



Chapter 9

Isolation and Identification of Group 2 Innate Lymphoid Cells in Settings of Type 2 Inflammation

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Abstract

The recent discovery of innate lymphoid cells (ILCs), a major source of canonical T cell cytokines, has prompted significant interest into understanding the role of these novel cells in immune responses. Unlike T cells, ILCs lack antigen receptors, instead receiving activation from locally derived tissue signals. Group 2 ILCs (ILC2s), which express the genes encoding GATA-3, interleukin (IL)-4, IL-5, IL-9, and IL-13, are distributed throughout nonlymphoid tissues. Although ILC2s lack antigen receptors, there are considerable similarities that they share with their Th2 cell counterparts, including receptors, secreted signals, and key transcription factors. Here we describe a method of isolating ILC2s for analysis from peripheral tissues of the mouse. The approach consists of digesting and mechanically dissociating harvested organs followed by staining with fluorescently labeled antibodies for flow cytometry or cell sorting. We suggest panels of antibodies for each tissue that can be used as positive and negative markers to selectively separate ILC2s from other cells, and we demonstrate marker specificity with example cells from a “cytokine reporter” mouse strain.

Key words Group 2 innate lymphoid cell (ILC2), Th2 cell, *Nippostrongylus brasiliensis*, Allergic inflammation, Type 2 inflammation

1 Introduction

The discovery of group 2 innate lymphoid cells (ILC2s) dramatically altered the understanding of type 2, or allergic, immunity [1–3]. First, an innate cellular source of what were believed to be signature T cell cytokines had been suggested in previous reports [4, 5] but never clearly defined. Second, the canonical transcription factors and signals of type 2 immunity evolved around the same time as did the antigen receptors of adaptive lymphocytes [6, 7]. Therefore, finding an innate cell that lacked antigen receptors but nevertheless was capable of coordinating an evolutionarily “advanced” type 2 immune response raised the possibility that these cells may represent an evolutionary intermediate between invertebrate immunity and our own.

Definitive identification of ILC2s was made possible by advances in transgenic mouse technology and flow cytometry, which revealed them to be a population of cells that expressed the genes encoding interleukin (IL)-4, IL-5, IL-9, and IL-13 but lacking most standard markers typically used to identify hematopoietic cell lineages. Furthermore, subsequent work has shown that, whereas other innate cells can produce type 2 cytokines in a T cell-dependent manner [8], ILC2s operate independently of T cell help and are the major source of IL-13 and IL-5 in unchallenged animals [9]. Their distribution in the periphery and the fact that ILC2s can be activated by innate signals such as IL-33, IL-25 and thymic stromal lymphopoietin (TSLP), as well as other mediators released during tissue damage, suggest they respond to low-level tissue stress by releasing cytokines that repair the tissue and return it to homeostasis [10]. But whatever their primary role in health may be, ILC2 numbers and activity are greatly amplified by the recruitment of T cells in disease states where an injury overwhelms innate immunity's ability to repair the damage [3, 11].

There is considerable interest in studying ILC2s in the context of type 2 inflammation, but isolating ILC2s without "contamination" by Th2 cells, which are generally present in the same space and time, has been a major challenge in the field. The defining characteristics of ILC2s are their reliance on the GATA3 transcription factor and their capacity for producing various type 2 cytokines, but it has been difficult to find additional markers for positively selecting ILC2s because they share many of these features with cytokine-expressing Th2 cells [12]. Furthermore, since the initial discovery of ILC2s, there is greater appreciation that ILC2s are heterogeneous, varying according to their location and/or the type of stimulus they receive [13, 14]. Here we describe an approach for enriching ILC2s from single-cell suspensions of the lung, small intestine lamina propria, and mesenteric lymph nodes of mice following infection with the model nematode *Nippostrongylus brasiliensis* [15]. We suggest positive and negative surface markers that can be used to separate ILC2s from other cells for analysis, and we demonstrate the specificity of these markers using example flow cytometry plots from wild-type and IL-5 "cytokine reporter" mouse strains.

