

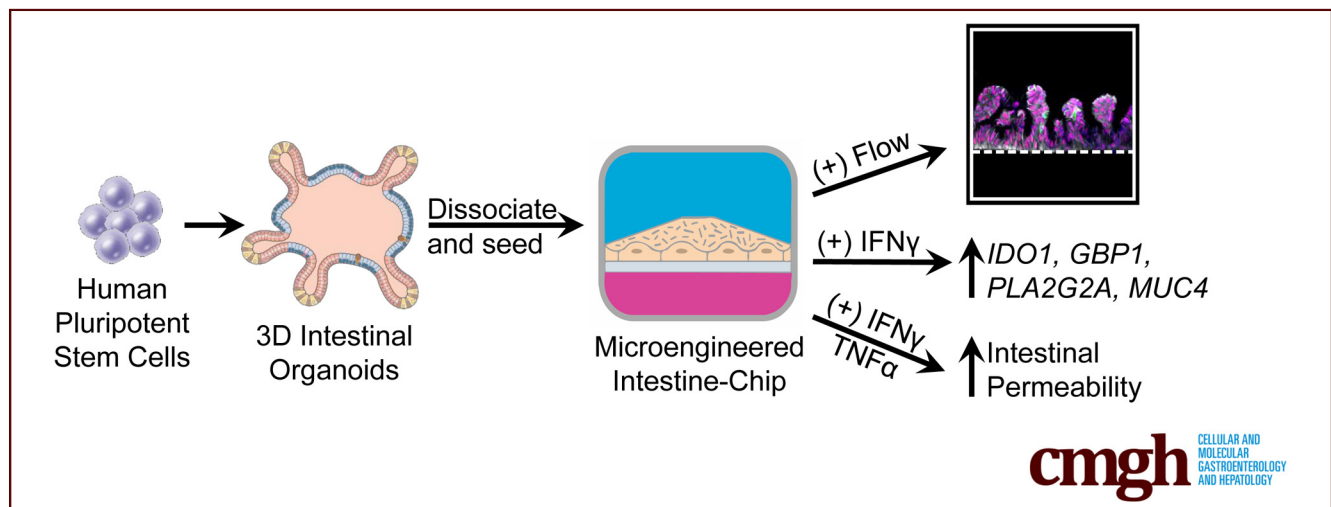
ORIGINAL RESEARCH

Enhanced Utilization of Induced Pluripotent Stem Cell–Derived Human Intestinal Organoids Using Microengineered Chips



Michael J. Workman,^{1,2} John P. Gleeson,¹ Elissa J. Troisi,¹ Hannah Q. Estrada,¹ S. Jordan Kerns,³ Christopher D. Hinojosa,³ Geraldine A. Hamilton,³ Stephan R. Targan,⁴ Clive N. Svendsen,^{1,2} and Robert J. Barrett^{1,4}

¹Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, California; ²Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California; ³Emulate Inc, Boston, Massachusetts; ⁴F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, California



SUMMARY

The 3-dimensional structure of human intestinal organoids makes them challenging to use. Here we describe how organoids, derived from induced pluripotent stem cells, can be incorporated into small microengineered Chips making them more amenable for study.

BACKGROUND AND AIMS: Human intestinal organoids derived from induced pluripotent stem cells have tremendous potential to elucidate the intestinal epithelium's role in health and disease, but it is difficult to directly assay these complex structures. This study sought to make this technology more amenable for study by obtaining epithelial cells from induced pluripotent stem cell–derived human intestinal organoids and incorporating them into small microengineered Chips. We then investigated if these cells within the Chip were polarized, had the 4 major intestinal epithelial subtypes, and were biologically responsive to exogenous stimuli.

METHODS: Epithelial cells were positively selected from human intestinal organoids and were incorporated into the Chip. The effect of continuous media flow was examined.

Immunocytochemistry and *in situ* hybridization were used to demonstrate that the epithelial cells were polarized and possessed the major intestinal epithelial subtypes. To assess if the incorporated cells were biologically responsive, Western blot analysis and quantitative polymerase chain reaction were used to assess the effects of interferon (IFN)- γ , and fluorescein isothiocyanate–dextran 4 kDa permeation was used to assess the effects of IFN- γ and tumor necrosis factor- α on barrier function.

RESULTS: The optimal cell seeding density and flow rate were established. The continuous administration of flow resulted in the formation of polarized intestinal folds that contained Paneth cells, goblet cells, enterocytes, and enteroendocrine cells along with transit-amplifying and *LGR5*⁺ stem cells. Administration of IFN- γ for 1 hour resulted in the phosphorylation of STAT1, whereas exposure for 3 days resulted in a significant upregulation of IFN- γ related genes. Administration of IFN- γ and tumor necrosis factor- α for 3 days resulted in an increase in intestinal permeability.

CONCLUSIONS: We demonstrate that the Intestine-Chip is polarized, contains all the intestinal epithelial subtypes, and is biologically responsive to exogenous stimuli. This represents a more amenable platform to use organoid technology and will be highly applicable to personalized medicine

and a wide range of gastrointestinal conditions. (*Cell Mol Gastroenterol Hepatol* 2018;5:669–677; <https://doi.org/10.1016/j.jcmgh.2017.12.008>)

Keywords: Human Intestinal Organoids; Induced Pluripotent Stem Cells; Small Microengineered Chips.

See editorial on page 634.

Studies examining human intestinal epithelial cell function have been severely hampered because primary intestinal epithelial cells rapidly undergo apoptosis when cultured *ex vivo*.^{1,2} Although adenocarcinoma lines, such as Caco-2 cells, recapitulate some aspects of intestinal function, namely barrier function, a substantial breakthrough in the intestinal epithelial field occurred when it was reported that 3-dimensional human intestinal “organoids” (HIO) could be generated from either human biopsy samples^{3,4} or induced pluripotent stem cells (iPSCs).⁵ Irrespective of how these organoids are derived, they contain all the intestinal epithelial subtypes, are polarized toward the lumen, and can be maintained for prolonged periods of time in a tightly controlled milieu. However, there are substantial technical challenges associated with this technology. Organoids are heterogeneous both in shape and size, which may lead to inconsistent findings. Access to the lumen, which is crucial for assessing intestinal permeability, microbial-epithelial interactions, and drug absorption are technically challenging. Coculture with other cell types, such as immune cell subtypes or endothelial cells, is also difficult given that organoids are typically embedded in a 3-dimensional matrix.

One potential way to overcome such challenges is to combine intestinal organoid culture with microengineering technology. Small microengineered Chips are integrated systems that place living human cells in precisely microengineered environments that can more accurately recapitulate human physiology and disease states. They allow unprecedented control over key physiological aspects, such as interactions between tissues, mechanical forces, blood and immune components, and the biochemical milieu.⁶ This engineering also allows the tuning and control of the microenvironment. Indeed, lung^{7,8} and Caco-2 cells^{9–11} have previously been incorporated into such Chips and studies examining epithelial-immune cell interactions, prolonged epithelial-microbial interactions, and permeability experiments have all successfully been performed.

