**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.