Journal of Visualized Experiments

A novel human epithelial enteroid model of necrotizing enterocolitis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59194R1
Full Title:	A novel human epithelial enteroid model of necrotizing enterocolitis
Keywords:	Enteroid; epithelial organoid; Necrotizing Enterocolitis; LPS model; intestinal crypt; stem cells; human tissue model
Corresponding Author:	Catherine J Hunter Ann and Robert H Lurie Children's Hospital of Chicago Chicago, IL UNITED STATES
Corresponding Author's Institution:	Ann and Robert H Lurie Children's Hospital of Chicago
Corresponding Author E-Mail:	CHunter@luriechildrens.org
Order of Authors:	Guillermo Ares
	Christie Buonpane
	Carrie Yuan
	Douglas Wood
	Catherine J Hunter
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Chicago

Ann & Robert H. Lurie
Children's Hospital of Chicago™

Division of Pediatric Surgery

Division Head
Fizan Abdullah, MD
Professor of Surgery

Katherine A. Barsness, MD
Associate Professor of Surgery

Anthony C. Chin, MD
Associate Professor of Surgery

Julia Grabowski, MD
Assistant Professor of Surgery

Catherine J. Hunter, MD
Associate Professor of Surgery

Rashmi Kabre, MD
Assistant Professor of Surgery

Timothy Lautz, MD
Assistant Professor of Surgery

Mary Beth Madonna, MD
Associate Professor of Surgery

Ann O'Connor, MD
Assistant Professor of Surgery

Marleta Reynolds, MD
Surgeon-in-Chief
Professor of Surgery

Erin E. Rowell, MD
Assistant Professor of Surgery

Riccardo Superina, MD
Director, Transplant Surgery
Professor of Surgery

Advanced Practice Nurses
Kerri Baldwin
Teri Coha
Kelly Finkbeiner
Suzanne Kujawa
Sarah Martin
Elizabeth Nanney
Alison Osborne
Laurie Sands
Susan Shumaker
Kathryn Walsh
Linda Zekas

RE: JoVE59194: A novel human epithelial enteroid model of necrotizing enterocolitis

Date: 11/15/2018

Dear Editors,

Thank you for considering our manuscript, "A novel human epithelial enteroid model of necrotizing enterocolitis" for publication. Our laboratory investigates the pathophysiology of NEC, and recently had developed a novel model of human NEC using enteroids generated from neonatal tissue. We appreciate the thoughtful critiques by the reviewers and have address each issue. We believe that our manuscript is greatly improved and we hope that it will be considered for publication. Please do not hesitate to contact me with any questions.

Sincerely,

Catherine J. Hunter, M.D., FACS, FAAP

Associate Professor of Surgery and Pediatrics
Feinberg School of Medicine, Northwestern University
Attending Pediatric Surgeon
Ann and Robert H. Lurie Children's Hospital of Chicago

TITLE:

A Novel Human Epithelial Enteroid Model of Necrotizing Enterocolitis

AUTHORS & AFFILIATIONS:

Guillermo J Ares[1,2], Christie Buonpane[2,3], Carrie Yuan[2], Douglas Wood[2], Catherine J Hunter[2,3]

[1]Department of Surgery, University of Illinois at Chicago, Chicago, IL
[2]Department of Pediatrics, Northwestern University, Chicago, IL
[3]Division of Pediatric Surgery, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL

Corresponding Author:
Catherine J Hunter
Chunter@luriechildrens.org

Email Addresses of Co-authors:
Guillermo Ares (gares1@uic.edu)
Christie Buonpane (cbuonpane@luriechildrens.org)
Carrie Yuan (yewen.yuan@northwestern.edu)
Doug Wood (d-wood6@northwestern.edu)

KEYWORDS:

Enteroid; epithelial organoid; Necrotizing Enterocolitis; LPS model; intestinal crypt; stem cells; human tissue model.

SUMMARY:

Enteroids are emerging as a novel model in the study of human disease. The protocol describes how to simulate an enteroid model of human necrotizing enterocolitis using lipopolysaccharide (LPS) treatment of enteroids generated from neonatal tissue. Collected enteroids demonstrate inflammatory changes akin to those seen in human necrotizing enterocolitis.

ABSTRACT:

Necrotizing enterocolitis (NEC) is a devastating disease of newborn infants. It is characterized by multiple pathophysiologic alterations in the human intestinal epithelium, leading to increased intestinal permeability, impaired restitution, and increased cell death. Although there are numerous animal models of NEC, response to injury and therapeutic interventions may be highly variable between species. Furthermore, it is ethically challenging to study disease pathophysiology or novel therapeutic agents directly in human subjects, especially children. Therefore, it is highly desirable to develop a novel model of NEC using human tissue. Enteroids are 3-dimensional organoids derived from intestinal epithelial cells. They are ideal for the study of complex physiologic interactions, cell signaling, and host-pathogen defense. In this manuscript we describe a protocol that cultures human enteroids after isolating intestinal stem cells from patients undergoing bowel resection. The crypt cells are cultured in media containing growth factors that encourage differentiation into the various cell types native of the human intestinal epithelium. These cells are grown in a synthetic, collagenous mix of proteins that serve as a

scaffold, mimicking the extra-cellular basement membrane. As a result, enteroids develop apical-basolateral polarity. Co-administration of lipopolysaccharide (LPS) in media causes an inflammatory response in the enteroids, leading to histologic, genetic, and protein expression alterations similar to those seen in human NEC. An experimental model of NEC using human tissue may provide a more accurate platform for drug and treatment testing prior to human trials, as we strive to identify a cure for this disease.

INTRODUCTION:

Human enteroids are an ex-vivo 3-dimensional culture system generated from stem cells isolated from intestinal crypts of human intestinal tissue samples. This ground-breaking model was pioneered by Hans Clevers et al. in 2007 following the discovery of Lgr5+ stem cells at the crypts of small intestine in mice[1]. Their work laid the foundation for establishing an ex vivo intestinal epithelial culture of multiple cell types that could be passaged without significant genetic or physiologic changes[2]. Since this discovery, enteroids have been used as a novel model to study normal digestive physiology, and the pathophysiology of intestinal diseases such as inflammatory bowel disease, host-pathogen interactions, and regenerative medicine[2].

