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

2 Functional Assessment of Intestinal Permeability and Neutrophil Transepithelial Migration in

3 Mice Using a Standardized Intestinal Loop Model

4

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17 **KEYWORDS:**

18 intestinal barrier function, mucosal immunology, intestinal epithelium, permeability assay,

19 leukocyte transepithelial migration assay, ileal loop, proximal colon loop, fluorescent

20 isothiocyanate (FITC)–dextran, chemokine, proinflammatory cytokine

21

22 **SUMMARY:**

23 Dysregulated intestinal epithelial barrier function and immune responses are hallmarks of

24 inflammatory bowel disease that remain poorly investigated due to a lack of physiological

25 models. Here, we describe a mouse intestinal loop model that employs a well-vascularized and

26 exteriorized bowel segment to study mucosal permeability and leukocyte recruitment in vivo.

27

28 **ABSTRACT:**

29 The intestinal mucosa is lined by a single layer of epithelial cells that forms a dynamic barrier

30 allowing paracellular transport of nutrients and water while preventing passage of luminal

31 bacteria and exogenous substances. A breach of this layer results in increased permeability to

32 luminal contents and recruitment of immune cells, both of which are hallmarks of pathologic

33 states in the gut including inflammatory bowel disease (IBD).

34

35 Mechanisms regulating epithelial barrier function and transepithelial migration (TEpM) of

36 polymorphonuclear neutrophils (PMN) are incompletely understood due to the lack of

37 experimental in vivo methods allowing quantitative analyses. Here, we describe a robust murine

38 experimental model that employs an exteriorized intestinal segment of either ileum or proximal

39 colon. The exteriorized intestinal loop (iLoop) is fully vascularized and offers physiological

40 advantages over ex vivo chamber–based approaches commonly used to study permeability and

41 PMN migration across epithelial cell monolayers.

42

43 We demonstrate two applications of this model in detail: (1) quantitative measurement of

44 intestinal permeability through detection of fluorescence-labeled dextrans in serum after

45　intraluminal injection, (2) quantitative assessment of migrated PMN across the intestinal
46　epithelium into the gut lumen after intraluminal introduction of chemoattractants. We
47　demonstrate feasibility of this model and provide results utilizing the iLoop in mice lacking the
48　epithelial tight junction-associated protein JAM-A compared to controls. JAM-A has been shown
49　to regulate epithelial barrier function as well as PMN TEpM during inflammatory responses. Our
50　results using the iLoop confirm previous studies and highlight the importance of JAM-A in
51　regulation of intestinal permeability and PMN TEpM in vivo during homeostasis and disease.
52
53　The iLoop model provides a highly standardized method for reproducible in vivo studies of
54　intestinal homeostasis and inflammation and will significantly enhance understanding of
55　intestinal barrier function and mucosal inflammation in diseases such as IBD.
56
57　**INTRODUCTION:**
58　The intestinal mucosa encompasses a single layer of columnar intestinal epithelial cells (IECs),
59　underlying lamina propria immune cells and the muscularis mucosae. Besides its role in the
60　absorption of nutrients, the intestinal epithelium is a physical barrier that protects the body
61　interior from luminal commensal bacteria, pathogens, and dietary antigens. In addition, IECs and
62　lamina propria immune cells coordinate the immune response inducing either tolerance or
63　response depending on the context and stimuli. It has been reported that the disruption of the
64　epithelial barrier can precede the onset of pathologic mucosal inflammation and contribute to
65　inflammatory bowel disease (IBD) encompassing both ulcerative colitis and Crohn's disease[1-7].
66　Individuals with ulcerative colitis present excessive transepithelial migration (TEpM) of
67　polymorphonuclear neutrophils (PMN) forming crypt abscesses, a finding that has been
68　associated with severity of disease[8,9]. Although compromised epithelial barrier function and
69　excessive immune responses are hallmarks of IBD, there is a lack of experimental in vivo assays
70　to perform quantitative assessments of intestinal permeability and immune cell recruitment into
71　the intestinal mucosa.
72
73　The most common methods used to study intestinal epithelial permeability and PMN TEpM
74　employ ex vivo chamber–based approaches using IEC monolayers cultured on semi-permeable
75　porous membrane inserts[10-12]. The epithelial barrier integrity is monitored either by
76　measurements of transepithelial electrical resistance (TEER) or the paracellular flux of
77　the Fluorescein isothiocyanate (FITC)-labeled dextran from apical to basal compartment[13-15].
78　Similarly, PMN TEpM is typically studied in response to a chemoattractant that is added in the
79　lower chamber[16]. PMN are placed in the upper chamber and after an incubation period, PMN
80　that have migrated into the basal compartment are collected and quantified. While these
81　methods are useful, easy to perform and very reproducible, they are obviously reductionist
82　approaches and do not necessarily represent an accurate reflection of in vivo conditions.
83
84　In mice, a common assay to study intestinal paracellular permeability is by oral gavage of FITC-
85　dextran and subsequent measurement of FITC-dextran appearance in the blood serum[13,17]. The
86　disadvantage of this assay is that it represents an assessment of overall barrier integrity of the
87　gastrointestinal tract rather than that of regional intestinal contributions. In addition, Evans blue
88　is commonly used to evaluate vascular leakage in vivo[18] and has also been employed to evaluate

89 intestinal mucosal permeability in mouse and rat[19-21]. The quantification of Evans blue in the
90 intestinal mucosa requires extraction from tissue employing incubation in formamide overnight.
91 Therefore, the same tissue cannot be used to study intestinal epithelial permeability and
92 neutrophil infiltration.
93
94 Here we highlight a simple protocol that reduces the number of animals needed to collect
95 reproducible data on colonic mucosal permeability and leukocyte transepithelial migration in
96 vivo. We, therefore, recommend the use of FITC-dextrans that are easily detectable in blood
97 serum without compromising the integrity of intestinal loops which can be harvested for further
98 analysis. Of note, the intestinal ligated loops have been used in various species (including mouse,
99 rat, rabbit, calf) to study bacterial infection (such as *Salmonella, Listeria monocytogenes and*
100 *Escherichia coli*)[22-25] as well as intestinal permeability[26]; however, to the best of our knowledge
101 there are no studies investigating mechanisms of PMN TEpM in specific regions in the intestine
102 such as ileum or colon that are commonly involved in IBD.
103
104 Here we describe the mouse intestinal loop (iLoop) model that is a robust and reliable
105 microsurgical in vivo method that employs a well-vascularized and exteriorized intestinal
106 segment of either the ileum or proximal colon. The iLoop model is physiologically relevant and
107 allows the assessment of intestinal barrier integrity and PMN TEpM on living mice under
108 anesthesia. We demonstrate two applications: 1) quantification of serum levels of 4 kDa FITC-
109 dextran after intraluminal administration in the iLoop 2) quantification of transmigrated PMN in
110 the iLoop lumen after intraluminal injection with the potent chemottractant Leukotriene B$_4$
111 (LTB$_4$)[27]. Moreover, utilizing the iLoop model with *Jam-a* –null mice or mice harboring selective
112 loss of JAM-A on IECs (*Villin-cre;Jam-a* $^{fl/fl}$) compared to control mice, we are able to corroborate
113 previous studies that have reported a major contribution for tight junction-associated protein
114 JAM-A to intestinal permeability and neutrophil transmigration[15,28-31].
115
116 The iLoop model is a highly functional and physiological method that can be used to corroborate
117 in vitro assays. Furthermore, this is a versatile experimental model that allows the study of
118 various reagents that can be injected into the loop lumen, including chemokines, cytokines,
119 bacterial pathogens, toxins, antibodies and therapeutics.
120
121 **PROTOCOL:**
122 All animal experiments were conducted in accordance with the guidelines and policies of the
123 National Institutes of Health and approved by the Institutional Animal Care & Use Committee at
124 the University of Michigan.
125
126 **1.   Preoperative preparation**
127
128 NOTE: This method was generated employing adult mice from C57BL/6 genetic background, aged
129 8 – 12 weeks. All mice were kept under strict specific pathogen free conditions with ad libitum
130 access to normal chow and water. Results were obtained using C57BL/6, *Jam-a* - null mice (*Jam-*
131 *a* $^{-/-}$) or mice harboring selective loss of JAM-A on IECs (Villin-cre;*Jam-a* $^{fl/fl}$) and littermate *Jam-a*
132 $^{fl/fl}$ controls as previously described[30].

