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Interrogating the small intestine tuft cell – ILC2 circuit using *in vivo* manipulations

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Abstract

Recent findings position tuft cells as key mediators of intestinal immunity through production of the cytokine interleukin (IL)-25 and activation of group 2 innate lymphoid cells (ILC2s). Though tuft cells are found in numerous epithelial tissues, the phenotype and function of tuft cells has been best characterized in the small intestine, where robust *in vivo* techniques have enabled dissection of cellular function, ontogeny, and key signaling pathways. We describe methods for identification, quantification, and manipulation of tuft cells, focusing on analysis of ILC2s as a readout of tuft cell function.

Basic protocol 1: *Ex vivo* analysis of small intestinal tuft cells and ILC2 by flow cytometry

Alternate protocol 1: *Ex vivo* analysis of small intestinal tuft cells and ILC2 by flow cytometry in the context of type 2 inflammation

Basic protocol 2: *Ex vivo* analysis of small intestinal tuft cells by imaging

Basic protocol 3: Tuft – ILC2 circuit activation by oral gavage of adult *N. brasiliensis* worms

Basic protocol 4: Circuit activation by colonization with *Tritrichomonas* spp.

Basic Protocol 5: Circuit activation by treatment with succinate in drinking water

Basic protocol 6: *In vivo* analysis of tuft cell function by cytokine manipulation

Keywords

Tuft cells; group 2 innate lymphoid cells (ILC2s); type 2 cytokine signaling; small intestine; IL-25; parasites; type 2 immunity; *N. brasiliensis*; *Tritrichomonas* spp.; succinate; helminth infection

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Introduction

Tuft cells were described over 50 years ago based on their unique morphological characteristics—namely, their striking apical "tuft" of microvilli (Jakob von Moltke, 2018). Tuft cells are found interspersed within the simple columnar epithelium of many mucosal surfaces, and are a conserved epithelial cell lineage in mammals (O'Leary, Schneider, & Locksley, 2019). Due to their rarity, and lack of distinguishing protein or gene markers, tuft cells remained understudied until a critical report identified numerous gene expression programs specific to tuft cells in the small intestine using a fluorescent reporter for expression of transient receptor potential cation channel subfamily M member 5 (TRPM5), a calcium-activated ion channel critical for signaling in type II taste cells, which detect bitter, sweet, and umami. These gene expression programs include transcripts related to chemosensation as well as immune and neural function (Bezençon et al., 2008). This, together with more recent work (Gerbe et al., 2016; Haber et al., 2017; Nadjsombati et al., 2018; J. von Moltke, Ji, Liang, & Locksley, 2016) has led to a working definition of tuft cells as secretory epithelial cells, dependent on the transcription factor *Pou2f3,* that express interleukin(IL)-25 and the enzymatic machinery for eicosanoid biosynthesis, and are $TRPM5^{+}$. Additional common markers for tuft cells include choline acetyltransferase (ChAT) and doublecortin-like kinase 1 (DCLK1); although highly specific for intestinal tuft cells among the epithelium, these proteins are also expressed by other (non-epithelial) cells (reviewed in (O'Leary et al., 2019)).

In 2016, three studies identified a role for tuft cells in the small intestine during anti-helminth response (Gerbe et al., 2016; Howitt et al., 2016; J. von Moltke et al., 2016), with follow-up work identifying an additional role for tuft cells in response to succinate production from the commensal protist *Tritrichomonas*, commonly found in many mouse vivaria (Lei et al., 2018; Nadjsombati et al., 2018; Schneider et al., 2018). Tuft cells are the dominant, if not the only, source of IL-25 in the small intestine (J. von Moltke et al., 2016). During helminth infection, or upon exposure to *Tritrichomonas*-dependent metabolites, IL-25 drives IL-13 production from small intestinal group 2 innate lymphoid cells (ILC2s), which in turn drives additional tuft cell differentiation from crypt cells. Tuft cells also produce leukotrienes during anti-helminth responses, which further enhances ILC2 activation (McGinty et al., 2020). These recent breakthroughs position the long-enigmatic tuft cell as a critical player in local type 2 immunity in the small intestine (reviewed in (Schneider, O'Leary, & Locksley, 2019).

Optimized protocols for identification, isolation, and manipulation of tuft cells and ILC2s have enabled a new field of intestinal tuft cell biology. Here, we summarize several protocols for *in vivo* and *ex vivo* analysis of tuft cells, as well as for examination of ILC2s, which are, to-date, the most well-established recipient immune cell population for tuft cell signaling in the small intestine. The use of reporter mice along with specific antibody staining has greatly improved the identification and isolation of rare tuft cells. We have developed and reported use of an $Il25^{tdTomato}$ reporter mouse strain for highly specific identification of tuft cells by flow cytometry and imaging approaches (J. von Moltke et al., 2016). Additional commonly used tuft cell reporter strains include the transgenic TRPM5-eGFP (Bezençon et al., 2008) and the choline acetyltransferase $ChAT^{BAC}$-eGFP (Panneck et al., 2014). In the

absence of reporter mice, expression of intracellular DCLK1 (readily observed by antibody staining, as described in Basic Protocol 1 and 2) is equally useful for tuft cell identification among small intestinal epithelial cells; a number of other tuft cell-specific markers were summarized in (O'Leary et al., 2019).

The most well-characterized role for small intestinal tuft cells is in activation of ILC2 cytokine production and proliferation. Reporter mice, including those generated in the Locksley lab, provide a very sensitive readout of ILC2 activation and cytokine production directly *ex vivo* without the need for *in vitro* restimulation and intracellular cytokine staining (Liang et al., 2011; Nussbaum et al., 2013). However, flow cytometric analysis of cells isolated from small intestine affected by type 2 inflammation has historically been problematic, with cell viability decreasing in tandem with increased type 2 cytokine production and associated remodeling such as goblet cell hyperplasia (Schneider et al., 2018). In addition to describing previously published methods for lamina propria cell isolation (Basic Protocol 1), suitable for use in unmanipulated mice, we report an optimized protocol (Alternate Protocol 1) for isolation of highly viable epithelial and lamina propria cells from mice during peak infection with *Nippostrongylus brasiliensis*, as well as other methods of activating the tuft cell – ILC2 circuit described here in detail (Basic Protocol 3-5).

Our analysis protocols—Basic Protocols 1 and 2, and Alternate Protocol 1—will result in quantitative and qualitative flow cytometry and imaging data, which is necessary to determine how the *in vivo* manipulations described in Basic Protocol 3-5 impact tuft cell and ILC2 status. Generally, increased circuit activation causes an increase in ILC2 abundance, ILC2 cytokine production, and frequency of tuft cells, the extent of which correlates with duration and strength of the initial signal, although the molecular underpinnings of this are just beginning to be unraveled (Schneider, O'Leary, et al., 2019). The *in vivo* manipulations we describe for activating this circuit are useful in determining if a specific treatment, or loss or gain of function mutations alter or interrupt the tuft cell – ILC2 circuit activity.

Ex vivo analysis of small intestinal tuft cells and ILC2s by flow cytometry

Here, we detail a method previously published by our lab (Schneider et al., 2018) to generate a single cell suspension of small intestinal epithelial cells—including tuft cells—and lamina propria cells, suitable for flow cytometry and other downstream analysis applications. We also detail the use of antibody staining or reporter mice for tuft cell and ILC2 identification and enumeration by flow cytometry. This isolation method is suited to unmanipulated mice, in which type 2 cytokines are not at high levels, either locally in the gut or systemically.

When possible, use littermate and sex-matched mice. Basic Protocol 1 should not be used under conditions of intestinal type 2 inflammation (such as following worm infection), in which case Alternate Protocol 1 should be followed. In general, we recommend starting with a maximum of 6–8 samples. If well-prepared and experienced, it is feasible to do 10–20 samples in one day, however, if doing more than 14 samples we advise splitting them into two groups and slightly staggered the processing from step 12 onwards. The number of donor mice needed should be tailored to the user's needs and abilities—if a control and experimental condition are compared we would recommend a minimum of three mice per

condition, with several repeats of the experiment for rigorous statistical analysis; if the end goal is sequencing or another analysis, fewer donor mice may be needed.

Before starting to dissect mice, calculate the required volumes for all buffers (see spreadsheet for calculations), then make and pre-warm all solutions to 37 °C. We usually add DTT and enzymes to buffers after all tissues are harvested and before the processing (step 12).

BASIC Protocol 1: Prepare single cell suspension of intestinal epithelial cells

Materials

Donor mice – protocol is suitable for mice at any developmental age

Suggested tuft cell reporter lines: Flare25 (*Il25*$^{\text{flox-tdTomato}}$, not commercially available, inquire with Dr. Richard M. Locksley at UCSF), TRPM5-eGFP (not commercially available, inquire with Dr. Robert F Margolskee at the Monnell Center)

Suggested ILC2 reporter lines: Smart13 (*Il13*$^{\text{hCD4}}$, available at Jax: B6.129S4(C)-Il13tm2.1Lky/J

Stock No: 031367), Red5 (*Il5*$^{\text{tdTomato-Cre}}$, available at Jax: B6(C)-Il5tm1.1(icre)Lky/J

Stock No: 030926)

PBS (Thermo Fisher 14190250)

HBSS w/o calcium and magnesium (ThermoFisher 14175103)

HBSS (ThermoFisher 14025134)

DTT (Sigma D0632-5G)

EDTA (ThermoFisher 15575020)

Fetal calf/bovine serum (FCS/FBS)

HEPES (Sigma H3537-100ML)

Roche LiberaseTM (Sigma 5401127001, 2 × 50 mg)

DNase I, grade II from bovine pancreas (Sigma 10104159001 100mg)

70% ethanol

Percoll (Sigma P4937-500ML)

Equipment and consumables

Squeeze bottle

50 ml syringe

High contrast glass rod (see protocol for preparation): Trypan blue solution (Sigma T8154) and Natelson tube (Kimble Chase, 42F604)

Gavage needle (e.g. Harvard Apparatus 75-0286)

Dissection board and tools (e.g.: scissors and two curved forceps)

50 ml conical tubes (we recommend flip-top tubes such as Nunc EZFlip Conical Centrifuge Tubes, ThermoFisher 362696)

100 um filters, such as BD Cell Strainers 100 um nylon mesh filters Fisher 352360

gentleMACS C Tubes Miltenyi Biotec 130-096-334

15 ml conical tubes (BD Falcon or similar)

MACS SmartStrainers (100μm) Miltenyi Biotec 130-110-917

1 ml syringes, such as TB syringe slip tip (no needle) Fisher 309659

Filtercap FACS tubes (e.g Falcon 352235)

Spray bottle

Ruler with centimeter markings

Flat edge razor blade (such as Fisherbrand Razor Blades 12-640)

Plastic dish with raised edges, such as 15 cm cell culture dish

Pipet box lid, or other flat raised surface

Kimwipes

Vortex mixer

Tube racks

Bunsen burner

Euthanasia chamber and CO_2 gas

Rotating incubator

Refrigerated table-top centrifuge

gentleMACS Octo Dissociator (Miltenyi Biotec 130-095-937)

Solutions (see Reagents and Solutions and calculation spreadsheet)

Buffer 1: DTT wash

Buffer 2: EDTA wash

Rinse Buffer

Digestion Buffer

FACS wash buffer (FWB)

DTT stock solution 1 mM

5 mg/ml Liberase TM stock solution

10 mg/ml DNase1 stock solution

Protocol Steps

1. Perform the preparation prior to the dissection:

   a. We use a high contrast glass rod (Fig. 1a), which provides excellent support and contrast for removal of fat/mesentery, and visualization/ removal of Peyer's patches. To make this, seal glass rod on one end using Bunsen burner. Use p1000 pipet to fill the rod almost completely with Trypan blue. Seal open end with Bunsen burner.