Given that iPSCs can be generated from any individual,¹² iPSC-derived HIOs were chosen for incorporation into this Chip system, thereby permitting the study of intestinal epithelial cells from virtually any patient or nondiseased control. It also allows for the generation of other patient-specific cell types, such as macrophages,¹³ dendritic cells,¹⁴ and neutrophils,¹⁵ that can also be incorporated into the Chip and used to study multicellular interactions. Furthermore, we have previously reported that lymphoblastoid cell lines can be reliably reprogrammed to form iPSCs.¹⁶ Given there are numerous lymphoblastoid cell lines available in well-characterized

worldwide repositories that are linked to patient clinical history and long-term genotype-phenotype data,¹⁷ studies into epithelial cells and their interactions with other cell types from well-characterized patient cohorts are now possible.

Materials and Methods

Ethics Statement

All the cell lines and protocols in the present study were carried out in accordance with the guidelines approved by the stem cell research oversight committee and institutional review board at the Cedars-Sinai Medical Center under the auspice of the institutional review board stem cell research oversight committee protocols Pro00027264 (Derivation of Intestinal Stem Cells). All authors had access to the study data and have reviewed and approved the final manuscript.

Cell Lines and Culturing Conditions

Two iPSC lines (CS83iCTR-33n1 and CS688iCTR-n5) were obtained from the iPSC Core at Cedars-Sinai. Both lines were fully characterized and were confirmed to be karyotypically normal. All iPSC lines were maintained in an undifferentiated state on Matrigel-coated plates in mTeSR1 media (Stem Cell Technologies, Vancouver, British Columbia, Canada) under feeder-free conditions. All iPSC cultures were tested monthly for mycoplasma contamination. Caco-2 (ATCC HTB-37) cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD).

Small Microengineering Chip Microfabrication

Chips were fabricated using modified methods for Chip microfabrication as previously described.¹⁸ Briefly, poly(dimethylsiloxane) (PDMS) prepolymer was mixed at a 10:1 ratio of PDMS base to curing agent, wt/wt using a planetary mixer (Thinky ARE-310). PDMS prepolymer was then cast onto molds forming the microchannels of the upper layer (1000 μm wide \times 1000 μm high) and lower layer (1000 μm wide \times 200 μm high). The membrane was cast onto a silicon mold that was fabricated using photolithography and deep reactive ion etching, resulting in 7- μm pores. The components were cured overnight and removed from the mold. The upper layer, membrane, and lower layer were permanently bonded via plasma bonding to form the complete Chip.

Generation of Human Intestinal Organoids From Induced Pluripotent Stem Cells

The generation of HIOs from iPSCs involves a multistep technique whereby iPSCs were directed to form definitive

Abbreviations used in this paper: GBP1, guanylate binding protein 1; HIOs, human intestinal organoids; IDO1, indolamine 2,3-dioxygenase 1; IFN- γ , interferon- γ ; iPSCs, induced pluripotent stem cells; PDMS, poly(dimethylsiloxane); TNF- α , tumor necrosis factor- α .

 Most current article

© 2018 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

2352-345X

<https://doi.org/10.1016/j.jcmgh.2017.12.008>

endoderm, epithelial structures, and ultimately organoids. To induce definitive endoderm formation, iPSCs were cultured with a high dose of Activin A (100 ng/mL, R&D Systems, Minneapolis, MN) with increasing concentrations of fetal bovine serum over time (0%, 0.2%, and 2% on Days 1, 2, and 3, respectively). Wnt3A (25 ng/mL, R&D Systems) was also added on the first day of endoderm differentiation. To induce hindgut formation, cells were cultured in Advanced DMEM/F12 with 2% fetal bovine serum and FGF4 (500 ng/mL, R&D Systems) and CHIR99021 (3 μ M, Tocris, Minneapolis, MN). After 3–4 days, free-floating epithelial spheres and loosely attached epithelial tubes became visible and were harvested. These epithelial structures were subsequently suspended in Matrigel and then overlaid in intestinal medium containing CHIR99021 (2 μ M, Tocris), noggin, and epidermal growth factor (both 100 ng/mL, all R&D Systems) and B27 (1X, Invitrogen, Carlsbad, CA). HIOs were passaged every 7–10 days thereafter.

Cell Sorting, Seeding, and Maintenance of Intestine-Chip

To seed intestinal epithelial cells into the Chip, HIOs were first dissociated and the intestinal epithelial cells were positively selected using fluorescent activated cell sorting. At 24 hours before sorting, ROCK inhibitor (10 μ M, Tocris) was added to HIO culture media. The following day, HIOs were removed from Matrigel and subsequently incubated in TrypLE Express (Life Technologies, Camarillo, CA) for 15–30 minutes until the organoids were completely disassociated to a single cell suspension. These cells were then passed through a 30- μ m filter and stained with CD326 (Biolegend, San Diego, CA) for 30 minutes. A minimum of 5×10^6 cells were then positively sorted for CD326. Cells were collected and resuspended to a density of 6.25×10^6 cells/mL in intestinal media containing ROCK inhibitor (10 μ M, Tocris), SB202190 (10 μ M, Tocris), and A83-01 (500 nM, Tocris). Before cell seeding, Chips were treated with plasma in 100% oxygen for 2 minutes using a CUTE series plasma system (Femto Science, Gyeonggi-Do, South Korea) and immediately coated with Matrigel (83 μ g/mL). Dead/nonadhered cells were removed after 3–6 hours by flushing media through the Chip. Flow was started 3–6 hours later at a rate of 30 μ L/h. ROCK inhibitor was removed from media 24 hours postseeding. To induce flow, gas-permeable tubing (PharMed BPT, 1/32 \times 5/32 inch tubing, Cole Palmer, Vernon Hills, IL) with a connector (Right angle tube 18RW, Fourslide Spring and Stamping, Bristol, CT) was inserted into the microchannels to supply cell culture medium. A Fusion 200 Touch Syringe Pump (Chemxyx, Stafford, TX) fitted with a modular 10 syringe rack was used to induce and maintain flow. All of the apparatus, the Intestine-Chip, and syringe pumps were stored in an incubator and all experiments were carried out at 37°C.

Quantitative Real-Time Polymerase Chain Reaction

Phosphate-buffered saline was flushed through the upper and lower channels and total RNA from the Intestine-Chip was extracted *in situ* with the RNeasy mini kit (Qiagen,

Germantown, MD). cDNA was generated from 1 μ g of RNA using the Omniscript RT Kit (Qiagen). Quantitative real-time polymerase chain reaction was performed using SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA) on a BioRad CFX384 Real-Time System. Primer sequences as follows; *IDO1* F-ACACTTTGCTAAAGGCGCTG, *IDO1* R-TGCCTTTCCAGCCAGACAAA, *GBP1* F-AAACTTCAGGAACAGGAGCAAC, *GBP1* R-GGTACATGCCTTTCTGTCGTCT, *PLA2G2A* F-CAAGTTTACCACTCGGGGA, *PLA2G2A* R-TCTAGCAAAACAGGTGGC AG, *MUC4* F-AGACGCACAGCCACATCAC, *MUC4* R-GGGAAAC TCCTCTCTCAGGC, *LYZ* F-TTTCTGTTACGGTCCAGGGC, *LYZ* R-ACACATCCAGTTTGTAGGCT.

