte-derived dendritic cells added to the basal compartment) for 3 days with each antibiotic, again dosing from the basal side. IL-8 secretion is the primary chemokine implicated in CDAD (5). IL-8 is required for neutrophil recruitment to contain the infection, yet neutrophils are also implicated in progression of disease (51). Thus, a delicate control over dissemination and clearance of neutrophils is likely required for resolution of infection.

We therefore assessed the effect of antibiotics on the ability of immune-competent mucosal barriers to induce *il8* expression and IL-8 secretion following LPS stimulation. LPS signals through TLR4 and *tlr4* gene expression should increase following its activation, yet *tlr4* expression did not increase with LPS stimulation following ciprofloxacin exposure (Fig 3D). Clindamycin treated barriers had lower levels of *tlr4* relative to vehicle, though this was not significantly lower than for tigecycline by student’s t test (Fig 3D). We found *il8* gene expression (Fig. 3E) is reduced following ciprofloxacin and clindamycin exposure but unchanged with tigecycline in LPS-treated barriers. IL-8 secretion (Fig. 3F) was reduced to a statistically significance extent in all exposure groups. It is likely the magnitude of change is important in the case of CDAD-associated antibiotics.

To test whether the immune cells are impaired in function, we performed phagocytosis and killing assays using GFP+ *E. coli*. We find that pre-treating macrophages with CDAD-associated antibiotics reduce both phagocytosis of *E. coli* (Fig. 3G) and subsequent killing of phagocytosed *E. coli* (Fig. 3H). Together, these data support the hypothesis that CDAD-associated antibiotics
lead to a loss of immune responsiveness, which one can imagine might contribute to outgrowth of *C. difficile*.

Antibiotic effects are recapitulated in primary tissue

Previous work has shown the importance of mucus—primarily made up of mucins—on preventing *C. difficile* toxins from entering gut cell lines in culture (52). CDAD’s pathology is driven by the cytotoxic effects of toxins, primarily TcdB (5). TcdB enters epithelial cells through receptor-mediated endocytosis (53), though none of the known toxin receptors (NECTIN3, CSPG4, or FZD1/2/7) had significantly altered expression following exposure to any antibiotic we tested. Following acidification of the vacuole, the toxin enters the cytosol where it glucosylates its GTPase targets Rho, Rac, and Cdc42. This leads to actin depolymerization, characterized by visible rounding of the cell, and eventually to cell death. The *in vitro* human mucosal barrier used in this work is affected by TcdB as expected, by cell rounding and areas of cell death as determined by holes in the monolayer after 48 hours of exposure to *C. difficile* spent media (Supplemental Fig S5), demonstrating the utility of the system for studying TcdB effects.

To understand the translation of these altered barrier properties to potential impact in CDAD, we apically exposed mucosal barriers with TcdB (following pretreatment with standard concentrations of antibiotics (Table 1) in the basal compartment) and measured its action by the loss of a cellular target, activated Rac-1, by western blot analysis. Both CDAD-associated antibiotics had deactivation of Rac1 at 24hrs while tigecycline and controls were still active by quantitative western blot (Fig. 4A, B), implicating a shared sensitization to *C. difficile* toxin from CDAD-associated antibiotics with a mechanism independent of commensals.
Discussion

Here we demonstrate the convergent changes to *in vitro* germ-free human gut models from two separate CDAD-associated antibiotics in the absence of commensal bacteria. CDAD has been suggested to be a multi-phase system, where germination, outgrowth, and toxin production each have distinct signals upon which they activate (54). Substantial work has shown that antibiotics contribute to CDAD by changing commensal structure and removing inhibition on both germination and outgrowth (55). Yet, the specific structural definition of a CDAD-inhibitory gut microbiome remains elusive.