1.1. Area preparation

1.1.1. Perform surgery in clean area. The intestinal Loop model, however, is a non-survival surgery that does not require aseptic/sterile technique. Observe veterinary sanitation practices and use cleaned surgical instruments (i.e., scrubbed with soap, rinsed with water followed by 70% ethanol).

1.1.2. Turn on a temperature-controlled surgical board (or heating pads) and adapted light source to keep animal from hypothermia during the anesthesia and surgery.

1.1.3. Prepare ligatures by cutting 6 cm segments of non-absorbable 4-0 silk surgical sutures.

1.1.4. Prepare cotton gauzes (5 cm x 5 cm) that are cut in the center following an ellipsoid shape. These will be used to cover the midline laparotomy and prevent direct contact between the exteriorized iLoop and animal fur. Soak the cut gauzes in warm Hanks' Balanced Salt Solution (HBSS) in a petri dish container.

1.1.5. Prepare wet cotton swabs soaked in warm HBSS that will be used to handle organs and exteriorized iLoop.

1.1.6. Prepare 10 mL syringe filled with warm HBSS and attach to a yellow feeding tube. This syringe will be used for moisturizing exposed tissues during surgery and to gently flush the iLoop of fecal content.

1.2. Animal preparation

1.2.1. Anesthetize the animal in accordance with the approved animal protocol. In this protocol a mix of isoflurane and oxygen is administered through an anesthesia vaporizer. According to manufacturer's instructions, adjust oxygen flow rate to 1 L/min. Set the vaporizer to 5% and pre-charge the induction chamber. After 5 min, reduce isoflurane vaporizer to 2% – 2.5%.

1.2.2. Place the animal in the induction chamber for 3 min – 5 min, then transfer animal to a heated surgery board and connect an anesthesia nosecone plug. Restrain the animal in supine position by the four limbs using adhesive tape.

NOTE: As an anesthesia alternative, a mixture of ketamine (80 mg/kg – 100 mg/kg) and xylazine (5 mg/kg – 10 mg/kg) diluted in saline solution (0.9% NaCl) can be administrated by intraperitoneal injection. 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. If available, an isoflurane anesthesia vaporizer is highly recommended to assure better reproducibility, survivability and prevent animal pain.

177   1.2.3.   Apply ophthalmic ointment to both eyes to prevent corneal desiccation.

178

179   1.2.4.   Perform a physical exam that includes heart rate (around 500 beats/min) and rhythm,
180 mucous membrane color (pink), capillary refill time (< 2 s), respiratory rate (not lower than 40 –
181 60 breaths/min), and temperature (36.5 °C)[32].

182

183   1.2.5.   Before proceeding to the next steps, assess anesthetic depth by pedal withdrawal reflex.
184 Employ a painful stimulus (pinch) of the skin between the toes and/or toe pads. Mouse will
185 respond by contracting and removing its leg. This pedal reflex disappears when animal is
186 anesthetized deeply.

187

188 NOTE: Monitoring of vitals and pedal reflex are recommended throughout anesthesia at
189 minimum every 15 min. Institutional Animal Care and Use Committee (IACUC) guidelines for
190 evaluation of anesthetic depth recommend monitoring of the following: (a) color of tail, foot and
191 mucous membrane (such as tongue). Color pink as normal and pale or blue as indicative of
192 decreased blood perfusion or respiratory distress; (b) evaluation of the breathing pattern as
193 regular versus irregular breaths. A rectal temperature probe, rodent oximeter and heart rate
194 monitors can be used for the assessment of body temperature, heart and respiratory rates,
195 respectively.

196

197 **2.   Generation of the ileal loop**

198

199 2.1.   Skin preparation: Scrub fur of the abdominal midline with alcohol swabs or gauze sponge
200 soaked with 70% Ethanol. Do not wet a wide area of fur with alcohol to prevent hypothermia.

201

202 2.2.   Using scissors, perform a midline laparotomy. Make a horizontal incision in the middle of
203 the abdomen (about 2 cm in length) and expose the peritoneum. Be careful to not injure intra-
204 abdominal organs.

205

206 2.3.   Place pre-cut wet cotton gauze over the exposed intra-abdominal cavity.

207

208 2.4.   Use wet cotton swabs to mobilize and exteriorize the caecum. Carefully place the caecum
209 on the wet cotton gauze.

210

211 NOTE: Caecum is localized in the left caudal quadrant of the abdominal cavity in a majority of
212 mice independent of the sex of the animal.

213

214 2.5.   Use wet cotton swabs to mobilize and gently exteriorize the ileum of which the terminal
215 section (distal end) is attached to the caecum (**Figure 1B**).

216

217 2.6.   Deploy at least 6 cm of terminal ileum on the wet cotton gauze without disruption of the
218 mesenteric vessels and blood supply. Blood supply is maintained if there is no bleeding and the
219 tissue maintains its pink color (**Figure 1B**).

220

NOTE: Avoid drying of exposed tissues by maintaining tissues moist at all times with warm HBSS (every 2 – 3 min) using 10 mL syringe attached to a yellow feeding tube (step 1.1.6).

2.7.   Close to the caecum, identify the major artery supplying the ileum in the mesentery. Then locate two ligation sites in the mesentery that are free of critical blood vessels.

2.8.   Using blunt tissue forceps, firmly grab the terminal ileum (closest to the caecum) and using fine tip forceps, fenestrate the mesentery avoiding blood vessels. Place silk suture across the perforation and tie a surgical knot to create the first ligation (distal end of the loop).

2.9.   Use the ruler to measure 4 cm away from the first ligature and create the second ligature (proximal end of the loop) as mentioned in step 2.8 (**Figure 1C**).

2.10.   With fine scissors carefully cut next to each ligation to isolate the 4 cm ileal loop, keeping intact blood supply and mesenteric membrane.

NOTE: Cut off both ends of the exteriorized segment of the iLoop, 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.

2.11.   Gently flush the content of the ileal loop segment with warm HBSS using a flexible yellow feeding tube attached to a 10 mL syringe (see step 1.1.6).

2.12.   Ligate the two cut ends of the flushed ileal loop using silk suture.

2.13.   Use a 1 mL syringe with 30 G needle to slowly inject 250 μL of reagent such as FITC-dextrans (step 4.2) or chemokine (step 5.3) into the intestinal lumen. The ileal loop will inflate causing a moderate distension of the mucosa (**Figure 1D**).

NOTE: Inject reagent into the loop lumen on the opposite side of the mesenteric artery. Be careful not to pull out the ileal loop from the animal while injecting to avoid tearing blood vessels and induce bleeding.

2.14.   Using wet cotton swabs, gently put back the ileal loop, proximal ileum and caecum.

2.15.   Use a needle holder, anatomical forceps and 3.0 non-absorbable silk sutures with reverse cutting needle to close the abdominal wall.

2.16.   Place the animal in a temperature-regulated anesthesia chamber for the incubation period.

### 3.	Generation of the proximal colon loop (pcLoop)

NOTE: For details about mice that were used for the generation of the pcLoop, see the information provided at the beginning of the protocol section.

3.1.	Perform steps 2.1. – 2.4. as described above for the ileal loop.

3.2.	Using wet cotton swabs, exteriorize the entire ileum and place it on the top of a wet cotton gauze. Identify the proximal colon and the blood supply located in the mesocolon. Mobilize the proximal colon and by using fine tip forceps create the first ligature in an area free of vessels in the mesocolon at about 0.5 cm distal from the caecum (**Figure 2B**).

3.3. Measure 2 cm from the first ligature and create a second ligature at an area free of blood supply in the mesocolon (**Figure 2C**).

3.4. Using fine scissors carefully cut next to each ligation to isolate a 2 cm long pcLoop.

NOTE: As mentioned in the note under step 2.10, it is important to cut off both ends to isolate a pcLoop that is gently cleaned of luminal contents. Carefully cut through the colonic tissue and mesocolon to prevent small vessels from bleeding into the intestinal lumen. If necessary, use thermal cautery to limit bleeding at the incision site.