2 Materials

2.1 Media (All Tissues; See Table 1)

1. Hank's buffered saline solution: Ca^{++} -/ Mg^{++} -free (HBSS-CMF)—400 mg/mL potassium chloride (KCl), 60 mg/mL potassium phosphate monobasic (KH_2PO_4), 8000 mg/mL sodium chloride (NaCl), 350 mg/mL sodium bicarbonate (NaHCO_3), 48 mg/mL sodium phosphate dibasic (Na_2HPO_4), and 1000 mg/mL D-glucose.
2. Dubelco's phosphate-buffered saline (D-PBS): 200 mg/mL KCl, 200 mg/mL KH_2PO_4 , 8000 mg/mL NaCl, and 2160 mg/mL Na_2HPO_4 .

Table 1
Media and buffers

	Final concentration	For each 15 cm intestine piece (μL)	For eight intestines with small additional volume (mL)
1: Mucus wash (2×)			
HBSS-CMF	—	7400	62.9
HI-FCS	2%	400	3.4
10× Sputolysin ^a	6.5 mM	2000	17
100× Pen/strep	100 U/mL; 100 μg/mL	200	1.7
2: Epithelial wash (3 × 15 mL washes)			
HBSS-CMF	—	43200	367.2
HI-FCS	2%	900	7.65
EDTA (0.5 M)	5 mM	450	3.825
100× Pen/strep	100 U/mL; 100 μg/mL	450	3.825
3: Rinse			
HBSS-CMF	—	9900	84.15
HEPES (1 M)	10 mM	100	0.85
4: Digest solution			
HBSS-CMF	—	5000	42.5
DNase I (10 mg/mL) ^a	30 μg/mL	15	0.128
Liberase TM (26 WU/mL) ^a	0.4 WU/mL	77	0.654

^aAdd these three components immediately prior to their respective incubations

3. FACS wash buffer (FWB): D-PBS plus 2% v/v heat-inactivated fetal calf serum (HI-FCS) and 0.5 mg/mL sodium azide (NaN₃).
4. 70% ethyl alcohol (EtOH) in water.
5. Pharm Lyse or similar red cell lysis buffer.

2.2 Additional Media
(Small Intestine Only;
See Table 1)

1. Sputolysin (Calbiochem): 65 mM solution (10× concentrate).
2. Ethylenediaminetetraacetic acid (EDTA): 0.5 M and pH 8.0.
3. Penicillin/streptomycin (pen/strep): 10,000 units/mL penicillin and 10,000 μg/mL streptomycin (100× concentrate).

Table 2
List of antibodies

Epitope reactivity	Clone	Concentration (mg/mL)	Volume/50 μ L
Positive stains			
Anti-KLRG1	2F1	0.2	1
Anti-T1/ST2	DJ8	1	1
Anti-IL17RB	MUNC33	0.2	1
Anti-CD25	PC61	0.2	1
Anti-CD44	IM7	0.2	0.5
Anti-CD45	30-F11	0.2	1
Anti-CD90.2 (Thy1)	53-2.1	0.2	0.25
Negative stains			
Anti-CD4	GK1.5 or RM4-5	0.2	1
Anti-CD5	53-7.3	0.2	0.25
Anti-CD8a	53-6.7	0.2	1
Anti-CD11b	M1/70	0.2	0.5
Anti-CD11c	N418	0.2	1
Anti-CD19	6D5	0.2	1
Anti-NK1.1	PK136	0.2	1
Anti-NKp46	29A1.4	0.2	1
Anti-F4/80	BM8	0.2	
Anti-Ly6G/Ly6C (Gr-1)	RB6-8C5	0.2	0.5

4. HEPES: 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (100 $\times$ concentrate).

2.3 Enzymes (See Table 1)

1. Liberase TM (Roche): 5 mg/mL (26 Wünsch U/mL) stock solution, stored at -20°C .
2. DNase I (Roche): 10 mg/mL stock solution, stored at -20°C .

