Manuscript: JoVE62093

Title: *"Functional Assessment of Intestinal Permeability and Neutrophil Transepithelial Migration in Mice Using a Standardized Intestinal Loop Model*"

We would like to thank the reviewers for their positive assessment of our manuscript. Below is a point-by-point response to the reviewers' thoughtful comments that have helped to improve this manuscript. Additional information and discussion points are included in the revised manuscript and are highlighted in **red color**.

Response to the reviewers' comments

Reviewer #1:

I only have some minor comments to improve the clarity of the content:

- ***Line 95: They state that the Evans blue assay is not suitable for analyzing passage of the into the blood stream. I think it will be helpful for the unexperienced reader to provide the explanation.***

***Response*:** We acknowledge that Evans blue is commonly used to evaluate vascular leakage in vivo[1] and that it has also been employed to evaluate intestinal mucosal permeability in mouse and rat[2-4]. The quantification of Evans blue in the intestinal mucosa requires extraction from tissue employing incubation in formamide overnight. Thus, the same tissue cannot be used to study permeability and neutrophil infiltration. Here, we highlight a simple protocol that reduces the number of animals needed to collect reproducible data on colonic mucosal permeability and leukocyte transepithelial migration in vivo. We thus recommend the use of FITC-dextrans that are easily detectable in blood serum without compromising the integrity of intestinal loops which can be harvested for further analysis. We have clarified this point in the revised manuscript on line 89.

- ***Lines 116-118 and 532-533: Use of different stimuli is suggested, but not discussed later. Do the authors have experience using different stimuli and could discuss difference/similarities, advantages/disadvantages?***

***Response*:** Here, we used Leukotriene B_4 (LTB_4) to model PMN TEpM given that it is a well-accepted potent and physiologic PMN chemoattractant. However, our loop model is adaptable to other relevant chemoattractants. We have reported the use of the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF) to induce significant recruitment of PMN into the colonic lumen[5]. Furthermore, this model is adaptable for use of CXCL1/KC, another potent physiologic chemoattractant that we have successfully used with this model. We have focused on LTB_4 (336.5 Da) because of its ability to induce TEpM at very low concentrations (1nM) in the physiologic range. fMLF (437.5 Da) is a lower affinity chemoattractant in mice which requires much higher concentrations to be effective (1μM). CXCL1/KC is expensive and a relatively large molecule (11 kDa) that is less efficient in crossing the epithelial barrier. Of note, ligated intestinal loops have also been used by others to study bacterial infection (such as Salmonella, Listeria monocytogenes and Escherichia coli), therefore we believe that the ease in adaptability of this iLoop model can be used for these studies as well. We discuss this point on lines 377 and 638.

- ***Lines 148-152: Any recommendable anesthesia alternatives? For example, would the loop be affected by ip injection of ketamine/xylazine?***

***Response*:** We have added on line 170/ step 1.2.2 the use of ketamine/xylazine as anesthesia alternative. There is no contraindication for using intraperitoneal injection (IP) of ketamine (80 - 100 mg/kg) and xylazine (5 - 10 mg/kg) to conduct the surgery. Anesthesia should be maintained throughout the surgery by intramuscular administration of ketamine/xylazine at 0.1 - 0.25 times of initial doses to ensure anesthetic depth. We highly recommend an isoflurane anesthesia vaporizer for this method, which is optimal to assure better reproducibility, survivability and prevent animal pain.

- ***Lines 194-204: Please explain the necessity to cut off both ends for flushing and isolating the loop (same for the colon section). Wouldn't injection of the flushing needle into one end of the loop followed by ligation here be enough? This section does not appear to be very clear for unexperienced experimenters. It may become clear with the accompanying video; but consider providing more details here.***

***Response*:** We cut off both ends of the exteriorized segment of ileum or proximal colon, then flush gently as a necessary step that prevents interference with luminal contents (fecal matter) thus facilitating even dispersion of FITC-dextrans or chemotactic stimuli across the entire length of the isolated segment as well as allowing for more accurate quantification of leukocytes by flow cytometry. This procedure also allows uniform distension of the mucosa after injection of specified volumes of reagent and better reproducibility between animals. We have clarified this information on lines 241 and 288.

- ***Line 267: 10,000 x g (also lines 332, 344…)***

***Response*:** Corrected

- **Lines 325-327: please add why EDTA is not required in the pcLoop wash buffer.**

***Response*:** Thank you for this comment. The wash buffer for the ileal and pcLoop is the same and contains 2mM EDTA and 2% FBS in sterile PBS without calcium and magnesium. Given that adding DTT does not interfere with the collection of the ileal loop luminal content, we have now simplified the protocol and recommend the same wash buffer complemented with 5mM DTT for the iLoops (ileum and colon) in step 5.6.1.