The use of enteroids as an ex vivo model for the study of intestinal pathophysiology has several advantages over alternative techniques. For the past several decades, animal models and immortalized intestinal cancer-derived cell lines have been used to study intestinal physiology[3-5]. Single-cell cultures do not represent the diversity of cell types present in normal intestinal epithelium, thereby lacking cell to cell cross-talk and segment-specificity in protein expression, signaling, and pathogen-induced disease[6]. Stem cells in enteroids differentiate into the major epithelial cell types such as enterocytes, Paneth cells, goblet cells, enteroendocrine cells and more[3]. They exhibit polarity, carry out epithelial transport functions, and allow for intestinal segment specificity[6]. Since enteroids can recapitulate the multiple cell types of human intestinal epithelium, they are able to overcome this recognized limitation of cancer cell-based systems. Over time, derivatives of cell lines are subcloned and evolve to exhibit greater diversity in protein expression and localization[3]. On the contrary, enteroids can be passaged without significant genetic or physiologic changes[2]. Although numerous animal models for NEC exist, response to injury and therapeutic interventions may be highly variable between species. As a result of these limitations, therapeutics derived from animal models fail 90% of the time when tested in human trials due to differences in toxicity or efficacy[3]. Enteroids serve as promising pre-clinical models that can overcome these deficiencies, leading to a better understanding of complex intestinal pathophysiology and therefore, more successful and cost-effective therapeutic innovations. There is also recent evidence that the age of the tissue that an enteroid is generated from remains biologically important[7]. This is an especially important detail for our model since enteroids are generated from neonatal tissue, thereby maintaining physiologic relevance to patients with NEC.

The utility of enteroids as models of human illnesses continues to expand, in hopes of finding cures to severe and pervasive conditions. Necrotizing enterocolitis (NEC) is a devastating intestinal disease of neonates characterized by intestinal necrosis and frequently leads to perforation of the bowel wall, septicemia, and death[8]. Due to the complex and multifactorial

pathophysiology of NEC, the exact mechanism of the disease has not yet been fully elucidated; however, increased intestinal permeability has been clearly implicated in the disease process [8]. Given that the study of NEC and potential therapeutic agents is ethically challenging in human subjects, especially children, it is highly desirable to utilize a biologically relevant enteroid model of NEC using human neonatal tissue. Thus far, enteroids have a limited role in the study of NEC. This protocol describes the use of enteroids derived from human intestinal tissue samples as a novel *ex-vivo* model for the study of necrotizing enterocolitis.

PROTOCOL:

Institutional review board approval was obtained (IRB #2013-15152) for collection of tissue samples from patients undergoing bowel resection at Ann and Robert H. Lurie Children's Hospital of Chicago, Chicago, IL. All protocols were performed in compliance with institutional and national guidelines and regulations for human welfare. Written informed parental consent was required and obtained prior to sample collection in all cases.

1. Reagent preparation

1.1. Prepare Culture Media stock solution for whole tissue collection: 1 L of Dulbecco's Modified Eagle Medium (DMEM), 110 mL of Fetal Bovine Serum (FBS), 11 mL of Penicillin-Streptomycin (final concentration 1%) and 1.1 mL of filter-sterilized insulin (final concentration 0.1%.) Store stock solution at 4 °C.

1.2. Prepare Chelating Buffer #1: 30 mL of Culture Media (as described in 1.1), 600 µL of 0.5 M Ethylenediaminetetraacetic acid (EDTA) (final concentration 10 mM), 300 µL of Gentamicin (final concentration 1%) and 60 µL of Amphotericin B (final concentration 0.2%). Store stock solution at 4 °C.

1.3. Prepare Chelating Buffer #2: 30 mL of Culture Media (as described in 1.1), 300 µL of 0.5 M EDTA (final concentration 5mM), 300 µL of Gentamicin (final concentration 1%) and 60 µL of Amphotericin B (final concentration 0.2%). Store stock solution at 4 °C.

1.4. Prepare Human Minigut Media: 41.4 mL of DMEM/F-12, 5 mL of FBS (final 10%), 500 µL of Penicillin-Streptomycin (final concentration 1%), 500 µL of L-glutamine (final concentration 1%), 500 µL of Gentamicin (final concentration 1%), 100 µL of Amphotericin B (final concentration 0.2%), 500 µL of 1 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (final concentration 10 mM), 500 µL of 100x N-2 supplement, and 1 mL of 50x B-27 supplement minus Vitamin A for a total volume of 50 mL. Store stock solution at -20 °C.

1.5. Prepare Human Minigut Media Complete: 10 mL of Human Minigut Media (prepared in 1.4), 10 µL of 100 µg/mL Wnt3a (final concentration 100 ng/mL), 10 µL of 100 µg/mL Noggin (final concentration 100 ng/mL), 10 µL of 1 mg/mL R-Spondin (final concentration 1 µg/mL), 10 µL of 500 µg/mL Epidermal Growth Factor (EGF) (final concentration 50 ng/mL), 10 µL of 1 M N-Acetylcysteine (final concentration 1 mM), 10 µL of 10 mM Y-27632 (final concentration 10 µM),

10 µL of 500 µM A-83 (final concentration 500 nM), 10 µL of 10 mM SB202190 (final concentration 10 µM), 100 µL of 1 M Nicotinamide (final concentration 10 mM) and 1 µL of 100 µM [leu] 15-gastrin 1 (final concentration 10 nM). Total volume 10 mL. Store stock solution at 4 °C.

NOTE: Solutions must be used within 48 hours.

2. Crypt isolation and plating from whole tissue

2.1. At the time of collection in the operative suite, place the human small intestinal tissue sample in cold Dulbecco's Phosphate Buffered Saline (DPBS). Wash the specimen in cold DPBS until clear of stool and blood. Store specimen at 4 °C in RPMI 1640 Medium until ready for crypt isolation.

NOTE: Tissue cannot be stored more than 24 hours.

2.1.1. Make sure that the specimen is clear of stool and blood. Using delicate dissecting scissors, remove any excess fat or surgical clips/staples etc. Weigh the specimen.

NOTE: Aim for a piece approximately 0.75-2.5 g.

2.2. Cut the specimen into 0.5 cm pieces and place in 30 mL of Chelating Buffer #1 (as prepared in step 1.2).

2.2.1. Shake at low speed for 15 min at 4 °C.

2.2.2. Filter tissue through 100 µm cell strainer and discard flow through.

2.3. Add the tissue to 30 mL of Chelating Buffer #2 (as prepared in step 1.3).

2.3.1. Shake at low speed for 15 min at 4 °C.

2.3.2. Filter tissue through a 100 µm cell strainer and discard flow through.

2.4. Thaw 500 mL of basement membrane matrix on ice for use in step 2.8.

2.5. Add tissue to 10 mL of cold DMEM in a 50 mL conical tube and shake vigorously by hand for 10 s.

2.5.1. Filter through a 100 µm cell strainer and collect flow through (Label #1). Keep tube #1 on ice.

2.5.2. Add tissue to another 10 mL of cold DMEM in a separate 50 mL conical tube and shake vigorously by hand for 10 s.

2.5.3. Filter through a 100 µm cell strainer and collect flow through (label #2).

2.5.4. Repeat two additional times until there are four conical tubes with flow through (labeled tubes #1- 4).

2.6. Filter tube #1 solution through a 100 µm cell strainer and transfer flow through into 15 mL conical tube (Label #1). Repeat for #2-4.