2.4 Staining Reagents

1. 4',6-diamidino-2-phenylindole (DAPI): 1 mg/mL stock solution in PBS, stored at 4°C .
2. FcBlock: 1 mg/mL stock of purified rat anti-mouse CD16/CD32 (clone 2.4G2).
3. Antibodies (*see* Table 2).

2.5 Other Equipment

1. Dissecting scissors and forceps.
2. Dissecting board with pins.

3. 20 mL Luer-Lok syringe.
4. 22-gauge needle.
5. gentleMACS tissue dissociator (Miltenyi Biotec).
6. gentleMACS C tubes (Miltenyi Biotec).
7. 37 °C orbital shaking incubators.
8. Swinging bucket centrifuge that can accommodate 50 and 5 mL tubes at $400 \times g$ -force.
9. 50 mL snap-top conical bottom polypropylene tubes.
10. 70 μ m cell strainers (insertable into standard 50 mL tubes).
11. Plunger from a 3 mL syringe.
12. 5 mL round bottom polystyrene tubes.
13. 5 mL round bottom polystyrene test tubes with 35 μ m cell strainer snap caps.
14. CountBright or similar flow cytometry counting beads.

2.6 Additional Equipment (For Small Intestine Only)

1. Feeding needle, 16-gauge $\times$ 3–4" (Roboz FN-7916).
2. Blunt-tipped scissors (Roboz RS-5990 or RS-5992).
3. 100 μ m cell strainers (insertable into standard 50 mL tubes).

3 Methods

1. Prepare D-PBS and chill at 4 °C (20 mL per lung, 50 mL per small intestine).
2. Prepare HBSS-CMF (5 mL per lung, 75 mL per intestine).
3. For lungs, pre-warm 2.5 mL per mouse to 37 °C.
4. For small intestine, prepare the solutions described below (see Subheading 3.5), and pre-warm to 37 °C.
5. Prepare FWB and chill at 4 °C (50 mL per mouse per tissue).
6. Prepare a separate mixture of FWB with 1 μ g/mL DAPI (200 μ L per mouse per tissue).

3.1 Mouse Dissection and Tissue Harvesting

1. For lungs, apportion 2.5 mL HBSS-CMF into gentleMACS C tubes, and place them on ice.
2. Sacrifice a mouse by CO₂ inhalation, and as soon as it has stopped breathing, immediately spray completely with 70% EtOH (*see Note 1*).
3. Using standard dissection technique, starting from the xyphoid process, cut laterally through the left and right thorax exposing the lungs and mediastinum (*see Note 2*).
4. With scissors, make a 2 mm hole in the right atrium.

5. Fill a 20 mL syringe with cold D-PBS, affix 22-gauge needle, and *slowly* infuse 12 mL of D-PBS into the left ventricle at the point of the apex. Withdraw the needle, reposition it to the right ventricle, and *slowly* infuse the remaining D-PBS (*see Note 3*).
6. Remove the lungs: grasp the proximal bronchi with forceps and cutting through the airways and pulmonary vasculature. Place the left lung into the chilled C tube with 2 mL HBSS-CMF. Separate the four lobes of the right lung into four pieces, and place them into the chilled C tube with HBSS-CMF. Leave the tube on ice until all lungs have been harvested.
7. Remove any desired regional lymph nodes by gently grasping with one pair of forceps and freeing the nodes by peeling away adherent vasculature and fat with a second pair of forceps.
8. Remove the small intestine with sharp scissors, transect the pylorus, and cut away the bile and pancreatic ducts. With one hand, grasp the duodenum gently with forceps, and pull slowly from the peritoneum. With scissors in the other hand, cut any adherent pancreas, mesenteric vasculature, and fat away from the intestine. Continue to move down the intestine, repositioning the forceps every 8–10 cm. Pull the intestine gently, and keep the scissor cuts as close as possible to the intestine, without making any holes in the intestine wall. Transect the terminal ileum at the insertion to the cecum. Place the entire intestine in 1 mL of HBSS-CMF on ice until all intestines have been harvested.

