Isolation and phenotype analysis of tissue-resident lymphocytes from mouse colon

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Chapter Outline

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Abstract

The intestinal epithelium consists of a single layer of cells that includes enterocytes, enteroendocrine and goblet cells. This monolayer is in close contact with the underlying lamina propria and spatially segregates the gut microbiota and other environmental stimuli from the mucosal immune system. Under certain circumstances, however, gut bacterial components and/or metabolites can perturb tissue homeostasis resulting in abnormal immune responses and tissue damage. In this context, some bacterial toxins, besides stimulating an inflammatory response, may contribute to the activation of pathways related to carcinogenesis. Since immunosurveillance represents a critical barrier to an emerging tumor, characterizing tissue resident immune cells can prove a relevant tool for translational research. The protocol described herein describes the required steps for the isolation and flow cytometry phenotype analysis of tissue resident immune cells from mouse colon following repeated exposure to a pathogenic *E. coli* toxin as model stimulus.

1 Introduction

The intestinal epithelium lining forms the luminal surface to the external environment of both the small and large intestines and consists of a single layer including several types of cells in which enterocytes are the predominant cells (Ali, Hui, Tan, & Kaiko, 2020). The enterocytic monolayer is interrupted by both enteroendocrine cells and goblet cells. The firsts are responsible for releasing peptide hormones, which are correlated with tissue repair, angiogenesis, enterocytes' differentiation and polarization. Conversely, goblet cells produce mucus that forms a protective layer over the epithelial barrier. All together these cells form a physical barrier to maintain host metabolism. Furthermore, the intestinal barrier includes the immune system that contains the largest reservoir of immune cells in the body because it is in close contact with the outside world, including microbiota, pathogens and environmental antigens. The mucosal immune system mainly includes organized tissues, such as the mesenteric lymph nodes and the Peyer's patches; diffuse lymphoid tissue, such as the cryptopatches of the lamina propria in the intestinal mucosa; and groups of immune cells, such as the intraepithelial lymphocytes (Ma, Qiu, & Yang, 2021; Olivares-Villagómez & Van Kaer, 2018).

The gut microbiota is not just a passive bystander, but actively impacts many host functions, including immunity (Zheng, Liwinski, & Elinav, 2020). In fact, mammals and their commensal microorganisms co-evolved toward mutualism and hemostasis and such correlation requires commensals bacteria to be controlled by a proper host immunity function (Chu & Mazmanian, 2013). Pathological changes in the gut microbiota composition (i.e., dysbiosis) can be induced by genetic or environmental factors. In these situations, dysbiosis modifies the burden of bacterial components and/or metabolites and can results in systemic dissemination of commensal microorganism, susceptibility to pathogenic invasion, and abnormal immune responses. In the context of gut microbiota dysbiosis, pathogenic bacteria are receiving increasing attention and among the others those able to produce toxins, due to their association with colorectal cancer (CRC) (Buc et al., 2013; Piciocchi et al., 2021). Some of these toxins stimulate inflammation which then

contributes to activating pathways related to carcinogenesis (Fiorentini et al., 2020). Therefore, characterizing the type of immune infiltrate associated to microbiota perturbations can prove useful to dissect the immune-mediated mechanisms of pathogenesis. This protocol describes the required steps for the isolation and flow cytometry analysis of tissue-resident immune cells from mouse colon following repeated exposure to a pathogenic *E. coli* toxin as model stimulus.