3.5. Gently flush the pcLoop with warm HBSS to remove feces using a flexible yellow feeding tube attached to 10 mL syringe (see step 1.1.6).

3.6. Ligate the two cut ends of the flushed pcLoop using silk suture.

3.7. Use a 1 mL syringe with 30G needle to slowly inject 200 μL of reagent such as FITC-dextrans (step 4.2) or chemokine (step 5.3) into the intestinal lumen. The pcLoop will inflate causing a moderate distension of the mucosa (**Figure 2D**).

NOTE: Inject the reagent into the pcLoop lumen on the opposite side of the mesenteric artery. Ensure consistency between animals and create a 2 cm long pcLoop to ensure equal distension of the mucosa.

3.8.	Utilize wet cotton swabs to gently place back the ligated pcLoop, ileum and caecum into the abdominal cavity.

3.9.	Use a needle holder, anatomical forceps and 3.0 non-absorbable silk sutures with reverse cutting needle to close the abdominal wall.

3.10.	Place the animal in a temperature-regulated anesthesia chamber for the incubation period.

4.   Quantitative assessment of intestinal permeability: 4kDa FITC-dextran assay

4.1.   Perform an ileal loop or pcLoop (as described above).

4.2.   Using a 1 mL syringe with 30 G needle, inject into the intestinal lumen either 250 µL (ileum – step 2.13) or 200 µL (colon – step 3.7) of 4 kDa FITC-dextran solution (1 mg / mL in HBSS). Keep the unused FITC-dextran solution protected from light to prepare the standard curve after serum collection.

4.3.   For the ileal loop follow steps 2.14 to 2.16, for the pcLoop steps 3.8 to 3.10. Briefly, put organs and iLoop back in place into the abdominal cavity, close the abdominal wall.

4.4.   Place the animal for 120 min in a heated anesthesia chamber.

4.5.   After the incubation period open the abdominal wall, gain access to the heart and perform cardiac puncture using a 1 mL syringe with 25G needle to collect the blood. Transfer blood into a 1.3 mL serum clot activator tube, mix gently, and keep on ice protected from light. Collect at least 500 µL of blood per mouse.

NOTE: Animals are euthanized when being under anesthesia by using a physical method such as decapitation or cervical dislocation, and in accordance with the approved animal protocol.

4.6.   Centrifuge serum clot activator tube for 5 min at 10,000 x *g* at room temperature according to manufacturer's recommendations. Collect the serum (supernatant) and transfer into a 1.7 mL centrifuge tube. Keep the tube on ice and protected from light.

4.7.   Quantification of fluorescence in blood serum

4.7.1.   Prepare a standard curve of FITC-dextran 4 kDa in serum of control mice (saline or HBSS is a valid alternative). Create a two-fold serial dilution with a starting concentration of 1 mg/mL FITC-dextran. FITC-dextran 4kDa concentrations that are measured range between 0.25 mg/mL and 2 µg/mL.

4.7.2.   Transfer equal volume of the sample and standards to a black 96-well plate (flat bottom) and measure FITC in a fluorescence plate reader (Excitation 490 nm, Emission 520 nm), according to manufacturer's instructions and published protocols[33]. Calculate the FITC concentration based on the standard curve or present permeability values as fold-change normalized to the experimental control group.

5.   Quantitative assessment of migrated PMN into the intestinal lumen after intraluminal stimulation with chemokines

353 NOTE: Very few PMN reside in the intestinal mucosa at the baseline level. Pretreatment of
354 animals with pro-inflammatory cytokines results in an inflammatory environment that
355 facilitates PMN recruitment from bloodstream into the intestinal mucosa.
356
357 5.1.   Using a 1 mL syringe with 30 G needle, perform intraperitoneal (i.p.) injection of a sterile
358 solution of 100 ng of Tumor Necrosis Factor-α (TNFα) and 100 ng of Interferon-γ (INFγ) in 200
359 μL of Phosphate Buffered Saline (PBS).
360
361 5.2.   After 4 – 24 h of pretreatment with pro-inflammatory cytokines, perform an ileal loop or
362 pcLoop (as described above).
363
364 5.3.   Using a 1 mL syringe with 30 G needle, inject into the intestinal lumen either 250 μL (ileum
365 – step 2.13) or 200 μL (colon – step 3.7) of chemoattractant solution Leukotriene B4 (LTB$_4$) 1 nM
366 in HBSS.
367
368 NOTE: Leukotriene B4 (LTB$_4$) is used in this protocol as a potent chemoattractant for PMN. Other
369 chemoattractants such as N-Formylmethionyl-leucyl-phenylalanine (fMLF) or chemokine (C-X-C
370 motif) ligand 1 (CXCL1/KC) can also be used to induce significant recruitment of PMN into the
371 colonic lumen[30].
372
373 5.4.   For the ileal loop follow steps 2.14 to 2.16. For the pcLoop follow steps 3.8 to 3.10. Briefly,
374 put organs and iLoop back in place into the abdominal cavity, close the abdominal wall.
375
376 5.5. Place the animal for 60 min in a heated anesthesia chamber.
377
378 5.6. Collection of the intestinal loop content
379
380 5.6.1.   Prepare solutions and store on ice: For the ileal loop and pcLoop, prepare a wash buffer
381 containing 2 mM Ethylenediaminetetraacetic acid (EDTA), 5 mM Dithiothreitol (DTT) and 2%
382 FBS in sterile PBS without calcium and magnesium.
383
384 5.6.2.   After the incubation period and under anesthesia maintenance, open the abdominal wall
385 and pull out the iLoop (ileal loop or pcLoop). Euthanize animals when being under anesthesia by
386 using a physical method such as decapitation or cervical dislocation, and in accordance with the
387 approved animal protocol.
388
389 5.6.3.   Rinse loop with cold PBS to remove any residual blood contaminant and absorb excess
390 of PBS with tissue wipes. Carefully collect the loop content into a 1.7 mL centrifuge tube (about
391 250 μL for ileal loop and 200 μL for pcLoop). Flush the loop with 500 μL cold wash buffer and,
392 immediately after collection, place the tube on ice.
393
394 NOTE: DTT helps to dissolve mucus. If iLoop luminal content is very viscous (depending on the
395 mouse genetic background), dilute it 1:2 or 1:3 with wash buffer containing DTT.
396

397   5.6.4.   Pass the luminal content solution through a 35 µm nylon mesh filter using a 5 mL round-
398   bottom tube with cell strainer cap. This step helps to remove tissue fragments and cell
399   aggregates. Rinse the cell strainer with 1 mL of wash buffer.
400
401   5.6.5.   Centrifuge tube at 400 x g for 5 min at 4 °C. Discard supernatant, rinse pellet with 500 µL
402   – 1 mL wash buffer, then centrifuge at 400 x g for 5 min, 4 °C.
403
404   5.6.6.   Resuspend iLoop luminal cell pellet in 200 µL of flow cytometry buffer (FCB) containing
405   2% FBS in sterile PBS without calcium and magnesium. Cells can be kept in a tube or transferred
406   to a 96-well round bottom plate for flow cytometry staining and analysis.
407
408   5.7.   Flow Cytometry staining and analysis
409
410   5.7.1.   Compensation controls: white blood cells
411
412   5.7.1.1. Prepare a 1 mL syringe with 25 G needle pre-filled with sterile 0.5 M EDTA (pH 8.0).
413   10% EDTA per expected blood volume (100 µL of EDTA for 1 mL blood).
414
415   5.7.1.2. Collect blood under anesthesia by cardiac puncture. Transfer blood into a 1.7 mL tube,
416   then centrifuge at 400 x g for 10 min, 4 °C.
417
418   NOTE: While the mouse is under anesthesia use a physical method to confirm death in
419   accordance with the approved animal protocol (such as cervical dislocation).
420
421   5.7.1.3. Aspirate the supernatant. Resuspend the pellet in 1 mL of Ammonium-Chloride-
422   Potassium (ACK) lysis buffer for the lysis of red blood cells. Incubate for 3 min – 5 min on ice.
423   Centrifuge 400 x g for 5 min, 4 °C. If the pellet is still red, repeat this ACK lysis buffer step until
424   pellet turns white.
425
426   5.7.1.4. Resuspend the pellet in 1 mL FCB and plate 0.5×10^6 – 1×10^6 of cells per well. Prepare
427   five wells of a 96-well round-bottom plate. Place the plate on ice.
428
429   NOTE: Use the same 96-well plate that contains the loop luminal contents (see step 5.6.6).
430
431   5.7.2.   Flow cytometry staining
432
433   5.7.2.1. Centrifuge the 96-well plate for 5 min at 400 x g, 4 °C. Discard the supernatant and
434   resuspend the pellets with 50 µL of Purified Rat Anti-Mouse CD16/CD32 as an Fc-Block (1 µg per
435   100 µL of FCB). Incubate for 5 min – 10 min on ice.
436
437   5.7.2.2. Immunostaining of the iLoop luminal content: Prepare a mixture containing all
438   fluorochrome-conjugated antibodies (1:50 dilution in FCB): anti-CD45-PerCP, anti-CD11b-PE and
439   anti-Ly-6G-Alexa Fluor 647. Add 50 µL of the combination per well, for a final volume of 100 µL.
440