Our data suggest a potential mechanism by which an already outgrown but microbiome-controlled population of *C. difficile* might be able to take hold and produce toxin following the host changes of CDAD-associated antibiotics: loss of mucin barrier, increased sensitivity to toxin, and reduced innate immune response. We found that both CDAD-associated antibiotics lead to increased toxin transport (Fig. 2D) and concomitant sensitivity to toxin B (Fig. 4).

Increased relative abundance of proteobacteria is associated with CDAD and has been proposed to be a risk factor (56). Proteobacterial bloom following antibiotic treatment might be accounted for by the loss of *E. coli* and LPS responsiveness we observed. This *in vitro* study is limited, however, by the relatively short time frame for investigation of effects. Additionally, the simplicity of the model, which lacks microbes and peripheral organs, does not capture the complex reciprocal interplay between the shifting microbial populations and the mucosal barrier.

Specifically, the presence of microbes contributes to mucus production in mouse models (57).

New microfluidic microphysiological systems are emerging that enable co-culture of anaerobic microbial populations with colonic mucosal barriers, and when these become validated and accessible, the combined effects on host and microbe populations can be assessed more fully.
Our work suggests that further study of how CDAD-associated antibiotics might influence CDAD pathology through commensal-independent effects on human tissue may yield insights important for clinical practice.

Antibiotics with high risk for CDAD may prime the host to be less prepared for combating C. difficile infection and pathogenesis by reducing barrier function and immune cell capability and increasing toxin sensitivity, suggesting possible implications for host-directed prophylactic or CDAD-treatment therapies. Encouragingly, host-directed anti-oxidant treatment concomitant with ciprofloxacin therapy showed mitigation of effects on host tissues in a mouse model (25).

There are several limitations to this pilot study. This work was done in a germ-free, in vitro setting. As such, its results may not be generalizable to in vivo settings. In addition to the simplicity of the cell populations used, we used added antibiotics only to the basal culture medium, which may not fully recapitulate the combined apical and basal exposure known to occur in vivo as all 3 of the antibiotics in the study are partially excreted in feces. While ciprofloxacin is known to be basal-apically transported in colon epithelia, thus likely mitigating lack of deliberate apical exposure in vitro, whether such transport occurs for tigecycline and clindamycin is unknown. This model also lacks reciprocal effects of microbe-host interactions that occur in vivo yet. The impact of antibiotics on the gut bacteria—and their impact on the host—cannot be extrapolated from this work and will need to be studied in future.

An additional limitation to this study is that we focused on the effects of antibiotics with the highest and lowest risks, and therefore did not test effects of commonly used classes, such as beta lactams. Further work is needed to understand the commensal-independent, host-dependent effects of this and other classes of antibiotics on gut barrier function.
Methods

Tissue culture: cell lines
Caco2 (clone: C2BBe1, passage 48–58, ATCC, Manassas, VA) and HT29-MTX (passage 20–30, Sigma–Aldrich, St. Louis, MO) were maintained in DMEM (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Flowery Branch, GA), 1% GlutaMax (Gibco), 1% Non-Essential Amino Acids (NEAA, Gibco), and 1% Penicillin/Streptomycin (P/S). Both cell lines were passaged twice post thawing before their use for transwell seeding. Briefly, the apical side of transwell membrane were coated with 50mg/mL Collagen Type I (Corning Inc., Corning, NY) overnight at 4°C. Caco2 at 80–90% confluence and HT29-MTX at 90–95% confluence were harvested using 0.25% Trypsin-EDTA and mechanically broken up into single cells. 9:1 ratio of C2BBe1 to HT29-MTX was seeded onto 12-well 0.4mm pore polyester transwell inserts (Corning, Tewksbury, MA) at a density of $10^5$ cells/cm². Seeding media contained 10% heat inactivated FBS, 1x GlutaMax and 1% P/S in Advanced DMEM (Gibco). Seven days post seeding, the media was switched to a serum-free gut medium by replacing FBS with Insulin-Transferrin-Sodium Selenite (ITS, Roche, Indianapolis, IN) and the epithelial cultures were matured for another 2 weeks. P/S was left out of media during experimental procedures.