Immunohistochemistry and Microscopy

HIOs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), transferred to 30% sucrose overnight at 4°C, embedded in Tissue-Tek O.C.T Compound (VWR, Radnor, PA), and cut into 10- μ m sections. Sections were blocked in 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) with 0.5% Triton X-100 and incubated with primary antibodies (Supplementary Table 1) for either 3 hours at room temperature or overnight at 4°C. Sections were then rinsed and incubated in species-specific AF488, AF594, or AF647-conjugated secondary antibodies (Life Technologies, Carlsbad, CA) followed by DAPI (0.5 μ g/mL; Life Technologies) to counterstain nuclei, and were imaged using a Leica DM6000 B microscope. Intestine-Chips were flushed through the upper and lower channels, and cells were fixed with 4% paraformaldehyde for 15 minutes without flow. Intestine-Chips cultured under static conditions were immunostained and imaged in a similar manner as previously mentioned. To obtain cross-section images, a Leica VT1200S vibratome or Leica CM3050S cryostat was used to obtain sections of the Intestine-Chip. These sections were blocked in 10% normal donkey serum with 0.5% Triton X-100 and incubated with primary antibodies (Supplementary Table 1) for 24 hours at 4°C. Sections were rinsed; incubated in species-specific AF488, AF594, and AF647 followed by DAPI; and were imaged using a Nikon A1R Eclipse Ti Confocal Microscope.

In Situ Hybridization

Intestine-Chips were fixed as previously mentioned in 4% paraformaldehyde for 15 minutes without flow. To obtain cross-section images, Intestine-Chips were sectioned with a Leica CM3050S cryostat at 10 μ m per section. These sections were prepared and treated using RNAscope In Situ Hybridization 2.5 HD brown assay kit (Advanced Cell Diagnostic, Newark, CA). In brief, the tissue underwent target retrieval, permeabilization, hybridization of LGR5 (Hs-LGR5 311021) and WDR43 (Hs-WDR43 472711), amplification, and visualization using DAB-A and DAB-B. Sections were imaged using a Leica DM6000 B microscope.

Permeability and Cytotoxicity Assay

Intestine-Chips were prepared as previously mentioned, and maintained under flow conditions (30 μ L/h) for 10 days. Each Intestine-Chip was microscopically assessed

to confirm barrier integrity before experimentation. After 10 days, 10 ng/mL interferon (IFN)- γ and 10 ng/mL tumor necrosis factor (TNF)- α (both from R&D Systems) was added to the lower channel of the Intestine-Chip for 3 days, whereas the control group was untreated. After 3 days, the media was removed and the system was flushed with transport buffer (HBSS supplemented with 12.5 mM glucose and 25 mM HEPES) and equilibrated for 1.5 hours. At time zero, fluorescein isothiocyanate-dextran 4 kDa was added to the top channel (10 μ g/mL) in transport buffer, and the lower channel transport buffer was either untreated or contained 10 ng/mL IFN- γ and 10 ng/mL TNF- α . Samples from the lower channel were taken every 1.5 hours for 6 hours and samples from the top channel were taken at 0 and 6 hours to determine the area under the curve. Fluorescence was measured in a spectrofluorometer (EnVision 2104, Perkin Elmer, Waltham, MA) with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Area under the curve was calculated using Prism-7 software (GraphPad, La Jolla, CA). After the final time point for the permeability experiments, the Intestine-Chip was flushed with transport buffer and the epithelium was treated with MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) to assess cytotoxicity induced by IFN- γ and TNF- α exposure. Intestine-Chips were incubated with MTS for 4 hours at 37°C in a humidified incubator with 5% CO₂ and 95% O₂. Optical density was measured at 490 nm. Each value presented was normalized against untreated control. Permeability and cytotoxicity experiments were calculated from 2 independent experiments, each of which included 6 replicates.

Western Blotting

Phosphate-buffered saline was flushed through both the upper and lower channels and total protein was extracted *in situ* using cell lysis buffer supplemented with protease and phosphatase inhibitor (# MSSAFE, Sigma, Carlsbad, CA). Protein concentration was then determined and equal amounts of protein lysates were heat denatured and separated on a 10% mini-protein precast gel (Bio-Rad, Hercules, CA). Gel was then transferred on to midi format 0.2 μ m Nitrocellulose Membrane (Bio-Rad). Blot was blocked in Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 1 hour and then incubated in primary antibody for STAT1, pSTAT1, and glyceraldehyde-3-phosphate dehydrogenase (Supplementary Table 1) overnight at 4°C. After incubation with infrared conjugated secondary antibodies (LI-COR) for 1 hour, blots were washed with TBST and bands were visualized by scanning membrane on Odyssey Infrared Imaging System (Li-COR). Image J analysis was used to quantify levels of phosphorylated STAT1 compared with glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analyses

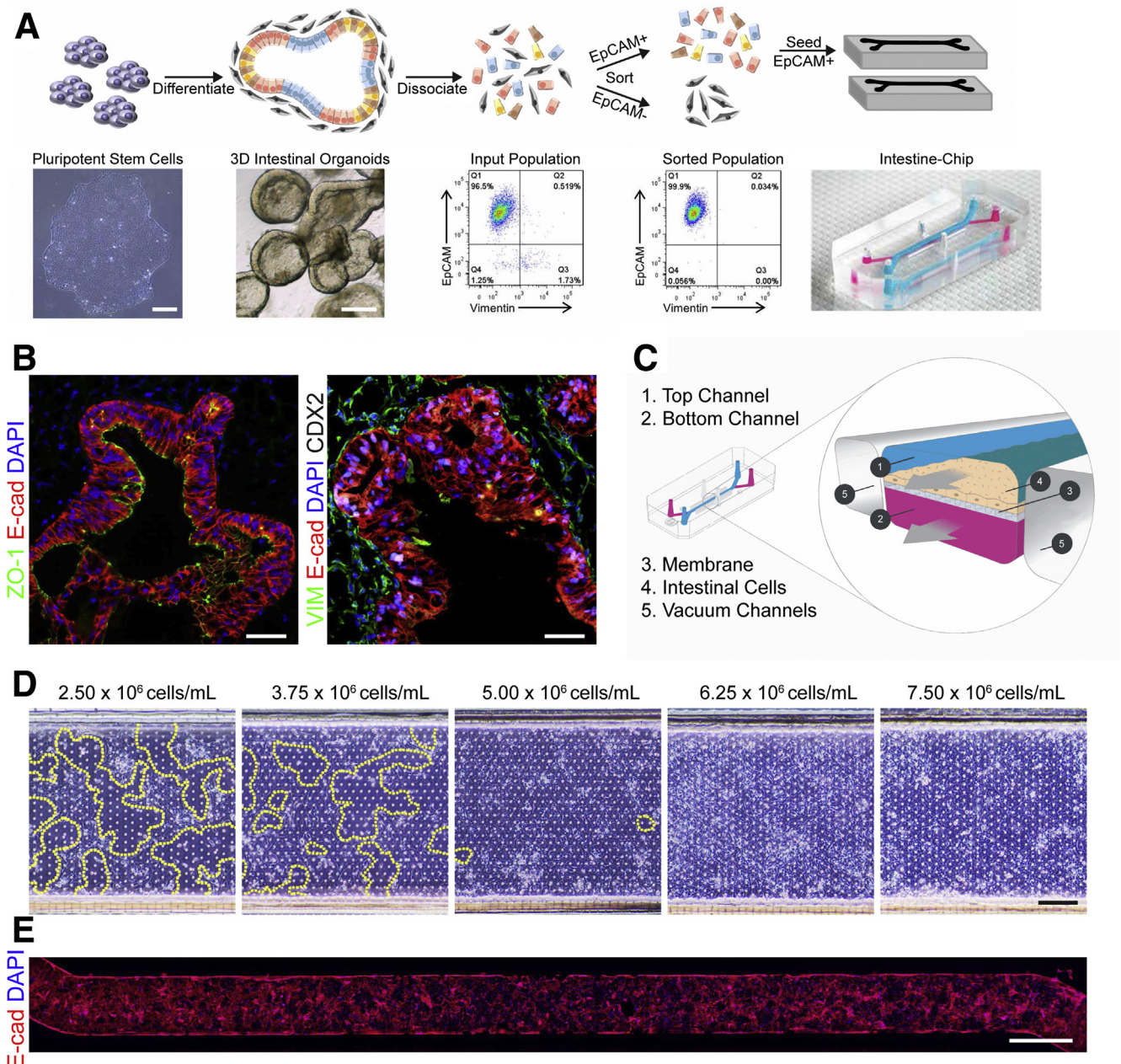
All data are represented as mean \pm standard error of the mean. An unpaired Student's *t* test was completed using Excel, and control and IFN- γ treated Chips were compared with each other in each condition. Differences

