Brief Report

Immunocompetent High-Throughput Gut-on-Chip Model for Intestinal Microbes–Host Interaction Studies

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Immunocompetent High-Throughput Gut-on-Chip Model for Intestinal Microbes–Host Interaction Studies

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BIOASTER, Lyon, 69007, France

* Correspondence: ibelotserkovsky@bioaster.org

Abstract

The intestinal microbiota and immune system are separated by an epithelial layer, whose barrier function is crucial for the healthy state of the host, while its impairment, usually associated with inflammation, leads to a variety of pathologies in humans. Different probiotics targeting the preservation of epithelial barrier homeostasis are thus currently being developed. Their selection and validation are a complex process involving *in-vitro* and *in-vivo* models. While the former lacks the complexity of the physiological system, the latter often fails to reflect the effect in humans. We used the commercial microfluidic high-throughput device OrganoPlate to set up a Gut-on-Chip model comprising human epithelial and peripheral blood mononuclear cells (PBMC). As a proof-of-concept, we co-cultured several intestinal anaerobic bacteria species in direct contact with the epithelial monolayer for two days to investigate their impact on epithelial barrier integrity and immune response upon induction of inflammation. We demonstrate here that *Bacteroides thetaiotaomicron* protects the epithelial barrier and dampens CCL2 secretion provoked by LPS-stimulated PBMC. Recapitulating the key features of intestinal inflammation, we suggest that the current Gut-on-Chip model enables an easy-to-use screen of next-generation probiotics and live biotherapeutics with homeostatic and immunomodulatory properties.

Keywords: immunomodulation; organ-on-chip; probiotics; epithelial barrier; inflammation; hypoxia; anaerobe; *Bacteroides thetaiotaomicron; Clostridium scindens;* CCL2

Introduction

The intestinal mucosa surface separates the host from the myriads of microorganisms found in the intestinal lumen. This barrier consists primarily of epithelial cells tightly connected thanks to different junction proteins, thus restricting the infiltration of external antigens between the cells [1, 2]. Impairment of this barrier, a phenomenon called the "leaky gut", and the associated inflammation underline and/or aggravate a variety of pathologies in humans starting from those manifested mainly within the gastrointestinal (GI) tract such as inflammatory bowel diseases (IBD), irritable bowel syndrome and celiac disease, and ending with those affecting distant organs throughout the human body, for instance rheumatoid arthritis, non-alcoholic fatty liver disease, diabetes, Parkinson's disease and others [3, 4]. Intensive research in the last decades unraveled the key role of the intestinal microbiota in the maintenance of the epithelial barrier homeostasis, while changes in the microbiome (also called "dysbiosis") are often associated with a variety of autoimmune, metabolic and neurodegenerative diseases [5-7]. On the other hand, some microbial structural components and metabolites were demonstrated to strengthen the intestinal epithelial barrier through modification of the expression and spatial organization of junction proteins of intestinal epithelial cells [8, 9] either directly or via the modulation of cytokines secretion (*e.g.* IFNγ, TNFα, IL-1, IL-6 and others) by epithelial and immune cells [10-13]. Therefore, "classical" probiotics and postbiotics, as well as next-

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generation probiotics such as live biotherapeutic products (LPBs), are extensively developed to aid in maintaining the intestinal epithelial barrier and dampening excessive and chronic inflammation [14, 15]. However, while numerous product candidates pass through pre-clinical development that includes both *in-vitro* and *in-vivo* models, only ~15% of these candidates in the field of inflammatory diseases succeed in clinical trials and get approved [16]. Specifically, for IBDs, the use of adequate pre-clinical models that take into account microbiota–host interactions was identified as one of the major targets to improve the success rate of novel treatment development (Ciorba 2024). In this light, microfluidics-based models offer several advantages over "standard" (one cell-type based) *in-vitro* assays as well as over animal models including:

- The use of flow, which mimics the physiological conditions in the intestine and also exerts shear stress on epithelial cells, promoting their differentiation [17, 18]
- The possibility of combining several cell types in one assay (*e.g.* epithelial and immune cells)
- The 3D spatial organization of the tissues
- The use of human-derived host cells

Here, we co-cultured human PBMC and several intestinal bacteria in a previously described high-throughput Gut-on-Chip model [19-21], which allows rapid maturation of the epithelial monolayer and easy monitoring of its barrier integrity as well as cytokine sampling and microscopic analysis. Using *E. coli* LPS to stimulate PBMC, we induced inflammation-mediated epithelial damage and then tested the capacity of two bacterial strains to reduce the inflammation and protect the epithelial barrier. We suggest that the current Gut-on-Chip model recapitulates the key features of intestinal inflammation and enables an easy-to-use screen of novel (bio) therapeutics in a high-throughput manner.