      Use appropriate precautions when handling Trypan blue and working with open flame. Wear safety goggles. Make sure to not heat Trypan blue solution to boiling temperature when sealing open end.

   b. For each sample, prepare one 50 ml conical tube with 2 ml of Buffer 1 (before adding DTT) in each tube and store on ice. Prewarm remaining Buffer 1 and remember to add DTT at step 12. (*Intestinal samples will be stored in those 2 ml until dissection is completed).*

   c. Prepare a squeeze bottle with cold PBS, fill a 50 ml syringe with cold PBS and gavage needle attached, and pre-chill on ice.

      This material will only be used after the dissection.

   d. Place a Kimwipe over the pipet box and wet with PBS. Prop the pipet box/Kimwipe on the edge of the plastic pan to collect waste, as seen in Fig. 1a.

      This material will only be used after the dissection.

2. Euthanize mouse following institutional guidelines.

   We generally use CO_2.

3. Spray mouse with 70% ethanol solution. Dissect open peritoneal cavity to expose intestines. Cut below the stomach at the pyloric sphincter and use forceps to carefully pull small intestine from mesentery. Avoid tearing.

4. Align the proximal most piece of small intestine with the ruler, remove and discard the most proximal 2 cm. Harvest the next 6 cm to obtain sample of proximal small intestine. Other similarly sized segments of gut can also be analyzed. Attempt to use identical lengths of intestine for each sample.

   This protocol can be used to process tissue from any intestinal region, including cecum or colon. In our experience, a 4–6 cm long piece of intestine yields enough epithelial and/or lamina propria cells for multiple flow cytometry staining panels. Optional: weigh tissue to be able to normalize cells per gram of tissue if needed.

5. Insert the gavage needle into one end of the intestine sample and flush the lumen to remove contents (Fig. 1b).

   Wearing a lab coat is advised. Flushing also facilitates the next step.

6. Mount the segment of gut on the glass rod using hands or forceps, exposing the serosal side. Use dissecting scissors to cut away residual fat and mesentery.

   Optional: Most of the times, approx. 1–3 Peyer's patches are clearly visible (size depends on mouse hygiene status, while the number depends on intestinal region and segment size) and can be removed by pinching surrounding tissue with forceps, lifting it up and cutting underneath the forceps using a razor blade. This step is not necessary if only epithelial fraction will be analyzed.

7. Lay glass rod vertically on the Kimwipe/pipet box. Hold it down carefully and slice open using razor blade (Fig. 1c). Rinse tissue, which is now fileted open with luminal side exposed, with the PBS squirt bottle and remove glass rod. Rinse off any intestinal content.

8. Transfer tissue to the 50 ml conical tube from step 1a. Store on ice until all samples are harvested.

9. Remove all tubes from ice, add DTT to pre-warmed buffer 1, mix well and add 18 ml to each tube containing tissue and 2 ml buffer 1. Incubate with rocking at 37 °C, 80 rpm for 20 minutes.

10. Vortex each tube at intermediate speed for 5–10 sec and discard supernatant.

11. Transfer tissue to new 50 ml tube containing 10 ml of pre-warmed buffer 2. Incubate with rocking at 37 °C, 80 rpm for 15 min.

12. Vortex each tube at intermediate speed for 5–10 sec and decant supernatant (including epithelial cells) through a 100 μm cell strainer into a 50 ml tube containing 10 ml cold FWB and store on ice.

13. Repeat steps 11 and 12 twice.

50 ml tubes can be re-used by adding buffer following decanting. Particularly after the first two EDTA wash steps, a lot of epithelial cells should be released and at the end, the tissue will appear considerably thinner.

14. After the third collection of EDTA wash, centrifuge epithelial cell fraction at 1500 rpm for 5 min, 4 °C, and resuspend pellet in 5 ml of FWB.

a. Proceed to stain for tuft cells as described in Part 2 or continue on with lamina propria cell isolation.

If staining is done in 96 well V-bottom plates, 5 ml is typically an appropriate volume to resuspend cells. Single cell suspension of villus epithelium is also suitable for non-flow cytometry applications including sequencing or protein analysis.

15. Transfer tissue to a new tube containing 20 ml pre-warmed rinse buffer. Incubate with rocking at 37 °C, 80 rpm for 5–10 min to rinse off any EDTA that could inhibit the digestion. While incubating prepare digestion buffer (step 16).

Tissue should now appear thin and slightly pink in color. Very pale appearance is generally associated with high degree of type 2 inflammation and will result in mostly dead cells using this protocol.

16. Add enzymes (amount calculated using the accompanying spreadsheet: 100 µl per sample 5 mg/ml Liberase TM stock, 15 µl per sample DNaseI stock solution) to digestion buffer, mix gently, and for each sample, add 1 ml of digestion buffer to a gentleMACS C tube.

17. Discard supernatant from step 15 and transfer tissue to C tube containing 1 ml digestion buffer. Mince tissue with blunt end scissors to avoid damage to the C tube. Rinse scissors while adding the additional 4 ml of digestion buffer to the minced tissue. Between samples, rinse scissors by dipping in a container with a large volume of water and clean with paper towel.

gentleMACS C tubes can be re-used multiple times by rinsing in water and ethanol for a few hours. In this case, tubes should be labeled with tape rather than a lab marker.

18. Digest tissue with rocking at 37 °C, 120 rpm for 30 min.

19. Place C tubes on gentleMACS Octo Dissociator and run protocol "Intestine".

20. Briefly centrifuge the C tubes at 1500 rpm, 4 °C, to remove any liquid from the lids. Place tubes on ice and add 5 ml of ice cold FWB. Strain cell suspension through a 100 µm MACS SmartStrainer into a 15 ml conical tube; rinse C tube and strainer with an additional 5–10 ml of FWB.

SmartStrainers can also be re-used multiple times—rinse in water or reuse only the plastic support, using another brand of 100 uM nylon mesh strainer on top. If no gentleMACS Octo Dissociator is available, use 25 or 50 ml tubes to digest tissue. Mince tissue finely prior to digestion using long blunt

end scissors, vortex repeatedly after digest period, and strain any visible tissue pieces through the 100 uM nylon mesh strainer using the flat head of a 1 ml syringe.

21. Spin cell suspension at 1500 rpm for 5 minutes, 4 °C. Decant supernatant and blot excess liquid.

Optional: To remove debris and dead cells, resuspend pellet in 5 ml 40% Percoll solution, centrifuge at 2000 rpm for 10 minutes with acceleration/deceleration set at 3–4. Aspirate supernatant, including the top layer containing debris and dead cells, and wash the pellet containing live lamina propria cells once using 15 ml FWB.

22. Resuspend pellet to a final volume of 0.5 ml FWB, filter through filtercap FACS tubes and transfer required volume to plate or tubes for staining as described below.

Proceed directly to analysis by flow cytometry to minimize cell death. Red blood cell lysis is not necessary. Percoll gradient separation of live and dead cells is also not typically necessary if appropriate live/dead cell dye will be used for flow cytometry.

This digest typically yields several millions of viable immune and non-immune cells. This single cell suspension is also suitable for non-flow cytometry applications including sequencing or protein analysis.

Analyze tuft cell frequency and ILC2 activation by flow cytometry (plate-based staining)

Materials—Epithelial and/or lamina propria cell single cell suspension

Anti-EpCAM PerCPCy5.5 (Biolegend 118219)

Anti-SiglecF AF647 (BD Biosciences 562680)

Anti-CD24 PECy7 (Biolegend 101822)

Anti-CD45 BUV395 (BD Biosciences 564279)

DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) (Sigma 10236276001)

Fixable live-dead stain (e.g. Life Technologies L-34964)

Anti-DCLK1 (DCAMKL1) (Abcam ab31704)

Donkey anti-rabbit IgG secondary antibody (AF488: Life Technologies A-21206; PE: Biolegend 406421)

Anti-Thy1.2 BV785 (Biolegend 105331)

Anti-human CD4 (PE Biolegend 300508; APC Biolegend 300514)

Anti-IL-17RB APC (Biolegend 146308)

Anti-KLRG1 eFluor710 (ThermoFisher 46-5893-82)

Anti-Gata3 PE (ThermoFisher 12-9966-42)

Anti-Ki67 FITC (ThermoFisher 11-5698-82)

Fc receptor blocking antibody (Bio X Cell BE0307)

Pacific Blue Lineage cocktail (see Reagents and solutions)

16% Paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences15710)

PBS (Thermo Fisher 14190250)

eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher 00-5523-00)

Counting beads (ThermoFisher C36950)

Equipment and consumables—V-bottom plate

Multichannel pipet and tips

Pipet basin

Vortex Mixer

Tube racks

Refrigerated table-top centrifuge

Microcentrifuge tubes (for staining master mix)

1.2 ml microtiter tubes and box (USA Scientific 1412-1000)

Flow cytometer (minimum 8 color)

Analysis software (Flowjo 10)

Solutions (See Reagents and Solutions)—FWB - stock solution described in reagents

4% PFA - stock solution described in reagents

FC block stock solution

DAPI 1 mg/ml stock solution

Tuft cell staining cocktail (surface)

ILC2 staining cocktail (surface)

Surface staining—All centrifugation is performed at 4 °C.

1. Transfer approx. 250 µl of intestinal epithelial or lamina propria single cell suspension (from basic protocol 1 steps 14 and 22) to 96 well V-bottom plate. Pellet cells in table top centrifuge at 3000 rpm for 1 min. Discard supernatant by flicking and pressing against paper towel. Cell pellet is readily visible in the V-bottom plate.

   In our experience 2 or more stainings can be done with lamina propria cells isolated in Basic Protocol 1.

2. Prepare enough volume of Fc receptor (FC) block in FWB (1 µl 1 mg/ml FC block stock solution in 50 µl of FWB per sample), and add 50 µl to each sample. Pipet with multichannel to mix.

3. Prepare tuft cell (for the epithelial fraction, see: Tuft cell staining cocktail (surface): Final dilutions in the Reagents and Solutions section) and/or ILC2 staining cocktail (for the digest fraction, see: ILC2 staining cocktail (surface): Final dilutions in the Reagents and Solutions section) in FWB using twice the final concentration of antibody. Add 50 µl to the appropriate wells. Mix with a multichannel. Final staining volume is 100 µl.

   Depending on the research question, it may be interesting to perform tuft cell staining, ILC2 staining, or both. Volumes can be cut in half to conserve reagents; we recommend assessing staining in 100 µl before reducing volumes, particularly for the epithelial fraction. It is necessary to prepare the staining master mix at twice the final antibody dilution to account for the fact that cells are in 50 µl of Fc block solution, and the staining master mix is directly added 1:1 to cells suspended in 50 µl of Fc block (step 2) to avoid an extra centrifugation step.

4. Incubate for 15–30 min on ice in the dark.

   Staining time and antibody concentration may be dependent on volume and number of cells. If staining is routinely very dim, use fewer cells or stain in larger volumes (e.g. in FACS tubes).

5. Pellet cells at 3000 rpm for 1 min. Discard supernatant and use multichannel to wash pellets with 200 µl FWB. Spin plate again, and discard supernatant..

6. Donor mouse-dependent: If tuft cells or ILC2s will be identified using fluorescent reporter alleles (e.g.: Flare25 or Red5), prepare 200 µl/sample of FWB with DAPI 1 µg/ml (1:1000 dilution of stock solution) and counting beads (10–20 µl per sample). Resuspend cells in 200 µl FWB/DAPI/bead solution and transfer to 1.2 ml racked microtubes in box using multichannel for direct acquisition on a flow cytometer.