Tissue culture: primary cells
Colon organoids (enteroids) used in this study were established and maintained as previously described (58, 59). Endoscopic tissue biopsies were collected from the ascending colon of de-identified individuals at either Boston Children’s Hospital or Massachusetts General Hospital upon the donors informed consent. Methods were carried out in accordance to the Institutional Review Board of Boston Children’s Hospital (protocol number IRB-P00000529) and the Koch
Institute Institutional Review Board Committee as well as the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. Tissue was digested in 2 mg ml\(^{-1}\) collagenase I (StemCell, cat. no. 07416) for 40 min at 37 °C followed by mechanical dissociation, and isolated crypts were resuspended in growth factor-reduced Matrigel (Becton Dickinson, cat. no. 356237) and polymerized at 37 °C. Organoids were grown in expansion medium (EM) consisting of Advanced DMEM/F12 supplemented with L-WRN conditioned medium (65% vol/vol, ATCC, cat. no. CRL-3276), 2 mM Glutamax (Thermo Fisher, cat. no. 35050-061), 10 mM HEPES (Thermo Fisher, cat. no. 15630-080), Penicillin/Streptomycin (Pen/Strep) (Thermo Fisher, cat. no. 15070063), 50 ng ml\(^{-1}\) murine EGF (Thermo Fisher, cat. no. PMG8041), N2 supplement (Thermo Fisher, cat. no. 17502-048), B-27 Supplement (Thermo Fisher, cat. no. 17502-044), 10 nM human [Leu15]-gastrin I (Sigma, cat. no. G9145), 500 μM N-acetyl cysteine (Sigma, cat. no. A9165-5G), 10 mM nicotinamide (Sigma, cat. no. N0636), 10 μM Y27632 (Peprotech, cat. no. 1293823), 500 nM A83-01 (Tocris, cat. no. 2939), 10 μM SB202190 (Peprotech, cat. no. 1523072) and 5 nM prostaglandin E2 (StemCell cat. no. 72192) at 37°C and 5% CO\(_2\). Organoids were passaged every 7 days by incubating in Cell Recovery Solution (Corning, cat. no. 354253) for 40 min at 4 °C, followed by mechanical dissociation and reconstitution in fresh Matrigel at a 1:4 ratio.

For 2D enteroid studies, at day 7 post passaging, colon organoids were collected, Matrigel was dissolved with Cell Recovery Solution for 40 min at 4 °C followed by incubation of Matrigel-free organoids in Trypsin (Sigma, cat. no. T4549) at 37 °C for 5 minutes. Organoids were mechanically dissociated into single cells, resuspended in EM without nicotinamide and 2.5 μM thiazovivin (Tocris, cat. no. 3845) in the place of Y27632, and seeded onto 24-well 0.4 μm pore polyester transwell inserts (Corning, 3493) coated with a 200 μg/mL type 1 collagen and 1% Matrigel mixture at a density of 1 x 10\(^5\) cells/transwell. After 3-4 days of incubation,
monolayers were confluent and differentiation was initiated. For differentiation apical media was replaced with Advanced DMEM/F12 plus HEPES, glutamax, and Pen/Strep and basal media with differentiation medium (DM), which is EM without L-WRN conditioned medium, nicotinamide, prostaglandin E2 and Y27632, but supplemented with 100 ng ml⁻¹ human recombinant noggin (Peprotech, cat. no. 120-10C) and 20% R-spondin conditioned medium (Sigma, cat. no. SCC111). Transepithelial electrical resistance (TEER) measurements were performed using the EndOhm-12 chamber with an EVOM2 meter (World Precision Instruments). At day 8 post seeding, the 2D enteroids were washed to remove P/S and used for further experimentation.