2 Materials

2.1 Common disposables

- Falcon® 6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate, with lid (Corning #353046) (see Note 1)
- Falcon® 96-Well (U-bottom) plates with lid (Corning #351177) (see Note 1)
- CLEARLine® 50 mL Conical Sterile Polypropylene Centrifuge Tubes (Biosigma #CL474) (see Note 1)
- CLEARLine® 15 mL Conical Sterile Polypropylene Centrifuge Tubes (Biosigma #CL482) (see Note 1)
- Eppendorf Safe-Lock 2 mL Microtubes (Eppendorf #0030120094)
- Corning® cell strainers 100 μm pore size, sterile (Corning # CLS431752)
- Transparent polypropylene tube 1.3 mL, round bottom (Sysmex #04-2010)

2.2 Mice and surgical instruments

- Mice strain C57BL/6 (Envigo, Italy) (see Note 2)
- Toughcut, straight scissors (see Note 1)
- Scissors Noyes/Castroviejo straight 120 mm (see Note 1)
- Tweezers (see Note 1)
- Gillis forceps (see Note 1)

2.3 Reagents and solutions

- Hank's Balanced Salt Solution (HBSS) (w/o Ca2+, Mg2+) (Gibco #14175095) (see Note 1, 3)
- Dulbecco's Phosphate Buffered Saline (DPBS) (w/o Ca2+, Mg 2+, Phenol Red), Sterile Filtrated (Gibco # 14190144)
- Heat-inactivated Fetal Bovine Serum (FBS) (Capricon Scientific, #FBS-HI-12A)
- Dithiothreitol (DTT) (Sigma-Aldrich, #D9779)
- DNAse I (Sigma-Aldrich #DN25)
- Dispase (MP Biomedicals #9001-92-7)
- Collagenase Type IV (Worthington-Biochemical Corporation #LS004209)
- Percoll (Sigma-Aldrich #P1644):
- 70 % Ethanol (v/v in distilled water)
- Invitrogen™ Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher #L34976,)

- Anti-CD19-FITC mAb (Clone:MB19-1, Biolegend #101506)
- Anti-TCR$\gamma\delta$-PE mAb (Clone GL3, Biolegend #118108)
- Anti-CD3-PECy7 mAb (Clone:17A2, Biolegend, #100220)
- Anti-CD4-BV420 mAb (Clone: GK1.5, Biolegend, #100443)
- Anti-CD45-BV510 mAb (Clone: C363-16-A, Biolegend, #103308)
- 0.4 % Trypan blue dye in sterile PBS
- 1 % paraformaldehyde (Fluka chemika, 76240) in PBS (see Note 1, 3, 11)
- DTT solution: HBSS supplemented with 1 mM DTT, 1 mM EDTA and 1 % FBS
- Digestion solution: RPMI medium supplemented with 1 mg/mL Dispase (8 units/mg stock), 0.4 mg/mL DNase I, 1 mg/mL Collagenase type IV (283 units/mg stock), 2 % FBS
- 80 % Percoll solution: 80 % Percoll and 20 % HBSS
- 40 % Percoll solution: dilute 1:2 80 % Percoll in RPMI supplemented with 2 % FBS
- FACS buffer: DPBS supplemented with 2 mM EDTA and 1 % FBS

2.4 Equipment

- Refrigerated centrifuge suitable for centrifugation of 50- and 15-mL conical tubes and 96-well plates up to 600g (see Note 1)
- Cell culture CO_2 incubator (see Note 1)
- Heated water bath (see Note 1)
- Laboratory biosafety cabinet (Class II), (see Note 1)
- Chemical fume hood, such as Hamilton SafeAire II Fume Hood (#HD03015,
- Hamilton) (see Note 1)
- Standard bench top centrifuge, such as Allegra X-14R Centrifuge, 120 V, 60 Hz
- (#A99464, Beckman Coulter) (see Note 1)
- Flow cytometer equipped with at least 3-lasers for multicolour analysis (Gallios Beckman Coulter) (see Note 1)
- Haematocytometer counting chamber with V-slash (see Note 1)
- Standard 25 binocular microscope (see Note 1)

2.5 Software

- Kaluza Analysis Software (vers. 2.1.00001.20653, Beckman Coulter)
- Excel or Graphpad Prism Software for graph design and statistical analysis

3 Methods

3.1 Mice treatment

1. Anesthetized (Ketamine 100 mg/Kg + Xilazine 10 mg/Kg) C57Bl/6 female mice received six intrarectal administrations of E.coli CNF1 toxin (10–11 M in 0.05 mL PBS per mice) or PBS every 3 weeks. Ten days after the last administration, mice were sacrificed for the analysis of tissue-resident lymphocyte composition.