2.6.1. Centrifuge 15 mL tubes #1-4 at 200 x *g* for 15 min at 4 °C.

2.7. In the laminar flow hood, remove the supernatant from tubes #1-4 and discard. Avoid disrupting the cloud of tissue immediately above the pellet, even if that means leaving some supernatant behind.

2.7.1. By pipetting slowly, mix together the pellet with the leftover supernatant in tubes #1-4.

2.7.2. Transfer the mixture from tubes #1-4 into one single 2 mL conical tube.

2.7.3. Centrifuge the conical tube at 200 x *g* for 20 min at 4 °C.

2.8. Remove the supernatant and re-suspend the pellet in 500 µL of basement membrane matrix.

NOTE: Keep the basement membrane matrix on ice at all times and work quickly for the next steps. This product polymerizes very quickly at room temperature.

2.8.1. Apply 50 µL of specimen/basement membrane matrix suspension to the center of a well in a 24-well plate. This should appear dome-shaped.

NOTE: Use of chilled pipette tips aids in smoother transfer of the suspension, minimizing polymerization.

2.8.2. Repeat 9 times to fill 10 total wells.

2.9. Place the 24-well plate in 37 °C, 5% CO_2 incubator for 30 min to allow polymerization.

2.10. Add 500 µL of Human Minigut Media Complete (as prepared in step 1.5) to each well. Replace every 2 days.

2.11. Collect enteroids after 5-10 days, when budding is visualized. See Steps 4 and 5 for collection instructions.

3. Induction of experimental NEC

3.1. Add 10 µL of 5 mg/mL of lipopolysaccharide (LPS) to 500 µL of Human Minigut Media Complete (as prepared in step 1.5) in each well on Day 0. Replace every 2 days until collection.

4. Preparation for paraffin embedding

4.1. Gently remove media.

4.1.1. Add 1 mL of PBS and gently pipette up and down to dissolve the basement membrane matrix with care not to lyse the enteroids.

4.1.2. Centrifuge PBS/enteroid mixture at <300 x *g* for 5 min to pellet and remove PBS.

4.2. Add 4% paraformaldehyde to fix at room temperature for 1 hour.

4.3. Centrifuge at <300 x *g* for 5 min to pellet, and then remove PBS.

4.3.1. Wash gently with 1 mL of PBS and centrifuge at <300 x *g* for 5 min to pellet, and then remove PBS.

4.3.2. Repeat step 4.3.1.

4.4. Melt a sufficient volume of the tissue processing gel (around 300 µL) by placing the desired amount in a conical tube and warming it in a dry bath incubator at 65 °C for 3-10 min until liquid.

4.4.1. Add the tissue processing gel to the pellet and mix gently.

4.4.2. On a coverslip, place a small cloning ring.

4.4.3. Pipette the tissue processing gel and enteroid mixture into a cloning ring mounted onto a coverslip.

4.5. Allow the tissue processing gel and enteroid mixture to solidify at 4 °C for 1 hour.

4.5.1. Submerge the cloning ring with solidified mixture into 70% ethanol in preparation for paraffin embedding.

5. Enteroid collection for RNA and protein extraction

5.1. Gently remove Human Minigut Media Complete from each well.

5.2. Add 1 mL PBS and gently pipette up and down to dissolve the basement membrane matrix. Be careful not to dissociate the enteroids.

5.2.1. Place PBS/enteroid mixture into a sterile 2 mL conical tube.

5.2.2. Centrifuge at <300 x *g* for 5 min to pellet.

5.2.3. Gently remove PBS, taking care not to remove the enteroids.

5.3. Repeat step series 5.2 two more times.

5.4. Freeze in -80 °C until ready for protein and/or RNA extraction.

5.5. Perform gene expression and protein isolation of the enteroids using well-established qRT-PCR and Western Blotting techniques.

REPRESENTATIVE RESULTS:

Immediately after plating, the freshly isolated intestinal crypts appear as elongated rods. Within hours, the enteroid will take on a round appearance (**Figure 1a**). Over the next several days, the enteroids will start forming spheres as seen in **Figure 1b**. Budding should occur between 5-10 days (**Figure 1c**) and enteroid collection should occur at that time.

The growing enteroids will also exhibit polarity, containing a centralized lumen, an apical border and a basolateral domain (**Figure 2**). Enteroids also show structural integrity, represented by a robust actin cytoskeleton (**Figure 3**). After several days in culture, the LPS treated enteroids experience more apoptosis and will have a lower yield than the control group (**Figure 4**). As seen in human NEC and murine models of NEC[9], an increased expression of Toll-like receptor 4 (TLR4) was found in LPS treated enteroids compared to controls (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1. Crypt culture and human enteroid formation from whole tissue. (**a**) Day 0 crypt culture with round, flat appearance. (**b**) Day 4 enteroids with spheroid formation. (**c**) Day 7 enteroids with budding (black arrow). Scale bars = 50 µm.

Figure 2: Histological appearance of an enteroid. Haemotoxylin and Eosin staining. The enteroid displays a centralized lumen and exhibits polarity, with both an apical and basolateral domain. The haemotoxylin (purple) represents the nucleus, whereas the eosin (pink) represents cytosolic components. Scale bar = 20 µm.

Figure 3: Enteroid actin cytoskeleton. Immunoflorescence micrograph of an enteroids. The enteroid displays structural integrity as demonstrated by the prominent actin cystoskeleton (magenta). Nuclei stained with DAPI (blue), scale bar = 100 µm.

Figure 4. Human enteroid culture yield is decreased with LPS treatment. Day 5 enteroids in culture, grown from the same whole tissue sample. (**a**) Enteroids treated with control culture media. Display robust growth. (**b**) Enteroids treated with LPS in culture media. Only a few

surviving enteroids. Scale bar = 200 µm.

Figure 5. Increased TLR4 expression in experimental NEC enteroids. (**a**) qRT-PCR showed increased TLR4 mRNA expression in enteroids exposed to LPS ($p = 0.03$). (**b**) Western blot analysis showing increased TLR4 expression in experimental human enteroid NEC ($p = 0.02$). Representative western blot depicted. Values are means ± SEM of 3 samples per group. *$p < 0.05$ by Student's *t* test.

DISCUSSION:

This novel ex vivo human intestinal enteroid model serves as a useful method for the study of intestinal barrier dysfunction in necrotizing enterocolitis (NEC). The enteroid processing methods presented here were adapted from the previous work of Drs. Misty Good, Michael Helmrath and Jason Wertheim[10-12].