Materials and Methods

Mammalian Cells

Human colon adenocarcinoma cell line Caco-2/TC-7 (Caco2) (Sigma-Aldrich) at passages between 2 and 12, were cultured in MEM medium (Gibco) supplemented with 10% FBS (Gibco), 1% Sodium Pyruvate (Gibco), 1% Non-essential Amino Acids (NEAA, Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. PBMCs from human blood were isolated using standard procedure using Ficoll-Paque Plus (Cytiva). Briefly, the whole blood was diluted 1:1 with PBS and centrifuged at room temperature (RT) for 30 min at 400 *g* without a break on top of the Ficoll layer. The interphase containing PBMC was then recovered, and the cells were washed in PBS by centrifugation at 600 *g* and then again at 450 *g*, 10 minutes each time. PBMC were set to 10^6 cells/mL in RPMI medium (Sigma-Aldrich) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich). The blood from healthy donors was supplied by the French Blood Establishment (EFS), which is responsible for the collection and distribution of blood in France. All donors provided informed consent to EFS. This sample collection does not require any ethical committee submission.

OrganoPlate Seeding

Epithelial tubule formation by Caco-2 cells in the 3-lane OrganoPlate (Mimetas) was performed as previously reported [20]. In short, $2*10^4$ Caco-2 cells were seeded against a pH-buffered 4 mg/mL Collagen I gel (Collagen-I rat tail, R&D systems) in a 3-lane OrganoPlate and allowed to attach against the gel for 4 h (37 °C, 5% CO_2). Caco-2 medium was then added to the inlets and outlets and bidirectional flow was created by placing the plate on an interval rocker (Perfusion Rocker, Mimetas) switching between a +7° and −7° inclination every 8 min (37 °C, 5% CO_2). Medium was changed 4 days after the seeding. When indicated, 5 days after Caco-2 seeding, the medium in the opposite channel was exchanged with 10^5 PBMCs in supplemented RPMI, with (or without) LPS mix of three

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previously communicated strains of *E. coli* (Sigma-Aldrich, cat. nos. L2637, 3012 and 3137, [22]) at 100 ng/mL each.

Bacteria co-Culture in OrganoPlate

Bacterial strains (Table 1) were cultivated first on previously described mGAM-CRIM agar plates [23] for 48 hours in the BACTRON600 (Sheldon) anaerobic chamber at 37 °C. Then a single colony of each strain was inoculated in 5 mL mGAM-CRIM liquid broth and incubated in the chamber for 24 additional hours. The next day, bacterial cultures were centrifuged at 10000 *g* for 10 minutes and the supernatants were filtered through a 0.22µm filter (Millex-GS, Millipore). Bacteria pellets were washed twice in PBS by repeating resuspension and centrifugation steps and then set to an optical density of 1 in a MEM medium supplemented with 1% Sodium Pyruvate (Gibco), 1% NEAA (Gibco), 1X Insulin-Transferrin-Selenium-Ethanolamine solution (Gibco), called here "MEM-co-culture medium", to support mammalian cell viability in the absence of serum. On day 5, upon Caco-2 seeding and simultaneously with PBMC (+/- *E. coli* LPS) administration in the bottom channel, the medium in the top channel was replaced by bacteria suspension in MEM-co-culture medium or with sterile medium supplemented with filtered bacteria culture at 30% final concentration. OrganoPlates were then incubated in hypoxic conditions: 4% O_2, 5% CO_2 at 37 °C. At indicated time-points, 10µl samples of medium from the top channels were taken, serially diluted in PBS, plated on mGAM-CRIM plates and incubated for 48h in the anaerobic chamber for colony-forming units (CFU) determination.

