

1 Prophylactic Lipoxin A₄ Attenuates 2 *Clostridioides difficile* Infection by 3 Augmenting Epithelial Barrier and 4 Resolving Inflammation

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14 Abstract

15 *Clostridioides difficile* infection (CDI) is a leading healthcare-associated diarrhea with high
16 recurrence rates, partially due to antibiotic-induced dysbiosis and dysregulated host
17 inflammation. Specialized pro-resolving mediators (SPMs), such as Lipoxin A₄ (LXA₄), offer
18 promise in controlling excessive inflammation and promoting tissue repair, yet their role in CDI
19 remains unexplored. Here, we developed a compact, gas-tight gut-on-a-chip (GOC) system that
20 reconciles the anaerobic requirements of *C. difficile* with the oxic needs of human intestinal
21 epithelium, enabling physiologically relevant co-culture within a standard incubator. A CDI *in vitro*
22 model was established based on this GOC system. Using the model, we demonstrated that
23 prophylactic administration of LXA₄ significantly preserved epithelial barrier integrity, attenuated
24 pro-inflammatory cytokine secretion (IL-8 and IFN-γ), and reduced bacterial colonization.
25 Transcriptomic analysis revealed that LXA₄ pretreatment upregulated genes involved in cell
26 junction organization while downregulated immune activation pathways. These protective effects
27 were validated in a murine CDI model, where LXA₄ pretreatment reduced weight loss,
28 pathological damage, and fecal bacterial burden. Furthermore, prophylactic administration of
29 LXA₄ synergized with vancomycin treatment further enhanced antibiotic efficacy while allowing a
30 50% dose reduction without compromising therapeutic outcomes. Our study establishes a robust
31 approach for CDI research and highlights the prophylactic and adjuvant potential of
32 inflammation-resolving strategies, offering a novel approach to mitigate CDI incidence and
33 improve treatment outcomes.

35 Introduction

36 *Clostridioides difficile* is an opportunistic intestinal pathogen and the primary cause of antibiotic-
37 associated diarrhea *Rupnik et al. (2009)*. Under the conditions of gut microbiota dysbiosis, *C. dif-*
38 *ficile* infection (CDI) exhibits high incidence rates and mortality, establishing it as one of the most

39 prevalent healthcare-associated infections *Barbut et al. (2000)*; *Guh et al. (2020)*.

40 Oral antibiotics such as vancomycin or fidaxomicin are the standard first-line therapy for CDI
41 *Bainum et al. (2023)*. However, this approach creates a therapeutic paradox. While reducing the
42 pathogen load, these agents further deplete the already compromised gut microbiota and exacer-
43 bate dysbiosis *Crowther and Wilcox (2015)*. Consequently, antibiotic treatment is associated with
44 a high recurrence rate, affecting between 20% and 30% of patients after an initial episode *Bainum*
45 *et al. (2023)*. Additionally, sustained antibiotic pressure raises concerns for the development of
46 antimicrobial resistance, presenting a significant long-term public health challenge *Murray et al.*
47 *(2022)*. This compels the search for more effective and safer therapeutic strategies.

48 The pathogenesis of CDI critically involves a dysregulated host inflammatory response that di-
49 rectly exacerbates intestinal injury *Wang et al. (2024)*. *C. difficile* secreted toxins not only disrupt the
50 epithelial barrier but also actively dysregulate host immune signaling within the intestinal tissue,
51 triggering an excessive release of inflammatory mediators *Pourliotopoulou et al. (2024)*. This pro-
52 cess can be amplified by pre-existing dysbiosis, which compromises innate immune surveillance
53 *Spigaglia (2024)*. This disproportionate inflammatory cascade inflicts significant collateral dam-
54 age on the mucosal architecture, compromising both tissue integrity and function *Kordus et al.*
55 *(2022)*. Importantly, the resulting damaged intestinal environment creates a pathophysiological
56 milieu that further facilitates *C. difficile* colonization and toxin production *Wang et al. (2024)*. Thus,
57 a self-perpetuating cycle is established within the host: toxin-driven inflammation causes tissue
58 damage, and the damage in turn promotes further bacterial proliferation and pathological pro-
59 gression. Consequently, we propose that a therapeutic strategy aimed at controlling excessive in-
60 flammation and re-establishing a balanced immune response may offer a breakthrough for more
61 effective CDI management.

62 Acute inflammation is typically self-limiting, followed by an active resolution phase mediated by
63 specialized pro-resolving mediators (SPMs) that restore tissue homeostasis *Serhan (2014)*; *Fuller-*
64 *ton and Gilroy (2016)*; *Serhan and Petasis (2011)*. Lipoxin A₄ (LXA₄) is one of the most well-characterized
65 SPMs, derived from arachidonic acid via transcellular biosynthesis. It potently inhibits neutrophil
66 chemotaxis and infiltration, stimulates non-phlogistic phagocytosis of apoptotic cells by macrophages,
67 and promotes mucosal repair and barrier function *Gronert et al. (1998)*; *Gewirtz et al. (2002)*. These
68 properties suggest that LXA₄ could be a promising therapeutic agent for suppressing excessive
69 acute inflammatory responses in CDI. Previous studies have shown that LXA₄ analogs can selec-
70 tively inhibit *Salmonella typhimurium*-induced secretion of neutrophil-attracting chemokines in
71 intestinal epithelial cells, thereby dampening the inflammatory cascade *Gewirtz et al. (1998)*. Addi-
72 tionally, LXA₄ was found to enhance bronchial epithelial barrier function in cystic fibrosis by promot-
73 ing tight junction assembly during *Pseudomonas aeruginosa* infection *Higgins et al. (2016)*. Based
74 on these evidences, we hypothesize that LXA₄ may alleviate CDI pathology by modulating host im-
75 mune responses. Therefore, targeted use of LXA₄ could offer a novel interventional strategy for
76 CDI. However, the specific effects of LXA₄ on CDI remain unexplored.

77 The pathophysiology of CDI has been extensively investigated using murine models *Fachi et al.*
78 *(2024)*. However, interspecies differences can significantly influence immune responses, host-pathogen
79 interactions, and the pharmacokinetics and efficacy of therapeutic agents *Calvigioni et al. (2023)*.
80 This limits the direct translatability of findings to humans and has motivated the development of
81 advanced in vitro human intestinal models. These models hold promise for more accurately reca-
82 pitulating the specific host-microbe interactions within the human gut *Ewin et al. (2023)*. Conven-
83 tional in vitro models for CDI primarily utilize static monolayer cultures of immortalized epithelial
84 cells, grown in multi-well plates or Transwell inserts *Jafari et al. (2016)*; *Janvilisri et al. (2010)*. A
85 fundamental limitation of this approach is the conflicting environmental requirements, as *C. diffi-*
86 *cile* thrives under strict anaerobic conditions while epithelial cells require normoxic conditions for
87 optimal growth. Consequently, such setups are restricted to short-term co-culture experiments fol-
88 lowing bacterial inoculation. This brief exposure window is insufficient for studying the progressive
89 cellular and molecular events that characterize CDI pathophysiology. Alternatively, many existing

models rely on the static application of purified *C. difficile* toxins rather than live bacteria *Kasendra et al. (2014); Leslie et al. (2015)*. While informative for toxin biology, this method fails to capture the dynamic and multi-faceted interactions between live bacteria and the host epithelium. These interactions are critical for a comprehensive understanding of infection dynamics.