Details surrounding the whole tissue collection and timing of crypt isolation are critical steps in this protocol. Tissue must be collected immediately at the time of operative resection and processed for crypt isolation as soon as the tissue arrives in the lab. We selected tissue from patients less than 3 months old for this model. Delayed processing can occur within 24 hours after whole tissue collection if needed. Additionally, the quality of the human tissue sample greatly affects the yield of crypt isolation. Healthy small intestine without underlying pathology and from younger patients (<2 months of age) have been found to yield the best results. Additionally, the collected tissue should be chosen from the healthiest area, typically at the distal ends of the resected specimen. Using a larger piece of intestine does not improve crypt isolation with the described solution volumes in the protocol above. Intestinal segments approximately 1-2 cm in length, weighing 0.75-2.5 g are optimal. The induction of experimental NEC via LPS exposure was modeled from well-established cell culture and animal models. The established work of Hackam et al. showed the critical role of TLR4 (LPS receptor) in NEC development[9,13-15]. LPS activation of TLR4 stimulates proinflammatory cytokines, a reduction in barrier integrity, and activation of subepithelial leukocytes that characterize the signaling events involved in human NEC. TLR4 has been implicated as a key molecule in promoting inflammation[7] and animals that lack functional TLR4 are have demonstrated protection from the development of NEC[15]. Circulating levels of LPS are elevated in patients with NEC and elevated in stool and plasma of animal models of NEC. LPS induces intestinal inflammation in animals that resembles human NEC, which highlights the significance of inflammation in this pathway. Mirroring the established cell culture and animal models of NEC, we believe that induction of experimental NEC via LPS administration in enteroid culture is a useful ex vivo human neonatal model for the study of NEC. In line with previous reports in other established models, our experimental NEC enteroids demonstrated increased expression of TLR4 compared to controls.

The protocol for induction of experimental NEC via LPS exposure has undergone several important modifications and improvements. Initially, enteroids were grown for 5 days, then inoculated with LPS for 24 hours and subsequently collected. The protocol now recommends LPS inoculation on day 0 with continued exposure in every culture media change until collection.

Additionally, the dosage of LPS was optimized. After performing a dose curve and trialing several different LPS concentrations, 5 mg/mL was selected. Experimentation with LPS inoculation directly into the basement membrane matrix/enteroid mixture at the time of plating was also trialed; however, this was cumbersome and did not improve results.

There are limitations that should be addressed as the utilization of the human enteroid model evolves. Since the discovery in 2007, several different protocols have been used to establish and culture enteroids. The numerous growth factors required for enteroid maintenance and differentiation lack standardization, which may affect reproducibility. Additionally, enteroid culture lacks mechanical forces that affect the human intestinal epithelium in physiologic conditions. Pressure from luminal flow and peristalsis may influence gene and protein expression, which is not accounted for in this model. This model also lacks nerves, immune cells, a microbiome, vasculature and mesenchyme that are present in human intestinal epithelium.

Exposure to LPS in this human intestinal enteroid model causes an inflammatory response leading to histologic, genetic, and protein expression alterations similar to those found in human NEC. Although there are several current animal models of NEC, response to intestinal injury and therapeutic interventions varies widely between species. Since it is ethically challenging to study disease pathophysiology or to test novel therapeutic agents directly in humans, especially infants, it is exceedingly important to continue to cultivate this novel ex vivo model of NEC using human tissue. Human enteroids are amenable to genetic modification and may be fundamental in the development of therapies for many human intestinal diseases[16]. Future directions should aim to culture human enteroids on a permeable scaffold in a perfusion chamber with apical and basolateral flow to in order to reproduce in vivo crypt-villus architecture as well as physiologic forces such as peristalsis and luminal flow.

ACKNOWLEDGMENTS:

This work was supported by the National Institute of Health Institute of Diabetes and Digestive and Kidney Disease Grant (K08DK106450) and the Jay Grosfeld Award from the American Pediatric Surgical Association to C.J.H.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature.* **459** (7244), 262-265, doi:10.1038/nature07935 (2009).
2. Zachos, N. C. et al. Human Enteroids/Colonoids and Intestinal Organoids Functionally Recapitulate Normal Intestinal Physiology and Pathophysiology. *The Journal of Biological Chemistry.* **291** (8), 3759-3766, doi:10.1074/jbc.R114.635995 (2016).
3. Foulke-Abel, J. et al. Human enteroids as an ex-vivo model of host-pathogen interactions in the gastrointestinal tract. *Experimental Biology and Medicine (Maywood).* **239** (9), 1124-1134, doi:10.1177/1535370214529398 (2014).
4. Hidalgo, I. J., Raub, T. J., Borchardt, R. T. Characterization of the human colon carcinoma

cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology.* **96** (3), 736-749 (1989).
5. Hilgers, A. R., Conradi, R. A., Burton, P. S. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. *Pharmaceutical Research.* **7** (9), 902-910 (1990).
6. In, J. G. et al. Human mini-guts: new insights into intestinal physiology and host-pathogen interactions. *Nature Reviews Gastroenterology & Hepatology.* **13** (11), 633-642, doi:10.1038/nrgastro.2016.142 (2016).
7. Senger, S. et al. Human Fetal-Derived Enterospheres Provide Insights on Intestinal Development and a Novel Model to Study Necrotizing Enterocolitis (NEC). *Cell and Molecular Gastroenterology and Hepatology.* **5** (4), 549-568, doi:10.1016/j.jcmgh.2018.01.014 (2018).
8. Moore, S. A. et al. Intestinal barrier dysfunction in human necrotizing enterocolitis. *Journal of Pediatric Surgery.* **51** (12), 1907-1913, doi:10.1016/j.jpedsurg.2016.09.011 (2016).
9. Neal, M. D. et al. A critical role for TLR4 induction of autophagy in the regulation of enterocyte migration and the pathogenesis of necrotizing enterocolitis. *The Journal of Immunology.* **190** (7), 3541-3551, doi:10.4049/jimmunol.1202264 (2013).
10. Lanik, W. E. et al. Breast Milk Enhances Growth of Enteroids: An Ex Vivo Model of Cell Proliferation. *Journal of Visualized Experiments.* (132), doi:10.3791/56921 (2018).
11. Mahe, M. M., Sundaram, N., Watson, C. L., Shroyer, N. F., Helmrath, M. A. Establishment of human epithelial enteroids and colonoids from whole tissue and biopsy. *Journal of Visualized Experiments.* (97), doi:10.3791/52483 (2015).
12. Uzarski, J. S., Xia, Y., Belmonte, J. C., Wertheim, J. A. New strategies in kidney regeneration and tissue engineering. *Current Opinion in Nephrology and Hypertension.* **23** (4), 399-405, doi:10.1097/01.mnh.0000447019.66970.ea (2014).
13. Leaphart, C. L. et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *The Journal of Immunology.* **179** (7), 4808-4820 (2007).
14. Neal, M. D. et al. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *The Journal of Immunology.* **176** (5), 3070-3079 (2006).
15. Sodhi, C. P. et al. Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology.* **143** (3), 708-718 e705, doi:10.1053/j.gastro.2012.05.053 (2012).
16. Koo, B. K. et al. Controlled gene expression in primary Lgr5 organoid cultures. *Nature Methods.* **9** (1), 81-83, doi:10.1038/nmeth.1802 (2011).