3.2 Lung Digestion

1. Add 50 μ L per mouse Liberase TM and 12.5 μ L per mouse DNase to the pre-warmed HBSS-CMF.
2. Apportion 2.5 mL of the warmed HBSS into each C tube to achieve a final concentration of 0.05 mg/mL (0.26 WU/mL) Liberase TM and 0.025 mg/mL in each 5 mL lung digest tube.
3. Screw the C tube cap firmly past the first the “first resistance.” Carefully insert the tube into its holder in the gentleMACS dissociator, and run “Lung_01.”
4. Unscrew the cap and dislodge any pieces from the rotors. If any lobes seem to remain intact, insert the tube into the gentleMACS dissociator, and run “Lung_01” again. Unscrew the cap again to dislodge any pieces impacted in the rotors (*see Note 4*).
5. Proceed through **steps 1–4** for all tubes. Ensure all caps are firmly tightened again past the “first resistance,” and secure the tubes horizontally in a 37 °C shaking incubator at 180 rpm for 30 min.

3.3 Lung Dissociation

1. Carefully insert the tube into its holder in the gentleMACS dissociator, and run “Lung_02.”
2. Add 3 mL FWB to each tube, and “pulse” in the centrifuge ($400 \times g$ -force $\times$ 30 s).
3. Remove the tubes and place them on ice.
4. Insert 70 μ m strainers into 50 mL tubes. One at a time, pour the tube contents over the strainer into the tube. Rinse the tube with 5 mL FWB, and pour this over the strainer.
5. Using the handle end of the plunger from a 3 mL syringe, scrape and sweep the sides and bottom of the strainer for 10–15 s to dissociate any remaining tissue pieces, working it all through to the tube. Rinse the strainer with 5 mL of FWB, and resume mashing the tissue and liquid through the strainer. Alternate between mashing and rinsing three times until the total volume in the tube is 45 mL (*see* **Note 5**).
6. Discard the strainer, place each 50 mL tube on ice, and proceed to the next sample until all gentleMACS contents have been dissociated through the strainers.
7. Spin the single-cell suspensions at $400 \times g$ -force for 5 min, and discard the supernatant by aspiration or by pouring in a single steady motion.
8. Close the tube, and dislodge the pellet by vortex or by running it across an Eppendorf rack. Resuspend the pellet in 5 mL FWB and vortex for 3 s. Place the tubes on ice until ready for staining.

3.4 Preparing Single-Cell Suspensions of Lymph Nodes

1. Insert a 70 μ m strainer into a 50 mL collecting tube for each mouse.
2. Going one by one, place each lymph node(s) in a strainer, and moisten the bottom with FWB.
3. Using the handle end of the plunger from a 3 mL syringe, scrape and sweep the sides and bottom of the strainer for 10–15 s to dissociate the tissue completely, working it all through to the tube. Rinse the strainer with 5 mL of FWB, and resume mashing the tissue and liquid through the strainer. Alternate between mashing and rinsing three times until the total volume in the tube is 45 mL.
4. Discard the strainer, place each 50 mL tube on ice, and proceed to the next sample.
5. Spin the 50 mL collecting tubes at $400 \times g$ -force for 5 min, and discard the supernatant by aspiration or by pouring in a single steady motion.
6. Close the tube, and dislodge the pellet by vortex or by running it across an Eppendorf rack. Resuspend the pellet in 5 mL FWB

and vortex for 3 s. Place the tubes on ice until ready for staining.