To reconcile the divergent oxygen demands, specialized devices featuring two chambers separated by a porous membrane have been developed *Bang et al. (2025); Anonye et al. (2019)*. In these systems, an oxic chamber supports epithelial cells and an adjacent anoxic chamber cultivates *C. difficile*. Furthermore, such configurations can provide epithelial cells with fluidic shear stress, a key mechanical cue that promotes phenotypic differentiation and maturation. Typically constructed from rigid plastics, these devices remain compatible with standard cell culture incubators. Also based on the two chambers oxygen segregation principle, gut-on-a-chip (GOC) models fabricated from elastic silicone elastomers have emerged as superior platforms for mimicking the native intestinal microenvironment *Jalili-Firoozinezhad et al. (2019); Meza-Torres et al. (2025)*. The elastic property enables GOC to integrate more essential physiological cues, such as cyclic peristalsis-like mechanical strain. This makes GOC systems uniquely drive intestinal epithelial development toward a more physiologically relevant state, closely mirroring the structural complexity and functional maturity observed *in vivo*. Nonetheless, a significant practical challenge remains due to the high gas permeability of the silicone elastomer. To maintain the stringent anoxia required for anaerobic bacteria, the entire GOC device must be housed within a custom-built anaerobic chamber. This requirement introduces practical challenges related to cost, complexity, and compatibility with routine laboratory workflows.

Herein, we developed a GOC-based system to establish an *in vitro* CDI model. The system is configured within a sealed chamber that can be placed in a standard cell culture incubator, offering a compact, cost-effective, and operationally straightforward approach for co-culture experiments with reduced risk of microbial dissemination. Employing this system, we investigated the role of the pro-resolving mediator LXA₄ in CDI. We found that while LXA₄ administration after infection did not show direct therapeutic efficacy, its prior application significantly alleviated the severity of CDI pathology, represented as LXA₄ treatment greatly strengthened the epithelial barrier and mitigated dysregulated inflammatory responses. This protective effect was corroborated in a murine model. Furthermore, we revealed a promising synergistic strategy wherein the combination of LXA₄ with standard antibiotics enhanced therapeutic efficacy beyond antibiotic monotherapy. Collectively, our work not only provides a robust and accessible platform for studying host-*C. difficile* interactions but also opens new avenues for developing inflammation resolution-based prophylactic and adjunctive strategies to augment current treatments for healthcare-associated infections.

Results

Establishment of a CDI model on a GOC system

A key advancement of our platform is its compact and fully integrated design, which reconciles the conflicting oxygen requirements for epithelial cells and *C. difficile* within a single, sealable chamber compatible with standard incubators. The setup of the GOC-based system is shown in *Figure 1A*. It consists of four main components: an anaerobic gas-generating sachet to establish an anoxic atmosphere, an oxygen indicator to monitor oxygen levels, a GOC microfluidic chip that mimics the human intestinal tract, and a gas-tight transparent chamber that isolates all components from the ambient normoxic environment. Culture media with different oxygen partial pressures are maintained outside the chamber and perfused into the GOC device through two sealed ports on its sidewall. Effluent media are collected separately inside the chamber to facilitate subsequent recovery and analysis. The entire assembly is compact, occupying an area of < 300 cm², and can be readily placed inside a conventional cell culture incubator. The gas-tight design prevents bacterial escape, allowing safe operation alongside other cell cultures in the same workspace.

We employed a widely adopted GOC design to establish the CDI model *Huh et al. (2013)*. As illus-

139 trated in **Figure 1B**, the GOC chip comprises two vertically stacked microchannels separated by a
 140 porous membrane (**Figure 1—figure Supplement 1A**). Caco-2 cells, derived from human colorectal
 141 adenocarcinoma, were seeded onto the membrane and cultured to form a confluent monolayer
 142 resembling the intestinal epithelium (**Figure 1—figure Supplement 1B**). This cell layer physically
 143 separates the two microchannels, permitting the maintenance of an anoxic environment in the
 144 upper channel and an oxic environment in the lower channel. The pore size and density of the
 145 membrane allow the free diffusion of oxygenated mammalian cell culture medium to the basal
 146 side of the Caco-2 monolayer while preventing cell migration into the lower channel. *C. difficile* sus-
 147 pended in anoxic medium was introduced into the upper channel to enable bacterial colonization
 148 and proliferation, thereby simulating infection. Culture medium was continuously perfused at a
 149 rate of 30 μ L/h to mimic intestinal fluid flow and shear stress.

150 The formation of intercellular tight junctions and the secretion of mucus are critical indicators
 151 of functional intestinal epithelium in vitro. To evaluate our GOC system, we characterized the tight
 152 junction protein Zonula Occludens-1 (ZO-1) and mucin production after 72 hours of oxic Caco-2
 153 culture. Mucin secretion confirmed Caco-2 cell differentiation, and robust ZO-1 expression indi-
 154 cated the establishment of a functional epithelial barrier (**Figure 1C**). The oxic medium was then
 155 replaced with anoxic medium to simulate the anaerobic luminal environment of the human intes-
 156 tine. After 24 hours under anoxic conditions, no adverse effects on epithelial cells were observed,
 157 as evidenced by comparable ZO-1 intensity (**Figure 1C, E**) and cell viability (**Figure 1—figure Supple-**
 158 **ment 1C**) between anoxic and normoxic cultures.

159 To establish the CDI model, *C. difficile* was inoculated into the upper channel of the GOC de-
 160 vice. Following 24 hours of coculture, bacterial counts increased near 6-fold (**Figure 1D**), confirming
 161 the suitability of the anoxic environment for *C. difficile* growth. Importantly, infection significantly
 162 disrupted epithelial tight junctions, as shown by markedly reduced ZO-1 signal (**Figure 1C, E**), indi-
 163 cating compromised barrier integrity. We further assessed the inflammatory response triggered
 164 by *C. difficile*. Levels of the representative pro-inflammatory cytokines IL-8 and IFN- γ increased
 165 approximately 14-fold and 12-fold, respectively, compared to the non-infection state (**Figure 1F**).
 166 These results demonstrate that our GOC system successfully recapitulates key pathophysiological
 167 features of CDI, confirming the establishment of a robust in vitro CDI model.