Cytokine Secretion

Media were collected separately from Lumen and Basal channels and stored at −20 °C until further assessment by Luminex method. The concentrations of CCL2, CCL20, CCL28, IFNγ, IL-2, IL-8, TNFα, CCL3, CCL25, CXCL10, IL-1β, IL-6 and IL-10 were quantified using a human multiplex assay (R&D Systems) according to manufacturer protocol using a BIOPLEX 200 reader (BIORAD). Only the results of quantitatively detected cytokines are presented. IL-8 concentration was assessed using ELISA method according to the manufacturer protocol (R&D Systems).

TEER Measurements

Transepithelial electrical resistance (TEER) was measured at different time points using an automated multichannel impedance spectrometer designed for use with the OrganoPlate (OrganoTEER, Mimetas) as described by Beaurivage *et al*. [20]. OrganoPlates were equilibrated at room temperature for 30 minutes before each measurement. Data was analyzed using OrganoTEER software, which automatically extracts the TEER contribution and reports values normalized to Ohm*cm^2.

Immunohistochemistry and Microscopy

At the end point of experiments, all cell cultures were fixed in 4% paraformaldehyde (VWR 100504-858) in DPBS (+Ca/+Mg, Gibco 14040-083) for 30 min. Cell membranes were permeabilized with a 0.1% Triton-X-100 solution in DPBS for 20 min and blocked with a 2% BSA solution in DPBS (Sigma A2153) for over 1h. Primary antibodies against ZO-1 (Invitrogen 61-7300, AB_138452) were then diluted in a 2% BSA solution in DPBS and incubated overnight at 4°C. Secondary antibodies (Invitrogen A-21429, AB_2535850), DAPI (Invitrogen, D1306), and Phalloidin (Sigma, P5282) were then also diluted in 2% BSA and incubated for 2h. Cell cultures were rinsed with DPBS prior to imaging. All steps, except when specified otherwise, were performed on chip and at room temperature. Confocal immunostaining images were acquired using a spinning disk microscope (Andor BC34 CF) using a 20X magnification air objective (NA 0.8). Z-stacks were acquired over a range of 200 µm with a Z resolution of 0.3 µm.

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Statistical Analysis

TEER measurements: repeated measures (RM) two-way or one-way Anova analysis was performed comparing each condition to a control conditioned decided for each type of experiment. Only statistically significant different results were considered when the p-value was lower than 0.05 and presented with asterisks.

IL-8 ELISA: ratio paired T test was performed comparing each condition to control condition. Only statistically significant different results were considered when the p-value was lower than 0.05 and presented with asterisks.

Multiplex cytokine analysis (Luminex): for each comparison considered in each dataset of interest, each assay was analyzed individually, comparing its expression in one condition compared to another using a non-parametric Wilcoxon test (the null hypothesis being that the mean expression of the considered assay is not significantly different between the two investigated conditions). To correct for the multiple testing effect, p-values were adjusted using the Benjamini-Hochberg approach [24]. The $\log_2$-fold-change associated with the comparison was calculated as the $\log_2$ ratio of mean expression levels between the two conditions. We considered that the expression of an assay was significantly different in the two investigated conditions when the adjusted p-value was lower than 0.05.

Results

A tubule-shaped monolayer of Caco2 cells within one of the channels ("Lumen" channel) was prepared within each chip of the microfluidic device called OrganoPlate®, as it was previously demonstrated by Beaurivage *et al.* [20]. Once the epithelial barrier (assessed by TEER) was established, 6 bacterial species (Table 1) commonly inhabiting the human intestine were introduced within the Lumen channel of the chips to survive in co-culture with the epithelial cells (Fig. 1A). Since these bacteria are anaerobic, experiments were conducted at 4% oxygen, which better reflects the physiological values ranging from ~6% in the vascularized submucosa to 1 - 2% near the crypt-lumen interface [25]. Except for *Roseburia intestinalis*, all 5 other species maintained a concentration of viable units above 10^6 CFU/chip (Fig. 1B) for 48 hours. At the same time, no decrease in epithelial barrier efficiency (Fig. 1D) and no significant increase in secretion of the main pro-inflammatory cytokine IL-8 [26, 27] was detected (Fig. 1C), suggesting that epithelial cells suffered no damage or inflammatory stress.