Monocyte-derived dendritic cells were used as the immune component of the gut when indicated. Briefly, peripheral blood mononuclear cells (PBMCs) were processed from Leukopak (STEMCELL Technologies, Vancouver, BC, Canada). Monocytes were isolated from PBMCs using the EasySep Human Monocyte Enrichment Kit (STEMCELL Technologies, 19058) and were differentiated in RPMI medium (Gibco) supplemented with 10% heat-inactivated FBS (Gibco), 50 ng/mL GM-CSF (Biolegend, San Diego, CA), 35 ng/mL IL4 (Biolegend), and 10 nM Retinoic acid (Sigma). After 7 days of differentiation (at day 19–20 post epithelial cell seeding), immature dendritic cells were harvested using PBS (Gibco) and seeded onto the basal side of the gut transwells in the absence of P/S 1 day prior to start of experiment. Macrophages were derived similarly, but with M-CSF (Biolegend) at 500 ng/mL.

RNA preparation, qPCR

RNA was prepared using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. DNA removal was done on column with PureLink DNase (Invitrogen). cDNA synthesis using High-Capacity RNA-to-cDNA Kit (Applied Biosystems,
Foster City, CA) according to product insert. qPCR was completed using TaqMan® assays with Fast Advanced Master Mix (Applied Biosystems) as per manufacturer’s guidelines.

3' DGE library preparation

RNA samples were quantified and quality assessed using an Advanced Analytical Fragment Analyzer. 20ng of totalRNA was used for library preparation with ERCC Spike-in control Mix A (Ambion 10-6 final dilution). All steps were performed on a Tecan EVO150. 3'DGE-custom primers 3V6NEXT-bmc#1-24 are added to a final concentration of 1.2uM. (5’-

/5Biosg/ACACTCTTTCCTACACGACGCTCTTCCGATCT[BC₆]N₁₀T₃₀VN-3’ where

5Biosg = 5’ biotin, [BC₆] = 6bp barcode specific to each sample/well, N₁₀ = Unique Molecular Identifiers, Integrated DNA technologies). After addition of the oligonucleotides, samples were denatured at 72C for 2 minutes followed by addition of SMARTScribe RT per manufacturer’s recommendations with Template-Switching oligo5V6NEXT (12uM, [5V6NEXT : 5’-
iCiGiCACACTCTTTCCCTACACGACGCrGrGrG-3’ where iC: iso-dC, iG: iso-dG, rG: RNA G ] ) and incubation at 42C for 90’ followed by inactivation at 72C for 10’. Following the template switching reaction, cDNA from 24 wells containing unique well identifiers were pooled together and cleaned using RNA Ampure beads at 1.0X. cDNA was eluted with 90 ul of water followed by digestion with Exonuclease I at 37C for 45 minutes, inactivation at 80C for 20 minutes. Single stranded cDNA was then cleaned using RNA Ampure beads at 1.0X and eluted in 50ul of water. Second strand synthesis and PCR amplification was done using the Advantage 2 Polymerase Mix (Clontech) and the SINGV6 primer (10 pmol, Integrated DNA Technologies 5’-/5Biosg/ACACTCTTTCCCTACACGACGC-3’). 12 cycles of PCR was performed followed by clean up using regular SPRI beads at 1.0X, and was eluted with 20ul of EB. Successful amplification of cDNA was confirmed using the Fragment Analyzer. Illumina libraries are then
produced using standard Nextera tagmentation substituting P5NEXTPT5-bmc primer (25μM, Integrated DNA Technologies, (5’-AATGATACGGGACACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG*A*T*C*T*-3’ where * = phosphorothioate bonds. ) in place of the normal N500 primer. Final libraries were cleaned using SPRI beads at 1X and quantified using the Fragment Analyzer and qPCR before being loaded for paired-end sequencing using the Illumina NextSeq500.

Sequencing data analysis

Post-sequencing, quality-control on each of the libraries was performed to assess coverage depth, enrichment for messenger RNA (exon/intron and exon/intergenic density ratios), fraction of rRNA reads and number of detected genes using bespoke scripts. The sequencing reads were mapped to hg38 reference using star/2.5.3a. Gene expression counts were further estimated using ESAT v1(60).