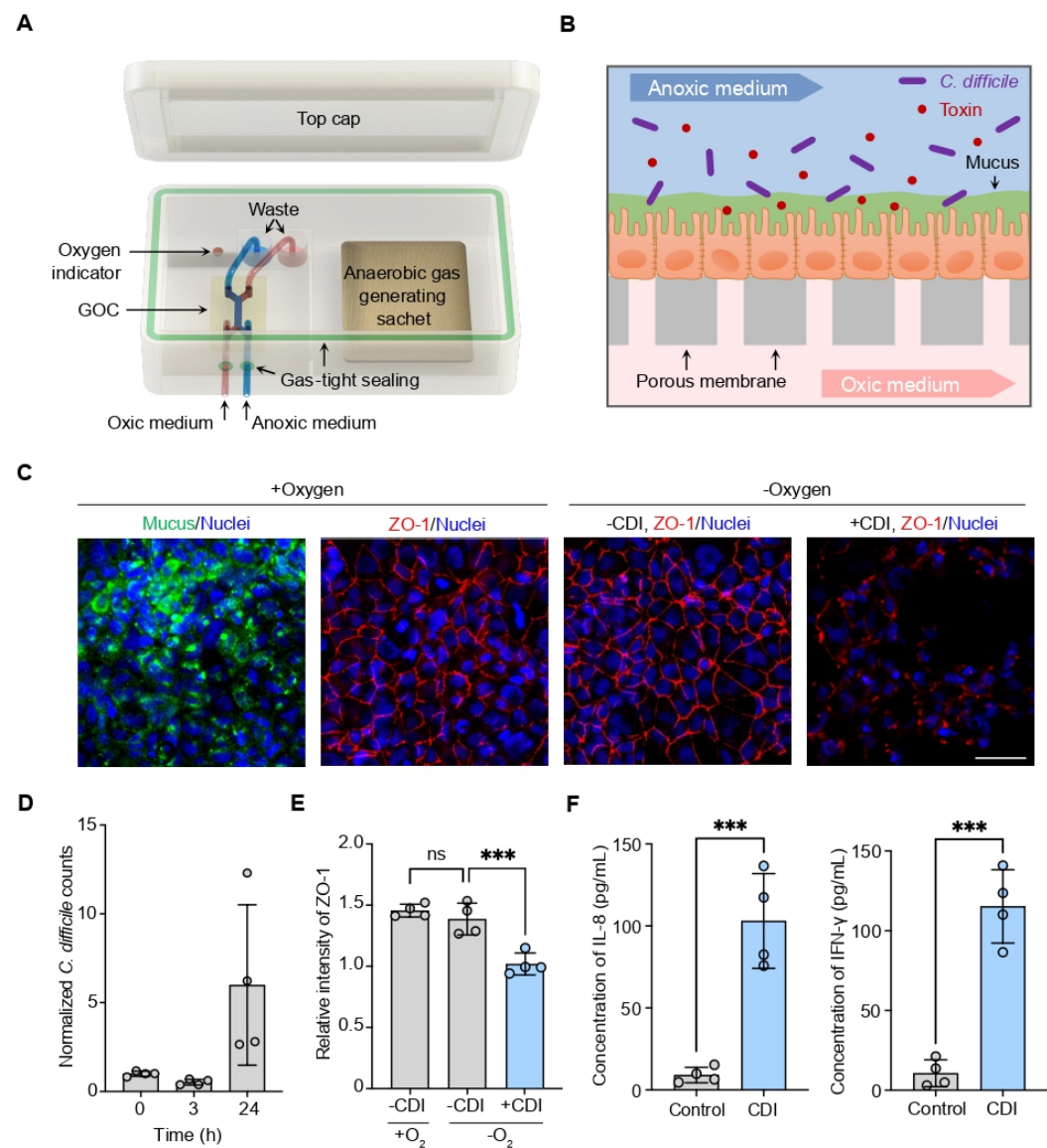


Figure 1. Modeling CDI using a GOC-based system. (A) Schematic of the GOC system design. (B) Schematic of the GOC-based *in vitro* CDI model. (C) Confocal micrographs of the intestinal epithelium cultured for 24 hours in the GOC. The upper microchannel was perfused with normoxic (+ Oxygen) or anaerobic (- Oxygen) culture medium. Under anaerobic condition, the intestinal epithelium was either infected (+ CDI) or not infected (- CDI) by *C. difficile*. Tight junctions were immunostained for ZO-1 (red), the mucus layer was labeled with wheat germ agglutinin (WGA, green), and nuclei were stained with DAPI (blue). Scale bar: 50 μ m. (D-F) Quantitative analysis of (D) *C. difficile* cell counts in the GOCs at 0, 3 and 24 hours post-infection; (E) the average ZO-1 fluorescence intensity of intestinal epithelium in GOCs; and (F) secretion of pro-inflammatory cytokines IL-8 and IFN- γ in GOCs with or without *C. difficile* infection. Data are presented as mean $\pm$ SD (n = 4 independent replicates). Significance was determined by unpaired t-test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Figure 1—figure supplement 1. Characterization of the GOC device and on-chip cell culture.

168 Prophylactic effect of LXA₄ against CDI in *in vitro* model

169 Building on the established GOC-based *in vitro* CDI model, we next sought to evaluate the thera-
 170 peutic and prophylactic potential of the pro-resolving mediator LXA₄ against CDI induced intestinal
 171 injury. To first determine if LXA₄ itself exerted any adverse effects on epithelial cells, we treated
 172 intestinal epithelial cells with 200 nM LXA₄ for 24 hours. The treatment did not affect intercellular

173 tight junctions indicated by ZO-1 expression remained unchanged compared with controls, proven
174 that LXA₄ is non toxic to epithelial cells (**Figure 2—figure Supplement 1A**). This result is consistent
175 with previous reports. We then assessed whether LXA₄ could cure or alleviate CDI mediated dam-
176 age to the intestinal epithelium. In the treatment regimen, LXA₄ was administered 6 hours post
177 infection, and outcomes were analyzed 24 hours after infection (**Figure 2A**). Immunofluorescence
178 staining for the tight junction protein ZO-1 revealed severe disruption of epithelial integrity fol-
179 lowing CDI, regardless of LXA₄ treatment (**Figure 2B**). Quantitative analysis showed no significant
180 difference in ZO-1 expression between the CDI control and LXA₄ treated groups (**Figure 2C**), indi-
181 cating that LXA₄ administration after infection did not confer therapeutic benefit.

182 We therefore examined the prophylactic potential of LXA₄. In the prevention regimen, LXA₄ was
183 added 6 hours before bacterial challenge, with data collected 24 hours post infection (**Figure 2A**).
184 Notably, pretreatment with LXA₄ markedly preserved tight junction organization, as evidenced by a
185 significantly stronger ZO-1 signal compared with both the untreated control and the post infection
186 treatment group (**Figure 2B, C**). Epithelial cell viability was also substantially maintained; the LXA₄
187 pretreated group exhibited approximately 2.5 fold higher viability than the untreated CDI control
188 (**Figure 2D**).

189 Given the well established role of LXA₄ as a pro-resolving mediator, we further asked whether
190 it could dampen the characteristic inflammatory response triggered by CDI. As anticipated, levels
191 of the key pro inflammatory cytokines IL 8 and IFN- γ were reduced by about 50% in the prevention
192 group relative to the untreated control (**Figure 2E**), demonstrating a pronounced anti inflammatory
193 effect of LXA₄ during CDI. These data suggest a direct link between the anti inflammatory activity
194 of LXA₄ and its prophylactic efficacy.

195 It has been reported that *C. difficile* exacerbates intestinal damage by driving an excessive in-
196 flammatory response, which not only disrupts the epithelial barrier but also releases host derived
197 nutrients and metabolites that can support bacterial proliferation *Wang et al. (2024)*. We thus hy-
198 pothesize that the prophylactic effect of LXA₄ may operate through two interrelated mechanisms:
199 first, by attenuating the inflammatory cascade, LXA₄ limits the availability of pro proliferative host
200 factors for *C. difficile*; second, the preserved epithelial barrier integrity, reflected in enhanced cell
201 viability and tight junction stability, likely hinders bacterial adhesion and colonization. Consistent
202 with this hypothesis, bacterial counts in the LXA₄ prevention group were approximately threefold
203 lower than those in the untreated control (**Figure 2F**).

204 To gain further insight into epithelial responses to LXA₄, we performed transcriptomic analysis
205 comparing LXA₄ pretreated and CDI infected epithelial layers. A total of 226 differentially expressed
206 genes (48 downregulated, 178 upregulated) were identified in LXA₄ exposed epithelia (**Figure 2G**,
207 **Figure 2—figure Supplement 1B**). Gene Ontology enrichment analysis revealed that upregulated
208 genes were significantly associated with cell recognition, regulation of chemokine production, cell
209 junction organization, and intercellular adhesion, indicating enhanced pathogen sensing and bar-
210 rier reinforcement. Conversely, downregulated genes were enriched in immune activation path-
211 ways, consistent with a restrained inflammatory response (**Figure 2H**). These findings demonstrate
212 that LXA₄ reprograms the intestinal epithelial transcriptome to bolster barrier function and temper
213 immune signaling.

214 Collectively, LXA₄ can protect against CDI through prophylactic administration by preserving
215 epithelial barrier integrity, reducing inflammation, and limiting *C. difficile* proliferation.