Encouraged by these observations, we tested the capacity of some of these bacterial species to mitigate inflammation-induced epithelial damage. We decided to proceed with two species that were proposed for live biotherapeutic development. The first is *B. thetaiotaomicron*, whose anti-inflammatory and epithelial barrier supporting features were demonstrated both *in vitro* and *in vivo* [28-31]. The second is *C. scindens,* which was not reported to possess such abilities but is capable of secondary bile acid production, preventing the emergence of important human pathogen *Clostridioides (Clostridium) difficile* [32, 33] and playing a significant role in steroid metabolism in humans [34]. To mimic epithelial damage mediated by the pro-inflammatory activity of immune cells, we introduced human peripheral blood mononuclear cells (PBMC) into the opposite ("Basal") channel of each chip and stimulated them with a lipopolysaccharide (LPS) from *E.coli* as we reported previously [19]. Simultaneously with PBMC addition to the Basal channel, washed *B. thetaiotaomicron, C. scindens* or their filtered spent culture medium from overnight cultures at 30% vol/vol were co-incubated in the Lumen channels (Fig. 2A). Similarly to the experiment without PBMC, *B. thetaiotaomicron* viability remained roughly unchanged during the two days of the co-culture experiment, while the CFU of *C. scindens* rapidly declined (Fig. 2B). Intriguingly, while LPS stimulation of PBMC provoked a decrease in TEER as previously described [19], co-culture with *B. thetaiotaomicron* or co-incubation with its supernatant protected the Caco-2 cells from the LPS-induced damage (Fig. 2C). On the other hand, the spent medium of *C. scindens*, but not bacteria themselves, demonstrated only a (not statistically significant) tendency to mitigate the epithelial barrier damage.

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To test whether the protective effect is associated with immunomodulation, we analyzed the cytokine profile in both channels at the end of the 48 hours of the experiment. As expected, LPS stimulation of PBMC induced a sharp increase in concentration of most tested cytokines, which was more profound in the Basal channel where the PBMC resided (Fig. 3A and 3B). Interestingly, bacteria and their supernatants had very limited and mostly statistically insignificant effect on the cytokine profiles in the Basal channel (Fig. 3C). On the other hand, the concentrations of CCL2 and CCL20 chemokines in the Lumen channel was strongly altered (Fig. 3D). Specifically, *B. thetaiotaomicron* and its spent medium strongly decreased the CCL2 concentration, while only the bacteria, but not the supernatant, reduced the concentration of CCL20. Co-culture of *C. scindens* with the Caco-2 cells provoked even more profound decrease (about 17 folds) in the concentration of CCL20, however this tendency was reverted when only the spend medium of *C. scindens* was used. Accordingly, the concentration of two pro-inflammatory cytokines IL-6 and IL-8 also showed moderate, but statistically significant, increase when only the supernatant of *C. scindens* was added to the Lumen channel.

Since the efficiency of the epithelial barrier is maintained mainly by tight junction proteins [1, 2], we investigated the spatial organization of the key component of this complex – Zonula Occludin-1 (ZO-1) – using immunofluorescence microscopy. Indeed, LPS stimulation of PBMC altered the homogeneous distribution of ZO-1 along Caco-2 cell edges and also impacted the general morphology of the epithelial monolayer as evidenced by the filamentous actin (F-Actin) staining (Fig. 4). In accordance with TEER measurements, co-culturing *B. thetaiotaomicron* in the Lumen channel mitigated the alteration of ZO-1 and F-actin spatial distribution. The spent medium of *B. thetaiotaomicron*, on the other hand, provoked a dramatic effect on the organization of both markers: ZO-1 often appeared in patches along the cell edges, while F-actin seemed to be concentrated almost exclusively at the cell edges (cortex) in contrast to a more homogeneous distribution seen in other experimental conditions. The morphology of epithelial cells upon incubation with *C. scindens* or its supernatant looked similar to the condition with LPS-stimulated PBMC without any additional treatment.

Discussion

To co-culture anaerobic bacteria with host cells *in vitro*, researchers usually use sophisticated models involving pumps and/or anaerobic chambers that allow control of oxygen tension in different compartments of the microfluidic device [35-40]. The current model demonstrates the feasibility of prolonged co-culturing of some anaerobic bacteria in an easy and high-throughput mode simply by reducing the oxygen concentration to the physiologically relevant value of 4%. It is also possible that the rapid oxygen consumption by epithelial cells creates a niche in their close vicinity with even lower oxygen concentration, allowing anaerobic bacteria to survive.