Figure 1

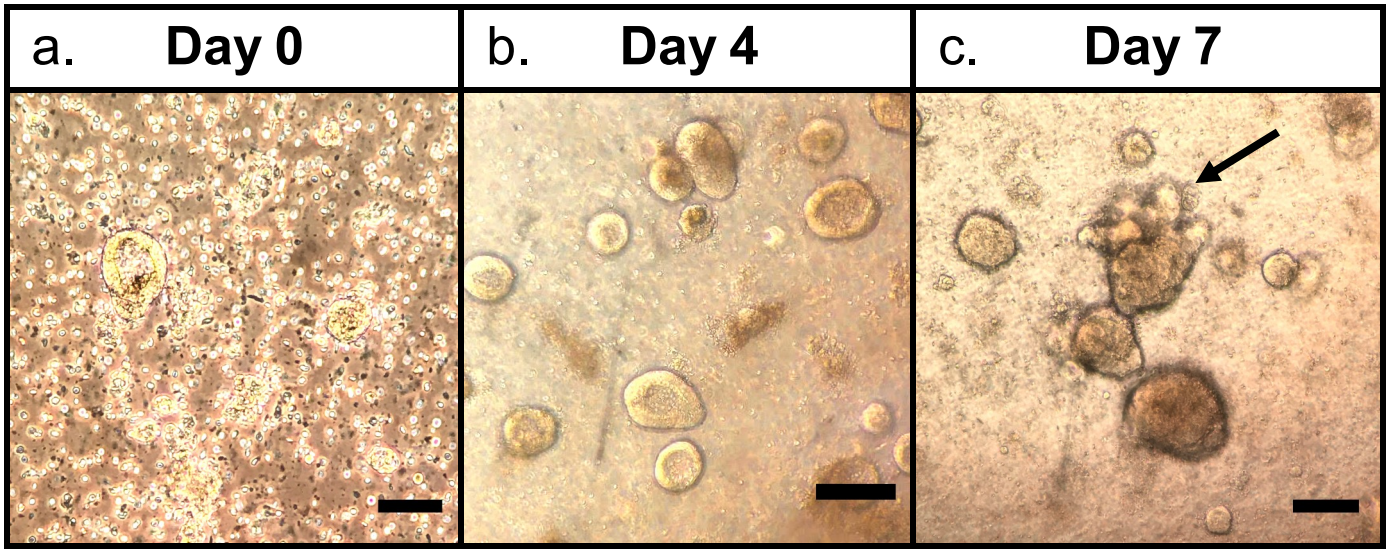


Figure 2

Click here to access/download;Figure;FIGURE 2.jpg

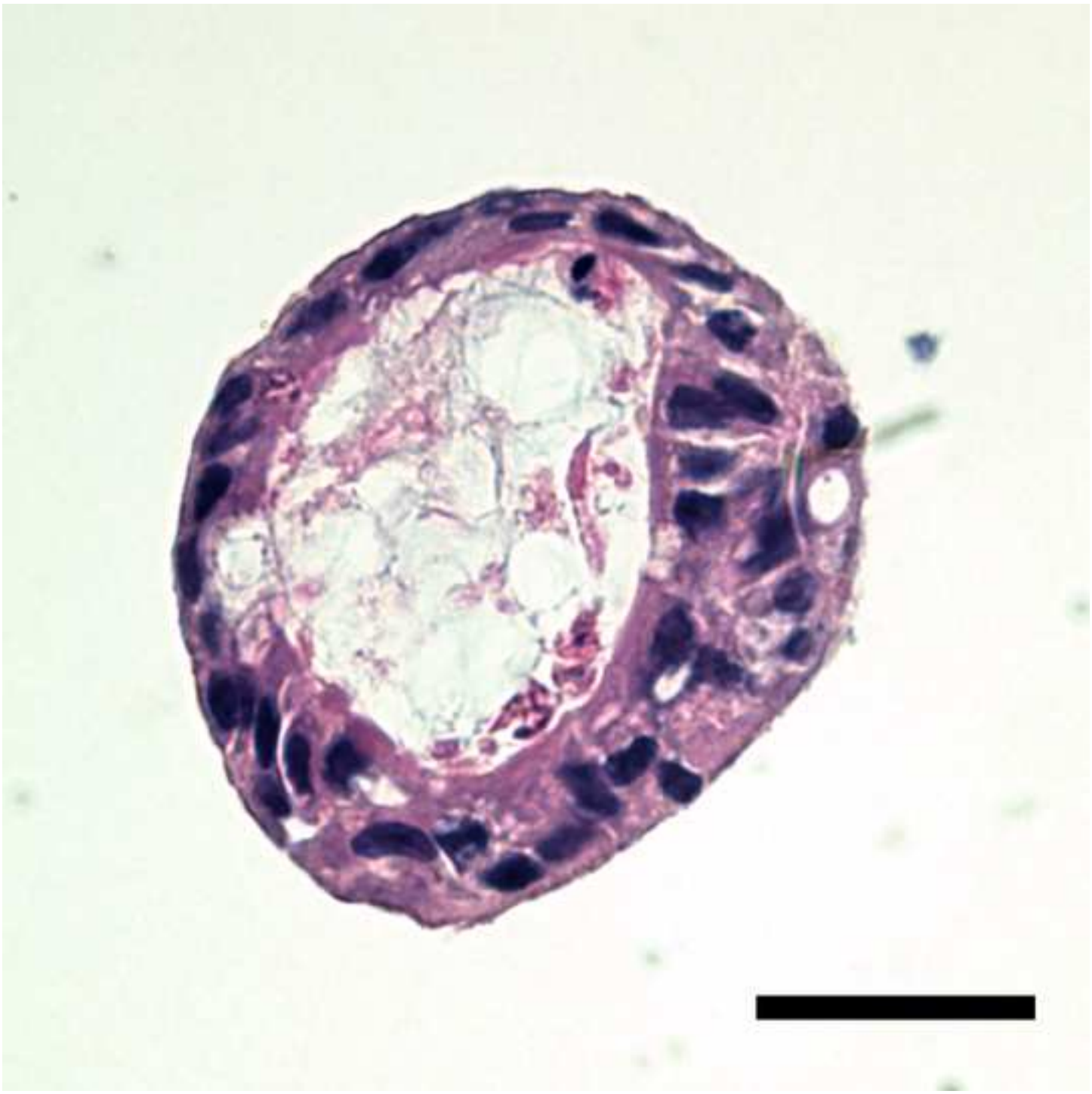


Figure 3

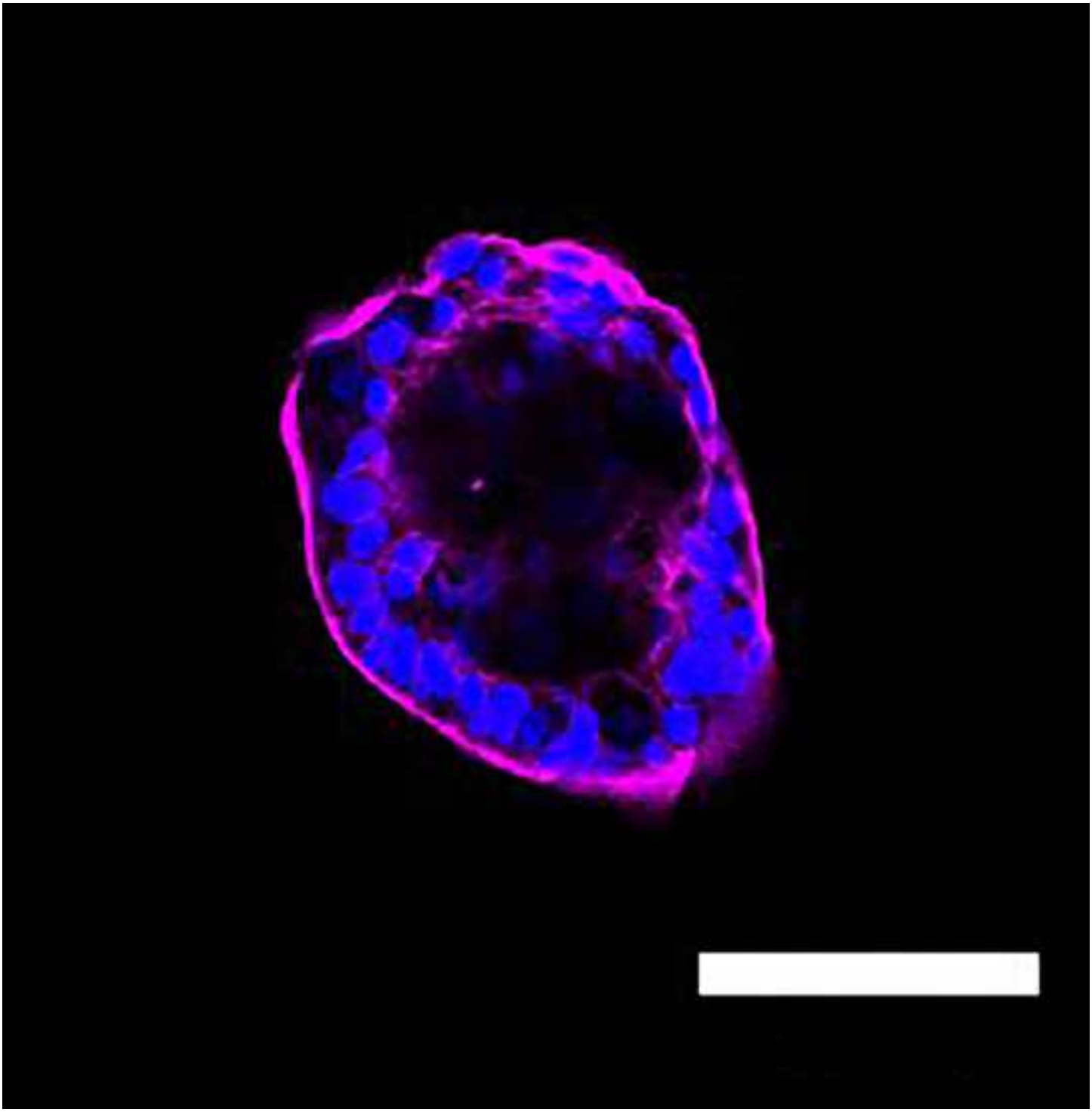


Figure 4

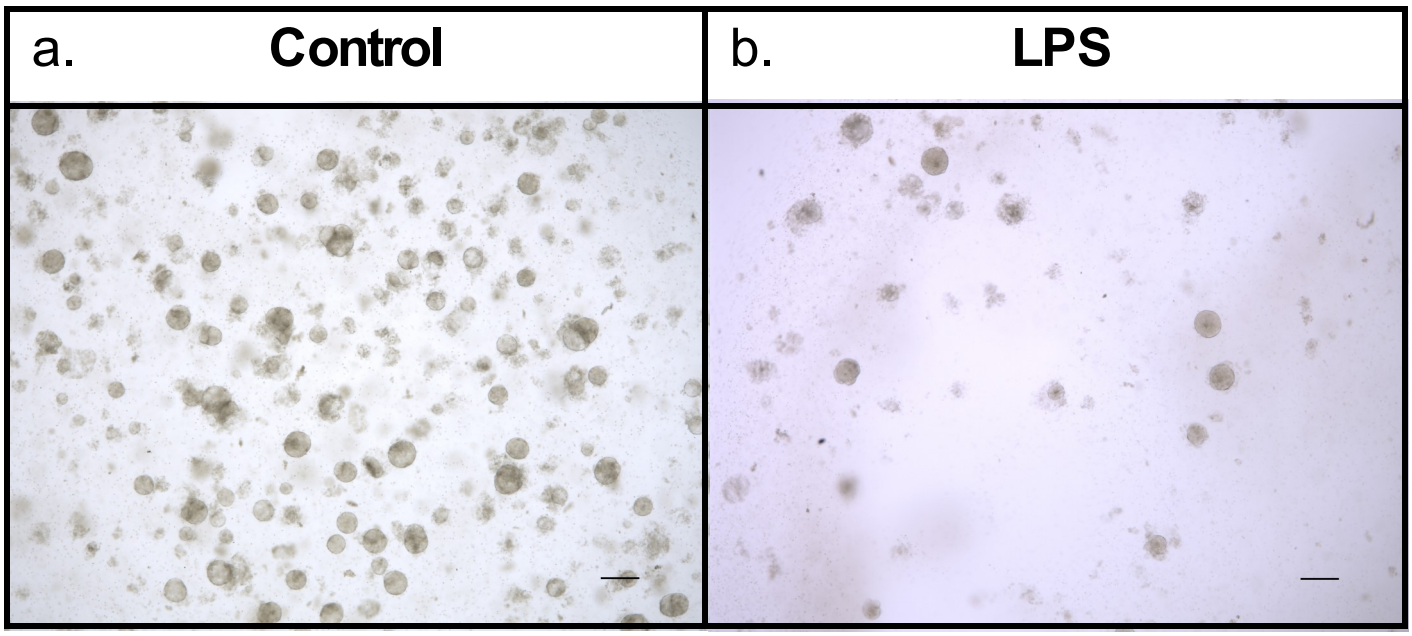
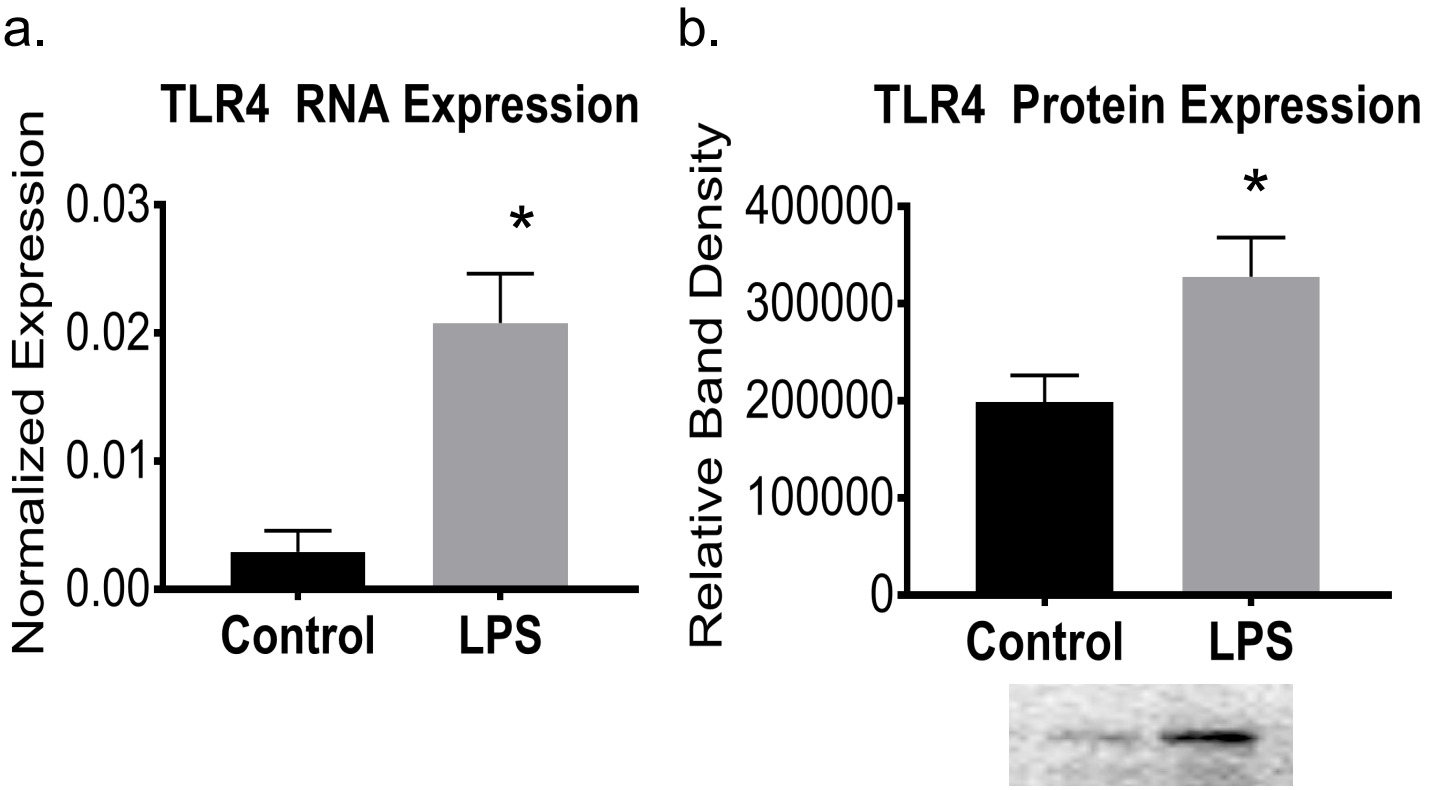


Figure 5



Name of Material/ Equipment	Company
4% Paraformaldehyde	ThermoFisher
A-83	R&D Tocris
Amphotericin B	ThermoFisher
B-27 supplement minus Vitamin A	ThermoFisher
Basement Membrane Matrix (Matrigel)	Corning
DMEM/F-12	ThermoFisher
Dulbecco's Modified Eagle Medium (DMEM)	ThermoFisher
Dulbecco's Phosphate-Buffered Saline (DPBS)	ThermoFisher
Epidermal Growth Factor (EGF)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Fetal Bovine Serum (FBS)	Gemini Bio-Pro
Gentamicin	Sigma
GlutaMAX (L-glutamine)	ThermoFisher
Insulin	Sigma
[leu] 15-gastrin 1	Sigma
Lipopolysaccharide (LPS)	Sigma
N-2 supplement	ThermoFisher
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES)	ThermoFisher
N-Acetylcysteine	Sigma
Nicotinamide	Sigma
Noggin	R&D Systems INC
Penicillin-Streptomycin	ThermoFisher