7. For intracellular staining of DCLK1 (tuft cells), or Ki-67 and GATA-3 (ILC2), skip step 6 and wash cells once with PBS after step 5.

Be careful to avoid contaminating any material with DAPI when doing intracellular staining as cells will otherwise be brightly stained following permeabilization.

8. Stain cells with fixable live/dead at 1:500 in 20 µl/well for 15 min on ice in the dark.

   Any commercially available fixable live/dead dye can be used according to manufacturer's instructions.

9. Spin the plate and wash once in FWB.

10. Fix cells

    a. For DCLK1 staining, fix cells in 200 µl 4 % PFA in PBS per well for 5–10 min.

       i. After PFA fixation, spin plate and discard fixative. Resuspend in FWB and store over night at 4 °C in the dark for staining on the next day or proceed to step 11 for immediate intracellular staining.

          4% PFA/PBS solution can be stored at 4 °C in the dark for up to four weeks.

    b. For ILC2 identification by transcription factor staining, or for Ki-67 analysis, fix in diluted FoxP3 staining buffer kit Fixation/ Permeabilization buffer

       i. Dilute 4x Fixation/Permeabilization Concentrate using provided Fixation/Permeabilization Diluent

       ii. Fixation can be as short as 10 min or performed overnight at 4 °C. After fixation, spin plate and discard fixative. Resuspend in FWB and store over night at 4 °C in the dark for staining on the next day or proceed to step 11 for immediate intracellular staining.

           Any endogenous fluorescence (such as reporters) will be quenched.

11. Dilute Permeabilization (perm) buffer in water, 1:10.

    1x buffer can stored at 4 °C. All subsequent intracellular stainings and washes are done using this 1x perm buffer.

12. Spin plate and wash cells once in 200 µl 1x perm buffer. Then stain for intracellular proteins

    a. For tuft cells, dilute rabbit anti-DCLK1 1:1000 in 1x perm buffer.

    b. For ILC2s, dilute anti-GATA3 and Ki-67 1:300 in 1x perm buffer.

13. Incubate cells in primary antibody for a minimum of 20 min.

14. Spin plate and wash 1x in perm buffer

    a. For tuft cells: Resuspend in anti-rabbit secondary (e.g. Invitrogen donkey anti-rabbit AF488 at 1:4000 or Biolegend donkey anti-rabbit PE 1:1000 in 1x perm buffer). Stain for 10 min.

        Staining for longer than 20 min will increase background.

    b. Spin plate and wash once in 1x perm buffer

15. Resuspend in 200 µl FWB buffer and transfer to 1.2 ml racked microtubes in box using multichannel for acquisition on a flow cytometer.

    Inclusion of counting beads is recommended, however quantification in fixed samples may be different from those obtained with fresh samples, i.e. using reporter alleles.

    Representative data for tuft cell and ILC2 gating from a successful and failed prep are found in Fig. 2. Notably, unlike other tissues, activated ILC2s in the small intestine often show low expression of Thy1, and usually express high levels of KLRG1 (see (Schneider et al., 2018) for more detailed gating and marker expression data). After gating on singlets and lymphocytes, we gate on live/lineage negative CD45+ cells and identify ILC2s as KLRG1 high and Red5+ (if using reporter mice) or GATA3+ (Fig. 2b) if using intracellular transcription staining. Smart13 (human CD4 reporter) is stained together with the rest of the surface staining cocktail and can be analyzed on both fresh and fixed cells. Staining for IL17RB, the IL-25 receptor, is optional: though it can be dim, it is specific for small intestinal ILC2.

Alternate Protocol 1: *Ex vivo* analysis of small intestinal tuft cells and ILC2 by flow cytometry in the context of type 2 inflammation

This alternate protocol was optimized by Victor Cortez in the Locksley lab for use in models with intestinal type 2 inflammation, as occurs in mice with increased levels of IL-13 from ILC2s due to experimental manipulations (many of which are described in subsequent protocols). This protocol is rapid and in unmanipulated mice can be used in place of Basic Protocol 1 at the investigator's discretion. A major difference in Alternate Protocol 1 and Basic Protocol 1 is the shorter total incubation time. Reduced release of epithelial cells in Alternate Protocol 1 can lead to increased abundance of epithelial cells in the final digested fraction as compared to Basic Protocol 1 (see also section 'Critical parameters and Troubleshooting').

Speed is essential in preserving viability of small intestinal tissue under type 2 inflammatory conditions. An experienced user can complete 6 intestinal preps using this protocol in less than 2.5 hours if all the buffers and tubes are prepared in advance. We recommend working with additional personnel to process more than 6 tissues. Prepare all tubes and reagents, including surface staining master mix, in advance. Pre-warm all solutions to 37 °C. As in

Basic Protocol 1, we usually add enzymes after all tissues are harvested and before the processing (step 2). When possible, use littermate and sex-matched mice.

Materials and reagents

All reagents, equipment and consumables described in Basic protocol 1 are utilized in Alternate Protocol 1. However, buffer 1 and 2 differ in their composition compared to Basic protocol 1.

Donor mice – protocol is suitable for mice at any developmental age

Suggested tuft cell reporter lines: Flare25 (*Il25*$^{\text{flox-tdTomato}}$, not commercially available, inquire with Dr. Richard M. Locksley at UCSF), TRPM5-eGFP (not commercially available, inquire with Dr. Robert F Margolskee at the Monnell Center)

Suggested ILC2 reporter lines: Smart13 (*Il13*$^{\text{hCD4}}$, available at Jax: B6.129S4(C)-Il13tm2.1Lky/J

Stock No: 03136), Red5 (*Il5*$^{\text{tdTomato-Cre}}$, available at Jax: B6(C)-Il5tm1.1(icre)Lky/J

Stock No: 030926)

PBS (Thermo Fisher 14190250)

HBSS w/o calcium and magnesium (ThermoFisher 14175103)

HBSS (ThermoFisher 14025134)

DTT (Sigma D0632-5G)

EDTA (ThermoFisher 15575020)

Fetal calf/bovine serum (FCS/FBS)

HEPES (Sigma H3537-100ML)

Roche LiberaseTM (Sigma 5401119001 2× 5mg)

DNase I, grade II from bovine pancreas (Sigma 10104159001 100mg)

70% ethanol

Percoll (Sigma P4937-500ML)

Equipment and consumables

Squeeze bottle

50 ml syringe

High contrast glass rod (see protocol for preparation): Trypan blue solution (Sigma T8154) and Natelson tube (Kimble Chase, 42F604)

Gavage needle (e.g. Harvard Apparatus 75-0286)

Dissection board and tools (e.g.: scissors and two curved forceps)

50 ml conical tubes (we recommend flip-top tubes such as Nunc EZFlip Conical Centrifuge Tubes, ThermoFisher 362696)

100 um filters, such as BD Cell Strainers 100 um nylon mesh filters Fisher 352360

gentleMACS C Tubes Miltenyi Biotec 130-096-334

15 ml conical tubes (BD Falcon or similar)

MACS SmartStrainers (100μm) Miltenyi Biotec 130-110-917

1 ml syringes, such as TB syringe slip tip (no needle) Fisher 309659

Filtercap FACS tubes (e.g Falcon 352235)

Spray bottle

Ruler with centimeter markings

Flat edge razor blade (such as Fisherbrand Razor Blades 12-640)

Plastic dish with raised edges, such as 15 cm cell culture dish

Pipet box lid, or other flat raised surface

Kimwipes

Vortex mixer

Tube racks

Bunsen burner

Euthanasia chamber and CO_2 gas

Rotating incubator

Refrigerated table-top centrifuge

gentleMACS Octo Dissociator (Miltenyi Biotec 130-095-937)

Solutions (see Reagents and Solutions and calculation spreadsheet)

Buffer 1: EDTA/DTT wash 5% serum

Buffer 2: EDTA/DTT wash 2% serum

Rinse Buffer

Digestion Buffer

FACS wash buffer (FWB)

5 mg/ml Liberase TM stock solution

10 mg/ml DNaseI stock solution

1. Perform the following preparations prior to the dissection of experimental mice (Fig. 1a).
   a. Prepare high-contrast glass rod as described in Step 1 of Basic Protocol 1
   b. For each sample, prepare one 50 ml conical tube with 20 ml of Buffer 1 (with DTT) in each tube and store at 37 °C.
   c. Prepare a 12 well plate filled with cold PBS for storing intestine samples; keep on ice.
   d. Prepare a squeeze bottle with cold PBS, fill a 50 ml syringe with cold PBS and gavage needle attached, and pre-chill on ice
   e. Place a Kimwipe over the pipet box and wet with PBS. Prop the pipet box/imwipe on the edge of the plastic pan to collect waste (Fig. 1a).

      This material will only be used after the dissection.
   f. Prepare a surface to align cut, but not cleaned or fileted, tissue during dissection.

      Keep this surface cold and covered in PBS. This material will only be used after the dissection.
2. Euthanize mouse following institutional guidelines.

   We generally use CO_2.
3. Dissect open peritoneal cavity to expose intestines. Cut below the stomach at the pyloric sphincter and use forceps to carefully pull small intestine from mesentery. Avoid tearing.

   Critical step: Any tears in this step will result in more cell death.
4. Align the proximal most piece of small intestine with the ruler, remove and discard the most proximal 2 cm. Harvest the next 6 cm to obtain sample of proximal small intestine (or any other similarly sized segment of small intestine). Attempt to use identical lengths of intestine for each sample.
5. **Critical step:** Store intestine sample in the 12 well plate in PBS on ice until all samples have been isolated

   In our experience, keeping tissue intact as long as possible improves viability greatly.

6. When all tissue has been dissected, begin processing as described in Basic protocol 1 (Part 1) steps 5-7

   Critical step: Avoid tearing tissue

7. After cutting open and rinsing luminal side, transfer tissue to pre-warmed buffer 1 (step 1a) and incubate with rocking at 37 °C, 80 rpm for 15 minutes.

8. Vortex each tube aggressively (max speed) for 30 seconds, and decant supernatant (including epithelial cells) through a 100 μm cell strainer into a 50 ml tube containing 10 ml cold FWB and store on ice.

9. Add 10 ml of prewarmed buffer 2 to the sample tube containing the gut tissue and incubate with rocking at 37 °C, 80 rpm for 15 min.

10. Vortex sample aggressively (max speed) for 30 seconds, and decant supernatant through 100 μm filter into 50 ml conical on ice. Rinse filter with approximately 5 ml of ice cold FWB.

11. Centrifuge at 1500 rpm for 5 min, 4 °C, and discard supernatant. Resuspend pellet in 2 ml of FWB and proceed to stain for tuft cells as described in the second half of protocol 1.

    a. Proceed to stain for tuft cells as described in Part 2 or continue on with lamina propria cell isolation

       Optional: wait until completing digest to pool EDTA wash and digest fractions at 1:1 ratio (see also section 'Critical parameters and Troubleshooting').

12. Add 20 ml of prewarmed Buffer Rinse to each sample tube (containing remaining gut tissue). Incubate with rocking at 37 °C, 80 rpm for 5 minutes.

13. Add enzymes to digestion buffer, mix gently, and for each sample, add 1 ml of to a gentleMACS C tube.

14. Discard supernatant from step 16 and transfer tissue to C tube containing 1 ml digestion buffer. Mince tissue with blunt end scissors to avoid damage to the C tube. Rinse scissors while adding the additional 4 ml of digestion buffer to the minced tissue.

    Between samples, rinse scissors by dipping in a container with a large volume of water and clean with paper towel.

    GentleMACS C tubes can be re-used multiple times by rinsing in water and ethanol for a few hours. In this case, tubes should be labeled with tape rather than a lab marker.