3.5 Processing Small Intestine for Lamina Propria Prep

1. For each intestine, prepare buffers, and pre-warm to 37 °C (*see Note 6*; Table 1).
2. Place one small intestine on a paper towel, and moisten liberally with cold PBS.
3. Crop to 12–15 cm length and discard the residual tissue (*see Note 7*).
4. Insert feeding needle, and gently perfuse the lumen with cold PBS to flush out fecal material (*see Note 8*).
5. With sharp scissors, trim any residual mesenteric fat, and excise and discard any visible Peyer's patches (*see Note 9*).
6. With blunt-point or ball-point scissors, start at one end, and fillet the intestine longitudinally, irrigating with cold PBS to uncurl the tube. It should now look like a single long rectangle, with the luminal surface facing up and the serosal surface adherent to the paper towel.
7. Holding the paper towel over a waste beaker, and without dislodging the intestine off of the paper towel, then gently irrigate with ~20 mL of PBS from a squirt bottle to remove any remaining fecal aggregates.
8. Cut the intestine into three pieces, and place them in a 50 mL tube with 10 mL HBSS-CMF on ice.
9. Proceed through **steps 2–8** until all intestines have been processed and the pieces placed into individual 50 mL tubes.

3.6 Wash and Digest Lamina Propria for Single-Cell Isolation

1. Add the Sputolysin to complete the pre-warmed 2× mucus wash (*see Note 6*).
2. Add 10 mL of 2× mucus wash to each tube, and secure the tubes horizontally in a 37 °C shaking incubator at 70 rpm for 20 min.
3. After 20 min, vortex each tube gently for 5 s, and pour out supernatant into a waste beaker, using a 100 µm strainer to catch the intestine pieces.
4. Return pieces to the same 50 mL tube. Add 15 mL of epithelial wash to each tube, and secure the tubes horizontally in a 37 °C shaking incubator at 70 rpm for 15 min. Return the unused epithelial wash to 37 °C incubator.
5. Repeat **steps 3** and **4** twice more, vortexing and pouring off the supernatant, using the same 50 mL tubes and 100 µm strainers, for a total of three epithelial washes (*see Note 10*).
6. After the third epithelial wash, as before, vortex gently, and pour out the supernatant, using the 100 µm strainer to catch the pieces. Place the pieces in a new 50 mL tube.

7. Add 10 mL of Rinse to each tube, and secure the tubes horizontally in a 37 °C shaking incubator at 70 rpm for 10 min.
8. Add the DNase and Liberase TM to complete the pre-warmed digest solution (*see Note 6*).
9. After 10 min, as before, vortex gently, and pour out the supernatant, using the 100 μ m strainer to catch the pieces. Place the pieces in a gentleMACS C tube.
10. Add 5 mL of digest solution to each tube, and secure the tubes horizontally in a 37 °C shaking incubator at 110 rpm for 30 min.

3.7 Lamina Propria Dissociation

1. Carefully insert the tube into its holder in the gentleMACS dissociator, and run “Intestine_01.”
2. Add 3 mL FWB to each tube, and “pulse” in the centrifuge ($400 \times g$ -force $\times$ 30 s), and then place the tubes on ice.
3. Insert 100 μ m strainers into 50 mL collecting tubes. One at a time, pour each tube’s contents over its strainer into its collecting tube. Rinse each C tube with 5 mL FWB, and pour this over its strainer. Discard the C tubes.
4. Rinse the strainer with additional FWB, bringing the total volume up to 40 mL. Discard the strainer.
5. Place each 50 mL tube on ice, and proceed to the next sample until all gentleMACS contents have been transferred through the strainers.
6. Spin the single-cell suspensions at $400 \times g$ -force for 5 min, and discard the supernatant by aspiration or by pouring in a single steady motion.
7. Close the tube, and flick the pellet or run it across an Eppendorf rack to loosen. Resuspend the pellet in 5 mL FWB and vortex for 3 s.