LPS stimulation of PBMC in the current Gut-on-chip model upregulated several cytokines that play a crucial role in modulating the intestinal epithelial barrier (Fig 3). These include chemokines that control the recruitment of immune cells to the inflamed tissue, such as CCL-20, CCL2 (MCP-1) and CCL3 (MIP-1α), as well as cytokines regulating the activation status of these cells: IL-1β, IL-8, IL-6, TNFα and IL-10 [11, 41]. Administration of live bacteria or their filtered supernatants in the Lumen channel mostly did not modulate the increase in cytokine concentration in opposite Basal channel, whose main source is PBMC (Fig. 3C). In the Lumen channel, however, the increase in CCL2 concentration was reverted by *B. thetaiotaomicron* and its supernatant, and the increase in CCL20 was reverted by both bacterial strains (Fig. 3D). As no modulation of these two chemokines was observed in the Basal channel, the impact of bacteria is most probably mediated via modulation of cytokine secretion from the Caco-2 cells. The decrease in CCL2, but not in CCL20, correlates with the improvement in epithelial barrier function assessed by TEER (Fig. 2B), suggesting that CCL2 plays a key role in epithelial barrier damage in this model. In agreement with this observation, elevated CCL2 concentrations were detected in intestinal specimens of patients suffering from inflammatory bowel diseases (IBD) in several studies [42-44]. While *C. scindens* did not significantly improve epithelial

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barrier integrity, its pronounced impact on CCL20 secretion, a central chemokine in intestinal inflammation, underscores its relevance for future studies as a biotherapeutic in the autoimmune disease area [45, 46]. However, the opposite effect of *C. scindens* supernatant on CCL20 as well as increase in IL-6 and IL-8 concentrations in the Lumen channel suggest ambivalent interaction of this species with intestinal epithelium.

On the cell morphology level, *B. thetaiotaomicron* co-culture with epithelial cells seems to prevent the LPS-induced decrease of ZO-1 localization at the cell edges (Fig. 4), which aligns well with a previous communication about the positive effect of this species on ZO-1 mRNA expression [30]. The fact that the bacterial supernatant alone also promoted the protection of epithelial barrier function suggests that the effect of *B. thetaiotaomicron* is mediated by a secreted factor. Indeed, tryptophan metabolites, including indole, produced by this bacterium, were demonstrated to enhance epithelial barrier function [29]. However, the effect of the supernatant on F-actin and ZO-1 organization looked strikingly different from the one provoked by the *B. thetaiotaomicron* co-culture, although both treatments protected the epithelial barrier from inflammation-induced deterioration. While the patchy ZO-1 staining is not usually associated with a strong epithelial barrier, we observed a noticeable enhancement of cortical actin cytoskeleton, which is critical for the integrity of the gut barrier [47] and might explain the positive effect of *B. thetaiotaomicron* supernatant after all. In general, the different results obtained with live bacteria and its supernatant suggest that *B. thetaiotaomicron* has more than one way of impacting epithelial homeostasis. In fact, at least one cell-associated factor of *B. thetaiotaomicron* – pirin-like protein – was demonstrated to reduce pro-inflammatory NF-κB signaling in Caco-2 cells [28]. More investigations are needed to elucidate the complete picture of the effect of this species.

We thus conclude that the current Gut-on-Chip model recapitulates the key features of intestinal inflammation – epithelial barrier damage and pro-inflammatory cytokine secretion – while supporting the co-culture of anaerobic bacteria. Established in the commercially available OrganoPlate platform, the model is easily standardizable and scalable to higher-throughput applications. As a proof of concept, we screened six anaerobic bacteria and demonstrated the protective effect of *B. thetaiotaomicron* on the epithelial barrier against inflammation-mediated damage, associated with CCL2 secretion prevention and modulation of ZO-1 and F-actin spatial organization, while also flagging *C. scindens* as a promising candidate for future studies.

Figures and legends

Table 1. Bacterial strains used in the study.