15. Digest tissue with rocking at 37 °C, 120 rpm for 10 min.

16. Follow steps 19–22 of Basic Protocol 1 (Part 1)

    For flow analysis, stain as described in Part 2 of Basic Protocol 1. See representative data in Fig. 3 for example of expected viability in mixed

epithelial/digest (for tuft cell staining) and digest alone (for ILC2 staining) from small intestine on day 9 of N. brasiliensis infection.

Basic protocol 3: Enumeration of tuft cells by imaging intestinal swiss roll

Examination of tuft cells by imaging can be done in tandem with flow cytometric approaches, and is often a more reliable way to observe tuft cell expansion at early timepoints following stimulation, when they are beginning to emerge in the intestinal crypt. We also recommend performing imaging in tandem with flow cytometry due to the problems with cell viability during tissue processing for flow cytometry. Any segment of small intestine or colon of more than 4 cm can be prepared in swiss roll for tuft cell staining.

Materials

Donor mice and dissection materials as described in Basic protocol 1

16% Paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences15710)

PBS (Thermo Fisher 14190250)

Anti-EpCAM AF488 (BioLegend 118210)

DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride) (Sigma 10236276001)

Rabbit anti-DCLK1 (DCAMKL1) (Abcam ab31704)

Goat anti-rabbit IgG secondary AF555 (ThermoFisher A21428)

TSA Blocking Reagent (PerkinElmer FP1012 (10 g))

Sucrose (Sigma S7903)

Optimal Cutting Temperature (OCT) Compound Tissue-Tek (VWR 25608-930)

Dry ice

Goat serum (Sigma G9023-10ml)

Vectashield (Vector Labs H-1000, or other fluorescence microscopy mounting medium)

Equipment and consumables

Cryostat

ImmEdge Hydrophobic Barrier PAP Pen (Vector Laboratories H-4000)

Aspirator

Polarized glass slides

Kimwipes (Fisher 06666A)

Toothpicks

Cryomolds (such as VWR Sakura Cryomold 15×15×15mm #4566)

Bench pad (VWR 56616-018)

Coverslips

Whatmann paper (VWR GE Healthcare 3030153 Whatman 3MM Chr Chromotagraphy Paper, 17.5cm length,15 cm width) cut to 10 cm square or circles

6 or 12 well tissue culture plate

Confocal or fluorescence microscope

Solutions (See Reagents and Solutions)

4% PFA - stock solution described in reagents

DAPI 1 mg/ml stock solution

TNB blocking buffer

30% sucrose

1. Harvest and prepare approximately 6–8 cm of small intestine as described in Basic Protocol 1 (steps 1–7). Replace Kimwipe with PBS-soaked filter paper (Fig. 1d). After fileting with razor blade and rinsing luminal contents with cold PBS, fold filter paper over the tissue and submerge in fixative (4% PFA solution) (Fig. 1e). This creates a flat tissue amenable to swiss rolling.

   Fixing between filter paper is particulary helpful when tissue is affected by strong type 2 inflammation as the tissue tends to curl inwards, which hampers formation of a nice swiss roll. Tissue from naïve mice may be directly immersed in fixative.

2. Fix for 2–3 h at 4 °C with gentle shaking. Alternatively, fixation at room temperature for several hours or at 4 °C overnight works equally well.

   Based on the experience of multiple lab members, fixation time is very flexible.

3. Wash three times in PBS.

   Stopping point: Tissue can be kept in PBS for an extended period at 4 °C, without shaking.

4. Replace PBS with 30% sucrose solution and incubate overnight with shaking at 4 °C

   Stopping point: Tissue can be kept in 30% sucrose for several days, removed from shaking.

5. "Swiss roll" following the technique demonstrated in Bialkowska et al (Bialkowska, Ghaleb, Nandan, & Yang, 2016), and embed tissue as follows:

    a. Label cryomolds and add OCT to each one

    b. Wet bench pad with sucrose and place tissue, serosal side facing up, on the wet pad.

    c. Rolling from top to bottom, roll tissue over the toothpick. Edges should be flush, and not "telescoping." Rolling too tightly will make removal of toothpick challenging.

    d. Place rolled tissue plus toothpick in OCT. Using forceps, gently push tissue down while removing toothpick. Add additional OCT to cover tissue

        We find that placing the roll in the corner of the mold can prevent it from tipping over. Avoid bubbles in OCT, as this will impair cutting

6. Freeze on a very flat piece of dry ice. Store at −80 °C in a bag or a box.

    Stopping point: Long-term storage of OCT-embedded fixed tissue is common.

7. Equilibrate blocks in cryostat with temperature set to −20 °C for at least 30 min before starting to cut. Cut several 8 μM sections, putting 2–3 on same slide if desired. Store slides at −80 °C or stain on the same day when sectioning is completed.

8. Air dry in black slide staining box 15 min at room temperature.

9. Scrape excess OCT off using clean razor blade, and outline section with wax PAP pen

10. Add 200 μl PBS to each section to rehydrate for 5 min.

    Here and in all subsequent steps, it is critical to not detach the tissue section from the slide. Do not pipet solutions directly on the section.

11. Aspirate off PBS and add 100 μl blocking solution to each section

    Use appropriate serum for blocking (host species of secondary antibody). We suggest 5% goat serum in TNB for the reagents used here. PBS instead of TNB appears to work equally well.

12. Incubate 1 h, room temperature

13. Aspirate off block

14. Add 100 μl primary antibody solution (1:1000 rabbit anti-DCLK1, 1:300 directly conjugated anti-EpCAM AF488 diluted in TNB) and incubate 1 h at room temperature.

15. Aspirate off primary antibody.

16. Wash with gentle shaking 5 min PBS.
17. Incubate 40 minutes with 100 µl secondary antibody solution (1:1000 goat anti-rabbit AF555 diluted in TNB).
18. Aspirate off and wash 5 min in PBS.
19. Incubate 5 min with 1:1000 DAPI in PBS.
20. Aspirate, and wash in PBS as above.
21. Aspirate off PBS, and dry excess PBS from slide using a Kimwipe without touching the tissue section.
22. Add one drop of Vectashield and cover with coverslip. Proceed with imaging or keep slides at 4 °C.

    For best results, we recommend to image on the same day.
23. Enumerate tuft cells per villus. See representative data from von Moltke et al (2016) in Figure 1a, Figure 2 a-d (J. von Moltke et al., 2016).

Basic protocol 4: Tuft – ILC2 circuit activation by oral gavage of *N. brasiliensis* adult worms

When infection with *N. brasiliensis* is performed via subcutaneous injection of the L3 larval stage, the typical infection route for this model helminth in mice, it induces a robust activation of the tuft cell–ILC2 circuit shortly after arrival of L4 stages in the small intestine, but also causes significant alveolar damage and induces a strong pulmonary type 2 inflammation (Bouchery et al., 2017). To bypass other worm life cycle stages and to focus exclusively on the local, tuft cell-driven response in the small intestine, mice can be infected with adult worms by oral gavage. This enables precise temporal control over the presence of luminal worms.

As noted in the section 'Critical parameters and Troubleshooting', we strongly recommend screening of experimental mice for Tritrichomonas. The screening procedure is described in Basic Protocol 4.

The use of cold buffers will reduce worm motility and delay the isolation procedure from rat intestine and should therefore be avoided.

Materials

Donor rats: 4 weeks old male Lewis rats (Jackson, LEW/Crl)

Recipient mice: 8–16 weeks-old

N. brasiliensis L3 larvae

Isoflurane

PBS

Deionized water

Penicillin/streptomycin 100x solution (Pen/Strep) (Gibco, cat No 15140122)

Equipment and consumables

Dissection tools (e.g. tweezer, scissor)

Artery scissors with ball tip (Fine Science Tools 14080-11)

Beaker

15 cm petri dish (e.g. P5981-100EA, Sigma)

50 ml conical centrifugation tubes (e.g. BD Falcon)

Desk lamp

1 ml syringe (e.g. Injekt-F, 0.01 ml/1.0 ml, BRAUN)

Isoflurane anesthesia apparatus for mice and rats

Mesh/filter fabric (e.g. Sefar Nytal, pa 5xxx 250 115cm)

Mesh-filter apparatus (see protocol for preparation)

Intravascular catheter (e.g. Cat 381247, BD Insyte)

37 °C water bath to pre-warm solutions

Stereomicroscope

1. Follow published protocols for establishment and maintenance of *N. brasiliensis* (Bouchery et al., 2017).
2. Collect *N. brasiliensis* infective third-stage larvae (L3) and infect two rats with 5000 L3 each.

   From two infected rats, more than 4000 adult worms can be harvested, sufficient for 40 recipient mice.
3. 7 days post infection, sacrifice the rats, isolate the small intestine and transfer to a 15 cm petri dish containing 100 ml PBS and penicillin/streptomycin solution (1x).

   Antibiotics will prevent overgrowth of bacteria during an extended incubation time. Pen/strep will be removed during subsequent washing steps to avoid microbiota perturbation in the recipient mice.
4. Cut open the intestine longitudinally using artery scissors with ball tip. After opening the gut, the adult worms are apparent as luminal red clusters and visible by eye or using a stereomicroscope.

5. Carefully transfer the tissue and PBS to a self-made mesh-filter apparatus containing 500–800 ml PBS (pre-warmed to 37 °C).

    a. For making the mesh-filter apparatus, adhere the mesh to a wire basket with a large bottom surface area. Tightly fix the mesh with tape and place the whole apparatus into a large glass dish. Make sure there is a support (e.g. a petri dish) in the large glass dish for apparatus to sit on, so that the apparatus is not directly on the bottom of the dish (schematized in Fig. 4a).

    b. Pour the solution containing tissue and worms slowly when transferring to the mesh apparatus.

6. Incubate for 40–60 min at RT under a desk light (Fig. 4b). During this time adult worms will pass through the mesh and accumulate at the bottom of the glass dish.

    Exposure to light stimulates the motility of worms, which migrate out of the tissue into the solution. This can be observed by eye or using a stereomicroscope. In our hands, a majority of worms have passed through the mesh within about one hour. Extended incubation up to several hours is possible and does not impair the viability of the worms.

7. Carefully remove the mesh and tissue on top, and transfer the PBS containing adult worms into a beaker and wait for them to sediment to the bottom.

    Sedimentation typically takes 2–3 min.

8. Once the worms have completely sedimented, carefully aspirate the supernatant.

9. Fill the beaker with 100 ml of pre-warmed PBS to wash the worms.

10. Repeat steps 8–9 twice.

    The washing steps are important to remove leftover tissue, intestinal content, bacteria and Pen/Strep.

11. After the final washing step, add 50 ml pre-warmed PBS and pour the solution containing the worms into a 15 cm petri dish.

    Depending on the size of the worm cluster, it may be distributed to 2 or more plates, which will save time during the next step.

12. Incubate for 40–60 min under a light to allow the worms to disperse (Fig. 4c). During the incubation, move large worm clusters to less dense areas; avoid shaking the plate during the incubation.

13. For each recipient mouse, transfer 100 worms into a 5 ml FACS tubes using a P1000 pipette (Fig. 4d). For accuracy, we prefer individually counted aliquots. However, if well dispersed, worm concentration can be estimated by counting them in a small volume and appropriate volumes containing approximately 100 worms may be then be distributed to 5 ml FACS tubes.

Conical bottom tubes are not recommended because the adult worms tend to cluster quickly, which may clog the gavage needle.

14. Mount a plastic gavage needle with a wide orifice to a 1 ml syringe and aspirate the worm pellet into the syringe.

For the plastic gavage needle, we use the tube of an intravascular catheter, which has a wide orifice (Fig. 4e).