3.8 Antibody Staining

1. From each 5 mL suspension of lungs, intestinal lamina propria, and lymph nodes, remove an appropriate volume into a 5 mL FACS tube such that each FACS tube contains $0.5\text{--}5 \times 10^6$ CD45+ cells for staining (*see Note 11*).
2. Add 2–3 mL FWB to the tube, spin at $400 \times g$ -force for 5 min, and discard the supernatant (*see Note 12*). Run the tube across a rack to loosen the pellet.
3. Add 1 μ L per tube of FcBlock and vortex for 3 s.
4. Incubate at 4 °C for 10 min.
5. Add 50 μ L per tube of staining cocktail (*see Note 13*; Table 2).
6. Incubate at 4 °C covered with aluminum foil for 25 min
7. If necessary (e.g., for hemorrhagic lungs), add 1 mL of Pharm Lyse, and incubate at room temperature for 5 min.

8. Add 2–3 mL FWB to the tube, spin at $400 \times g$ -force for 5 min, discard the supernatant by pouring in a single steady motion, and, while inverted, blot the tubes gently once on a paper towel. Run the tube across a rack to loosen the pellet.
9. (Optional: if performing a streptavidin-biotin or other secondary stains, add 50 μ L per tube of secondary staining cocktail and repeat **steps 6–8**.)
10. Add 50 μ L of CountBright beads and 100 FWB + DAPI to the tubes.
11. Immediately prior to acquiring each tube on the cytometer, add 300 μ L of FWB, pipet up and down two to three times, and transfer to a new tube through an affixed 35 μ m cell strainer snap cap.

3.9 Flow Cytometry Acquisition and Gating:

1. Acquire enough events to isolate 500–1000 ILC2s. As discussed in the Introduction above, no combination of markers are perfectly specific for ILC2s, so using cytokine reporter mouse strains dramatically improves the isolation of ILC2s from other cells expressing similar surface markers.
2. Flow cytometry files can be analyzed according to the gating scheme shown, using positive and negative selection markers specific for the lung (Fig. 1), mesenteric lymph nodes (Fig. 2), and small intestine lamina propria (Fig. 3).

4 Notes

1. Maintaining a short time from sacrifice to perfusion of the systemic and pulmonary circulations ensures the blood does not have time to pool and clot, which can negatively impact the ability to flush out circulating cells from the tissue.
2. This protocol assumes blood will not be needed for analysis. However, if planning to harvest blood for serum and/or cell analysis, remove desired volume of blood from the right atrium with a syringe/needle (1 mL/27-gauge) before the perfusion step.
3. Infusion should be no faster than 1 mL/s. Faster perfusion forces fluid across the capillaries into the alveolar space, flushing bronchoalveolar cells up the trachea and out the mouth and nose. These cells will therefore be lost from the analysis. Maintaining identical perfusion technique from mouse to mouse ensures consistent and reproducible results.
4. “Lung_01” shears apart collagenous regions where immune cells reside. Running “Lung_01” more than twice (or using other mechanical dissociation techniques) prior to digestion