441 5.7.2.3. Immunostaining of the white blood cells for compensations (1:50 dilution in FCB): Use
442 50 µL of FCB alone (unstained sample, well 1), 50 µL of each individual fluorochrome-conjugated
443 antibody (wells 2 – 4), 50 µL of the combination of all fluorochrome-conjugated antibodies (well
444 5). Final volume of 100 µL.

446 5.7.2.4. Incubate the plate for 30 min on ice protected from light.

448 5.7.2.5. Centrifuge the plate for 5 min at 400 x g, 4 °C. Discard the supernatant and wash with
449 200 µL of FCB. Repeat this washing step twice.

451 5.7.2.6. Add FCB 150 µL/well to the blood samples.

453 5.7.2.7. Add 100 µL/well FCB to the iLoop luminal content sample. Then 50 µL/well of fluorescent
454 counting beads.

456 5.7.3. Flow cytometry analysis

458 5.7.3.1. Gate for CD45 positive events and for the expression of Ly-6G-/Gr-1 and CD11b[30].

460 5.7.3.2. Use 100 µL of the sample volume as a stop condition.

462 5.7.3.3. Calculate the absolute number of PMN that has migrated into the iLoop lumen following
463 the information provided by the manufacturer of the fluorescent counting beads.

465 NOTE: Data may be presented as (1) total number of PMN in the lumen[30,34,35], (2) number of PMN
466 per gram of tissue as well as (3) number of PMN per mm^3 by using the formula for volume of a
467 cylinder: V= π(pi) r 2 h (V for volume, r for radius and h for height).

469 **REPRESENTATIVE RESULTS:**
470 A schematic representation of the ileal loop and pcLoop models is depicted in **Figure 1** and **Figure**
471 **2**, respectively. The anatomical pictures display the critical steps of the procedure including
472 exteriorization of the intestinal segment (**Figure 1B** and **Figure 2B**), identification of an
473 appropriate location for ligations that allows minimal disturbance of blood supply (**Figure 1C** and
474 **Figure 2C**) and cleaning followed by ligation of cut ends of the iLoop that can be filled with
475 reagent solution (**Figure 1D** and **Figure 2D**). Importantly, the iLoop model preserves vital blood
476 supply and allows physiological absorption of applied reagents such as FITC-dextrans or the
477 potent PMN chemoattractant LTB_4. At the end of the assay, the iLoop should be inflated (as seen
478 in **Figure 1D** and **Figure 2D**) and display normal mucosal perfusion with bright-red mesenteric
479 vessels. Depending on the assay, blood is collected to measure FITC-dextran in serum or iLoop
480 luminal contents are processed for quantification of PMN TEpM prior to euthanizing the animal.

482 In order to verify the accuracy of the iLoop model for the assessment of intestinal permeability,
483 a FITC-dextran pcLoop assay was performed to evaluate the role of TJ-associated protein JAM-A
484 in the regulation of intestinal barrier function in vivo. Of note, it has been reported that JAM-A

485 deficiency lead to increased epithelial intestinal permeability in vitro[28] and after oral gavage in
486 vivo[29]. Herein, using the pcLoop model, a 2.5-fold increase in 4 kDa FITC-dextran serum levels
487 was quantified in *Jam*-a-null mice (*Jam-a* [-/-]) compared to controls (*Jam-a*^{+/+}) (**Figure 3A**)[30].
488 Furthermore, similar results were obtained with mice harboring selective loss of JAM-A on IECs
489 (*Villin-cre;Jam-a* [fl/fl]) compared to littermate controls (*Jam-a* [fl/fl]) (**Figure 3B**)[30]. Therefore, the
490 pcLoop model was able to corroborate previous studies that have reported a positive
491 contribution for JAM-A to the intestinal barrier function.
492
493 Then pcLoop model was employed to study PMN recruitment into the intestinal mucosa and
494 subsequent TEpM in vivo. As shown in **Figure 4A**, the number of PMN in the luminal content of
495 the pcLoop was quantified by flow cytometry analysis. PMN were defined as cells positive for
496 each of the cell-surface makers CD45, CD11b and Ly6G[36]. Circulating white blood cells were used
497 as positive control for gating strategy. As expected, the number of PMN present in a segment of
498 proximal colon similar to the pcLoop was low under physiological conditions (**Figure 4B**).
499 Pretreatment with pro-inflammatory cytokines TNFα and IFNγ prior to surgery resulted in
500 augmented numbers of PMN recruited in the pcLoop lumen. The administration of the PMN
501 chemoattractant LTB$_4$ led to a dramatic increase in PMN counts supporting a LTB$_4$-dependent
502 PMN recruitment (**Figure 4B**). Immunohistochemical staining of PMN in the colonic mucosa
503 corroborate the elevated recruitment of PMN following stimulation with cytokines and LTB$_4$
504 when compared with cytokine treatment without LTB$_4$ (**Figure 4C**)[30]. The pcLoop model was
505 employed to study the contribution of JAM-A to PMN TEpM by using *Villin-cre;Jam-a* [fl/fl] mice.
506 Loss of epithelial JAM-A led to a reduced number of transmigrated PMN in the colonic lumen
507 compared to littermate controls (**Figure 4D**)[30]. These findings strongly support a role for JAM-A
508 in facilitating PMN migration across the intestinal epithelium and provide complementary
509 insights to studies that have reported the involvement of JAM-A in PMN migration across vascular
510 endothelium in various models of inflammation[31,37,38].
511
512 **FIGURE AND TABLE LEGENDS:**
513 **Figure 1: The ileal loop model.** (**A**) Schematic overview of the ileal loop model. Median
514 laparotomy is performed on mice under anesthesia and placed on a temperature-controlled
515 surgery board. (**B**) Exteriorization of the caecum (*****), ileum and mesentery. Two adequate sites
516 for ligation are identified (1,2). (**C**) Isolate a segment of 4 cm length: the first ligature (1) is placed
517 close to the ileo-caecal junction and a second ligature (2) is placed 4 cm away from the first
518 ligature. (**D**) Two small incisions are made in the mesentery (1, 2) to create a 4 cm length ileal
519 loop. After removal of luminal content and ligation of cut-ends, reagents such as fluorescent
520 markers and chemoattractants can be injected into the lumen. The ileal loop is well vascularized
521 (black arrowheads).
522
523 **Figure 2: The proximal colon loop model.** (**A**) Schematic overview of the pcLoop model. Median
524 laparotomy is performed on mice under anesthesia placed on a temperature-controlled surgery
525 board. (**B**) Exteriorization of the caecum (*****), proximal colon, mesocolon and ileum. Two adequate
526 sites for ligation are identified (1,2). (**C**) The first ligature (1) is placed close to the caecum and a
527 second ligature (2) is placed 2 cm more distal from the first ligature. (**D**) The pcLoop is
528 exteriorized, cleaned of luminal content and inflated with reagents such as fluorescent markers

529 and chemoattractants. The pcLoop is a well-vascularized 2 cm segment of proximal colon (black
530 arrowheads indicate blood supply).