15. By inverting the syringe, worms are easily sedimented by gravity. Remove air bubbles and adjust the volume to 200 µl.

16. Restrain the mouse and carefully infect with adult worms by oral gavage with the syringe containing worms (Fig. 4e).

This step should be performed immediately to avoid the formation of worm clusters, which can also be further prevented by agitating the syringe (e.g.: by inversion).

17. Two days later, sacrifice mice and isolate cells from the duodenum. For flow cytometry analysis, perform the isolation of epithelial and lamina propria cells using Alternate Protocol 1, and stain as described in Part 2 of Basic Protocol 1. Alternatively, or concurrently, analyze tuft cells by imaging as described in Basic Protocol 2.

See Fig. 5 for representative FACS plots showing ILC2 activation (proliferation and IL-13 reporter expression) following colonization.

Basic protocol 5: Circuit activation by colonization with *Tritrichomonas* spp.

Mice from different animal facilities, and even different strains within one facility, can have highly variable tuft cell percentages in the small intestine, with accompanying variation in ILC2 activation state. A simple explanation for this is often the presence or absence of the protist *Tritrichomonas,* which is an effective driver of the tuft cell – ILC2 circuit via production of succinate (Howitt et al., 2016; Nadjsombati et al., 2018; Schneider et al., 2018). Experimental colonization with *Tritrichomonas* has proven a simple and robust way of assessing various components of this cellular circuit in the small intestine.

As described in section 'Critical parameters and Troubleshooting', we strongly recommend screening of experimental mice for Tritrichomonas. While there may be facility to facility variation in the specific protist species, gross inspection of cecal content appears sufficient to confirm the presence of Tritrichomonas spp.; results from labs at distinct locations suggest that inter-vivarium differences in species are unlikely to have major impacts on tuft cell – ILC2 circuit activation. Here we describe both simple screening for cecal protozoans as well as colonization of naïve mice with Tritrichomonas.

Materials

Donor mice: *Tritrichomonas* spp.-colonized mice

Many colonies in standard barrier animal facilities often have Tritrichomonas unless there is a strict rederivation policy for importing mice. If the latter is the case, it is possible there will be no internal source of the protist. In this case, a dissected cecum from a Tritrichomonas-colonized donor mouse can be shipped in PBS on wet ice and used for colonization.

Recipient mice

Reporter mice, as noted in Basic Protocol 1 materials, can aid in analysis of ILC2 activation, but must be *Tritrichomonas* negative

Cold PBS

Equipment and consumables

100 μm cell strainers

50 ml conical centrifugation tubes

Pipette

Gavage needle (e.g. Harvard Apparatus 75-0286)

Hemocytometer

Glass slide

Centrifuge

Microscope

Disposable polypropylene feeding tubes (Instech Laboratories, FTP-20-38).

1. Euthanize the *Tritrichomonas*-colonized donor mouse. Typically, one cecum contains 100–200 million protists.
2. Carefully cut open the cecum and squeeze out the luminal contents directly into a 50 ml conical tube.

   If simply checking for the presence of Tritrichomonas, it is sufficient to scoop a small sample of cecal content using a 200 μl pipette tip, mix in 100 μl PBS and perform step 4.
3. Resuspend well using cold PBS and a 10 ml pipette. Fill up to 50 ml with cold PBS and mix thoroughly.
4. Pipette 10 μl of the solution on a glass slide and check under a regular brightfield microscope (20x) if flagellated *Tritrichomonas* protists are present (Fig. 6).

   When observed under a microscope, Tritrichomonas protists are observed as teardrop shaped large eukaryotic cell-sized structure that are in constant tumbling motion. In negative cecal content, undigested dietary components and rod-shaped bacteria are visible.

5. Filter the cecal content suspension from step 3 through a 100 μm cell strainer into a new 50 ml conical tube.
6. Centrifuge at 1000 rpm for 10 minutes.
7. A pellet of several mm in height should be visible, which consists of protists.
8. Resuspend the pellet with PBS using a 10 ml pipette. Fill up to 50 ml with PBS.
9. Repeat steps 6–8 twice.
10. These washing steps greatly reduce the concentration of bacteria, as can be determined by qPCR for 16S rRNA.
11. Count the motile protists using a hemocytometer and dilute the pellet to the required concentration with PBS (e.g. 1 million/200 μl).
12. Colonize recipient mice with 10^6 protists by oral gavage using a regular gavage needle.
    a. We have observed complete colonization within a few weeks following transfer of as little as 20 protists. Generally, we colonize with 1–10 Million protists to reduce the time until complete colonization of the cecal niche.
    b. To mimic natural vertical transmission from the parents, 3–4 week old weanlings can be used. In that case, volume and gave needle size should be adjusted. In our hands, disposable polypropylene feeding tubes have been working well.
13. Sacrifice mice and isolate cells from the ileum 4 weeks later. Depending on the degree of tuft-ILC2 activation, either Basic Protocol 1 or Alternate Protocol 1 can be used. Perform staining and analysis as described above (representative data, obtained using Basic Protocol 1, can be found in Figure 5 (Schneider et al., 2018))

Basic Protocol 6: Circuit activation by treatment with succinate in drinking water

Small intestine tuft cells express the succinate G protein-coupled receptor SUCNR1 (Gpr91), and their activation and associated with this, that of ILC2s, can readily be achieved by supplying succinic acid in the drinking water of experimental mice.

As described in section 'Critical parameters and Troubleshooting', we strongly recommend screening of experimental mice for Tritrichomonas.

Materials

Mice: 4–16 weeks-old

Reporter mice, as noted in Basic Protocol 1 materials, can aid in analysis of ILC2 activation.

Succinic acid (Sigma S3674); similar results were reported using sodium succinate hexahydrate (Lei et al., 2018; Nadjsombati et al., 2018))

Deionized water

NaOH (such as Sigma S8045)

Equipment

Water bottles

Magnetic stirrer

1. If necessary, experimental mice can be accustomed to water bottles prior to the start of the experiment.
    a. If mice receive drinking water via an automated watering system, this step can avoid variation due to individual differences in adjustment of drinking behavior. It also helps to recognize leaking water bottles (see step 3).
2. Prepare 100 mM solution of succinic acid in deionized water. Mix thoroughly with a magnetic stirrer until fully dissolved.
    a. Succinic acid dissolves well in water at room temperature within just a few minutes.
    b. Optional: Adjust the pH to 5.5 using NaOH.
        i. Most animal facilities acidify the drinking water for mice to a pH of 2.5–3 to control Pseudomonas species. The pH of a 100 mM succinic acid solution is approximal 3.5 and therefore well tolerated by mice. It does not necessarily have to be increased; however, it should be matched to that of the control group's regular drinking water.
3. Provide mice with succinate water for 4 days. Check water level daily as water bottles can occasionally leak.
    a. Mark the bottles or cages accordingly to avoid replacement with regular water by animal caretaker or other research personnel. Succinic acid solutions are completely transparent and cannot be distinguished from regular water by eye.
4. After 96 hours, euthanize mice and proceed to dissection.
    a. Activation of the tuft-ILC2 circuit is rapid and robust. However, the level of activation is much lower than following helminth infection and we have successfully used Basic Protocol 1 and Alternate Protocol 1 for small intestine lamina propria cell isolation.
    b. Activation of ILC2s can already be detected on d3 or later. We have not tested shorter stimulation periods.

c. ILC2 activation can be detected in the proximal and distal small intestine and the preferred region may depend on the research question. We did not detect ILC2s activation in other intestinal regions (i.e. stomach, cecum, or colon) or non-intestinal tissues (lung)(Schneider et al., 2018).

5. Collect small intestinal lamina propria cells and perform the staining as described above (representative data, obtained using Basic Protocol 1, can be found in Figure 6d of (Schneider et al., 2018).

Basic protocol 7: Circuit activation by treatment with recombinant IL-25

In the small intestine, IL-25 is a critical mediator of type 2 responses to infection with helminths and colonization by protists. Here, we describe a reductionist method to activate small intestinal ILC2 responses using recombinant IL-25 (J. von Moltke et al., 2016). This approach is sufficient to activate ILC2s bypassing the need for luminal signals that activate tuft cells. Treatment results in an IL-4Rα-dependent acute increase in the frequency of small intestinal tuft cells.

Materials

Mice: 6–16 weeks-old

Reporter mice, as noted in Basic Protocol 1 materials, can aid in analysis of ILC2 activation

Recombinant IL-25 (R&D Systems, 1399-IL-025/CF)

1. Reconstitute lyophilized rIL-25 at 100 μg/ml in sterile PBS, aliquot and freeze stock at −80 °C.
2. Dilute stock in sterile PBS to a final concentration of 2.5 μg/ml.
3. Inject 200 μl (500 ng) per mouse intraperitoneally on days 0, 1, 2, and 3.
4. On day 4, euthanize mice and proceed to dissection.
   a. Activation of the tuft-ILC2 circuit is rapid and robust. We recommend using Alternate Protocol 1 if analyzing tuft cell expansion and/or ILC2 activation by flow cytometry.
5. Collect small intestinal lamina propria cells and perform the staining as described above (*See* Fig. 7 *for representative FACS plots showing increased tuft cell frequency following rIL-25 treatment*).

Reagents and Solutions

See also supplementary table "Buffer calculation sheets". Unless otherwise indicated, buffers may be stored at 4 °C for up to 1–2 weeks.

4% PFA

10 ml of 16% PFA

30 ml of PBS

Protect from light

30% sucrose

30 g sucrose

PBS to 100 ml

For long term storage of larger volumes, we recommend sterile filtration and handling.

Alternate Protocol 1: Buffer 1 (20 ml per sample)

5% FCS

10 mM HEPES

10 mM DTT (1:100 dilution of stock)

10 mM EDTA

HBSS no Ca/Mg

Alternate Protocol 1: Buffer 2 (10 ml per sample)

2% FCS

10 mM HEPES

10 mM DTT (1:100 dilution of stock)

10 mM EDTA

HBSS no Ca/Mg

Basic Protocol 1: Buffer 1 (20 ml per sample)

2% FCS

10 mM HEPES

5 mM DTT (1:200 dilution of stock)

HBSS no Ca/Mg

Basic Protocol 1: Buffer 2 (30 ml per sample)

2% FCS

10 mM HEPES

5 mM EDTA

HBSS no Ca/Mg

Buffer Rinse (20 ml per sample)

2% FCS

10 mM HEPES

HBSS (with Ca/Mg)

DAPI 1 mg/ml stock solution

10 mg DAPI

10 ml deionized water

Aliquot and store at −20 °C

Digestion Buffer (5ml per sample)

3% FCS

10 mM HEPES

30 ug/ml DNase (15 µl stock solution per sample)

100 µg/ml LiberaseTM (100 µl stock solution per sample)

HBSS (with Ca/Mg)

DNaseI Stock solution

100 mg lyophilized DNaseI

Reconstitute in 10 ml deionized water to make a 10 mg/ml stock solution

Aliquot 150 µl per tube and store at −20 °C

DTT 1 mM stock solution

5 mg DTT

32.4 ml deionized water

Aliquot and store at −20 °C

FACS wash buffer (FWB) 500 ml

500 ml PBS

3% FCS

0.5% sodium azide (optional; from 10 % sodium azide in H_2O stock solution)

FC block

Dilute 25 mg antibody to 1 mg/ml working stock with sterile PBS

Aliquot and store at −20 °C

ILC2 staining cocktail (surface): Final dilutions

Anti-Thy1 BV785 Biolegend 105331 1:1000

Anti-human CD4 PE Biolegend 300508; APC Biolegend 300514 1:50

For detection of IL-13 in Smart13 reporter mouse. If planning to perform intracellular staining for GATA3, make sure to choose non-overlapping fluorophore conjugates.