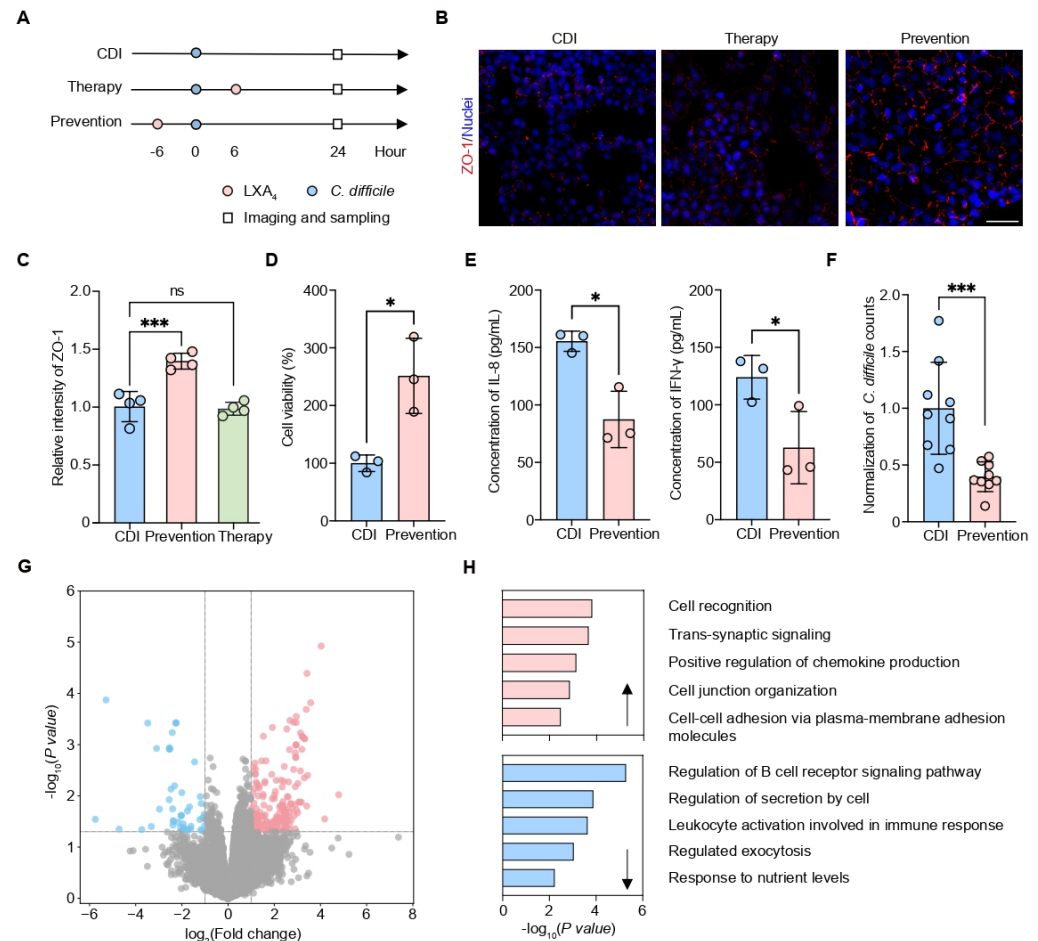


Figure 2. Prevention effect of LXA₄ against CDI in the GOC-based *in vitro* system. (A) Schematic of experimental timeline for LXA₄ administration in the control (CDI), prevention and therapy groups. (B) Confocal micrographs of the intestinal epithelium in GOCs subjected to prevention and therapy groups. Tight junctions were immunostained for ZO-1 (red) and nuclei were stained with DAPI (blue). Scale bar, 25 μm. (C-F) Quantitative analysis of (C) the average ZO-1 fluorescence intensity in the intestinal epithelium in CDI control, LXA₄ prevention and therapy groups (n = 4 independent replicates); (D) epithelial cell viability with or without LXA₄ prevention (n = 3 independent replicates); (E) secretion of the pro-inflammatory cytokines IL-8 and IFN-γ with or without LXA₄ prevention (n = 3 independent replicates); and (F) *C. difficile* cell counts in GOCs with or without LXA₄ prevention (n = 9 independent replicates). (G) Differentially expressed genes of intestinal epithelium cells from GOCs with or without LXA₄ prevention. Genes with |log₂(fold change)| ≥ 1 and adjusted P value < 0.05 were considered significantly differentially expressed. (H) GO analysis of up- and down-regulated genes in the LXA₄ prevention group. The top five enriched terms are displayed. Data in (C-F) are presented as mean ± SD; significance was determined by unpaired t-test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Figure 2—figure supplement 1. Cytotoxicity assessment of LXA₄ and its impact on the transcriptomic landscape.

Validating the prophylactic effect of LXA₄ against CDI in an *in vivo* model

To further validate the prophylactic effect of LXA₄ that had been initially observed under *in vitro* experimental conditions, we subsequently employed a murine model of CDI in order to comprehensively evaluate its protective efficacy within a living organism (**Figure 3A**). In mice that were infected with *C. difficile* but did not receive any pretreatment, the infection resulted in a pronounced and progressive decline in overall health, marked by a substantial body weight loss exceeding 5%. By contrast, those mice that had been prophylactically administered LXA₄ exhibited a significant

223 preservation of physiological condition, maintaining body weights that were closely comparable to
 224 those of the uninfected control group throughout the observation period, and demonstrating no
 225 measurable reduction attributable to the infection (**Figure 3B**). Furthermore, quantitative micro-
 226 biological analysis revealed that pretreatment with LXA₄ led to a remarkable reduction in fecal *C.*
 227 *difficile* bacterial loads, achieving an approximate 10-fold decrease compared to the infected but
 228 untreated control animals (**Figure 3C**).

229 Histopathological examination results showed that the intestinal tissues of mice infected with *C.*
 230 *difficile* exhibited obvious pathological changes, mainly manifested as severe submucosal edema,
 231 massive neutrophil infiltration, and significant epithelial structural damage, including the loss of
 232 intestinal crypts and disruption of mucosal tissue architecture (**Figure 3D, E**). In contrast, the ex-
 233 perimental group of mice that received preventive administration of LXA₄ showed significantly
 234 improved clinical disease scores, indicating that LXA₄ has an obvious protective effect against
 235 infection-induced pathological damage. In mice pretreated with LXA₄, the degree of pathological
 236 changes in intestinal tissues was significantly decreased, with both the extent of epithelial damage
 237 and the number of inflammatory cell infiltrations reduced by approximately 50% to 60% (**Figure 3D,**
 238 **E**).

239 Together, these in vivo results corroborate our in vitro findings, further validating the prophy-
 240 lactic efficacy of LXA₄ against CDI and its ability to alleviate associated clinical symptoms.