531

532 **Figure 3: JAM-A regulates intestinal permeability in vivo.** (**A**) JAM-A deficiency (*Jam-a*^{-/-}) led to
533 increased colonic permeability to 4 kDa FITC-dextran. *Jam-a*^{-/-} (13x animals; black dots) were
534 compared with *Jam-a*^{+/+} controls (12x animals; white dots). 4 kDa FITC-dextran (1 mg/mL) in HBSS
535 was injected into the pcLoop lumen. Fluorescence was measured in blood serum after a 120 min
536 incubation period. Data are expressed as means ± SEM; *n = 3* independent experiments. ****P* <
537 0.0001; Mann–Whitney U test. (**B**) Increased colonic permeability to 4 kDa FITC-dextran in *Villin-*
538 *cre; Jam-a*^{fl/fl} (18x animals, black dots) compared to controls (*Jam-a*^{fl/fl}, 12x animals, white dots).
539 Data are means ± SEM; *n = 4* independent experiments. ****P* < 0.0001; Mann–Whitney U test.
540 This figure has been modified from Flemming S, Luissint AC et al.[30].

541

542 **Figure 4: JAM-A promotes LTB$_4$-dependent recruitment of PMN into the lumen of the pcLoop.**
543 (**A**) Gating strategy to quantify PMN (CD45$^+$, CD11b$^+$, and Ly-6G/Gr1$^+$ cells) in luminal content by
544 flow cytometry with fluorescent counting beads. Leukocytes from blood samples were used as a
545 positive control for the gating strategy. (**B**) Number of PMN recruited into the pcLoop lumen after
546 cytokine (TNFα+IFNγ, 100ng each) treatment (10x animals; white dots) or after a combination of
547 cytokines and 1 nM LTB$_4$ (10x animals; black dots). Black squares represent the number of PMN
548 at baseline as assessed in an intact colonic segment identical in length to the pcLoop that was
549 not subjected to any surgery or treatment with proinflammatory cytokines and LTB$_4$ (9x animals).
550 Data are the mean ± SEM (*n = 3* independent experiments), Kruskal–Wallis test with Dunn's
551 multiple comparison test. **P* < 0.05, ****P* < 0.0001. (**C**) Immunohistochemical staining of PMN
552 (anti-Ly6G/Gr1 antibody) in the epithelium of the pcLoop after treatment with cytokines alone
553 (left panel, TNFα +IFNγ) or a combination of cytokines and LTB$_4$ (right panel). The number of PMN
554 recruited in the pcLoop is increased in the presence of LTB$_4$ (black arrowheads). Scale bar: 100
555 μm. (**D**) Number of PMN recruited in the pcLoop lumen in *Villin-cre; Jam-a*^{fl/fl} mice (11x animals;
556 black dots) compared to *Jam-a*^{fl/fl} mice (10x animals; white dots) in response to 1 nM LTB$_4$. Data
557 are means ± SEM; *n = 3* independent experiments. **P* < 0.05; 2-tailed Student's t test. This figure
558 has been modified from Flemming S, Luissint AC et al.[30].

559

560 **DISCUSSION:**
561 The mechanisms responsible for dysregulation of intestinal barrier function and immune cell
562 recruitment under pathologic conditions such as IBD are incompletely understood due to a lack
563 of experimental in vivo methods. Here, we detail a robust in vivo murine model that employs a
564 well-vascularized exteriorized intestinal segment of either ileum or proximal colon and allows for
565 assessment of intestinal permeability, neutrophil migration studies as well as other applications.

566

567 The iLoop is a non-recovery surgery that is performed on live animals. Anesthesia must be
568 continuously monitored over the course of the experiment and evaluation of depth of sedation
569 is mandatory. The most critical steps include (1) the isolation of the iLoop, (2) ligation of cut ends
570 and, (3) the inflation of the iLoop by intraluminal injection of reagent solution. In each of these
571 steps, bleeding can occur, compromising the blood supply of the iLoop and affecting accuracy of
572 the results. Of note, in rare instances of intraluminal bleeding during the PMN TEpM assay, the

573 flow cytometry gating strategy presented here will help to distinguish transmigrated PMN from
574 PMN originating directly from the bloodstream (non-migrated PMN). Transmigrated PMN
575 collected in the iLoop lumen express high levels of surface marker CD11b[10] compared to
576 circulating PMN (**Figure 4D**).
577
578 Given that the iLoop allows quantitative analyses of intestinal permeability and migration of
579 blood PMN into the intestinal lumen, it is important to standardize the size of the absorbing
580 mucosal area and the blood supply. In order to ensure consistency between animals, it is essential
581 that a correct length of intestinal segment is exteriorized. The iLoop should be 4 cm for the ileal
582 loop and 2 cm for the pcLoop and be perfused by comparable blood supply. Inconsistency in
583 these parameters will also result in unequal distension of the iLoop after intraluminal injection
584 of reagents and augment variability inter and between experimental groups. Furthermore, to
585 avoid over-distension of the iLoop, we recommend that no more than 250 μL of reagent solution
586 be injected in the lumen for the ileal loop and 200 μL for the pcLoop, respectively.
587
588 There are some limitations inherent with the nature of the procedure. The iLoop is a non-
589 recovery surgery that is performed on live animals. This is a technically challenging microsurgical
590 method; however, personnel can acquire surgical skills through practice. The average duration
591 of the surgery should be short (maximum 15 min). We recommend 120 min as an ideal incubation
592 time for measuring intestinal permeability and 60 min for the PMN TEpM. Incubation times can
593 be reduced, but extended timepoints might affect the overall inflammatory state of the animal
594 under anesthesia. In addition, the protocol from the start of the surgical procedure to sample
595 collection / analysis cannot be paused.
596
597 This iLoop model presents key advantages with respect to existing methods: (1) the iLoop is fully
598 vascularized and is more physiologically relevant, (2) in contrast to the oral gavage method that
599 assesses the overall gastrointestinal tract integrity and depends on gastrointestinal motility[13], the
600 iLoop allows to study the properties of specific localized areas in the intestine (terminal ileum or
601 proximal colon) that are commonly involved in IBD, (3) the iLoop is the first in vivo model that
602 allows the quantitative study of PMN TEpM into the gut lumen as well as other parts of the
603 intestinal mucosa, including lamina propria and epithelial factions[30,35]. It is possible to employ
604 high versus low molecular weight FITC-labeled dextrans (4 to 150 kDa) to evaluate both size
605 selectivity and/or severity of epithelial barrier defects in knockout/knock-in mice or various
606 experimental models including, but not limited to, intestinal inflammation. In addition FITC-
607 labeled dextrans can be quantified in other organs such as the liver[39] or as a novel approach for
608 studies of the blood brain barrier providing insights into the role of intestinal permeability in gut-
609 liver and gut-brain axes[40-42]. Furthermore, this method offers the possibility to perform two loops
610 in parallel (ileal loop and pcLoop in the same animal) and instill two different fluorescent labeled
611 probes for analyses of barrier properties in distinct areas in the intestine. Along similar lines,
612 generation of two loops in parallel can be employed to specifically evaluate ileum versus colon
613 for differences or similarities in recruitment of immune cells in response to the same reagent.
614
615 Here, by using the pcLoop with *Jam-a* –null mice or mice harboring selective loss of JAM-A on
616 IECs (*Villin-cre;Jam-a* [fl/fl]), we corroborate findings from previous studies that have reported a

positive role for the TJ-associated protein JAM-A in intestinal permeability and PMN TEpM. The applications of the iLoop can be expanded to various reagents including antibodies, microbial pathogens and therapeutic drugs[30,34,35]. Of note, we used LTB4 (336.5 Da) to model PMN TEpM given that it is a well-accepted potent and physiologic PMN chemoattractant and its ability to induce TEpM at low concentrations (1 nM) in the physiologic range. 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[30]. fMLF (437.5 Da) is a lower affinity chemoattractant in mice which requires much higher concentrations to be effective (1μM). This model is adaptable for use of CXCL1/KC, another potent physiologic chemoattractant that we have successfully used, yet CXCL1/KC is expensive and a relatively large molecule (11 kDa) that is less efficient in crossing the epithelial barrier. We have also demonstrated that neutralizing antibodies against leukocyte-specific integrin CD11b/CD18 (αMβ2) that were injected into the loop lumen prior administration of chemoattractant LTB4 resulted in reduced PMN TEpM corroborating results from in vitro studies[10,30,35]. Furthermore, the pcLoop was recently employed to study the effect of PMN versus epithelial glycans in controlling the rate of PMN TEpM[43]. Reagents were injected into the pcLoop lumen prior administration of chemoattractant LTB4. Therefore, with its broad spectrum of applications, the iLoop can complement and confirm findings obtained via in vitro assays. Ligated intestinal loops have also been used by others to study bacterial infection (such as *Salmonella, L. monocytogenes and E. coli*), therefore we believe that the ease in adaptability of this iLoop model can be used for these studies as well.