Anti-KLRG1 eFluor710 (ThermoFisher 46-5893-82) 1:300

Anti-IL-17RB APC (Biolegend 146308 1:100)

Pacific Blue Lineage cocktail 1:300 all

NK1.1 Biolegend 108722

DX5 Biolegend 108918

CD3 Biolegend 100214

CD4 Biolegend 100531

CD8α Biolegend 100725

Ter119Biolegend 116232

F4/80 Biolegend 123124

CD11b Biolegend 101224

CD11c Biolegend 117322

FcεR1α Biolegend 134314

CD19 Biolegend 115523

Gr-1 Biolegend 108430

Liberase TM Stock solution

50 mg lyophilized Liberase TM

Reconstitute in 10 ml deionized water to make a 5 mg/ml stock solution

Aliquot 500–1000 μl per tube and store at −20 °C

40% Percoll solution (5 ml per sample)

0.5 ml 10x PBS

2 ml Percoll

2.5 ml deionized water

TNB blocking buffer

0.1 M TRIS-HCl, pH 7.5

0.15 M NaCl

0.5% TSA Blocking Reagent (PerkinElmer FP1012 or FP1020)

Slowly dissolve blocking reagent in buffer with stirring, while heating to 55 °C. Store at 4 °C up to several weeks.

Tuft cell staining cocktail (surface)

Anti-EpCAM PerCPCy5.5 Biolegend 118219 1:300

Anti-SiglecF AF647 BD Biosciences 562680 1:300

Anti-CD24 PECy7 Biolegend 101822 1:4000

Anti-CD45 BUV395 BD Biosciences 564279 1:300

Commentary

Background information

Tuft cells have gained significant attention since the discovery that they promote type 2 responses in the small intestine as part of a feedforward circuit that is accompanied by a pronounced increased in their abundance in the epithelium (Gerbe et al., 2016; Howitt et al., 2016; J. von Moltke et al., 2016). These observations, followed by the identification of succinate as a metabolite signal—derived from the protist *Tritrichomonas*—for circuit activation (Lei et al., 2018; Nadjsombati et al., 2018; Schneider et al., 2018), also revealed critical effector mechanisms and uncovered novel ways to study the biology of tuft cells and ILC2s. Notably, the pioneering tuft cell work of the last several years was predicated on the initial studies identifying helminths and IL-25 as potent activators of ILC2s (Moro et al., 2010; Neill et al., 2010; Price et al., 2010), which resulted in a decade of intense research activity on the roles of innate type 2 immune responses and promoted the development of many exquisitely helpful genetic mouse tools, including the ones used in our protocols.

Our protocols represent a collection of methods, of which many were reported by us and others in prior studies. In addition to those mentioned above, Chudnovskiy & Mortha et al. optimized a protocol for the purification *Tritrichomonas* spp. (Chudnovskiy et al., 2016), that we modified in order to generate bacteria free-inocula of *Tritrichomonas* isolated from our UCSF mouse colonies, which were used to monocolonize germ-free mice (Schneider et

al., 2018). Infection with the widely used helminth model *N. brasiliensis* has been covered in detail in a prior *Current Protocols* article (Bouchery et al., 2017). Isolation and infection of mice with different *N. brasiliensis* life cycle stages was described by Harvie et al. (Harvie et al., 2010), and we recently reported that the gavage of adult worms potently activates the tuft cell–ILC2 circuit in the small intestine while bypassing the lung-damaging life cycle stages (Ricardo-Gonzalez et al., 2020). Notably, infection with this protocol efficiently transfers live worms that can establish persistent infections in mice with impaired type 2 immunity (X. Feng and C. Schneider, unpublished). A similar helminth-mediated acute and local activation of the circuit was recently reported by McGinty et al., who described induction of IL-13 expression in ILC2 within less than 24 h following oral gavage of *H. polygyrus* L3 and which was mediated by IL-25 and leukotrienes (McGinty et al., 2020).

Protocols for the isolation of lamina propria cells from intestine typically involve sequential steps of mucus depletion using reducing agents such as DTT, removal of epithelial cells with EDTA, and enzymatic digestion of the lamina propria (Sheridan & Lefrancois, 2012). Procedures for the isolation and detection of mouse intestinal ILCs were recently summarized in detail (Burrows, Chiaranunt, Ngai, & Mortha, 2020; Romera-Hernández, Mathä, Steer, Ghaedi, & Takei, 2019). However, the fast and extensive cell death when isolating intestinal lamina propria cells from tissues with strong type 2 immune activation, including following helminth infection, has greatly limited the study of cellular processes in the small intestinal tissue by subsequent downstream applications such as multiparameter flow cytometry and scRNA-seq, which rely on tissue dissociation and generation of single-cell suspensions. Two recent studies reported optimized protocols for isolating lamina propria leukocytes from the small intestine of *H. polygyrus*-infected mice (Ferrer-Font et al., 2020; Webster, Andrusaite, Shergold, Milling, & Perona-Wright, 2020). While these protocols vary from each other and from the protocol presented here in several aspects, key steps include gentle handling of tissues and enhanced processing speed achieved by shortening multiple steps of common methods of isolating lamina propria cells. While we have not directly compared our protocol with those by Ferrer-Font et al. and Webster et al., it is important to note that *N. brasiliensis* induces even more pronounced type 2 inflammation than *H. polygyrus* (Howitt et al., 2016; Schneider et al., 2018), suggesting that our protocol should also work for the latter situation.

Although not described here, tuft cells are present at low frequencies in intestinal 3D organoids cultured under published EGF, Noggin, R-spondin conditions (Mizutani & Clevers, 2020). Intestinal organoid cultures are amenable to investigating the molecular requirements for tuft cell differentiation, as has been done for conditions of increased IL-13 signaling through IL-4Ra^{+} progenitor cells (J. von Moltke et al., 2016). 3D intestinal organoid cultures can be maintained long term under appropriate maintenance culture conditions, including frequent passaging. To test induction of tuft cell differentiation under high IL-13 conditions, fresh or newly passaged cells should be stimulated with 20 ng/ml recombinant IL-13 on day one and day four following plating or passaging, as previously described (J. von Moltke et al., 2016). We advise utilizing tuft cell deficient donor mice, or reporter negative mice, as staining controls.

Critical parameters and Troubleshooting

We have noted multiple critical steps, particularly for Alternate protocol 1, throughout the method procedures, as well as in the strategic planning sections. Knowing whether the animals used are *Tritrichomonas*-colonized or not is critical for maximum sensitivity and success when performing *in vivo* circuit activation, given that ILC2s of colonized mice have higher basal ILC2 activity status and tuft cell frequencies. For this reason, and to reduce inter-cage and inter-experimental variability, we recommend the use of mice that are free of *Tritrichomonas* spp. Before working with a new strain, we suggest performing the basic visual screening described in Basic Protocol 4 to examine cecal contents for protists. To deplete these protists, various labs reported the use of highly concentrated metronidazole (2.5 g/L) in the drinking water. A three-week treatment regimen is typically sufficient to deplete protist completely, although this should be verified by visual inspection upon euthanasia. Because metronidazole broadly affects anaerobic bacteria and protozoa, such treatment might cause significant and long-lasting microbiota alterations in mouse colonies. To circumvent this, we developed a simple protocol to generate *Tritrichomonas*-free mouse colonies by fostering pups from colonized parents to clean dams (such as JAX C57BL/6J, Stock No: 000664). Because vertical transmission of *Tritrichomonas* spp. from the parents occurs when offspring start to show coprophagic activity, fostering can be started several days after birth and we have so far never observed protist contamination when pups were transferred to foster mothers up until postnatal day 7. Fostering is widely done by researchers and we do not aim to provide extensive information here. However, we generally try to use foster mothers which carry a representative microbiota (i.e. wild-type mice bred in-house) and with a litter of approximately similar age as the foster pups; additionally, we separate out the male breeder and most of the original pups, leaving 1–2 of the foster mother's own pups in the cage, which can later be used to screen for *Tritrichomonas*. Screening for *Tritrichomonas* is described in Basic Protocol 4. Notably, we have never observed inter-cage transmission of *Tritrichomonas*, and colonized and uncolonized mice can be housed in the same room/rack.

Low cell viability in flow staining—If low cell viability is an issue following Basic Protocol 1, switch to Alternate Protocol 1. Assess experimental mice for *Tritrichomonas* colonization. If using Alternate Protocol 1 to prepare tissue following manipulations described in Basic Protocol 2-5, low viability may be expected, and should correlate with highly expanded tuft cells and ILC2 activation. Work quickly, keep tissue cold, and avoid tearing the small intestine during dissection. Reduce the number of samples to enhance speed.

High variability experiment to experiment—We highly recommend using littermate or cage-matched mice, as possible. We caution against using vendor-purchased mice as "controls" for any mouse strains that are maintained in-house. Assess experimental mice for *Tritrichomonas* colonization. In addition, mouse age can strongly affect the percentage of proliferative (Ki-67$^+$) ILC2, which is very high after birth and decreases gradually over the first 2 months before reaching a plateau with less than 10% Ki-67$^+$ ILC2 in the small intestine lamina propria of a naïve mouse (Schneider, Lee, et al., 2019). Age-related

increases in small intestinal tuft cells have also been observed in *Tritrichomonas* positive mice (not tested in negative mice). Age-matching is therefore advised.

No tuft cell expansion following in vivo manipulations—While induction of IL-13 expression in ILC2s is rapid, effects on tuft cell frequencies typically require a few days. If using Alternate Protocol 1 to assess circuit activation shortly after induction of circuit activation when tuft cell differentiation is induced in the intestinal crypt, we have found that the expected tuft cell expansion can be challenging to observe; notably, there is an increased abundance of epithelial cells in the final digested fraction as compared to Basic Protocol 1, which may be enriched for crypt epithelial cells that are less efficiently washed off using the short protocol. As noted above, under these conditions and to get a representative epithelial composition, we recommend to pool EDTA wash and digest fractions at 1:1 ratio when staining for tuft cells. Additional validation by imaging (Basic Protocol 2) may be particularly important in this case.

No tuft cells observed using intracellular anti-DCLK1 staining—Always include the surface markers, CD24 and Siglec-F, as alternate markers of tuft cells in the small intestine. Remake PFA, and increase intracellular staining time.

Contact the authors for specific troubleshooting related to in vivo approaches. Questions regarding flow cytometry/compensation as well as microscopy should be directed to institutional facilities.

Understanding results

Expected results are indicated in each Basic Protocol section and shown in Figures 1–7.

Time considerations

Preparation of single cell suspensions of intestinal epithelial cells takes 2–2.5 hours for 8 samples (time needed prior to transferring all the samples to a V-bottom plate for staining). Dissection/organ collection takes 40–50 min, followed by approximately 60–80 min for washing/digestion for Alternate protocol 1. Basic protocol 1 takes 30–60 min longer.

The staining for analyzing tuft cell frequency and ILC2 activation by flow cytometry can be completed in 2 hours. Most staining and incubation times vary between labs and are relatively flexible without causing major differences in the final result. Additional time on the day of the experiment is required for live-dead staining and fixation if intracellular staining will be performed, but in this case fixed cells do not need to be analyzed immediately. We recommend planning to acquire almost the entire stained lamina propria sample—when resuspended in 200 ul, running on high speed this takes approximately 2 min. For the epithelial fraction we recommend acquiring a minimum of 500 tuft cells.

For enumeration of tuft cells by imaging, the entire protocol takes 24 hours. If done in parallel with collecting tissues for flow cytometry, samples for imaging can be stored on ice for at least an hour following dissection or should be processed by another person. To process 8 intestinal samples for imaging takes 10–20 min until fixation for 2–3 hours. The incubation of samples in 30% sucrose is done overnight and can be longer if convenient.