Species	Strain
Bacteroides thetaiotaomicron	DSM 72079
Akkermansia muciniphila	DSM 22959
Christensenella minuta	DSM 22607
Roseburia intestinalis	DSM 14610
Clostridium scindens	DSM 5676
Adlercreutzia equolifaciens	DSM 19450

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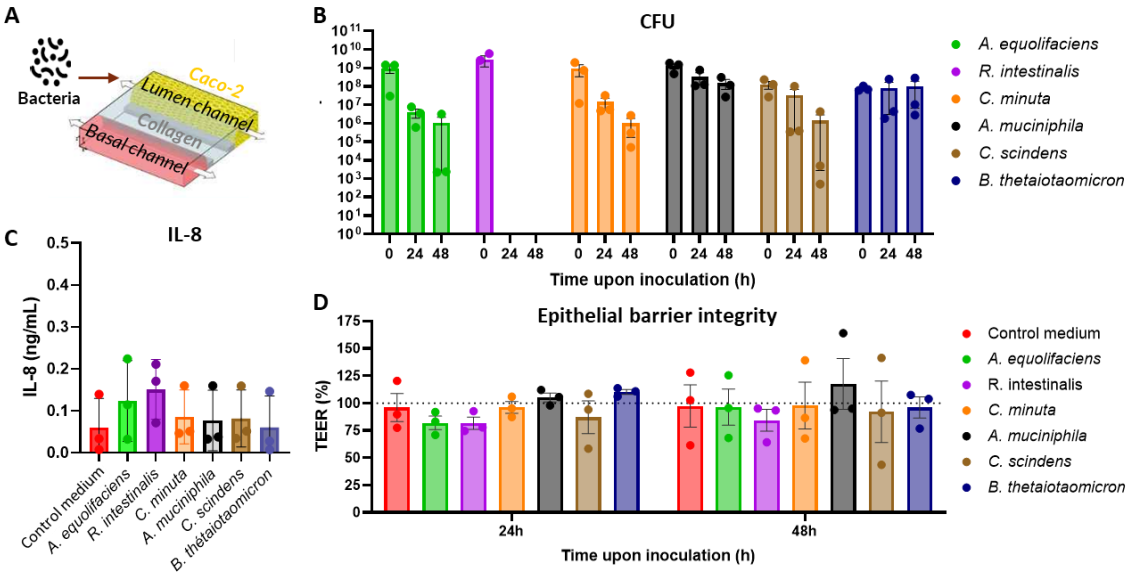

Figure 1. Co-culture of anaerobic bacteria with Caco-2 in Gut-on-chip model. (**A**) Graphical representation of the experimental system with one microfluidic module (chip) within the OrganoPlate. (**B**) Colony forming units (CFU) of each bacterial species retrieved from the Lumen channel at the indicated time points. (**C**) IL-8 concentrations in the Lumen channel after 48 h co-culture with the different bacteria. Ratio paired T test was performed comparing each condition to Control medium and no condition obtained *p*-values <0.05. (**D**) TEER values along the experiment normalized to the T=0 h measured just prior to PBMC introduction and defined as 100%. RM two-way Anova analysis was performed comparing each condition to Control medium for each time point and no condition obtained *p*-values <0.05.

Each experiment was repeated three times with three technical replicates. Means and standard deviations are presented except for CFU data for which the standard error of the mean (SEM) is used.