- **Figures 1 and 2 should be mentioned in the protocol where appropriate.**

***Response*:** Figures 1 and 2 are now mentioned in the protocol section

- **Line 411: Number of PMN**

***Response*:** Corrected (line 510)

- **Line 425: epithelium instead of epithelial**.

***Response*:** Corrected (line 524)

- **Line 467: what does "similar to the pcLoop at baseline" mean? Is baseline supposed to mean with cytokines only? But then it is not similar because it's significantly different (*). Or is baseline not shown here? Please clarify.**

***Response*:** On line 566, baseline condition means “no surgery and no treatment”. We have now clarified that the black squares represent the number of PMN in an intact colonic segment similar to the pcLoop that was not subjected to any surgery or treatment with proinflammatory cytokines and LTB_4.

- **Lines 500-507: is there a way to express the data differently to compensate for variations such as PMN/mm3 tissue/injected volume; or a similar formula?**

***Response*:** We do not expect major variations between experimental groups that need to be compensated if the length of the iLoop as well as the volume of reagent solution injected into the lumen are strictly maintained between animals. However, we agree that the data may be presented as number of PMN per mm^3 by using the formula for volume of a cylinder: V= π(pi) r 2 h (V for volume, r for radius and h for height) as well as number of PMN per gram of tissue. We have added this information on line 479.

- **Lines 536-538: the link between permeability and TEpM is interesting and should be discussed in some more detail. What kind of link do you expect, if any? Are the mechanisms much different to those in**

endothelial cells, where both phenomena are separated and explained by different mechanism (i.e. increased permeability does not necessarily mean increased transmigration!)?

***Response*:** We thank the reviewer for pointing out this important point of discussion. We have recently reported by employing the pcLoop model that enhanced intestinal paracellular permeability to 4 kDa FITC-dextran following treatment with a high dose of proinflammatory cytokines TNFα and IFNγ (1 mg of each) resulted in enhanced PMN recruitment into the pcLoop lumen in response to LTB_4, in comparison to low dose cytokines (100 ng of each)[5]. Interestingly, here we show that increased epithelial permeability secondary to *Jam-a* deficiency did not lead to enhanced PMN TEpM but diminished it. Therefore, it is likely that the intestinal paracellular permeability affects the rate of PMN TEpM but the correlation is not direct. We believe that the pcLoop enables future studies aiming to determine how increased intestinal permeability controls immune cell response and contributes to pathologic mucosal inflammation such as inflammatory bowel disease. We have discussed this point in the revised manuscript on line 661.

Reviewer #2:

- **Major Concerns: on the presented data not. However, as has been published by others (Sorribas M et al. J.Hepatology 2019) this method could be widened in its application by i) using different sizes of dextrans being FITC-labeled (e.g. 40 or 150 kDA) to characterize the severity of permeability changes in any pathological disease state or as here the JAM-/- and ii) as secondary read out use the liver thus, evaluating the gut-liver-axis, which is easy to achieve even within the same Experiment by harvesting liver tissue and analyse fluorescence per gramm tissue.**

***Response*:** We thank the reviewer for the constructive comments that broaden the application of our iLoop model. We have addressed these points of discussion on lines 621 - 632.

- **Finally, by utilizing differently labeled probes within the same mouse both loop could be generated and so the main site of translocated be tested. This at least could be stated in the discussion to stimulate other researches to do so since, it is not yet clear where translocation is most predominant along the GI-tract.**

***Response*:** We agree and thank the reviewer for the suggestion. We have now included this comment in the discussion on lines 621- 632.

Reviewer #3:

I believe that the manuscript is ready for the prime time and I do not have any meaningful comments or corrections.

***Response*:** We thank the reviewer for the positive evaluation of our manuscript.

Respectfully,

Charles A. Parkos, MD, PhD

References

1 Wick, M. J., Harral, J. W., Loomis, Z. L. & Dempsey, E. C. An Optimized Evans Blue Protocol to Assess Vascular Leak in the Mouse. Journal of Visualized Experiments. 10.3791/57037 (139), (2018).

2 Tateishi, H., Mitsuyama, K., Toyonaga, A., Tomoyose, M. & Tanikawa, K. Role of cytokines in experimental colitis: relation to intestinal permeability. Digestion. 58 (3), 271-281, (1997).

3 Mei, Q., Diao, L., Xu, J. M., Liu, X. C. & Jin, J. A protective effect of melatonin on intestinal permeability is induced by diclofenac via regulation of mitochondrial function in mice. Acta Pharmacologica Sinica. 32 (4), 495-502, (2011).

4 Vargas Robles, H. et al. Analyzing Beneficial Effects of Nutritional Supplements on Intestinal Epithelial Barrier Functions During Experimental Colitis. Journal of Visualized Experiments. 10.3791/55095 (119), (2017).

5 Flemming, S., Luissint, A. C., Nusrat, A. & Parkos, C. A. Analysis of leukocyte transepithelial migration using an in vivo murine colonic loop model. JCI Insight. 3 (20), (2018).