Basic protocol 3 requires 10 days, including maturation of *N. brasiliensis* larvae in rats and infection of mice by gavage of adult worms. Preparations on d0 take 2–3 hours. Harvesting of adult worms on day 7 and infection of mice takes 4–5 hours, followed by analysis 48 hours later.

The isolation and washing of *Tritrichomonas* spp. takes about 1 – 1.5 hours, followed by a 4 weeks colonization period.

Circuit activation through treatment with succinate in drinking water takes 5 days. On day 0, the succinate-containing water is provided to the mice and the small intestine is collected on day 4.

Circuit activation by treatment with recombinant IL-25 requires 5 days. rIL-25 is injected daily over 4 days and the small intestine is collected on day 4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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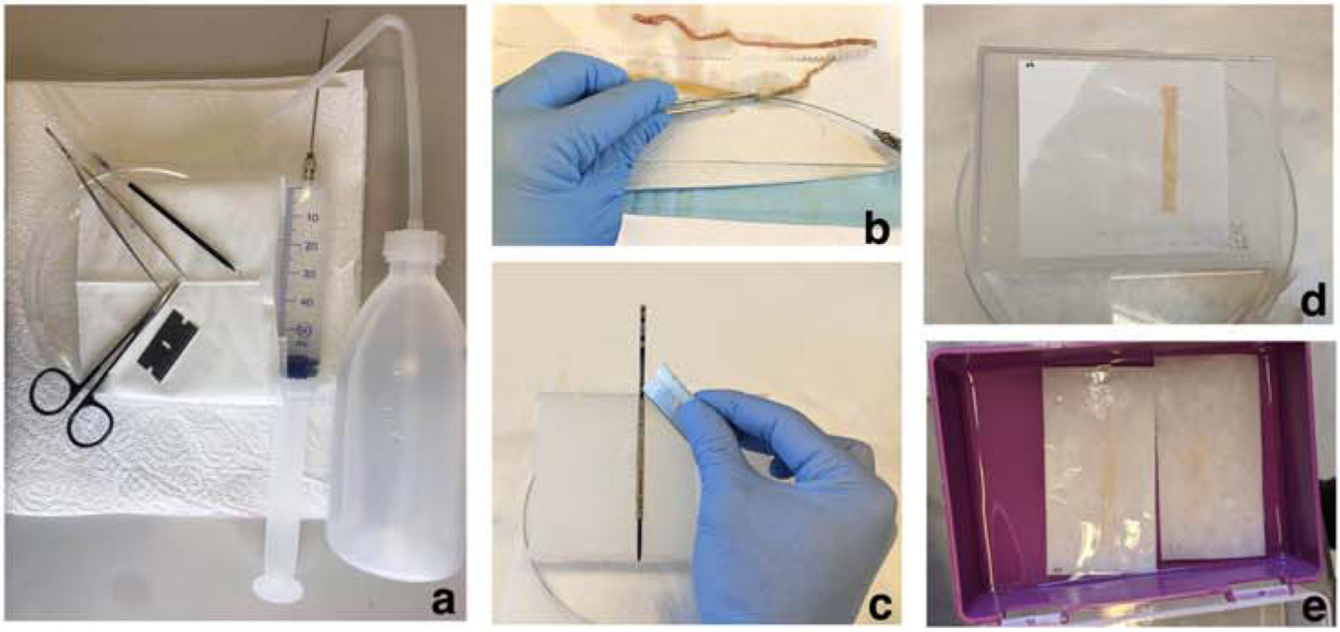

Figure 1: Prepare for small intestine processing.

a) Prior to euthanasia and dissection, make a high contrast glass rod, and fill squeeze bottle and syringe with cold PBS. Prepare a hard surface for fileting the intestine, such as the top of a pipet box propped on a large petri dish. Cover with a Kimwipe which will be wetted with PBS. **b**) Cut desired length of small intestine and insert gavage needle to flush luminal contents with cold PBS. **c**) After mounting the small intestine piece on the high contrast rod, filet open on the propped pipet box top using a fresh razorblade, in a single motion. **d**) For imaging, we recommend cutting filter paper for each intestine piece and using this in place of a Kimwipe. **e**) Multiple pieces of intestine can be fixed between filter paper in a bath of 4% PFA.

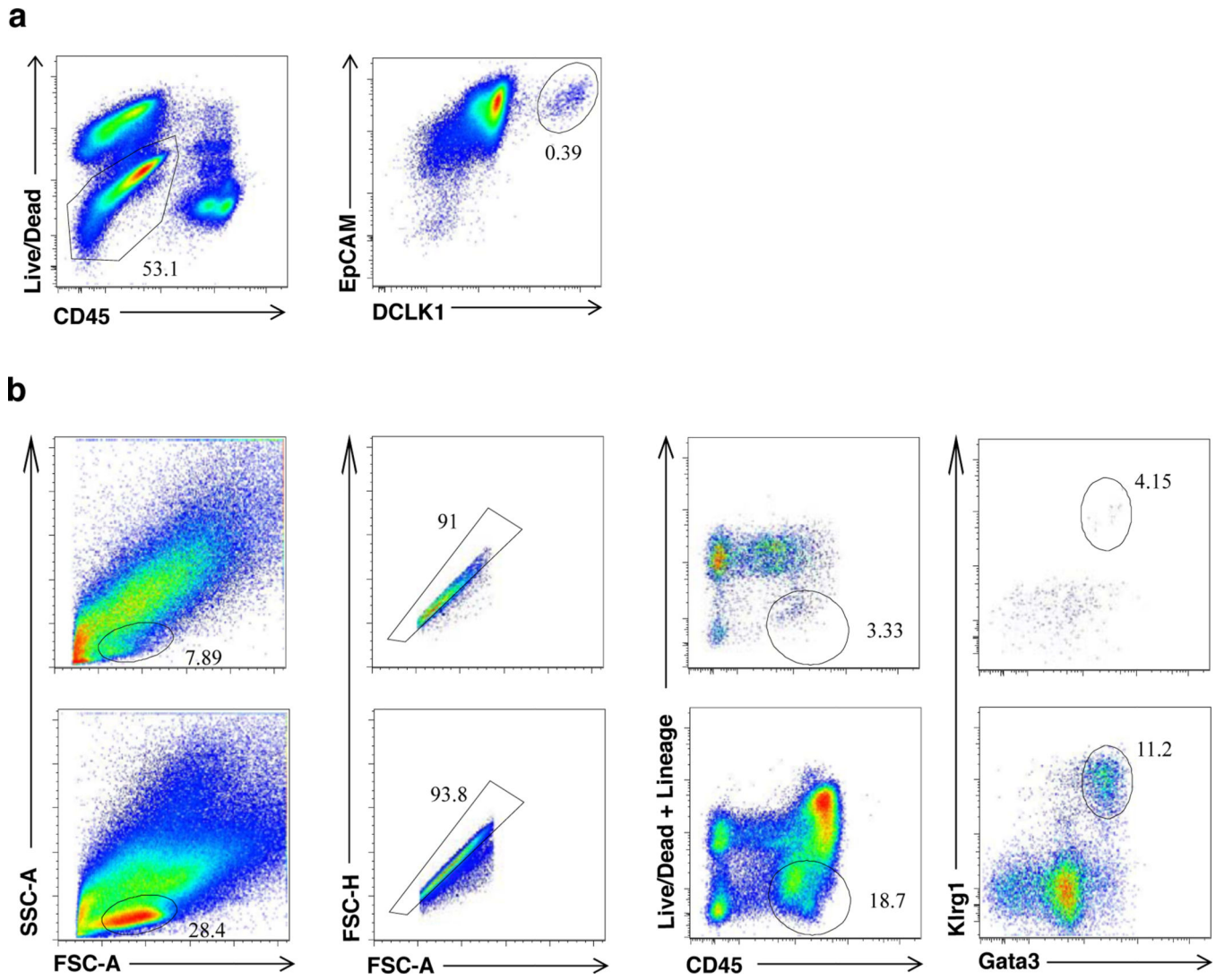

Figure 2: Representative gating for small intestinal tuft cells and ILC2s using Basic Protocol 1.

a) Epithelial single cell suspension obtained using Basic Protocol 1 from wildtype small intestine (*Tritrichomonas* negative mouse) and stained for tuft cells using intracellular staining for DCLK1. Previously gated on FSC-A x SSC-A. Recommended tuft cell gating: singlets, live cells (viability dye negative, FSC-A x SSC-A), EpCAM+CD45−, DCLK1+. **b**) Lamina propria single cell suspension obtained using Basic Protocol 1, demonstrating a failed (top panels) and successful (bottom panels) digest. Recommended ILC2 gating: singlets, live cells (viability dye negative, FSC-A x SSC-A lymphocytes), CD45+ Lineage negative, GATA3+KLRG1+. A robust population of live, lineage negative CD45+ cells is observed in viable cell preparations. If failed digests are repeatedly a problem, check mice for *Tritrichomonas* and/or utilize Alternate Protocol 1.

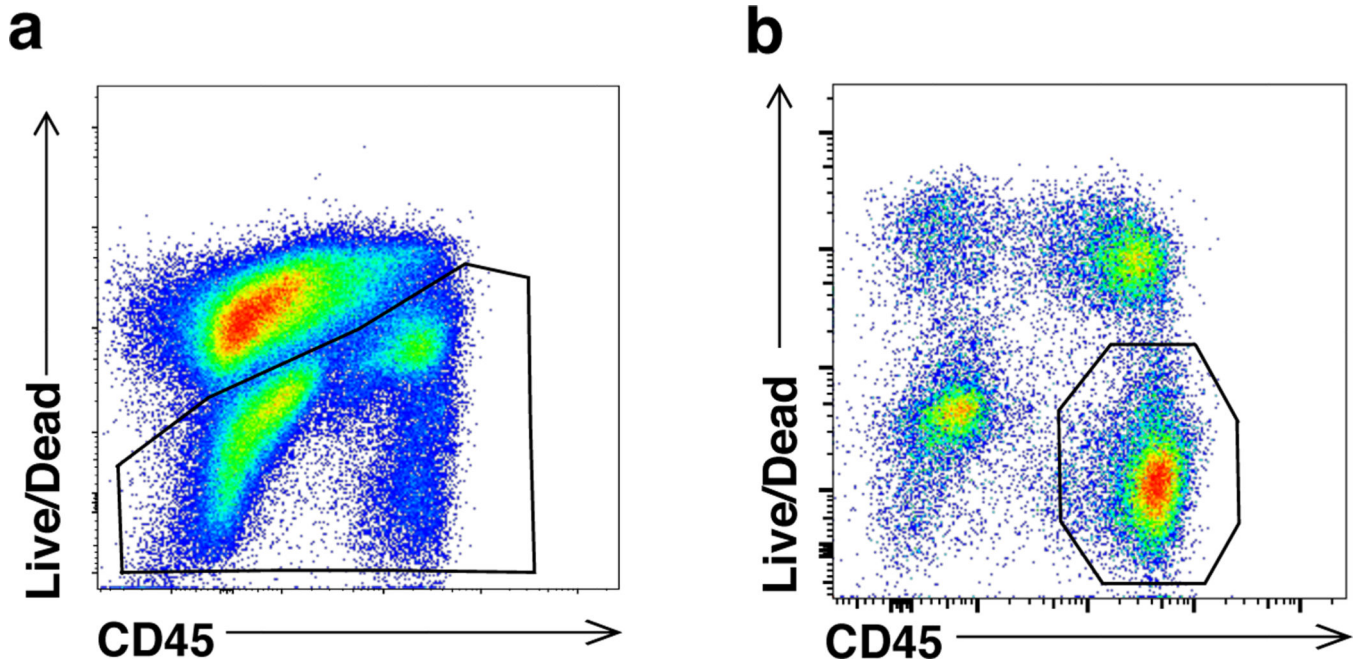

Figure 3: Alternate Protocol 1 can be used to generate viable single cell suspensions from inflamed intestine.