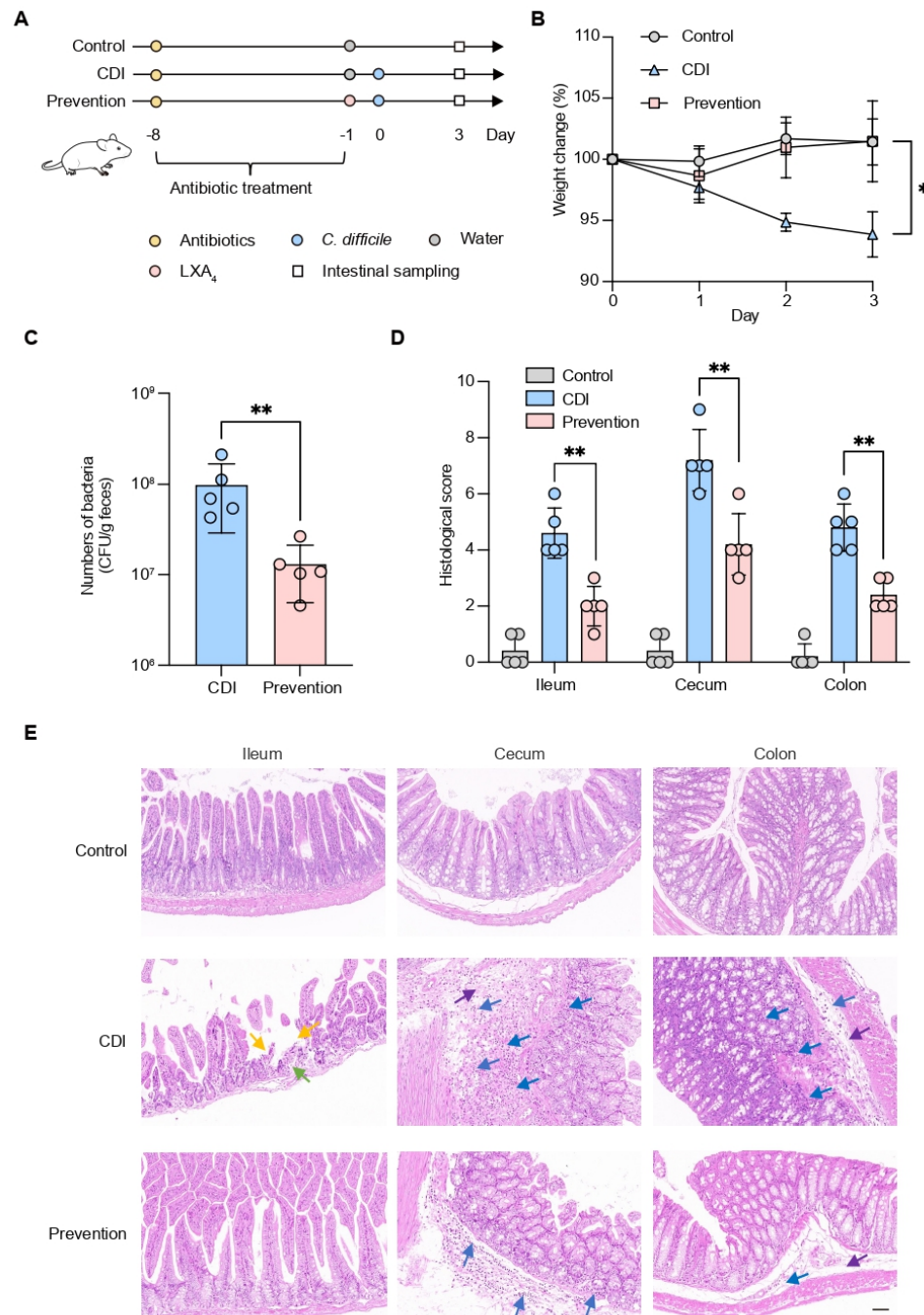


Figure 3. Protective effect of LXA₄ against CDI in a murine model. (A) Experimental scheme for establishing the CDI model in mice, including the timeline of LXA₄ administration. (B) Body weight changes in the CDI control and LXA₄ prevention groups. (C) *C. difficile* bacterial loads in feces at day 3 post-infection with or without LXA₄ prevention. (D) Histopathological scores of ileum, cecum, and colon from H&E-stained sections with or without LXA₄ prevention. (E) Representative H&E-stained images of ileum, cecum, and colon tissues with or without LXA₄ prevention. Arrows indicate key pathological features: immune cell infiltration (blue), villous epithelium loss (yellow), edema (purple), and crypt loss (green). Scale bar: 50 μ m. Data in (B–D) are presented as mean $\pm$ SD (n = 5 mice per group). Statistical significance was determined by unpaired t-test (* P < 0.05, ** P < 0.01).

241 Synergistic efficacy of LXA₄ combined with antibiotics against CDI

242 While LXA₄ monotherapy demonstrated limited therapeutic efficacy when administered after infec-
243 tion, it exhibited pronounced prophylactic potential against CDI-induced pathology. Given that an-
244 tibiotics remain the first-line clinical treatment for CDI, we hypothesized that a combined strategy,
245 which leveraging the preventive capacity of LXA₄ alongside antibiotic therapy, could offer a more
246 effective approach to managing healthcare-associated CDI. To test this, we selected vancomycin, a
247 cornerstone antibiotic routinely employed in the clinical management of CDI.

248 To establish the baseline therapeutic response, we first evaluated vancomycin monotherapy in
249 our GOC-based CDI model (**Figure 4A**). Vancomycin alone effectively ameliorated CDI-related dam-
250 age, consistent with its established clinical activity. Compared to the infected control, vancomycin
251 monotherapy significantly increased tight-junction protein ZO-1 expression (**Figure 4B, C**) and re-
252 duced levels of the pro-inflammatory cytokines IL-8 and IFN- γ (**Figure 4D**), confirming that our *in*
253 *vitro* model reliably recapitulates the antibiotic response observed *in vivo*.

254 Building on this validated model, we next asked whether LXA₄ pretreatment could further en-
255 hance therapeutic efficacy of vancomycin. In the combined regimen, LXA₄ was administered 6
256 hours before *C. difficile* inoculation and vancomycin was added 12 hours post-infection, with read-
257 outs taken 24 hours after bacterial challenge (**Figure 4A**). The combination markedly improved
258 outcomes relative to vancomycin alone. Specifically, the ZO-1 expression increased 1.4-fold (**Fig-
259 ure 4B, C**), while levels of the pro-inflammatory cytokines IL-8 and IFN- γ fell by approximately 3-
260 fold and 2.6-fold (**Figure 4D**). These results demonstrated that LXA₄ pretreatment synergized with
261 vancomycin to strengthen epithelial barrier integrity and more effectively suppress inflammation
262 during CDI. Thus, prophylactic LXA₄ indeed enhanced the therapeutic effect of vancomycin against
263 CDI *in vitro*.

264 Clinically, severe CDI requires higher antibiotic dosages, which increases the risk of patient tox-
265 icity and potential organ damage **Johnson et al. (2021); McDonald et al. (2018)**. Therefore, thera-
266 peutic strategies that enhance antibiotic efficacy without raising dosage are highly desirable. We
267 investigated whether a reduced antibiotic dosage combined with prophylactic LXA₄ could achieve
268 efficacy comparable to that of a full-dose regimen. Experimentally, the vancomycin concentration
269 was halved (from 0.5 to 0.25 $\mu\text{g/mL}$) in the presence of LXA₄ pretreatment, the combination reg-
270 imen achieved therapeutic outcomes comparable to those of full-dose vancomycin alone, with
271 no statistically significant differences in ZO-1 expression or pro-inflammatory cytokine levels (**Fig-
272 ure 4E, F**).

273 Taken together, prophylactic LXA₄ enhanced the therapeutic profile of vancomycin against CDI,
274 enabling a substantial reduction in antibiotic dosage without compromising efficacy.