between groups were considered statistically significant when $P < .05$. No statistical method was used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment. No randomization was made.

Results and Discussion

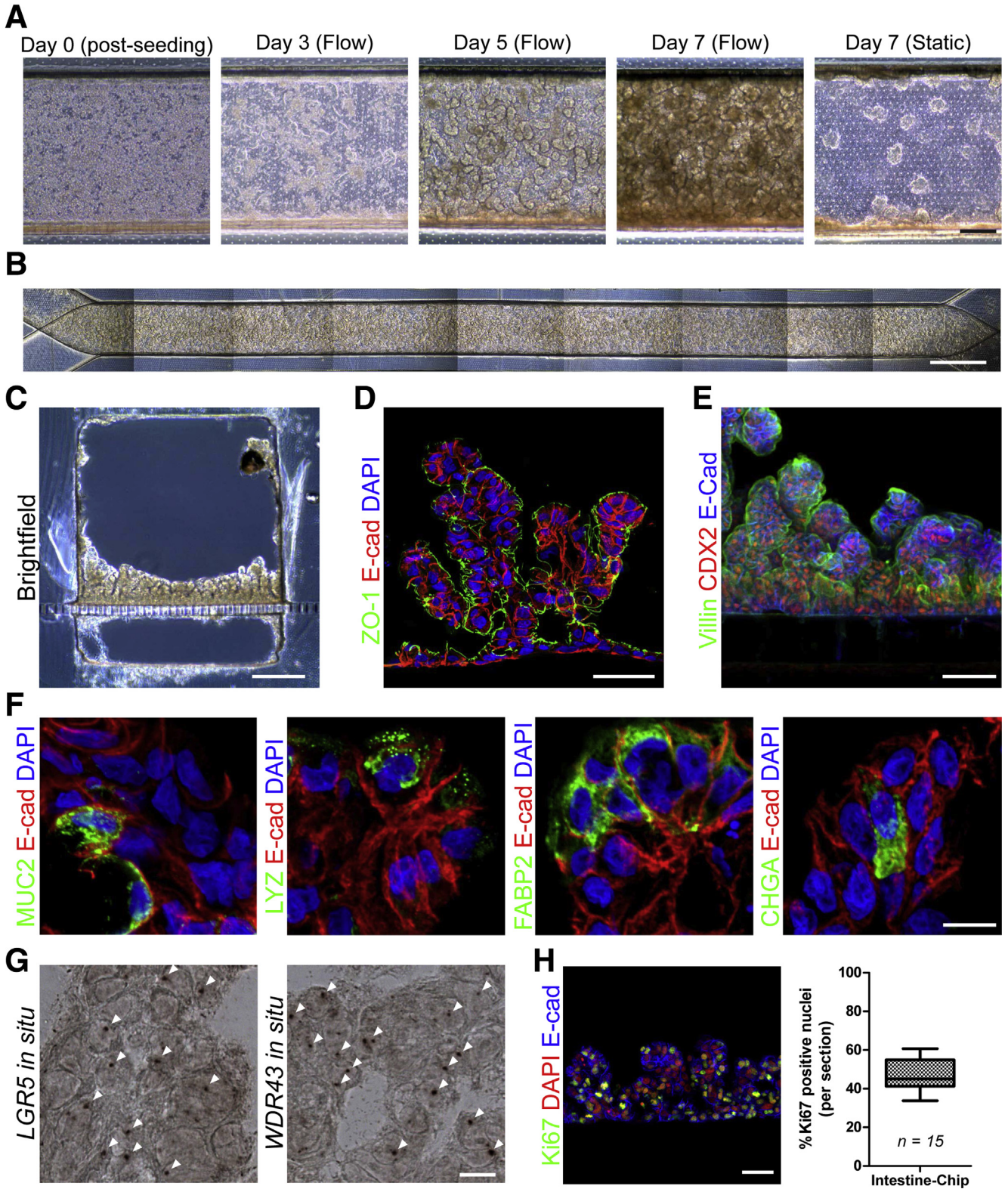
To develop the Intestine-Chip, we established a multistep technique whereby iPSCs were differentiated into HIOs, dissociated to single cells, sorted on an epithelial cell marker, and subsequently incorporated into small microengineered Chips (Figure 1A). iPSCs were directed to form 3-dimensional HIOs by culturing them with Activin A to induce definitive endoderm formation; CHIR99021 and fibroblast growth factor 4 to induce hindgut formation; and ultimately epidermal growth factor, Noggin, and CHIR99021 to induce and maintain organoid formation. This protocol resulted in the robust generation of intestinal organoids that were polarized towards the lumen, were CDX2/E-cadherin-immunopositive and, in contrast to biopsy-derived organoids, contained mesenchymal cells as previously reported (Figure 1B).⁵ The Chips used in this study were fabricated as previously described.¹⁸ Briefly, the Chips are made of PDMS and contain a top channel with a height and width of 1 mm separated from a parallel bottom channel (0.2 mm high \times 1 mm wide) by a thin (50 μ m), porous (7- μ m pores) PDMS membrane coated on the upper side with Matrigel (Figure 1C). Initially, we attempted to place intact HIOs into the top channel of the Chip. Although some HIOs adhered to the PDMS membrane and formed a partial monolayer of intestinal epithelial cells (Supplementary Figure 1A and B), most did not adhere and some HIOs gave rise to partial monolayers of mesenchymal cells (Supplementary Figure 1C) that physically impeded the expansion of the epithelial cells (data not shown). Given that the number of epithelial and mesenchymal cells within HIO cultures can vary enormously and the presence of mesenchymal cells seems to impede the expansion of epithelial cells, we sought to refine our approach. HIOs were dissociated to a single cell suspension and epithelial cell adhesion molecule (EpCAM/CD326) was used to positively select for HIO-derived epithelial cells using fluorescent activated cellular sorting. Concentrations of positively selected cells ranging from 2.5–7.5 \times 10⁶ cells/mL were added to the Matrigel-coated top channel of each Chip and it was found that 6.25 \times 10⁶ cells/mL gave a near continuous monolayer after 24 hours (Figure 1D). After 3 days, we observed a fully confluent monolayer of intestinal epithelial cells along the entire top channel of the Chip and thus this cell concentration was added in all future experiments to incorporate HIO-derived epithelial cells into the Intestine-Chip (Figure 1E).