Following treatment with pro-inflammatory immune mediators, the iLoop can be used as an acute model of intestinal inflammation. Furthermore, the iLoop may enable studies elucidating the link between increased intestinal permeability and immune cell recruitment after exposure to intraluminal pathogens or in chronic inflammatory experimental models. Of note, we have recently observed by employing the pcLoop model that in response to high dose of proinflammatory cytokines TNFα and IFNγ (1 mg of each) intestinal paracellular permeability to 4 kDa FITC-dextran resulted in enhanced PMN recruitment into the pcLoop lumen in response to LTB4 in comparison to low dose cytokines (100 ng of each)[30]. Interestingly, here we show that increased epithelial permeability secondary to *Jam-a* deficiency did not lead to enhanced PMN TEpM but diminished it. All together these results suggest that intestinal paracellular permeability affects the rate of PMN TEpM but the correlation is not direct and depends on factors such as the expression of adhesion molecules (similar to JAM-A) that play an important role in both epithelial barrier function and leukocyte migration[16]. Future studies are needed to investigate the fine tuning of immune cell responses by the intestinal epithelium, and contributions to pathologic mucosal inflammation such as inflammatory bowel disease.

In conclusion, the iLoop model provides a major improvement to existing approaches for the assessment of intestinal permeability and PMN TEpM in vivo that will significantly aid in understanding mechanisms underlying the regulation of intestinal inflammation and IBD.

665

666   DISCLOSURES:

668

669   REFERENCES:

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768

Figure 1: The ileal loop model

Figure 2: The proximal colon loop model

Figure 3: JAM-A regulates intestinal permeability in vivo

Figure 4: JAM-A promotes LTB4-dependent recruitment of PMN into the lumen of the p.oop

A

Blood — SSC-H vs FSC-H
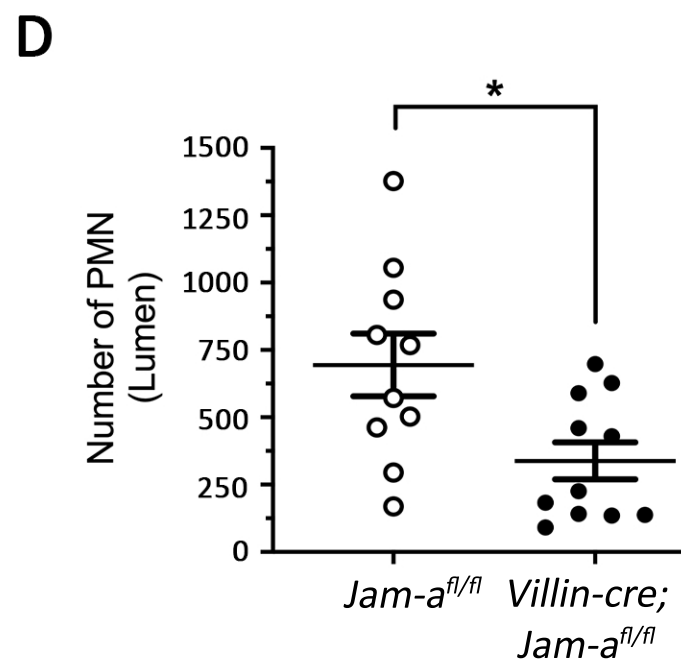Blood — SSC-H vs CD45
Blood — Ly6G vs CD11b

Luminal content — SSC-H vs FSC-H (beads 1.91)
Luminal content — SSC-H vs CD45
Luminal content — Ly6G vs CD11b

B

Number of PMN (Lumen)

Surgery	−	+	+
Cytokines	−	+	+
LTB$_4$ 1nM	−	−	+

****
*
*

C

Surgery + Cytokines   Surgery + Cytokines + LTB4

D

Number of PMN (Lumen)