3.2 Experiment set up

1. HBSS is prewarmed at 37 °C before use (see Note 1, 3)
2. DTT solution is prepared and used on the day of experiment (see Note 1, 3)
3. DNAse I, Dispase and Collagenase type IV are added to the Digestion solution immediately prior to use. Complete dissolution should be obtained before use and solution should be prewarmed at 37 °C (see Note 1, 3, 5, 6, 7)
4. 80 % and 40 % Percoll solutions are freshly prepared and allowed to warm up at 20–22 °C before use (see Note 4)
5. FACS buffer can be prepared in advance and stored at 4 °C until use for up to one month (see Note 1, 3)

3.3 Dissection of murine colon

1. Mice are euthanized by cervical dislocation and laid down on a surgery board and the abdomen is sprayed with 70 % ethanol (see Note 8, 12)
2. The skin of the abdomen is cut and lift with tweezers making a V-shaped incision. Skin is removed to set out the abdominal cavity (see Note 13)
3. Intestine is taken out using forceps, while the mesenteric fat is removed using another set of forceps (see Note 14)
4. The colon is excised and collected from the rest of the intestinal tract (see Note 15)
5. The colon tissue is placed in a 10-cm Petri dish and flushed through with a 5 mL-syringe filled with ice-cold HBSS to remove the intestinal content (see Note 16)
6. The colon is longitudinally cut with blunt-end scissors and rinsed thoroughly in ice-cold HBSS (see Note 17)
7. The colon tissue is placed in a 15 mL conical tube containing 5 mL of HBSS + 2 %FBS and kept on ice until all samples are collected (see Note 18)

3.4 Colon tissue digestion

1. Each colon sample is transferred to one 2 mL Eppendorf tube containing 1 mL of DTT solution each
2. Tissues are minced into small pieces with Noyes/Castroviejo scissors (see Note 21)
3. Tissues are then incubated for 30 min at 37 °C under agitation (see Note 19)
4. For optimal use of the final Digestion Solution, tissue enzymes are added to the pre-warmed Digestion solution (RPMI and FBS only) just before use (see Note 1, 3, 5, 6, 7, 20)
5. After incubation (see point 3), 5 mL final Digestion Solution are added to a 15 mL polypropylene tube where tissue fragments are collected and transferred
6. Samples are incubated for 20 min at 37 °C under agitation (see Note 19)
7. Tubes are vortexed and the entire content of the tube is decanted into a 100 μm cell strainer by flushing with 5 mL of HBSS (see Note 22) and transferred in 15 mL polypropylene tube
8. Tubes are centrifuged (300 g for 10 min, 21 °C) and the supernatant discarded (see Note 23 and 24)

9. Pellets are resuspended in 5 mL of 40 % Percoll (see Note 4) and layered onto 2.5 mL of 80 % Percoll in 15 mL conical tube for a total volume of 7.5 mL (see Note 25)
10. Gradients are centrifuged (860 g for 20 min, 21 °C - mandatory) without acceleration and deceleration (see Note 26)
11. New 15 mL conical tubes are prepared with 10 mL of HBSS (see Note 27)
12. Top half of the gradient is aspirated to within ~2 cm of the interface. The ring is harvested at the interface with a 1 mL Gilson pipet and placed in new 15 mL conical tubes prepared in the previous step (see Note 28)
13. Cells are rinsed by centrifugation (300 g for 10 min, 4 °C). Then, supernatants were discarded and pellets resuspend in 1 mL FACS Buffer (see Note 29)
14. Cells are ready for counting and staining using 0.4 % trypan blue dye exclusion (see Note 30)