After establishing the conditions to successfully incorporate HIO-derived epithelial cells into the Intestine-Chip, we then ascertained the optimal rate that media should continuously be flowed through the Chip. Continuous media flow over cells mimics some of the *in vivo* microenvironmental conditions and not only permits prolonged exposure to microbes, but enhances the differentiation of cells.^{6,9–11} Indeed, it was previously shown that continuous media flow caused Caco-2 cells to spontaneously develop intestinal



folds.^{9–11} We first tested a similar flow rate of 30 μL/h that was previously reported^{9–11} and found that after 72 hours, the HIO-derived epithelial cells exhibited a more crenulated appearance along the Intestine-Chip, which became more pronounced after 5 and 7 days (Figure 2A). Under static conditions, some small folds were observed after 7 days, although they primarily remained as a monolayer. Similarly,

cells seeded at a lower density (2.5–5 × 10⁶) failed to generate intestinal folds despite exposure to continual flow (data not shown). A stitched phase contrast image of HIO-epithelial cells that were exposed to continual media flow at 30 μL/h for 5 days demonstrated homogenous and near complete coverage of the Intestine-Chip (Figure 2B). An increased flow rate of 60 μL/h did not seem to accelerate



the process and so a flow rate of 30 $\mu\text{L/h}$ was used in all subsequent studies (Supplementary Figure 2). After 14 days, a cross-section of the Intestine-Chip revealed a substantially thickened layer that seemed to have organized

into villous-like projections (Figure 2C). The presence of E-cadherin and polarized ZO-1 immunostaining toward the apical surface demonstrates these are polarized epithelial cells and illustrates that the top channel is representative of

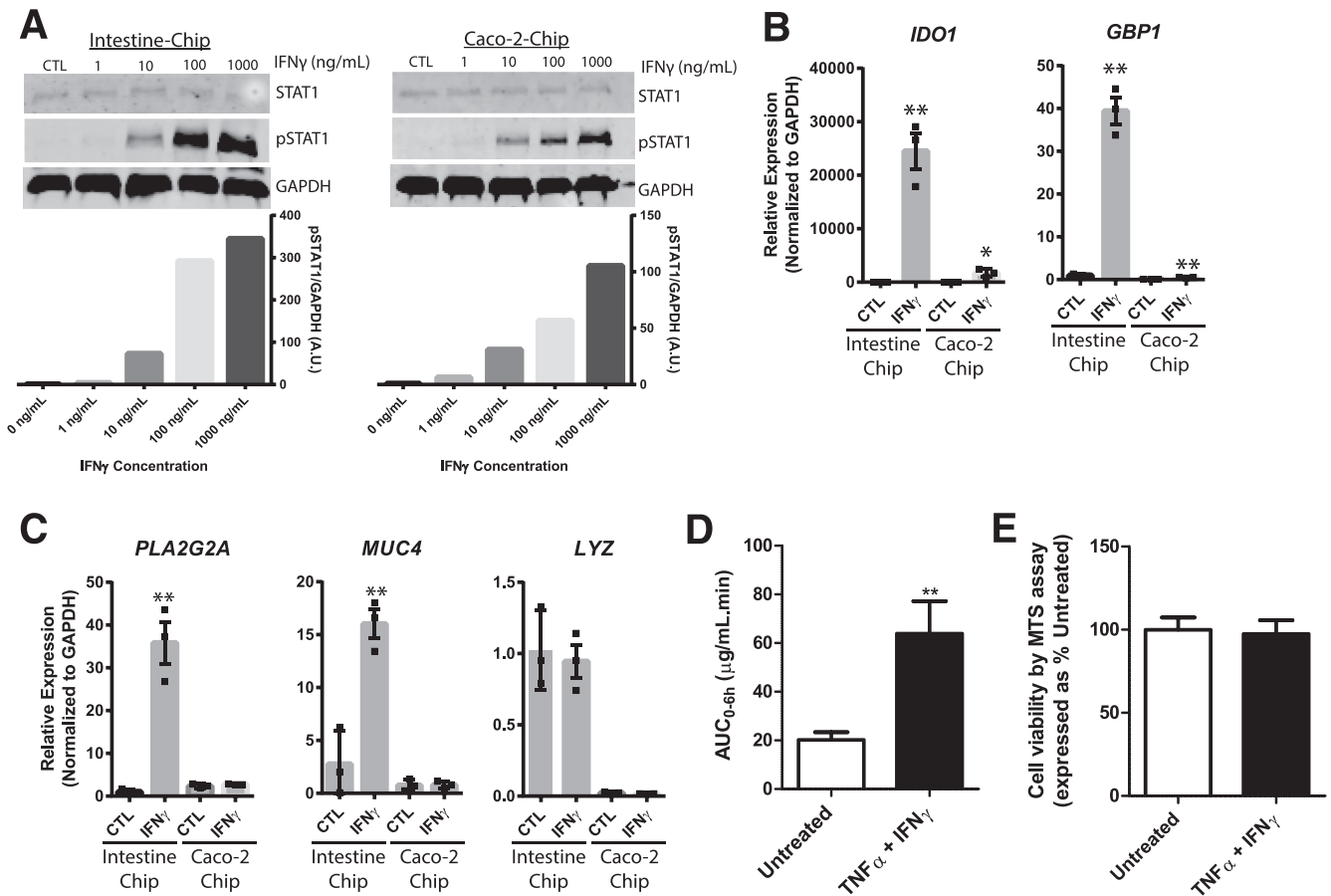


Figure 3. (A) Western blot analyses and quantification of phosphorylation of STAT1 in Chips containing either HIO-derived intestinal epithelial or Caco2 cells in response to exposure of increasing concentrations (0–1000 ng/mL) of IFN- γ in the lower channel for 1 hour. Glyceraldehyde-3-phosphate dehydrogenase and STAT1 were used as loading controls. Image J analysis was used to quantify levels of phospho-STAT1 compared with glyceraldehyde-3-phosphate dehydrogenase. **(B)** Quantitative reverse-transcription polymerase chain reaction analyses of *IDO1* and *GBP1* and **(C)** *PLA2G2A*, *MUC4*, and *LYZ* in Chips incorporating either HIO-derived epithelial or Caco-2 cells in response to exposure of 10 ng/mL of IFN- γ in the lower channel for 3 days. Three independent experiments were carried out; data represent mean \pm SEM; * $P < .05$, ** $P < .01$ compared with respective untreated controls. **(D)** Area under the curve of fluorescein isothiocyanate-dextran 4 kDa permeation over 6 hours was statistically higher ($P < .01$) in Intestine-Chip treated with IFN- γ and TNF- α compared with untreated. **(E)** Following the permeability studies, MTS cytotoxicity assay was carried out and showed no statistical difference between Intestine-Chips treated with IFN- γ and TNF- α or untreated. Two independent experiments were carried out; data represent mean \pm SEM. ** $P < .01$. AUC, area under the curve; CTL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the luminal aspect of the intestine (Figure 2D). Additionally, the presence of CDX2 and villin staining demonstrates that these cells are of intestinal lineage with brush borders (Figure 2E). Given that it was previously reported that