*

Jam-a$^{fl/fl}$ Villin-cre; Jam-a$^{fl/fl}$

Table of Materials

Click here to access/download;Table of Materials;Boerner_et_al_revised table JoVE62093_Materials.xls ⬇
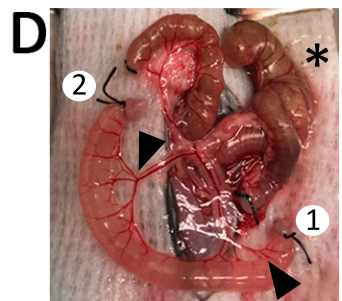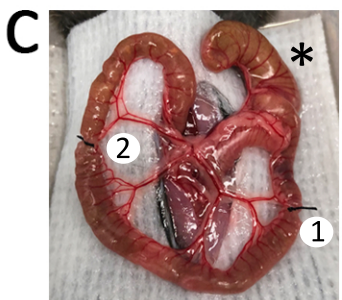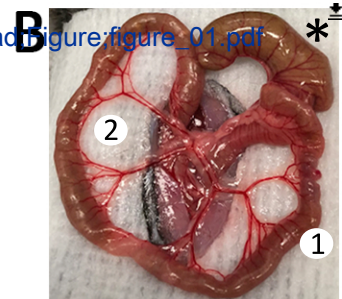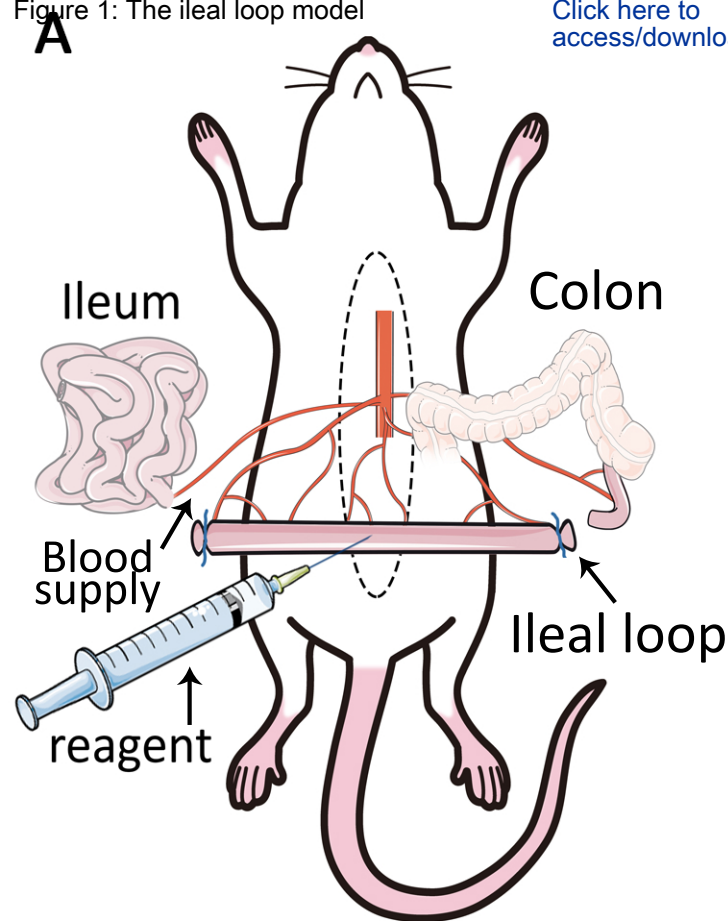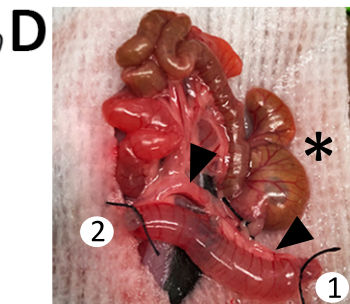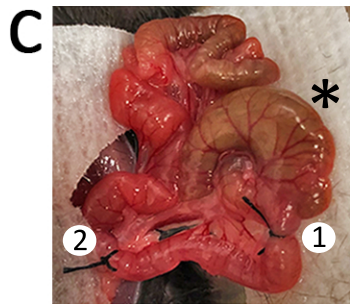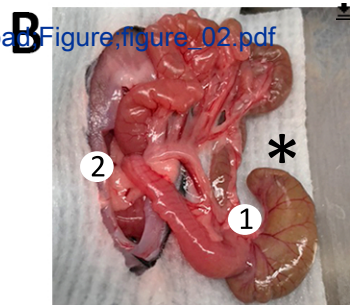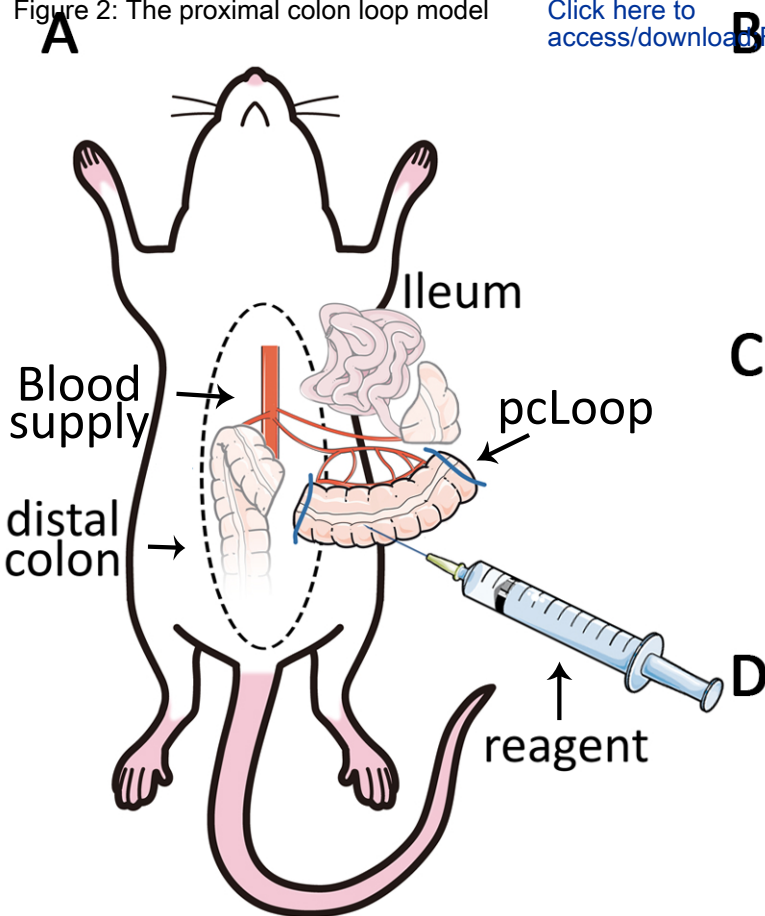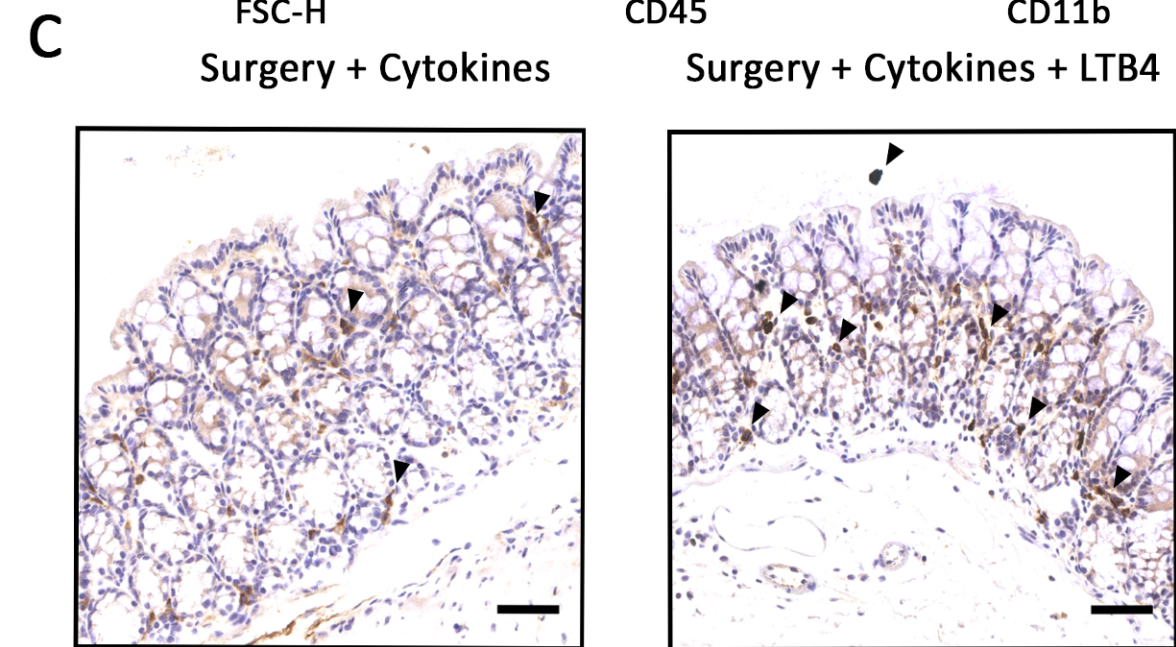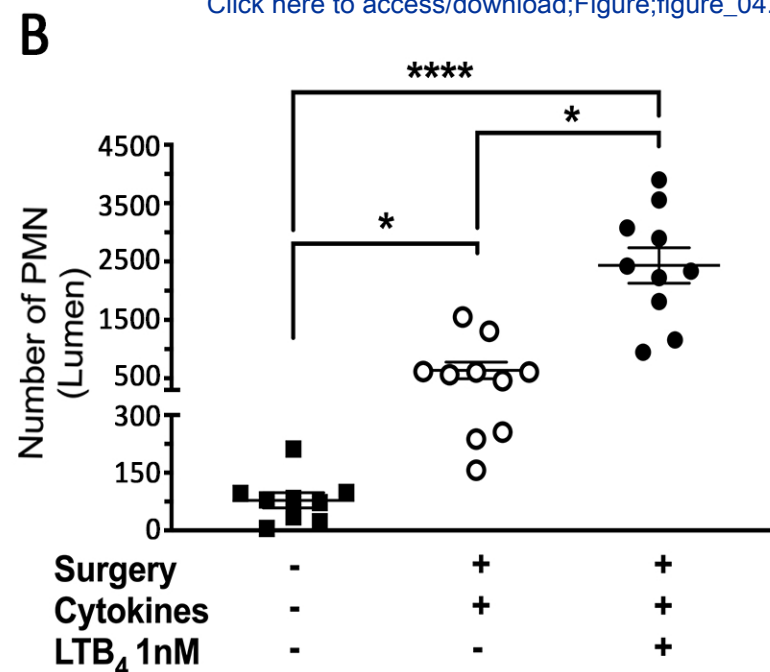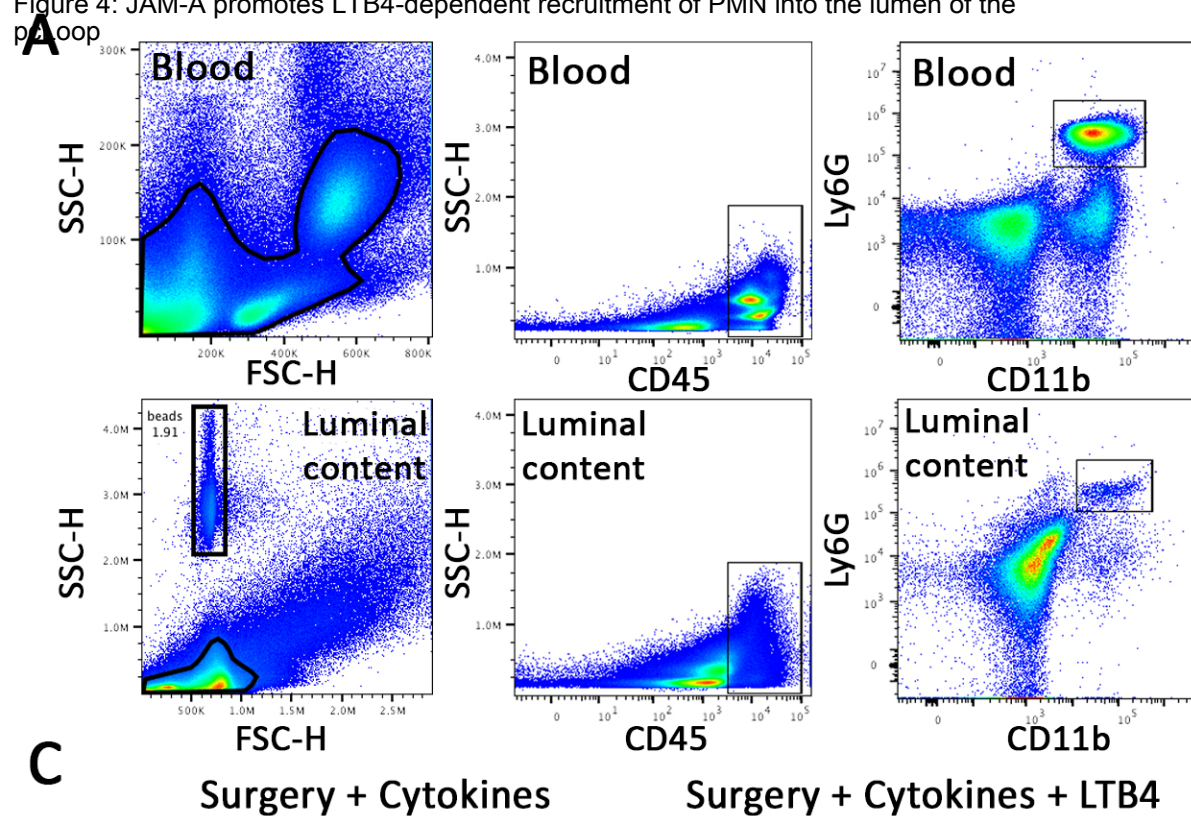
Name of Material/ Equipment	Company	Catalog Number
Equipment and Material		
BD Alcohol Swabs	BD	326895
BD PrecisionGlide Needle, 25G X 5/8"	BD	305122
BD PrecisionGlide Needle, 30G X 1/2"	BD	305106
BD 1ml Tuberculin Syringe Without Needle	BD	309659
15ml Centrifuge Tube	Corning	14-959-53A
Corning 96-Well Solid Black Polystyrene Microplate	FisherScientific	07-200-592
Corning Non-treated Culture Dish, 10cm	MilliporeSigma	CLS430588
Cotton Tip Applicator (cotton swab), 6", sterile	FisherScientific	25806 2WC
Dynarex Cotton Filled Gauze Sponges, Non-Sterile, 2" x 2"	Medex	3249-1
EZ-7000 anesthesia vaporizer (Classic System, including heating units)	E-Z Systems	EZ-7000
Falcon Centrifuge Tube 50ml	VWR	21008-940
Fisherbrand Colored Labeling Tape	FisherScientific	15-901-10R
Halsey Needle Holder (needle holder)	FST	12001-13
Kimwipes, small (tissue wipe)	FisherScientific	06-666
1.7ml Microcentrifuge Tubes	Thomas Scientific	c2170
Micro Tube 1.3ml Z (serum clot activator tube)	Sarstedt	41.1501.105
Moria Fine Scissors	FST	14370-22
5ml Polystyrene Round-Bottom Tube with Cell-Strainer Cap (35 μm nylon mesh)	Falcon	352235
Puralube Vet Ointment, Sterile Ocular Lubricant	Dechra	12920060
Ring Forceps (blunt tissue forceps)	FST	11103-09
Roboz Surgical 4-0 Silk Black Braided, 100 YD	FisherScientific	NC9452680
Semken Forceps (anatomical forceps)	FST	1108-13
Sofsilk Nonabsorbable Coated Black Suture Braided Silk Size 3-0, 18", Needle 19mm length 3/8 circle reverse cutting	HenrySchein	SS694
Student Fine Forceps, Angled	FST	91110-10
10ml Syringe PP/PE without needle	Millipore Sigma	Z248029
96 Well Cell Culture Plate	Corning	3799
Yellow Feeding Tubes for Rodents 20G x 30 mm	Instech	FTP-20-30
Solutions and Buffers		
Accugene 0.5M EDTA	Lonza	51201
Ammonium-Chloride-Potassium (ACK) Lysing Buffer	BioWhittaker	10-548E
Hanks' Balanced Salt Solution	Corning	21-023-CV
Phosphate-Buffered Saline without Calcium and Magnesium	Corning	21-040-CV
Reagents		
Alexa Fluor 647 Anti-Mouse Ly-6G Antibody (1A8)	BioLegend	127610
CD11b Monoclonal Antibody, PE, eBioscience (M1/70)	ThermoFisher	12-0112-81
CountBright Absolute Counting Beads	Invitrogen	C36950
Dithiotreitol	FisherScientific	BP172-5
Fetal Bovine Serum, heat inactivated	R&D Systems	511550
Fluorescein Isothiocyanate-Dextran, average molecular weight 4.000	Sigma	60842-46-8
Isoflurane	Halocarbon	12164-002-25
Leukotriene B$_4$	Millipore Sigma	71160-24-2
PerCP Rat Anti-Mouse CD45 (30-F11)	BD Pharmingen	557235
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD FC Block)	BD Bioscience	553142
Recombinant Murine IFN-γ	Peprotech	315-05
Recombinant Murine TNF-α	Peprotech	315-01A