Data availability statement

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus(61) and are accessible through GEO Series accession number GSE135383 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135383).

Phagocytosis and Intracellular Killing assays

Escherichia coli GFP (ATCC® 25922GFP™) (E. coli) was grown to early log in LB media plus ampicillin, then washed and resuspended in RPMI without antibiotic at the appropriate density. PBMC-derived macrophages were treated with low dose (Table 1) of indicated antibiotic for 3
days in RPMI with heat-inactivated FBS (Atlanta Biologicals). Antibiotic was removed and antibiotic-free RPMI with GFP+ \textit{E. coli} was added at an MOI of 10:1. Extracellular \textit{E. coli} were washed off at indicated time. For phagocytosis, at 30 minutes post infection, cells were fixed and permeabilized, DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher) and ActinRed\textsuperscript{TM} 555 ReadyProbes\textsuperscript{TM} (Molecular Probes, Life Technologies, Carlsbad, CA) stained, and imaged. Percent macrophages with at least one GFP+ bacterium was calculated from fluorescent microscopy images. For intracellular survival assay, macrophages were lysed in water, and supernatants were plated for CFU.

Self-organizing map

Gene expression fold changes from controls across antibiotics were considered as a function of CDAD risk and were normalized to be between 0 and 1 across the 3 antibiotics used. Genes without expression fold changes across all 3 conditions were omitted. The map was initialized with a 2-dimensional 3×3 square grid and implemented using the MATLAB (MathWorks, Natick, MA) R2017b Neural Network Toolbox.

Gene Ontology Enrichment Analysis

Gene ontology enrichment on all GO terms was performed using the free online PANTHER overrepresentation test (62-64). FDR was set to <0.05.
Prism 8 software (GraphPad Software, La Jolla, CA) was used to graph all data except SOMs. Statistical tests of measurements were used from the Prism suite as noted in figure legends. Statistical significance is indicated by as follows: *<0.05, **<0.01, ***<0.001, ****<0.0001.

Viability/Cytotoxicity analysis

Viability of monolayers post-antibiotic exposure was assessed using the Viability/Cytotoxicity Assay for Animal Live & Dead Cells kit (Biotium, Fremont, CA) according to package insert. Ratio of red to green cells was measured using ImageJ.

Secreted and cell-bound mucin quantification by Alcian blue colorimetric assay

Apical medium (secreted mucin) or supernatant from the lysed cell pellet (cell-bound mucin) was stained for 2 h at room temperature with 1% Alcian blue (Electron Microscopy Sciences) at a ratio of 1:4 with the sample. Dye-treated mucin was sedimented by a 30-min centrifugation, followed by two wash steps in wash buffer (290 mL 70% ethanol, 210 mL 0.1 M acetic acid, and 1.2 g MgCl₂). Dye-treated mucin was resuspended in 10% SDS and absorbance was read on a plate reader at 620 nm. Calculations were made based on a known standard prepared in parallel.

Western blotting

Western blotting was performed under reducing conditions using iBlot 2 dry blotting system (Invitrogen) standard procedures. Primary antibodies were incubated at 4°C overnight diluted as noted in Odyssey blocking buffer: rabbit monoclonal anti-Vinculin antibody (abcam, Cambridge, MA [EPR8185]) at 1:3000, mouse monoclonal anti-Rac1 antibody (abcam, [23A8]) at 1:750,
and purified mouse Anti-Rac1 antibody (BD Transduction Laboratories, San Jose, CA [clone 492/Rac1]) at 1:750. For detection, LI-COR (Lincoln, NE) goat anti-rabbit or anti-mouse IR800-conjugated secondaries at 1:8,000 were incubated for 30 minutes at room temperature in Odyssey blocking buffer (TBS) with 0.1% Tween 20. Imaging of membrane using LI-COR Odyssey imager with settings as follows: 24 μm resolution and high quality, laser intensity of 2.0 on the 800 channel.