HIOs contained the major epithelial subtypes,^{5,16} we confirmed the presence of lysozyme⁺ cells, MUC2⁺ cells, chromogranin A⁺ cells and FABP2⁺ in the Intestine-Chip (Figure 2F), which suggests that this platform may be

Figure 2. (See previous page). **(A)** Representative phase contrast image of HIO-derived epithelial cells seeded into the Chip. Cells were exposed to continual media flow at 30 $\mu\text{L/h}$ and imaged after 0, 3, 5, and 7 days. Static Chip was imaged after 7 days. Scale bar = 200 μm . **(B)** Stitched phase contrast image of HIO-epithelial cells that were exposed to continual media flow at 30 $\mu\text{L/h}$ for 5 days. Scale bar = 1 mm. **(C)** Representative brightfield image of cross-section of Chip that was exposed to continual media flow at 30 $\mu\text{L/h}$ for 14 days. Scale bar = 250 μm . Representative fluorescent images showing **(D)** E-cadherin/CDH1 (red), ZO-1 (green), DAPI (blue), and **(E)** E-cadherin (blue), CDX2 (red), and Villin (green), in cross-section of Chip under conditions similar to C. Both scale bars = 50 μm . **(F)** Representative fluorescent images showing E-cadherin/CDH1 (red), and MUC2, lysozyme, FABP2, and chromogranin A (all green), counterstained with DAPI (blue), in cross-section of Chips that were exposed to continual media flow at 30 $\mu\text{L/h}$ for 7 days. Scale bar = 10 μm . **(G)** Representative images of *in situ* hybridization for *LGR5*⁺ and *WDR43*⁺ (white arrows) in conditions similar to F. Scale bar = 10 μm . **(H)** Representative fluorescent image showing E-cadherin/CDH1 (blue), Ki67 (green), and DAPI (blue) in conditions similar to F. Scale bar = 100 μm . Graph shows percent Ki67⁺ nuclei per section.

useful for studying Paneth cells, goblet cells, enterocytes, and enteroendocrine cells. We also confirmed the presence of *LGR5*⁺ stem cells and *WDR43*⁺ transit-amplifying cells via *in situ* hybridization (Figure 2G).¹⁹ Although the number of differentiated cells were relatively low, we did find a large percentage of proliferative (Ki67⁺) cells, which were distributed throughout the intestinal folds (Figure 2H).

To demonstrate that the intestinal epithelial cells in the Intestine-Chip were biologically responsive to exogenous stimuli and therefore suitable for modeling epithelial responses, we chose to examine the response to IFN- γ , a cytokine known to be upregulated in patients with inflammatory bowel disease.^{20–22} Because Caco-2 cells are routinely used to assess intestinal epithelial responses and have previously been incorporated into Chips,^{9–11} we included these cells as a direct comparison with the new Intestine-Chip. Exposure to IFN- γ at concentrations ranging from 10–1000 ng/mL resulted in the robust phosphorylation of STAT1 in both cell types when added to the bottom channel for 1 hour (Figure 3A). Thus, both cell types were incubated with 10 ng/mL of IFN- γ for 3 days and assessed for upregulation of indoleamine 2,3-dioxygenase 1 (IDO1) and guanylate binding protein 1 (GBP1). These proteins were chosen because they are both upregulated in various cell lines in response to IFN- γ exposure and are also upregulated in the inflamed intestinal epithelium of patients with inflammatory bowel disease.^{23,24} We found that IDO1 and GBP1 were significantly increased in IFN- γ -treated Intestine-Chips as compared with respective untreated controls, validating results from these previous reports (Figure 3B). Although IDO1 and GBP1 were upregulated in Caco-2-Chips, this biologic affect was attenuated in comparison with the Intestine-Chip. We then looked for changes in expression of various genes that are specific to Paneth and goblet cells. There was significant upregulation of the antimicrobial, *PLA2G2A*, and the mucin, *MUC4*, although no differences were observed in *LYZ* (Figure 3C). Although Caco-2 cells in the Intestine-Chip were immunopositive for the intestinal epithelial subtypes (Supplementary Figure 3), corroborating a previous study,¹⁰ there was no significant upregulation of the aforementioned genes. These findings indicate that HIO-derived intestinal epithelial cells may provide a more faithful model to predict human intestinal epithelial responses. We then performed barrier function studies on the Intestine-Chip whereby IFN- γ and TNF- α were added to the bottom channel for 3 days and the permeability of fluorescein isothiocyanate-dextran 4 kDa was subsequently assessed over 6 hours. The area under the curve of fluorescein isothiocyanate-dextran 4 kDa was significantly increased in Intestine-Chips exposed to IFN- γ and TNF- α compared with those untreated (Figure 3D). Following the permeation assay, a cytotoxicity assay (MTS) was carried out and revealed that there was no change in the viability between groups, which demonstrates that this increase in permeability was not a result of increased cell death (Figure 3E).

Conclusions

The Intestine-Chip combines the strengths of both human iPSC-derived intestinal organoids and small micro-engineering technologies. The intestinal organoid component permits the incorporation of biologically responsive intestinal epithelial cells from almost any individual into the system. Previous studies have shown that various immune cell types and microbes can be incorporated into Chips,^{7–11} and the microengineering component now allows for an examination of how an almost unlimited combination of cytokines, microbes, and immune cells affects the functioning of intestinal epithelial cells in a patient-specific manner. The Chips further allow the establishment and control of a more physiologically relevant microenvironment, such as recapitulating the *in vivo* Wnt and BMP signaling gradients that regulate the location and differentiation of intestinal epithelial cells,²⁵ or establishing an anaerobic upper channel model to potentiate epithelial-microbial studies.¹⁸ All of these approaches would be highly applicable in the gastrointestinal field and will likely have major implications for personalized medicine.