3.5 Staining protocol

1. Cells (up to 10^6 cells/sample) are placed a 96well round bottom plate and washed once in cold FACS Buffer (300 g for 5 min, 4 °C) (see Note 30)
2. Each sample is resuspended in 50 µL FACS Buffer containing 0.25 µL NIR and incubated 20 min at room temperature in the dark (see Note 31).
3. Cells are washed in FACS Buffer and centrifuged (300 g for 5 min, 4 °C). After centrifugation, FACS buffer removed and 50 µL/well of FBS added to each well (see Note 32)
4. Cells are incubated 10 min at 4 °C in the dark (see Note 33)
5. Samples are washed twice with 0.15 mL of FACS Buffer and centrifuged (300 g for 5 min, 4 °C) (see Note 34, 35)
6. Fluorescent mAbs are properly diluted and 20 µL of the staining mix are added to each well (see Note 36)
7. Samples are incubated 15-20 min at 4 °C in the dark (see Note 33)
8. Samples are centrifuged (300 g, 5 min, 4 °C) and resuspended in 0.2 mL of FACS buffer
9. Samples are centrifuged again (300 g, 5 min, 4 °C) and resuspended in 0.2 mL of FACS Buffer or 0.2 mL FACS Buffer + 1 % paraformaldehyde (See note 11, 37)
10. Samples are stored at 4 °C in the dark until acquisition at Gallios (Beckman Coulter) (see Note 1, 38)

3.6 Flow cytometry acquisition and analysis

1. Samples are transferred from the 96-well plate to the transparent polypropylene 1.3 mL tubes and kept protected from light before acquisition
2. Multispectral flow cytometry is performed on Gallios flow cytometry analyzer (Beckman Coulter) equipped with 3 lasers

3. Unlabelled cells, cells labelled with only NIR and samples labelled with NIR, anti-CD45 anti-CD19, anti-CD3 were used as FMO samples to adjust lasers intensity and fluorochrome compensation
4. During acquisition setup, gates are created. Specifically, doublets are excluded by plotting FSC-A vs FSC-H and designing the "singlets" gate (see Fig. 1)
5. At least 20,000 viable CD45 + should be acquired for each sample with a flow rate between 300 and 500 events
6. Collected data are analysed by Kaluza Analysis software.
7. Each.fcs file is analyzed according to the gating strategy described in Fig. 1.
8. For each.fcs file, a gate statistics file is generated by Kaluza software and exported to an Excel file or equivalent software.
9. When plotting data, it is recommendable to choose a scatter plot or an equivalent plot showing the distributions of the single in each treatment group (see Fig. 2). Statistical significance of results can be calculated by Graphpad Prism or equivalent software.

4 Notes

1. Catalog number and provider are indicated as a reference, but an equivalent product can be purchased.
2. C57BL/6 female mice are used for this protocol. They were over 20 gr of body weight on arrival.
3. RPMI, FBS, PBS, EDTA, HBSS, Hepes are considered as non-hazardous (NONH). Anyway, they should be used with certified personal protective equipment (PPE).

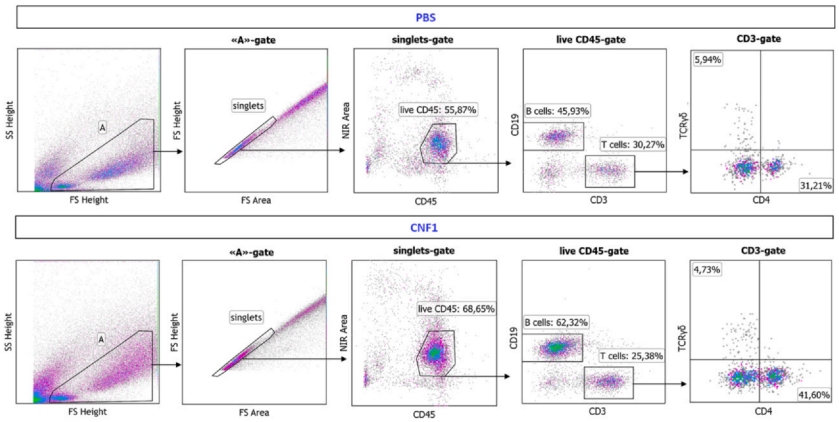

FIG. 1

Representative dot plots, including gating strategy, of tissue-resident lymphocyte percentages from colon tissue of C57Bl/6 female mice exposed to intrarectal CNF1 or PBS as control.