**Chemokine quantification**

Secreted IL-8 was measured by Quantikine® ELISA Human IL-8/CXCL8 Immunoassay (R&D Systems, Minneapolis, MN) per manufacturer’s guidelines.

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**Author Contributions Statement**

JCK and LGG conceived of the project. JCK designed the experiments. JCK acquired and analyzed the data, with technical support from JV and CW. JCK and DKB performed self-organizing maps analysis, with critical validation from DAL. JCK drafted the original manuscript. All authors contributed to manuscript revisions. LGG and DAL provided oversight and leadership for the project. LGG provided grant support for all study materials and reagents.
Competing interests

The authors declare no competing interests.
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### Table 1: Antibiotics used in this study

| Antibiotic       | Conc used (µg/mL) | Odds ratio# | Typical dosage* | C<sub>max</sub> in serum* (µg/mL) | Half-Life* (hours) |
|------------------|-------------------|-------------|-----------------|----------------------------------|-------------------|
| Tigecycline Std  | 1                 | 0.91, 95%   | 100mg load, 50mg| 0.87                             | 15-44             |
| Tigecycline High | 20                | CI 0.57–1.45| q12h, intravenous|                                  |                   |
| Ciprofloxacin Std| 5                 | 5.65, 95%   | 400mg q12h, oral| 5.4                              | 3.5-4             |
| Ciprofloxacin High| 200              | CI 4.38–7.28|                 |                                  |                   |
| Clindamycin Std  | 3                 | 20.43, 95%  | 600mg q6h, oral | 10.8                             | 2                 |
| Clindamycin High | 300               | CI 8.50–49.09|                |                                  |                   |

*Values from FDA drug inserts; #Values from meta-analysis studies in refs (26),(27)

### Figures

**Fig 1:** RNA-seq identifies differential gene expression changes following 24hr antibiotic treatment. 
A) Schematic of RNA-seq procedure. 6 transwells per condition. B) Venn diagram and color key of gene expression patterns for high dose treatment. C-E) Volcano plots of gene expression changes for high concentrations of indicated antibiotic compared to vehicle. Highlighted points have at least a 2-fold change with an adjusted p value of <0.01.
Fig 2: Self-organizing map (SOM) predicts CDAD-associated antibiotics may alter barrier and immune functions. A) SOM of 606 genes with statistically significant expression changes by RNAseq. B) SOM neighbor weight distances indicate level of similarity between each node pair: C) Line graphs of all genes in each node, plotted as increasing risk of CDAD on the x-axis by standardized fold change on the y-axis. D) Bubble chart showing overrepresented GO terms for clusters as indicated using PANTHER overrepresentation test, FDR <0.05.
Fig 3: CDAD-associated antibiotics reduce mucosal barrier and immune functions. A) Cell-bound and B) secreted mucin quantification. C) Expression fold changes of *muc17* in primary cell-derived 2D enteroids relative to vehicle by qPCR. D-F) Immune-competent monolayers: D) *tlr4* and E) *il6* gene expression fold change with LPS stimulation relative to vehicle by qPCR. F) Total IL-8 basal secretion with LPS stimulation by ELISA. G) Quantification of phagocytosis and H) intracellular killing of *E. coli* by primary macrophages pre-treated for 3 days with indicated antibiotic. Primary cell data in open bars, cell line data in filled bars. Representative data, experiments repeated 2-3 times with similar results, n=3 per experiment. Statistical significance determined by student's t test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
**Fig 4.** CDAD-associated antibiotics reduce mucosal barrier function in primary tissue. 

A) Immunoblots against active Rac1 following 24hrs of apical TodB exposure in primary cell-derived monolayers pre-treated with standard concentration of antibiotics (Table 1) for 48hrs. 

B) Quantification of blots. Rac1-vinculin normalized to vehicle control. n=3, paired t-test, *p<0.05, **p<0.01.