References

1. Strater J, Wedding U, Barth TF, Koretz K, Elsing C, Moller P. Rapid onset of apoptosis *in vitro* follows disruption of beta 1-integrin/matrix interactions in human colonic crypt cells. *Gastroenterology* 1996;110:1776–1784.
2. Grossmann J, Mohr S, Lapentina EG, Fiocchi C, Levine AD. Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. *Am Physiol* 1998;274(6 Pt 1):G1117–G1124.
3. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD, Clevers H. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011;141:1762–1772.
4. VanDussen KL, Marinsaw JM, Shaikh N, Miyoshi H, Moon C, Tarr PI, Ciorba MA, Stappenbeck TS. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. *Gut* 2015;64:911–920.
5. Spence JR, Mayhew CN, Rankin SA, Kuhar MF, Vallance JE, Tolle K, Hoskins EE, Kalinichenko VV, Wells SI, Zorn AM, Shroyer NF, Wells JM. Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*. *Nature* 2011;470:105–109.
6. Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol* 2014;32:760–772.
7. Huh D, Leslie DC, Matthews BD, Fraser JP, Jurek S, Hamilton GA, Thorneloe KS, McAlexander MA, Ingber DE. A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip micro-device. *Sci Transl Med* 2012;4:159ra47.
8. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. *Science* 2010;328:1662–1668.
9. Kim HJ, Huh D, Hamilton G, Ingber DE. Human gut-on-a-chip inhabited by microbial flora that experiences

- intestinal peristalsis-like motions and flow. *Lab Chip* 2012;12:2165–1274.
10. Kim HJ, Ingber DE. Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differentiation. *Integr Biol (Camb)* 2013;5:1130–1140.
 11. Kim HJ, Li H, Collins JJ, Ingber DE. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc Natl Acad Sci U S A* 2016;113:E7–E15.
 12. Shi Y, Inoue H, Wu JC, Yamanaka S. Induced pluripotent stem cell technology: a decade of progress. *Nat Rev Drug Discov* 2017;16:115–130.
 13. van Wilgenburg B, Browne C, Vowles J, Cowley SA. Efficient, long term production of monocyte-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. *PLoS One* 2013;8:e71098.
 14. Senju S, Haruta M, Matsumura K, Matsunaga Y, Fukushima S, Ikeda T, Takamatsu K, Irie A, Nishimura Y. Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy. *Gene Ther* 2011;18:874–883.
 15. Morishima T, Watanabe K, Niwa A, Fujino H, Matsubara H, Adachi S, Suemori H, Nakahata T, Heike T. Neutrophil differentiation from human-induced pluripotent stem cells. *J Cell Physiol* 2011;226:1283–1291.
 16. Barrett R, Ornelas L, Yeager N, Mandefro B, Sahabian A, Lenaeus L, Targan SR, Svendsen CN, Sareen D. Reliable generation of induced pluripotent stem cells from human lymphoblastoid cell lines. *Stem Cells Transl Med* 2014;3:1429–1434.
 17. Wheeler HE, Dolan ME. Lymphoblastoid cell lines in pharmacogenomic discovery and clinical translation. *Pharmacogenomics* 2012;13:55–70.
 18. Huh D, Kim HJ, Fraser JP, Shea DE, Khan M, Bahinski A, Hamilton GA, Ingber DE. Microfabrication of human organs-on-chips. *Nat Protoc* 2013;8:2135–2157.
 19. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegerbarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* 2007;449:1003–1007.
 20. Sasaki T, Hiwatashi N, Yamazaki H, Noguchi M, Toyota T. The role of interferon gamma in the pathogenesis of Crohn's disease. *Gastroenterol Jpn* 1992;27:29–36.
 21. Noguchi M, Hiwatashi N, Liu Z, Toyota T. Enhanced interferon-gamma production and B7-2 expression in isolated intestinal mononuclear cells from patients with Crohn's disease. *J Gastroenterol* 1995;30-(Suppl 8):52–55.
 22. Camoglio L, Te Velde AA, Tigges AJ, Das PK, Van Deventer SJ. Altered expression of interferon-gamma and interleukin-4 in inflammatory bowel disease. *Inflamm Bowel Dis* 1998;4:285–290.
 23. Ciorba MA. Indoleamine 2,3 dioxygenase in intestinal disease. *Curr Opin Gastroenterol* 2013;29:146–152.
 24. Schnoor M, Betanzos A, Weber DA, Parkos CA. Guanylate-binding protein-1 is expressed at tight junctions of intestinal epithelial cells in response to interferon-gamma and regulates barrier function through effects on apoptosis. *Mucosal Immunol* 2009;2:33–42.
 25. Bloemendaal AL, Buchs NC, George BD, Guy RJ. Intestinal stem cells and intestinal homeostasis in health and in inflammation: a review. *Surgery* 2016;159:1237–1248.

Received June 19, 2017. Accepted December 21, 2017.

Correspondence

Address correspondence to: Robert J. Barrett, PhD, Board of Governors Regenerative Medicine Institute and F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Advanced Health Sciences Pavilion 8308, 8700 Beverly Boulevard, Los Angeles, California 90048. e-mail: Robert.Barrett@cshs.org; fax: (310) 248-8066.

Acknowledgements

The authors thank Dr Nur Yucer for her technical assistance and Dr Soshana Svendsen for assistance in editing this manuscript.

Author contributions

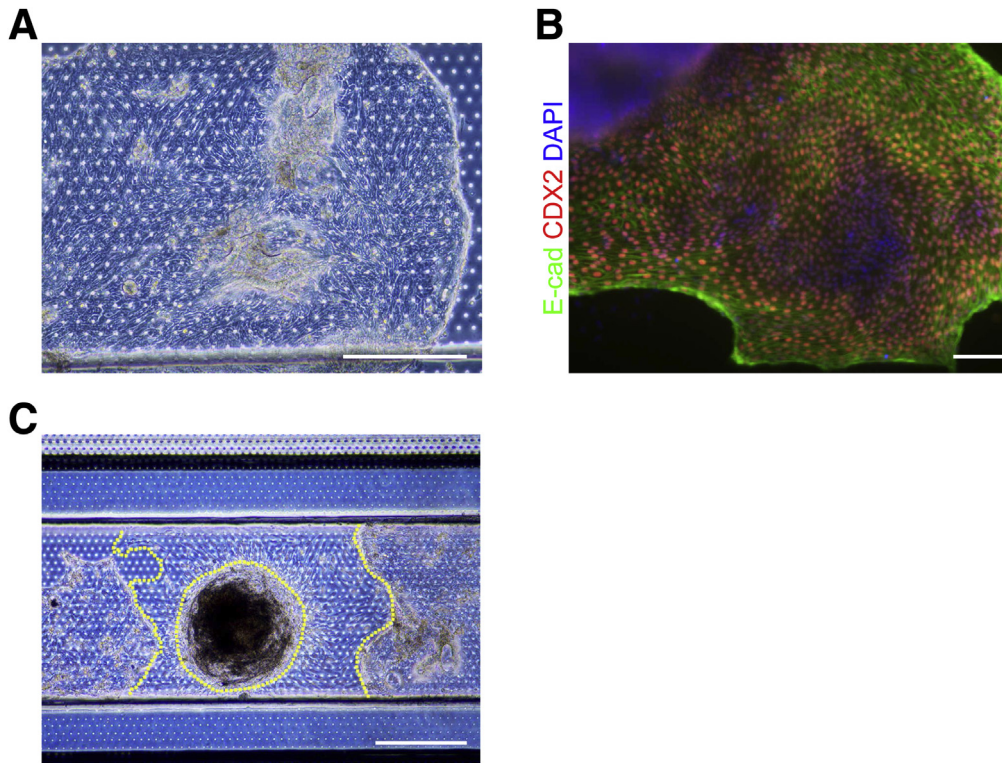
Study concept and design: Michael J. Workman, Stephan R. Targan, Clive N. Svendsen, and Robert J. Barrett. Acquisition of data: Michael J. Workman, John P. Gleeson, Elissa J. Troisi, Hannah Q. Estrada, and Robert J. Barrett. Statistics: Michael J. Workman and John P. Gleeson. Analysis and interpretation of data: Michael J. Workman, John P. Gleeson, S. Jordan Kerns, Christopher D. Hinojosa, Stephan R. Targan, Clive N. Svendsen, and Robert J. Barrett. Drafting of the manuscript: Robert J. Barrett. Review and editing: Michael J. Workman, Geraldine A. Hamilton, S. Jordan Kerns, Stephan R. Targan, Clive N. Svendsen, and Robert J. Barrett. Study supervision: Robert J. Barrett.