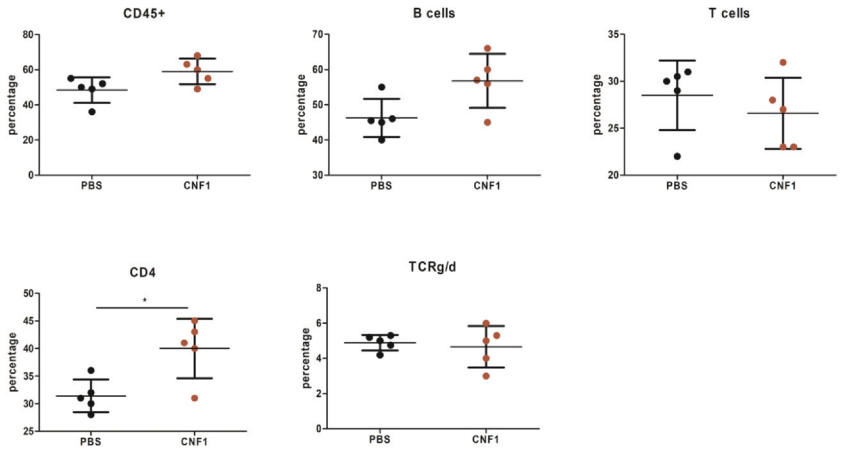

FIG. 2

Scatter plot graphs of the indicated immune subsets in the colon of C57Bl/6 female mice exposed to intrarectal CNF1 or PBS as control (n = 5). Lines represent mean and SD values (*$p < 0.05$).

4. Percoll is as non-hazardous chemical solution. Anyway, it should be used with certified PPE.
5. DNAse I may cause an allergic skin reaction (H317) and allergy or asthma symptoms or breathing difficulties if inhaled (H334).
6. Dispase may cause allergy or asthma symptoms or breathing difficulties if inhaled (H334).
7. Collagenase type IV is a chemical considered hazardous. It causes skin irritation (H315) and causes serious eye irritation (H319). It may cause an allergic skin reaction (H317) and respiratory irritation (H335). Their use with certified PPE.
8. Ethanol is highly flammable in liquid and vapor (H225) and causes serious eye irritation (H319), and hence should be stored in dedicated cabinets for flammables and manipulated by wearing appropriate certified PPE at distance from open flames as well as potential sources of heat and sparkles.
9. Trypan blue may cause cancer (H350). Protective gloves and protective clothing are needed (P280).
10. Fluorescent mAb are not considered hazardous (HNOC). PPE is however required.
11. Paraformaldehyde is flammable solid (H228). It is harmful if swallowed (H302) and inhaled (H332). It causes skin irritation (H315) and serious eye damage (H318). It may cause an allergic skin reaction (H317), cancer (H350) and respiratory irritation (H335). It is suspected of causing genetic defects (H341). Keep away from heat, hot surfaces, sparks, open flames and other ignition sources, no smoking. Wear correct certified PPE. Perform all operations in a certified chemical fume hood or other approved ventilated area.