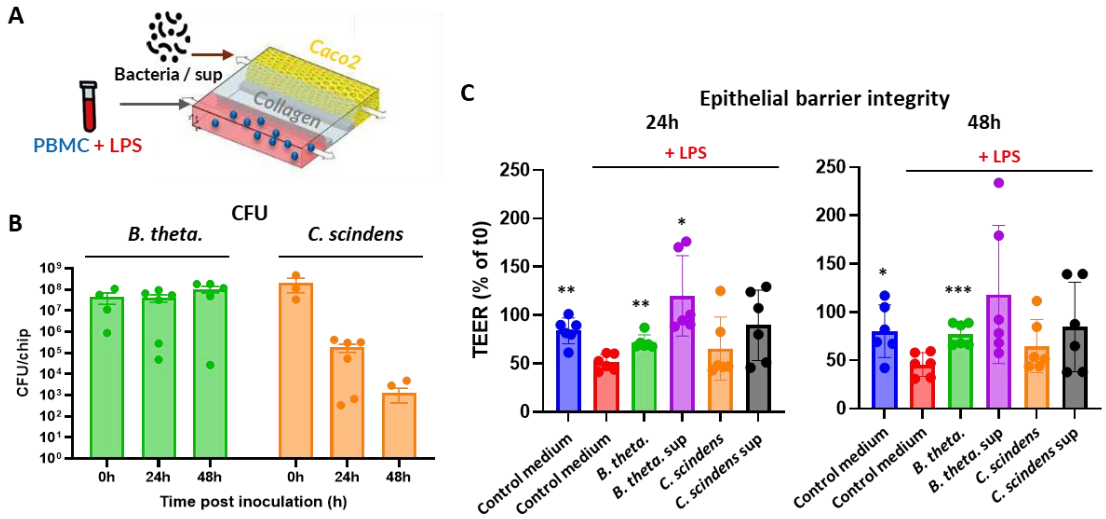

Figure 2. Co-culture of *B. thetaiotaomicron (B. theta.)* and *C. scindens* or incubation of their supernatants (sup) in the Gut-on-chip model with LPS-stimulated PBMC. (**A**) Graphical representation of the experimental system. (**B**) Colony forming units (CFU) of each bacterial species retrieved from the Lumen channel at the indicated time points. (**C**) TEER values along the experiment normalized T=0 h measured just prior to PBMC introduction and defined as 100%. RM one-way Anova analysis was performed with *, ** and *** representing statistical significance with *p*-values < 0.05, 0.01, and 0.001, respectively, when comparing each treatment to "Control medium" + LPS.

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Each experiment was repeated three times with PBMC from two donors each time (6 PBM donors in total) and at least three technical replicates. Means and standard deviations are presented except for CFU data for which SEM is used.