Mice infected with *N. brasiliensis* were euthanized 9 days post infection and intestine was processed using Alternate Protocol 1. Epithelial and digest fractions were mixed 1:1 prior to staining for tuft cells (a) or digested fraction alone was stained for analysis of ILC2s (b). Successful preparations have a robust population of live CD45+ cells. Cells were previously gated on FSC-A x SSC-A.

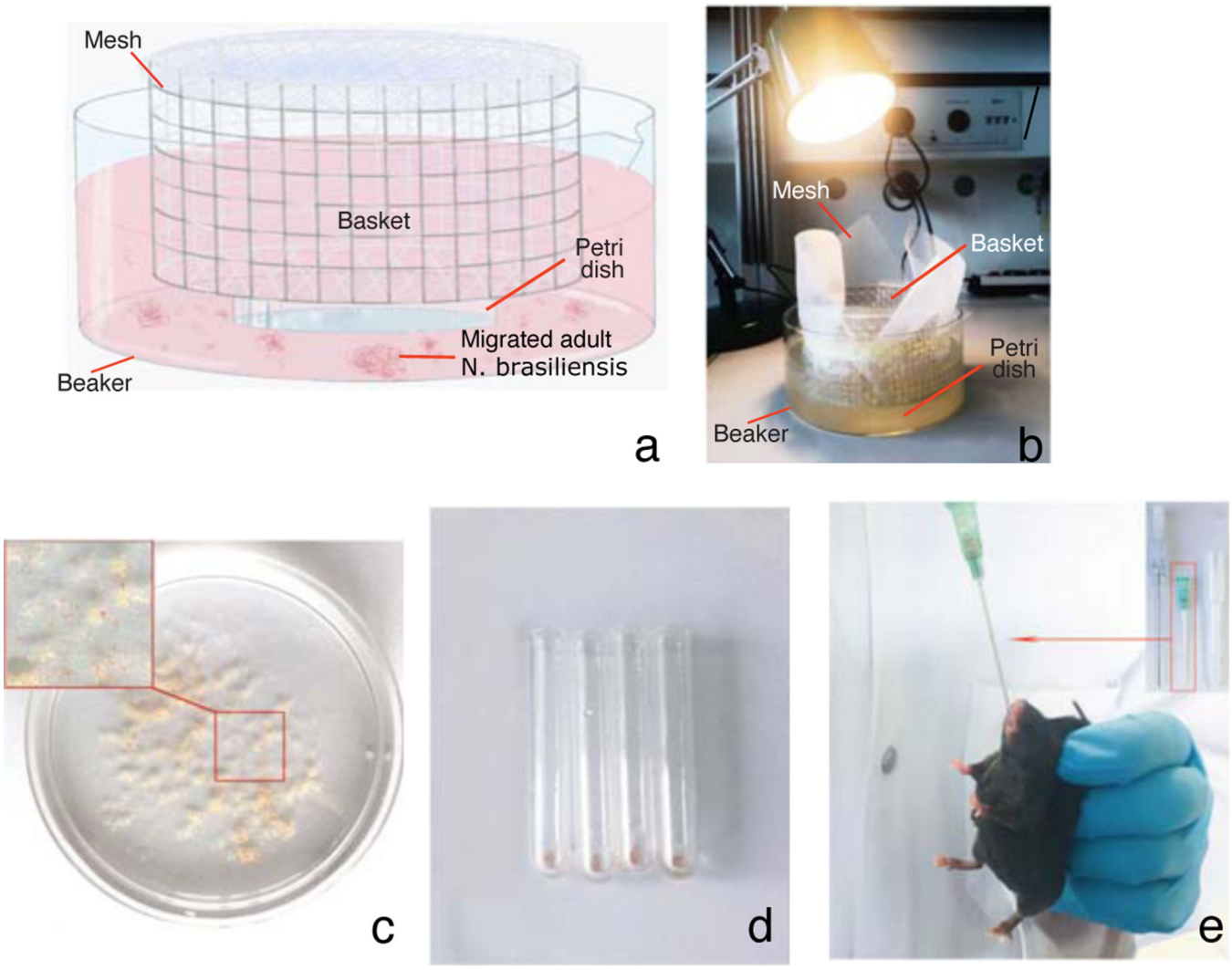

Figure 4: Infection of mice with adult *N. brasiliensis* by oral gavage.

a) Schematic of mesh-filter apparatus for harvesting worms from rat small intestine by gravity sedimentation and light-induced migration. **b**) Incubate the small intestinal contents in the mesh-filter apparatus under a desk lamp to promote migration. **c**) Disperse clusters of adult worms after washing by incubated for 40–60 min under light in a large petri dish with 50 ml warm PBS. **d**) Prepare for inoculation by transferring 100 worms into a 5 ml FACS tubes, preparing individually counted aliquots per recipient mouse. **e**) Use the protective cover of intravascular catheter mounted to a wide orifice 1 ml syringe to aspirate the worm pellet and infect mice by oral gavage.

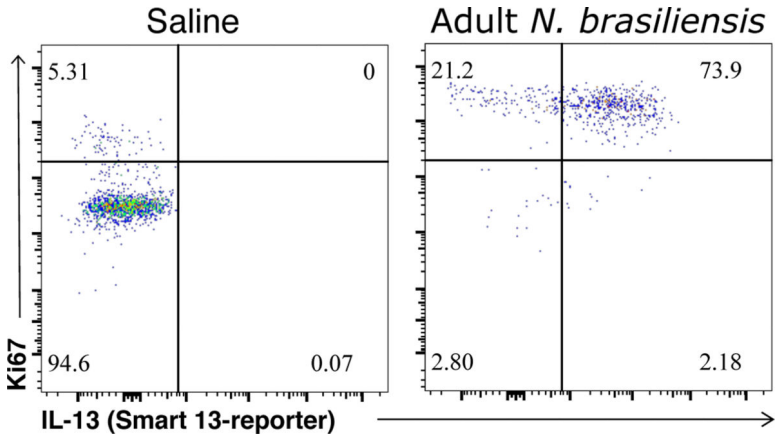

Figure 5: ILC2 activation following adult *N. brasiliensis* infection.

Mice were treated with PBS (saline) or infected with 100 adult *N. brasiliensis* worms by oral gavage; 48 hrs later, small intestine was harvested and ILC2s were isolated from small intestine of control mice or infected mice following Alternate Protocol 1. Lamina propria cells were stained according to Part 2 of Basic Protocol 1. Cells shown were previously gated on: lymphocyte FSC-A vs SSC-A, live CD45+, lineage negative (serially gated as CD3-CD4−, followed by CD11b-SiglecF-), Gata3+KLRG1+ ILC2. Staining for Ki67 and Smart13 reveals robust activation of ILC2 in infected mice.

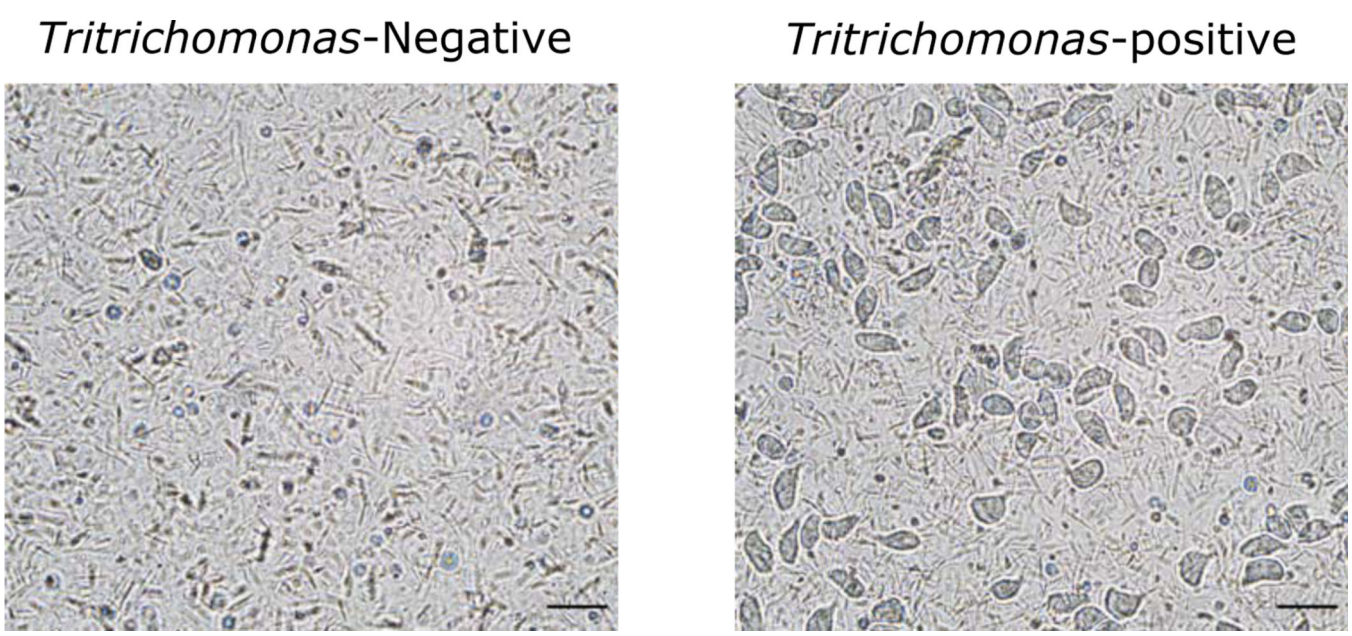

Figure 6: Visual screening for *Tritrichomonas.*

Tritrichomonas are readily observed in a PBS slurry of cecal content examined on a glass slide using the 20x objective of a regular light microscope. The protists are highly motile.

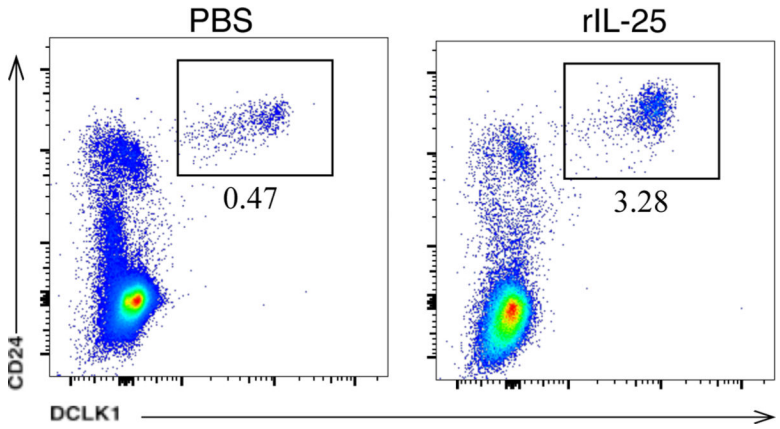

Figure 7: Expansion of tuft cell in mice treated with rIL-25.

a) Mice received 500ng rIL-25 by i.p. injection on four consecutive days; the control group received equal volume of PBS. 18–24 hrs after the last injection, small intestines were collected and processed to a single cell suspension using Alternate Protocol 1. The epithelial fraction was stained according to Part 2 of Basic Protocol 1 in section 1. The population was pre-gated on FSC-A x SSC-A, live cells, CD45-EpCAM+. Tuft cells are identified among EpCAM+ cells as DCLK1+CD24+.