12. To limit any microbial contamination, it is appropriate to spray mice abdomen with 70 % ethanol before surgical removal of colon.
13. Be careful during this operation to avoid damaging the abdominal wall.
14. Make sure to remove as much mesentery/fat as possible for optimal cell yield and viability.
15. Be careful during this operation.
16. This step is important to be sure that tissue will be clean at the beginning of the dissociation and digestion tissue protocol.
17. Curved blunt-edged forceps is necessary for this step.
18. Dissociation buffer should be prepared and used on the day of experiment. Keep on ice colon samples during the processing of the other mice. Colon fragments could be placed also in 6-well plates on ice.
19. If not possible, tissue fragments could be incubated for 30 min in a heated water bath at 37 °C and shaked every 5 min.
20. DNAse I, Dispase, Collagenase type IV should be added to the pre-warmed Digestion solution just before the use. Make sure the powders are well dissolved in the solution.
21. This step is necessary to facilitate the epithelium dissociation process
22. To facilitate the tissue filtering, it is advisable to rinse the filter with 5 mL of HBSS.
23. This step is necessary to pellet cell suspension.
24. During the centrifugation, 15 mL conical tubes with 2.5 mL of 80 % Percoll should be prepared.
25. The 15 mL conical tube should be inclined during the stratification. Be careful not to mix the gradient interface. For this step, you could also use pipette controller set to “slow” or “dropwise” mode.
26. These parameters shouldn’t be changed.
27. During the centrifugation, you can prepare new 15 mL conical tubes with 10 mL of HBSS.
28. Be careful with interface collection. For this step, you could also use pipette controller set to “slow” mode.
29. Finally, cell pellet is resuspended in 1 mL of FACS Buffer. Only viable cells are used for the staining. Haematocytometer counting chamber with V-slash and standard 25 binocular microscope are used during this step.
30. 96-Well (U-bottom) plate is needed for the next staining protocol.
31. NIR cell sample and the Fluorescent Minus One (FMO) cell sample should be included to optimize the flow cytometry analysis. The dark incubation is required to protect the fluorescence. In details, NIR (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit) is a specialized tool designed for assessing cell viability prior to the fixation and permeabilization processes necessary for intracellular antibody staining. FMO (Fluorescence Minus One) controls are crucial tools in multicolor flow cytometry. They play a key role in accurately setting gates for distinguishing between positive and negative populations, especially when dealing with low expression levels.

32. Full serum is added to saturate Fc receptor (FcR).
33. The protection from the light is required for these steps.
34. FACS buffer should be used cold.
35. You could also use PBS instead of FACS buffer.
36. A mix containing anti-CD19, anti-TCR$\gamma\delta$, anti-CD3, anti-CD4, anti-CD45 properly diluted according to previous titration is prepared calculating a volume of 20 μL to each sample. For anti-CD19, anti-CD3 the used dilution is 1:20. For anti-TCR$\gamma\delta$, anti-CD4 and anti-CD45 the used dilution is 1:40.
37. For long-term storage (up to one week), samples can be resuspended in 0.2 mL of FACS Buffer + 1 % paraformaldehyde. You can use PBS in the place of FACS buffer to dilute paraformaldehyde. Be careful with the use of paraformaldehyde.
38. You can keep the samples in a fridge or on ice until acquisition at Gallios (Beckman Coulter). You can use an alternative 3-lasers flow cytometer for the acquisition.

5 Concluding remarks

The protocol described herein offers an easy workflow to isolate and perform a phenotype analysis of tissue-resident lymphocytes from mouse colon. When going for the analysis of intestinal immune cells, one should be aware that these immune subsets are greatly influenced by animal housing, diet and pathological conditions as well as by the age of animals. Although time-consuming, this methodology has the advantage of isolating viable cells from murine intestinal tissue that can be further sorted and exploited in functional assays, including cytokine expression analysis and in vitro stimulation. The reduced number of markers and the lack of spatial information represent the main limitations; an extensive characterization of the intestinal immune system would require more markers to allow a comprehensive analysis of its complexity and heterogeneity. Thus, we recommend exploiting this protocol for the phenotypic characterization or the functional analysis of defined immune subsets relevant for a defined pathology or mechanisms rather than for wide screenings.

Conflicts of interest

All the authors have no conflicts to declare.

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