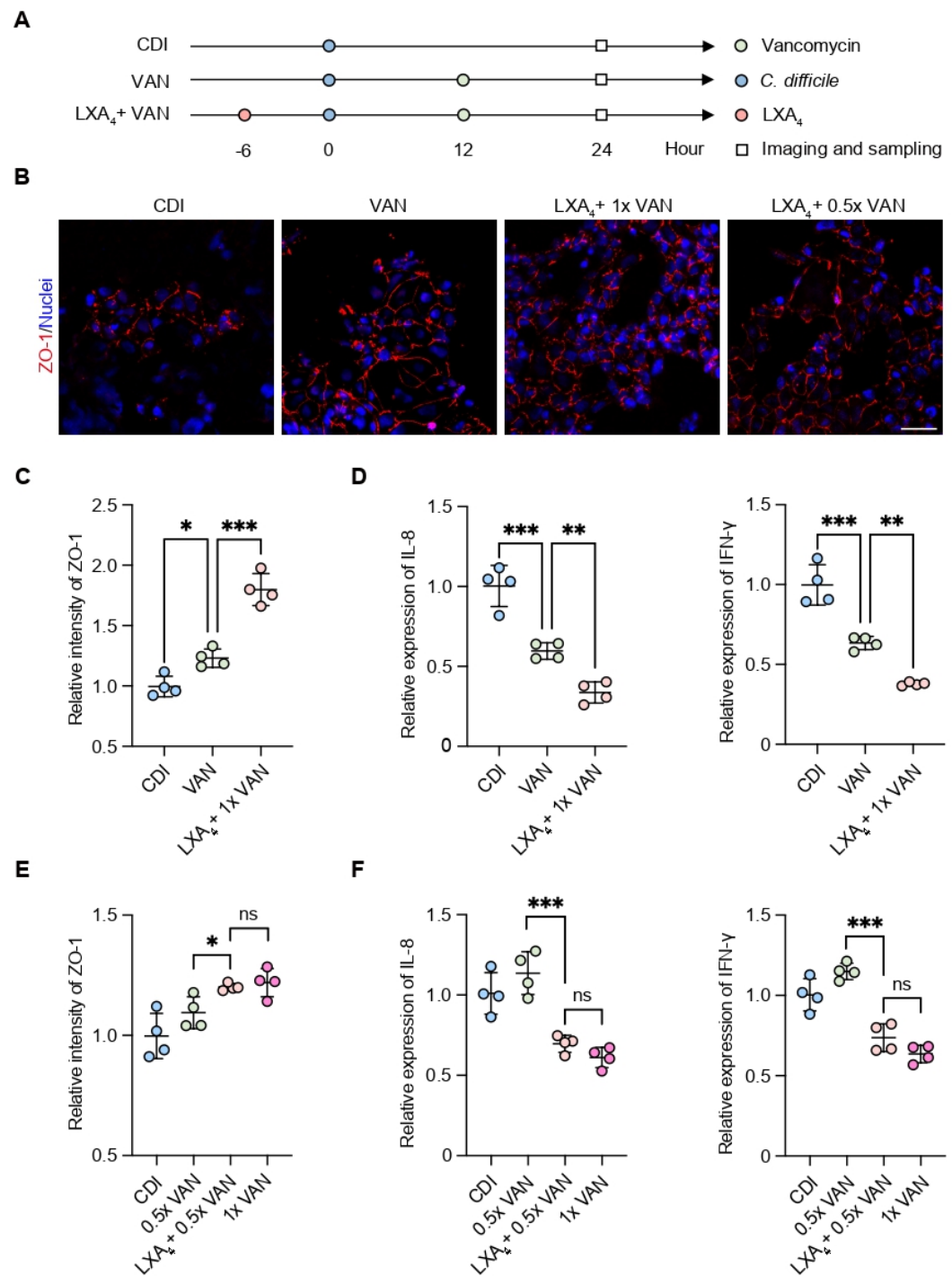


Figure 4. Adjunctive effect of LXA₄ with vancomycin for the treatment of CDI. (A) Experimental scheme for the combined administration of LXA₄ and vancomycin. (B) Confocal micrographs of the intestinal epithelium in GOCs under different conditions: CDI control, vancomycin monotherapy (VAN), LXA₄ combined with full-dose vancomycin (LXA₄ + 1× VAN), and LXA₄ combined with half-dose vancomycin (LXA₄ + 0.5× VAN). Tight junctions are immunostained for ZO-1 (red) and nuclei are stained with DAPI (blue). Scale bar: 50 μm. (C-D) Quantitative analysis of (C) the average ZO-1 fluorescence intensity and (D) the secretion of pro-inflammatory cytokines (IL-8 and IFN-γ) in GOCs from the CDI control, VAN, and LXA₄ + 1× VAN groups. (E-F) Quantitative analysis of (E) the average ZO-1 fluorescence intensity and (F) the secretion of IL-8 and IFN-γ in GOCs from the CDI control, VAN, LXA₄ + 1× VAN, and LXA₄ + 0.5× VAN groups. Data in (C-F) are presented as mean ± SD (n = 4 independent replicates). Statistical significance was determined by unpaired t-test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Discussion

In this study, we developed a compact, integrated gut-on-a-chip platform that reconciles the divergent oxygen requirements of aerobic intestinal epithelium and anaerobic *C. difficile* within a single gas-tight chamber compatible with standard cell culture incubators. This design not only prevents microbial dissemination but also offers a scalable and user-friendly system for host-pathogen co-culture. Using this platform, we established a robust *in vitro* model of CDI that successfully recapitulates key pathophysiological features of the infection, including disruption of epithelial tight junctions, elevated secretion of pro-inflammatory cytokines, and progressive bacterial colonization, thus providing a physiologically relevant tool for mechanistic investigation.

Our findings demonstrated that the specialized pro-resolving mediator LXA₄ exerted significant protective effects against CDI when administered prophylactically. LXA₄ pretreatment strengthened epithelial barrier integrity, as evidenced by preserved ZO-1 expression and enhanced cell viability, while simultaneously dampening the release of pro-inflammatory cytokines such as IL-8 and IFN- γ . These dual actions, barrier protection and inflammation resolution, collectively limit *C. difficile* colonization and mitigate infection induced tissue damage. Notably, LXA₄ synergized with the frontline antibiotic vancomycin, improving epithelial repair and inflammatory suppression beyond antibiotic monotherapy. Moreover, LXA₄ pretreatment allowed a substantial reduction in vancomycin dose without compromising therapeutic efficacy, highlighting its potential as an adjuvant to decrease antibiotic exposure and associated risks.

From a clinical perspective, prophylactic LXA₄ administration could address a critical unmet need in hospitalized patients at high risk for CDI as well, particularly those receiving broad-spectrum antibiotics, for whom no approved preventive strategies are currently available. Although several candidate vaccines targeting high-risk populations have entered clinical development, most advanced candidates (e.g., toxoid-based vaccines) require multiple-dose regimens (typically three doses) and, as demonstrated in recent phase 3 trials, have thus far shown limited success in preventing primary CDI [Donskey et al. \(2024\)](#); [Kuijper and Gerding \(2024\)](#). In this context, a readily administrable prophylactic agent such as LXA₄, or its stable analogs, could offer a practical strategy to reduce the incidence of primary CDI in vulnerable inpatient cohorts.

Beyond LXA₄, other SPMs, including resolvins, protectins, and maresins, represent promising candidates for modulating host responses in CDI. These mediators exhibit diverse pro-resolving and tissue reparative functions that could be harnessed to prevent or treat infection-driven intestinal damage. Future studies should systematically evaluate SPM libraries in relevant preclinical models to identify compounds with optimal efficacy, pharmacokinetics, and safety profiles for clinical translation.

Looking forward, the presented GOC-based CDI model offers a robust platform for studying host-pathogen interactions, yet its physiological relevance could be further enhanced by incorporating key components of the gut ecosystem. Integration of commensal microbiota would allow investigation of microbial community dynamics in shaping CDI susceptibility and the efficacy of SPMs such as LXA₄. Additionally, inclusion of immune cells, such as macrophages or neutrophils, would enable more comprehensive analysis of mucosal immune responses and the immunomodulatory mechanisms of SPMs. Such an advanced system would better recapitulate the intestinal microenvironment, providing a more holistic tool for dissecting the multifaceted mechanisms of CDI pathogenesis and resolution.

Methods and Materials

Fabrication of the GOC microfluidic device

The microfluidic chip was fabricated using soft lithography. Briefly, a template featuring raised SU-8 photoresist microstructures was first created on a silicon wafer via UV lithography. Polydimethylsiloxane (PDMS) prepolymer was prepared by mixing the base and curing agent at a 10:1 weight ratio (w/w) (Dow Corning). The thoroughly mixed prepolymer was then poured onto the template,

degassed under vacuum to remove bubbles, and cured at 80 °C for 30 minutes to obtain a PDMS block containing the embossed microchannel structures. The upper and lower microchannels of the chip were designed with identical dimensions of 1 mm (width) × 10 mm (length) × 0.2 mm (height). Inlet and outlet ports were punched prior to bonding. The two channels were separated by a porous PDMS membrane. To fabricate the membrane, a silicon wafer template with an array of cylindrical pillars (10 μm in diameter, 30 μm in height, 25 μm spacing) was fabricated using SU-8 photoresist. The template was then surface-treated with a silanizing agent. PDMS prepolymer was poured onto the wafer, and after degassing, a flat PDMS block was pressed onto the pillar array to allow the prepolymer to fill the inter-pillar spaces without covering the pillar tops. Curing was performed at 80 °C for 30 minutes to obtain a through-pore PDMS membrane. The upper PDMS layer was plasma-treated and bonded to the membrane; the bonded stack was then peeled off and finally sealed to the lower PDMS layer via plasma treatment *Huh et al. (2013)*.

Cell culture and seeding in the GOC microfluidic device

Caco-2 cells were maintained in T25 flasks using Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco) at 37 °C in a humidified 5% CO₂ incubator, and passaged at a 1:3 ratio every 2–3 days. Before cell seeding, the PDMS chip was treated with ultraviolet light and ozone for 30 minutes. Microchannels were then coated with 30 μg/mL collagen type I (Sigma) for 1 hour at 37 °C. Caco-2 cells were resuspended in DMEM containing 20% FBS and 1% penicillin–streptomycin at a density of 1×10⁷ cells/mL and seeded into the upper microchannel. The chip was kept static in a CO₂ incubator at 37 °C for 1 hour to allow cell attachment. Thereafter, cells were perfused with medium at a constant flow rate of 30 μL/h for 3 days to form a confluent epithelial monolayer.