Phosphate Buffered Saline (PBS)	Sigma
RPMI 1640 Medium	Invitrogen
R-Spondin	PEPROTECH INC
SB202190	Sigma
Tissue Processing Gel (Histogel)	ThermoFisher
Wnt3a	R&D Systems INC
Y-27632	Sigma

Catalog Number	Comments/Description
AAJ19943K2	
2939/10	
15290026	
17504-044	
CB-40230C	
MT-16-405-CV	
11-965-118	
14190-144	
E9644-.2MG	
EDS-500G	
100-125	
G5013-1G	
35050-061	
I9278-5mL	
G9145-.1MG	
L2630-25MG	
17502-048	
15630-080	
A9165-5G	
N0636-100G	
6057-NG/CF	
15140-148	
P5368-5X10PAK	
11875093	
120-38	
S7067-5MG	
22-110-678	
5036-WN-010	
Y0503-1MG	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	A novel human epithelial enteroid model of necrotizing enterocolitis
Author(s):	Guillermo Ares, Christie Buonpane, Carrie Yuan, Doug Wood, Catherine J. Hunter

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Catherine Hunter, MD
Department:	Pediatric Surgery
Institution:	Ann and Robert H. Lurie Children's Hospital of Chicago
Title:	Assocaite Professor of Surgery and Pediatrics
Signature:	
Date:	10/02/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

JoVE59194 "A novel human epithelial enteroid model of necrotizing enterocolitis" point-by-point response.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you. We have reviewed the manuscript and corrected any spelling or grammar issues.

2. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies and information that can help readers to determine if the method is appropriate for their application.

Response: Thank you for pointing out this deficit in the introduction. We have now revised the introduction to include more specific advantages over alternative techniques with applicable references to previous studies in order to help readers determine if this method is appropriate for their application.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Eppendorf, Matrigel, Histogel, etc.

Response: We have now removed all commercial language from the manuscript.

4. 2.1: Please specify the type of tissue collected.

Response: Thank you for pointing out that this was unclear. We have revised section 2.1 to specify that human small intestinal tissue is collected.

5. 2.1.1: What is used to cut?