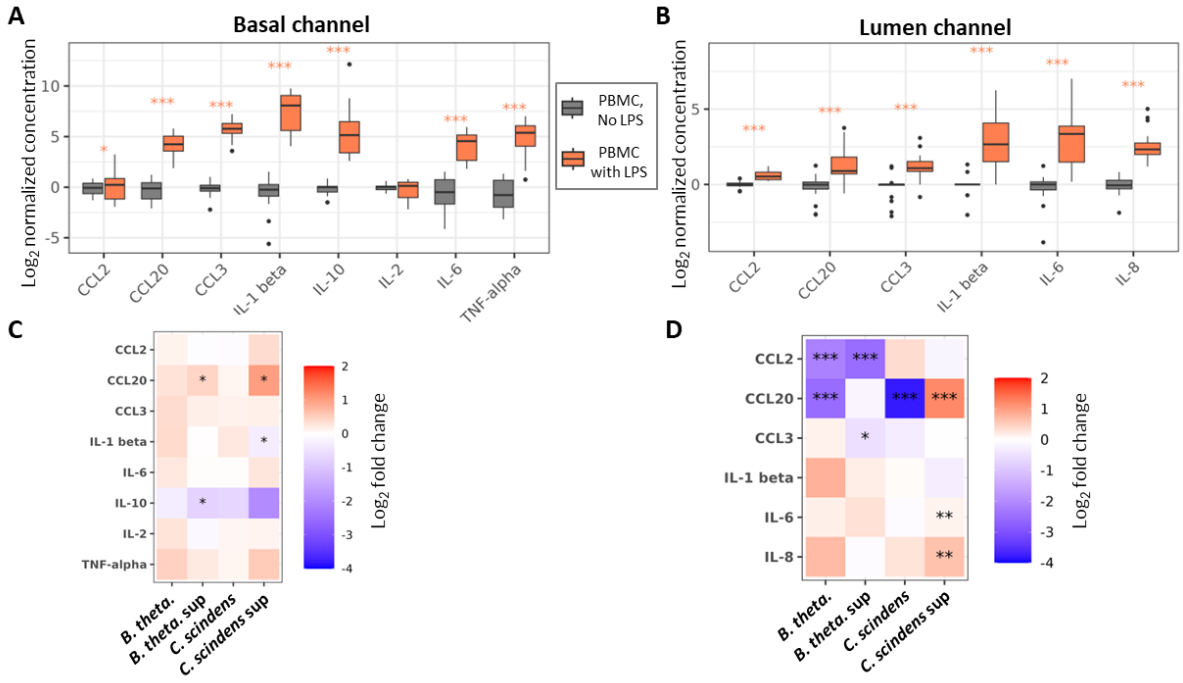

Figure 3. Cytokine secretion in immunocompetent Gut-on-Chip model after 48h of the experiment start. (**A** and **B**) Relative change in cytokines concentration in Lumen (A) and Basal (B) channels after LPS-stimulated (or non-stimulated) PBMC administration normalized to "PBMC, No LPS" group using non-parametric Wilcoxon test. (**C** and **D**) Heat-map of relative change in cytokine concentration in Lumen (C) and Basal (D) channels comparing each treatment to the control (fresh) medium in the presence of LPS-stimulated PBMC in the Basal channel using non-parametric Wilcoxon test. The concentration of IL-8 in basal channel upon LPS stimulation was too high for quantitative assessment and therefore could not be plotted in panels A and C. *, ** and *** represent statistical significance with p-value < 0.05, 0.01, and 0.001, respectively. Each experiment was repeated three times with PBMC from two donors each time (6 PBM donors in total) and at least three technical replicates.

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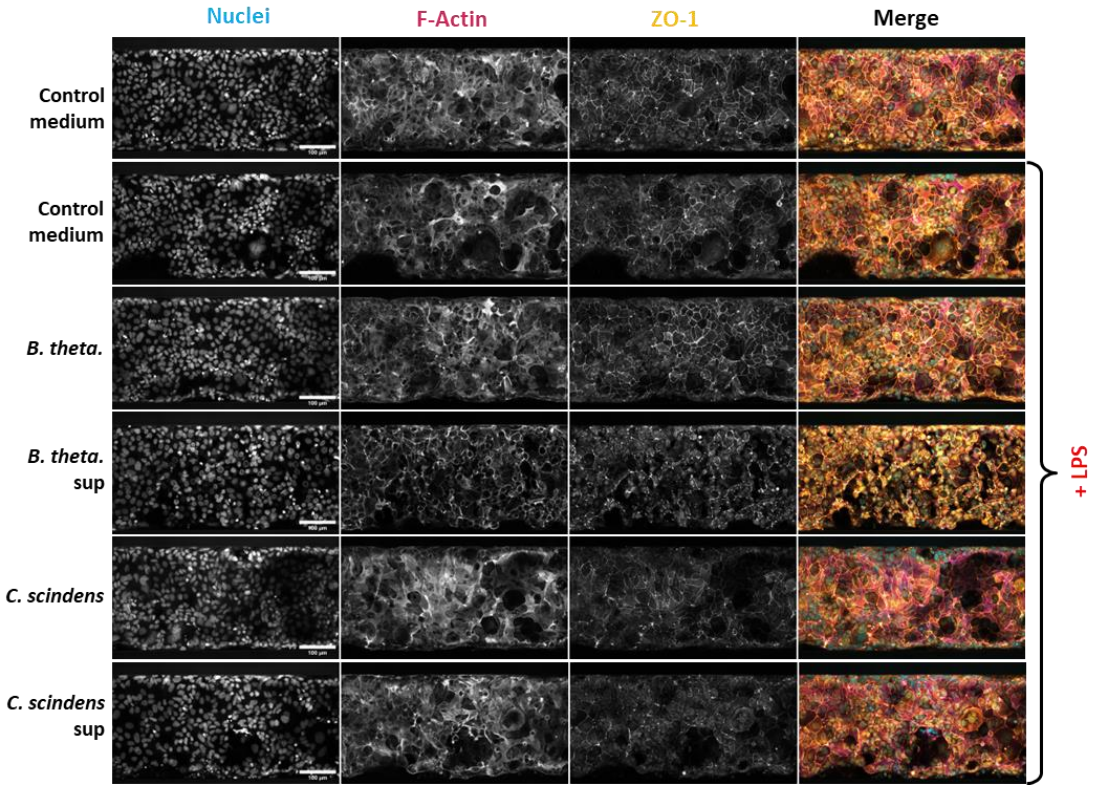

Figure 4. Effect of *B. thetaiotaomicron, C. scindens* and their supernatants on Caco-2 morphology. Representative immunofluorescence images. Shown images are the mean projection of 5 consecutive slices of Z-stacks, centered on the bottom cell layer of the Lumen channel, acquired by confocal microscopy. Individual channels are shown separately in grayscale. A color image with all channels merged shown on the right with the following color code: nuclei (cyan), F-actin (magenta) and ZO-1 (orange). Scale bars represent 100 µm.

Conflict of interest statement: The authors declare no conflict of interest

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