Culture and infection with *C. difficile* in the GOC microfluidic device

The obligate anaerobe *C. difficile* strain 1482 was routinely cultured on brain heart infusion (BHI) agar or in BHI broth under anaerobic conditions (90% N₂, 5% CO₂, 5% H₂). To induce CDI on-chip, an overnight bacterial culture was diluted in fresh medium to an OD₆₀₀ of 0.4. All equipment was placed inside an anaerobic chamber. Under static conditions, bacterial suspension (MOI = 1:10) was introduced into the upper channel (designated as 0 hours). After a 30 minutes settlement period, the chip was transferred to the transparent anaerobic chamber containing an anaerobic gas-generating sachet and an oxygen indicator (AnaeroPack™ System, Mitsubishi Gas Chemical). Tubing from medium-filled syringes was connected to the chip inlets through gas-tight holes on the side-wall of the chamber. Then the chamber was sealed by locking the top lid. The entire assembly was transferred from the anaerobic chamber to a standard cell culture incubator, where medium perfusion (30 μL/h) was resumed and maintained for 24 hours.

For LXA₄ pretreatment experiments, the upper channel medium was replaced with anoxic BHI medium containing 200 nM LXA₄ (Cayman) 6 hours before infection and perfused for 6 hours. For the control group, anoxic BHI medium without LXA₄ was perfused during the same period.

For antibiotic treatment experiments, 12 hours after bacterial inoculation, the upper channel medium was switched to anoxic BHI medium containing vancomycin (0.25 or 0.5 μg/mL, Sigma) and perfused for an additional 12 hours. Control groups received anoxic BHI medium without antibiotic for the same duration.

Quantification of bacterial load in the GOC microfluidic device

To quantify adherent *C. difficile*, the upper channel was gently flushed with 1 mL PBS at a flow rate of 50 μL/min. Effluent was collected, serially diluted, fixed, and stained with DAPI. Bacterial counts were determined using flow cytometry.

Immunofluorescence staining

For ZO-1 staining, the upper microchannel was washed with PBS and fixed with 4% paraformaldehyde for 15 minutes, followed by PBS rinses. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, blocked with 5% BSA in PBS for 1 hour, and incubated overnight at 4 °C with Alexa Fluor 594 conjugated mouse anti-human ZO-1 monoclonal antibody (Thermo Fisher, 339194, 1:100 dilution). After washing, nuclei were counterstained with DAPI (Keygen, KGA215-10).

For mucus staining, fluorescein labeled wheat germ agglutinin (WGA-Alexa Fluor 488 conjugate, Thermo Fisher, W11261) was diluted to 25 µg/mL in culture medium and introduced into the epithelial channel. After 20–30 minutes incubation at room temperature in the dark, the channel was gently washed three times with PBS (10 minutes per wash) to remove unbound WGA. Nuclei were counterstained with DAPI. All samples were imaged using a confocal laser scanning microscope.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit 8 (CCK-8, Sigma Aldrich). Briefly, CCK-8 solution was introduced into the upper channel and incubated for 2 hours. Effluent was collected, and absorbance at 450 nm was measured using a microplate reader.

Cytokine measurement

For all experiments involving cytokine analysis, effluent from the two outlets corresponding to the upper and lower microchannels was collected separately into microcentrifuge tubes. Collection started 6 hours before *C. difficile* infection and continued until 24 hours post infection. Collected samples were stored at –80 °C until analysis.

Levels of TNF-α and IL-8 in the effluent were measured using commercial ELISA kits (Biolegend) according to the manufacturer's instructions. Absorbance was read on a microplate reader for concentration analysis.

For each experimental condition, the reported cytokine concentration represents the average of the values obtained from the separately collected and analyzed effluents of the upper and lower microchannels of the same chip.

Live/dead staining

A live/dead staining kit (Invitrogen, R37601) was used. Live Green (Component A) and Dead Red (Component B) dyes were mixed 1:1, then combined with an equal volume of culture medium. The mixture was perfused into the chip and incubated for 15 minutes in the dark before imaging under a fluorescence microscope.

Murine in vivo CDI model

Germ free female C57BL/6 mice (6–8 weeks old) were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). Mice received an antibiotic cocktail (0.4 mg/mL kanamycin, 0.035 mg/mL gentamicin, 0.035 mg/mL colistin, 0.215 mg/mL metronidazole, 0.045 mg/mL vancomycin) in drinking water for 6 days, followed by a single intraperitoneal injection of clindamycin (10 mg/kg) one day before infection. Mice were then inoculated with *C. difficile* (1×10⁹ CFU per mouse) via oral gavage. For LXA₄ prophylaxis, mice received drinking water containing 200 nM LXA₄ starting one day before infection. Fecal pellets were collected on the third day post infection, homogenized in PBS, serially diluted, and plated on cycloserine cefoxitin fructose agar (CCFA) for bacterial enumeration. Mice were euthanized on the third day, and intestinal tissues were collected and fixed in 10% formalin for histopathological analysis.

Histopathological analysis

Formalin fixed intestinal tissues were embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E). Blinded scoring was performed to evaluate immune

cell infiltration (0–3), mucosal damage (0–3), and submucosal edema (0–2). The total score (range 0–8) was classified as follows: 0–1, normal; 2–4, mild; 5–8, severe.

RNA isolation and sequencing

Intestinal epithelial cells were harvested from the microchannels by cold PBS rinses. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured with a Qubit 2.0 Fluorometer (Invitrogen, Q32866) and the Qubit RNA Assay Kit (Life Technologies, Q32855). RNA integrity and genomic DNA contamination were assessed by agarose gel electrophoresis. After quality control, RNA was quantified using the Qubit RNA Broad Range Assay Kit (Life Technologies). Strand specific mRNA libraries were constructed from 100 ng total RNA using the Hieff NGS™ MaxUp Dual Mode mRNA Library Prep Kit for Illumina® (YEASEN). Libraries with insert sizes of 300–500 bp were quantified and sequenced on an Illumina HiSeq X Ten platform.

RNA seq data analysis

Raw reads were quality trimmed using Trimmomatic (v0.36). Clean reads were aligned to the Homo sapiens reference genome (NCBI) using HISAT2 (v2.1.0). Reads mapped to exonic regions were counted with StringTie (v1.3.3b). Differentially expressed genes (DEGs) were identified using the DEP R package (limma based) with thresholds of $P \leq 0.05$ and $|\log_2(\text{fold change})| \geq 1$. Gene Ontology (GO) enrichment analysis of significant DEGs was performed using the Metascape web tool.

Statistical analysis

Data are presented as mean $\pm$ SD. Comparisons between two groups were performed using Student's t test in GraphPad Prism 9 (GraphPad Software Inc.). $P < 0.05$ was considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Fluorescence intensity was quantified using ImageJ software.