Response: We have now clarified that delicate dissecting scissors should be used in step 2.1.1.

6. 2.7.1: This step is unclear. Resuspend 4 pellets in what solvent and in what volume? Please specify.

Response: Thank you for pointing out that this step was unclear. We have revised the instructions in section 2.7.1.

7. 4.4: Please specify how to melt Histogel.

Response: We have updated section 4.4 with specific instructions on how to melt the tissue processing gel.

8. As PCR and Western blot data are presented in the Representative Results section, please describe how to obtain these data in the protocol.

Response: As per JoVE instructions, we utilized well-established qRT-PCR and Western Blotting techniques without any modifications. This has been clarified in section 5.5 of the protocol.

9. Line 268: Is Table 1 equal to Table of Materials? If so, please remove this line from the manuscript. Otherwise please upload Table 1 individually to your Editorial Manager account as an .xls or .xlsx file.

Response: Yes, Table 1 has the same information as the Table of Materials and we agree was repetitive. This has been removed from the manuscript.

10. Figures 2 and 3: Please describe the figures in slightly more detail. For instance, explain what different colors represent.

Response: We have now added further description to Figure legends 2 & 3.

11. References: Please do not abbreviate journal titles.

Response: We have updated our reference list with full journal titles.

12. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Response: Thank you. We have now arranged the items in the Table of Equipment and Materials in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this manuscript Ares et al. discuss the development of an enteroid model of necrotizing enterocolitis. However, this is a model of inflammation by endotoxin rather than actual NEC. The pathogenesis and causes of NEC are poorly understood, but prematurity, initiation of feeding and establishment of the microbiota seem to be central to the development of this disease. Since none of these factors are included in the enteroid model, the title of this work does not reflect the actual model. Regardless, I believe that enteroids are a useful model and worth studying.

Response: Thank you for this important comment. We agree that the pathogenesis of NEC is complex and multifactorial. NEC develops after disruption in the intestinal barrier, leading to translocation of bacterial endotoxin (LPS). Our experimental NEC model via LPS administration was based on the established, fundamental importance of LPS in other NEC models (both cell and animal models. The established work of Hackam *et al*. showed the critical role of TLR4 (LPS receptor) in the development of NEC. LPS activation of TLR4 stimulates proinflammatory cytokines, a reduction in barrier integrity, and activation of subepithelial leukocytes that characterize the signaling events involved in human NEC. TLR4 activation by LPS has been implicated as a key molecule in promoting inflammation and animals that lack functional TLR4

are have demonstrated protection from the development of NEC. Circulating levels of LPS are elevated in patients with NEC and elevated in stool and plasma of animal models of NEC. LPS induces intestinal inflammation in animals that resembles human NEC, which highlights the significance of inflammation in this pathway. Mirroring the established cell culture and animal models of NEC, we believe induction of experimental NEC via LPS administration in enteroid culture is a useful ex-vivo human model for the study of NEC. In line with previous reports, our experimental NEC enteroids showed increased expression of TLR4 compared to controls. Since we are not able to simulate all the multifactorial predisposing factors of NEC within the cuture environment we chose to focus on LPS. We also believe that using neonatal tissue to generate enteroids from allows the response to be more specific to neonatal populations rather than adult sepsis. We have added a paragraph to our discussion to discuss this topic.

Major Concerns:

Line 308 The time of LPS administration defines two different models and the readers should be made aware of this. If you introduce it early on, enteroids will grow slowly and less 'mature cells' will be present. If introduced late, mature cells will predominate. Gene expression, for example, it is given not only by what cells are doing, but by what cells are present.

Response: We appreciate this very important comment. The timing of LPS administration, method of LPS administration (in the basement membrane matrix versus in the media) and timing and amounts of growth factors have a vast effect on the growth and maturation of the enteroid. Our data from early LPS exposure corresponded with our prior findings in human, cell-culture and rat studies while the late LPS exposure did not (1-3).

References:
1. Grothaus JS, Ares G, Yuan C, Wood DR, Hunter CJ. Rho kinase inhibition maintains intestinal and vascular barrier function by upregulation of occludin in experimental necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol. 2018;315(4):G514-G28.
2. Blackwood BP, Wood DR, Yuan C, Nicolas J, De Plaen IG, Farrow KN, et al. A Role for cAMP and Protein Kinase A in Experimental Necrotizing Enterocolitis. Am J Pathol. 2017;187(2):401-17.
3. Blackwood BP, Yuan CY, Wood DR, Nicolas JD, Grothaus JS, Hunter CJ. Probiotic Lactobacillus Species Strengthen Intestinal Barrier Function and Tight Junction Integrity in Experimental Necrotizing Enterocolitis. J Probiotics Health. 2017;5(1).

Minor Concerns:

Line 40 I don't think they are so readily available.

Response: Thank you for this comment. After consideration, we agree and have removed this from our manuscript.

Line 43 "we propose using human enteroids to study NEC." I would be more specific since many of the components of NEC may be missing. What about the epithelial response to NEC, or even better inflammation?

Response: Thank you for pointing this out. We agree that this sentence is misleading. With the length limitation of the abstract, we have removed this sentence from the abstract but have expanded on this topic in our discussion.

Line 196 Medium, media?

Response: Thank you for this comment. We have revised our manuscript to be consistent throughout with our reference to reagents prepared in step 1. Step 2.10 has been revised to read, “Add 500 µL of Human Minigut Media Complete (as prepared in step 1.5) to each well. Replace every 2 days.”

Line 260 what does 'with a luminal side lined with an epithelium' mean? The enteroid IS the epithelium.

Response: Thank you for pointing out that this was confusing. We have edited this part of the results section and the associated figure legend (Figure 2).

Line 303 Please indicate that the tissue collected was from a healthy section. It is possible that stem cells from pathological tissue underwent changes (epigenetic?) that will modify the phenotype of the lineage. I think this is very interesting, but no need to go into all this here.

Response: We agree that the tissue collected must be from a healthy section. We have elaborated on this point in our discussion.

Line 329 I am not sure that just a single marker can 'validate' a model.

Response: Thank you for your comment. We removed the isolated sentence regarding “validation” of our model and instead expanded our discussion to include the rationale for using LPS administration for our experimental NEC model in enteroids.

Reviewer #2:

Manuscript Summary:
Thank you for the opportunity to review the manuscript titled A novel human epithelial enteroid model of necrotizing enterocolitis. The authors describe an ex-vivo human entered model. The authors have established an enteroid model of human necrotizing enterocolitis using media inoculated with lipopolysaccharide (LPS) over 5-10 days. Collected enteroids demonstrate inflammatory changes akin to those seen in human necrotizing enterocolitis. The manuscript is well written and has suffering mechanistic denial. The manuscript will be a valuable addition to the field of NEC research and epithelial biology. I have no concerns.

Response: Thank you for your comments.