November 23ʳᵈ, 2020

Reference: JoVE62093

Dear Dr. Bajaj

We thank you for your recent email inviting to resubmit a revised version of our manuscript entitled *"Functional Assessment of Intestinal Permeability and Neutrophil Transepithelial Migration in Mice Using a Standardized Intestinal Loop Model"*.

Below is a point-by-point response to the editorial's comments and attached as a separate document a point-by-point response to the reviewers' comments.

All additional information and discussion points are included in the revised manuscript and are highlighted in red color.

Response to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Response: We have proofread and corrected the manuscript. Abbreviations are defined at first use.

2. Please shorten your title to "Functional Assessment of Intestinal Permeability and Neutrophil Transepithelial Migration in Mice Using a Standardized Intestinal Loop Model".
Response: As requested, we have shortened our title.

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.*
For example: EZ-7000 anesthesia vaporizer; Kimwipe etc
Response: We have edited our manuscript to comply with JoVE's publication requirements.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: We have included more details (including references) to our protocol in order to facilitate reproducibility of the methods. In addition, the specific points raised by reviewer #1 in the protocol section have also been addressed (see response to reviewers).

5. Please specify the number, strain, sex, and age of the mice. Please mention (even as notes) in the beginning if you are using Villin-cre;Jam-aᶠˡ/ᶠˡ and Jam-a-/- mice.
Response: We have provided more detailed information about the number, strain, sex, and age of the mice at the beginning of the protocol section (on line 125).

6. Please specify the method of euthanasia without highlighting it.
Response: We have clarified that animals are euthanized when being under anesthesia by using a physical method such as decapitation or cervical dislocation, and in accordance with the approved animal protocol (see note after step 4.5 and step 5.6.2).

7. Note after 1.2.5: What should the vitals and pedal reflex be (to help readers monitor)?
Response: We have now detailed what is expected for vitals and the pedal reflex when mice are under deep anesthesia and can undergo surgery (see steps 1.2.4 and 1.2.5).

8. Please use comma and not period as decimal (line 4.6).
Response: Done

9. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
Response: Done

10. Please do not abbreviate journal names in the reference list.
Response: Done

Respectfully,

Charles A. Parkos, MD, PhD

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.*
<u>**Response**</u>: 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?*
<u>**Response**</u>: 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?*
<u>**Response**</u>: 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: $V = \pi(pi) \, r \, 2 \, h$ (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).

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