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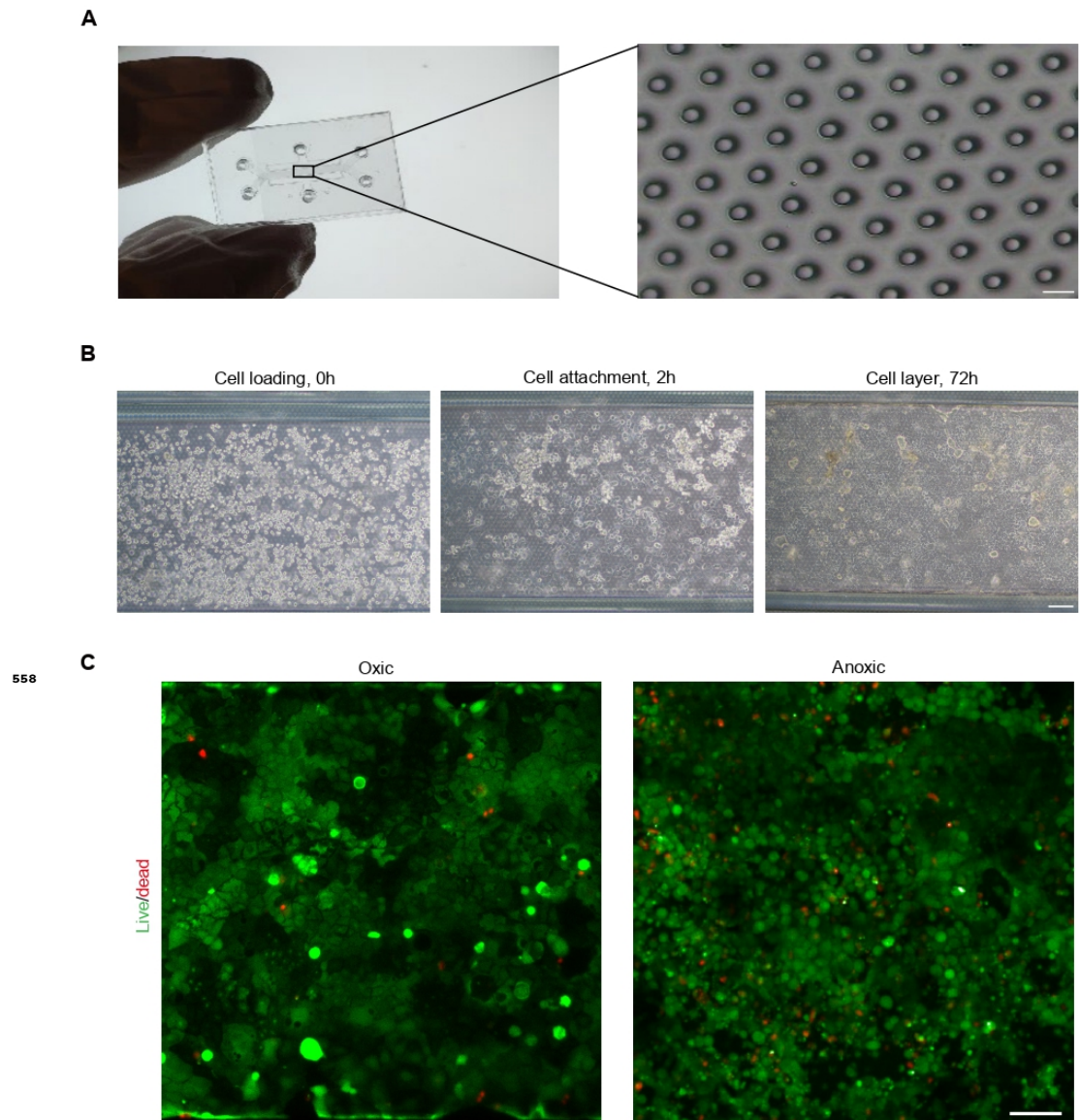


Figure 1—figure supplement 1. Characterization of the GOC device and on-chip cell culture. (A) Photograph of the fabricated GOC device (left) and a micrograph of its porous membrane (right). Scale bar: 20 μm . (B) Brightfield images depicted the endothelial cell layer formation procedure in the GOC. Scale bar: 100 μm . (C) Fluorescence images from a Live/Dead assay under normoxic and anaerobic conditions. Live and dead cells were stained green and red, respectively. Scale bar: 100 μm .

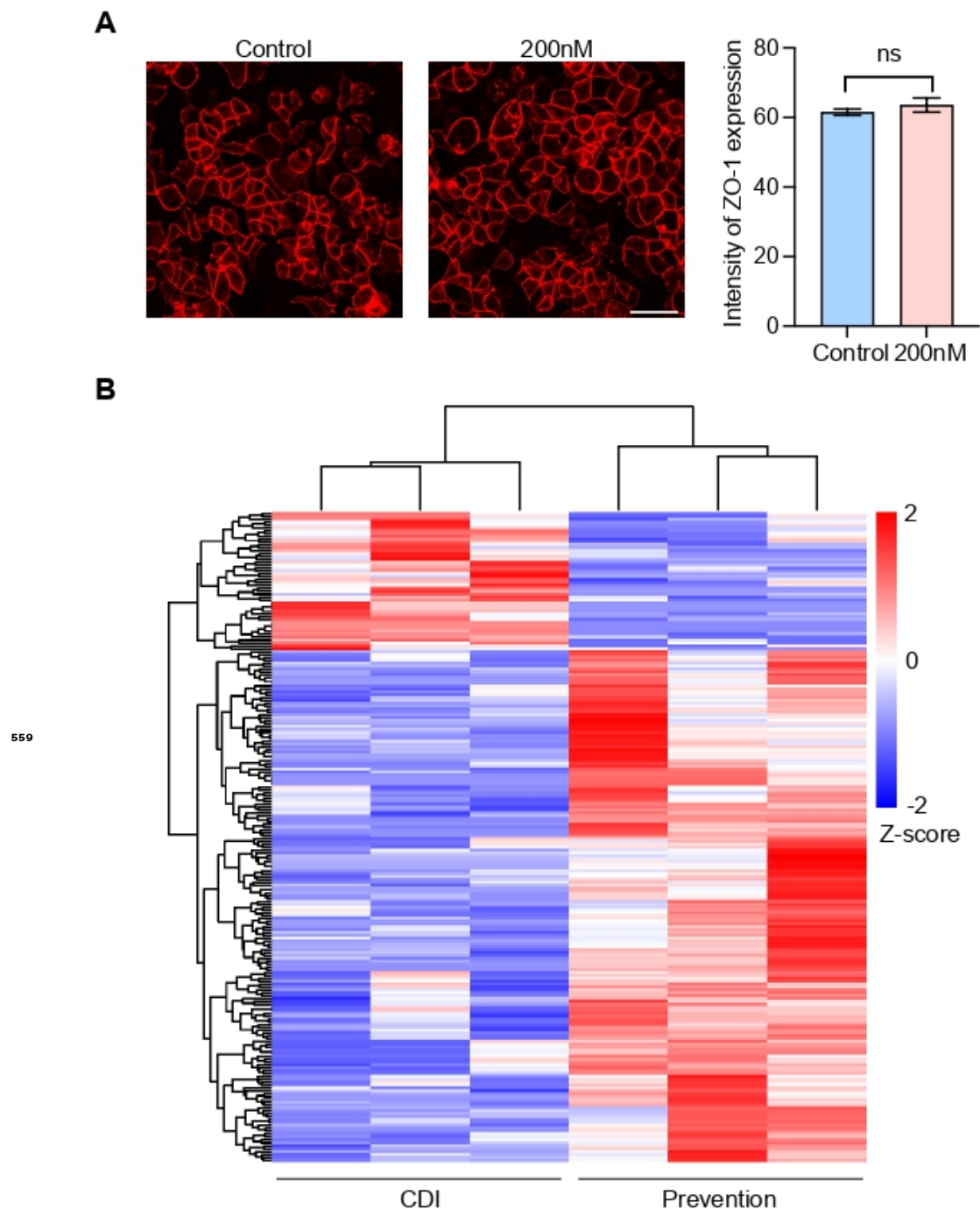


Figure 2—figure supplement 1. Cytotoxicity assessment of LXA₄ and its impact on the transcriptomic landscape. (A) Confocal immunofluorescence images of ZO-1 (red) in Caco-2 cells cultured in the presence or absence of 200 nM LXA₄. Scale bar: 50 μ m. The quantitative analysis (right) shows no significant change in ZO-1 expression, indicating that LXA₄ didn't cause observable toxicity to epithelial cells in the GOC. Data are mean $\pm$ SD (n = 6 independent replicates). (B) Heatmap of RNA-sequencing data illustrating distinct gene expression profiles between the CDI and LXA₄ prevention groups. Color scale corresponds to row-scaled Z-score (red: upregulated; blue: down-regulated).