Conflicts of interest

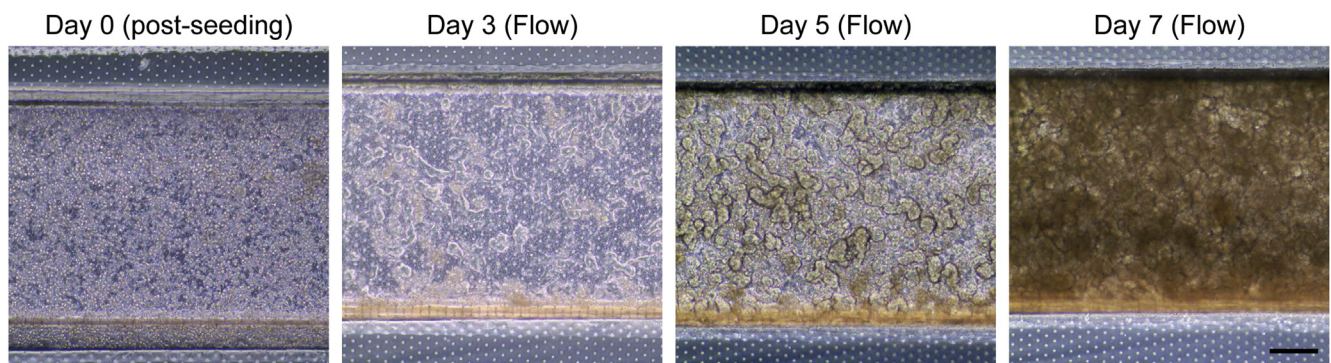
These authors disclose the following: Michael J. Workman, Stephan R. Targan, Clive N. Svendsen, and Robert J. Barrett are named as inventors on a patent application (PCT/US2017/016098) entitled "Systems And Methods For Growth Of Intestinal Cells In Microfluidic Devices" and owned by Emulate Inc and Cedars-Sinai Medical Center. Cedars-Sinai is an investor in Emulate Inc. The remaining authors disclose no conflicts.

Funding

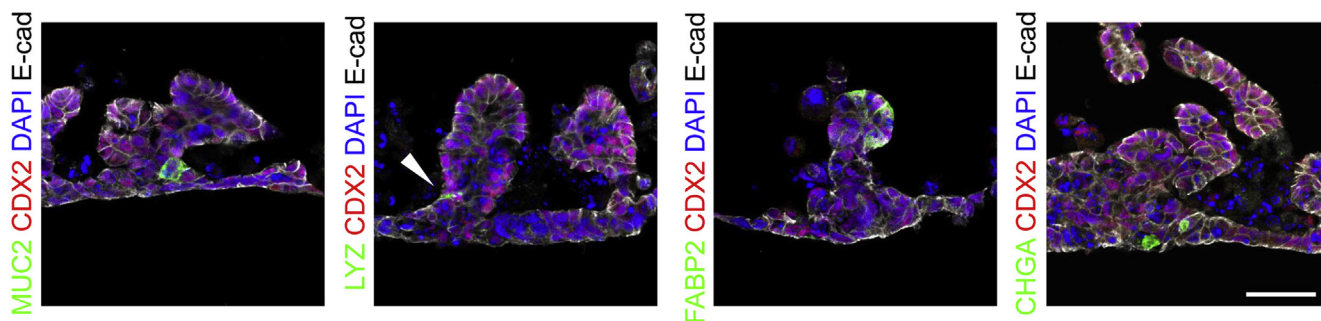
Supported by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (R56DK106202-01, S.R.T. and C.N.S.). Funded by the Board of Governors Regenerative Medicine Institute, the F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, and the Drown Foundation. The study sponsors played no role in the study design, collection, analysis, or interpretation of data.



Supplementary Figure 1. (A) Phase contrast image (scale bar = 500 μm) and (B) fluorescent image (scale bar = 100 μm) showing E-cadherin/CDH1 (green), CDX2 (red), counterstained with DAPI (blue) of intact HIO that was incorporated into the Chip and imaged 3 days later. (C) Phase contrast image showing epithelial and mesenchymal cells that arose from intact HIOs that were incorporated into the Chip and imaged 4 days later. Yellow dotted lines represent mesenchymal cells in Chip. Scale bar = 500 μm .



Supplementary Figure 2. Representative phase contrast images of HIO-derived epithelial cells seeded into the chip. Cells were exposed to continuous media flow of 60 $\mu\text{L/h}$ and imaged after 0, 3, 5, and 7 days. Scale bar = 200 μm .



Supplementary Figure 3. Representative fluorescent images showing E-cadherin (gray), CDX2 (red), and MUC2, lysozyme, FABP2, and chromogranin A (all green) in cross-section of Chips incorporating Caco-2 cells that were exposed to continual media flow of 30 $\mu\text{L/h}$ and imaged after 8 days. Scale bar = 50 μm .

Supplementary Table 1. Table of Antibodies Used in Study

Antigen	Dilution	Catalogue #	Isotype	Manufacturer
CDX2	1:500	NBP1-40553	Rabbit IgG	Novus Biologicals
Chromogranin A(CGA414)	1:500	NBP2-29428	Mouse IgG1	Novus Biologicals
E-cadherin	1:1000	AF648	Goat IgG	R&D Systems
FABP2/1-FABP(9A9B7B3)	1:500	NBP1-51589	Mouse IgG1	Novus Biologicals
GAPDH	1:1000	sc-25778	Rabbit IgG1	Santa Cruz
Ki67 (SP6)	1:200	RM-9106-S0	Rabbit IgG	Thermo Fisher
Lysozyme (BGN/0696/5B1)	1:500	NB100-63062	Mouse IgG2A	Novus Biologicals
Mucin-2 (CCP58)	1:500	NBP2-25221	Mouse IgG1	Novus Biologicals
P-Stat1(Y701) (58D6)	1:1000	9167S	Rabbit mAb	Cell Signaling
Stat1(9H2)	1:1000	9176S	Mouse IgG1	Cell Signaling
Villin 1 (1DC2C3)	1:500	NB600-1349	Mouse IgG1	Novus Biologicals
Vimentin	1:1000	550513	Mouse IgG1	BD Biosciences
ZO-1 (ZO1-1A12)	1:500	33-9100	Mouse IgG1	Life Technologies

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.