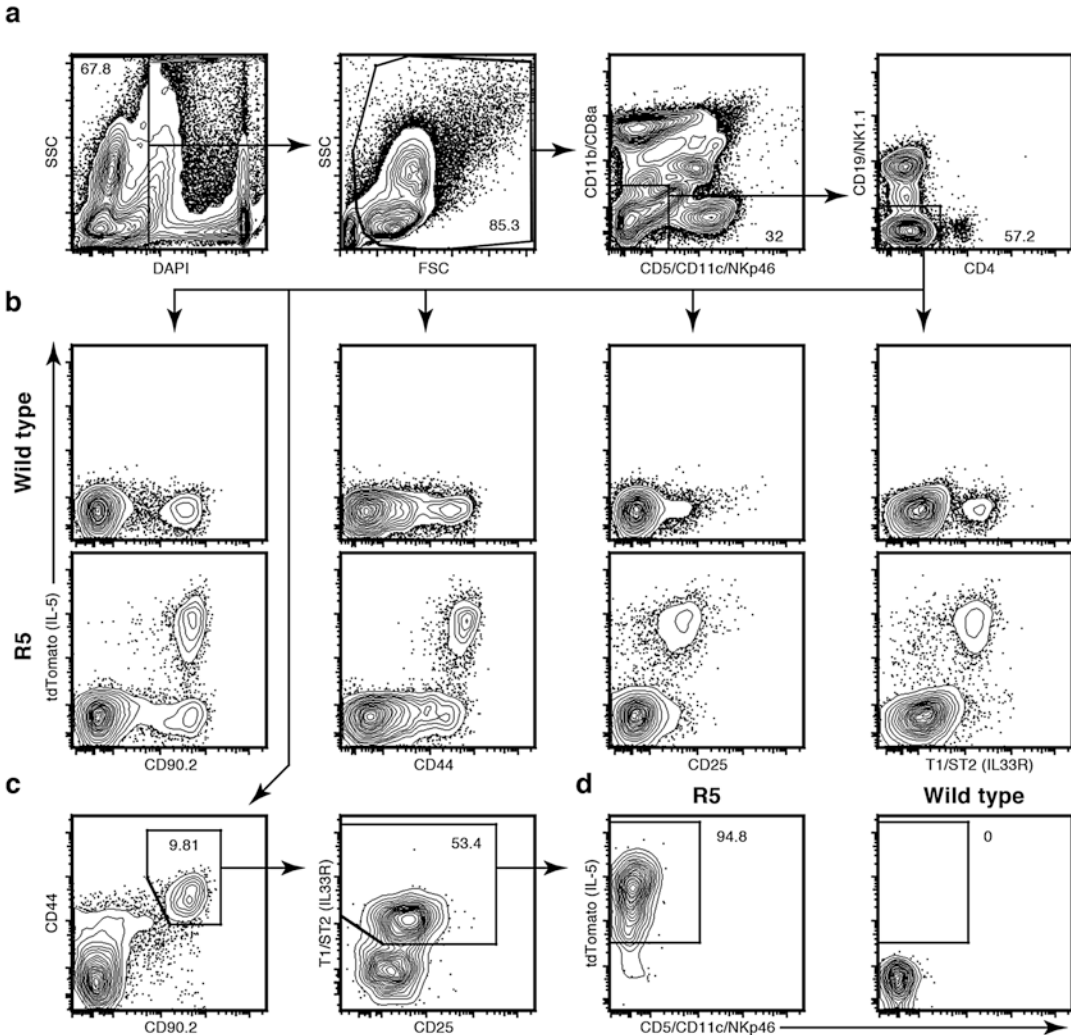


Fig. 1 Flow cytometry plots of lung single-cell suspensions 10 days after infection with 500 L3 larvae of *N. brasiliensis*. **(a)** Initial gating on live single cells and subsequent negative selection by gating of T cells, B cells, NK cells, and myeloid cells (negative for CD4, CD5, CD8a, CD11b, CD11c, CD19, NKp46, and NK1.1). **(b)** Panels show expression of the IL5-RFP (R5) reporter in wild-type and R5/+ heterozygous mice, compared with expression of the indicated markers on the X-axes. **(c)** Subsequent gating from the right panel in **(a)**, showing selection of CD90.2+ CD44+ CD25+ T1/ST2+ cells. **(d)** Panels show expression of the R5 reporter in wild-type and R5/+ heterozygous mice, previously gated from the right panel in **(c)**

causes undue structural damage that negatively impacts immune cell viability and yield.

5. Lungs from mice subjected to an immune challenge will be significantly more collagenous and fibrous, and the strainer might clog at first. Longer and more vigorous scraping may be needed to force the tissue through the strainer.

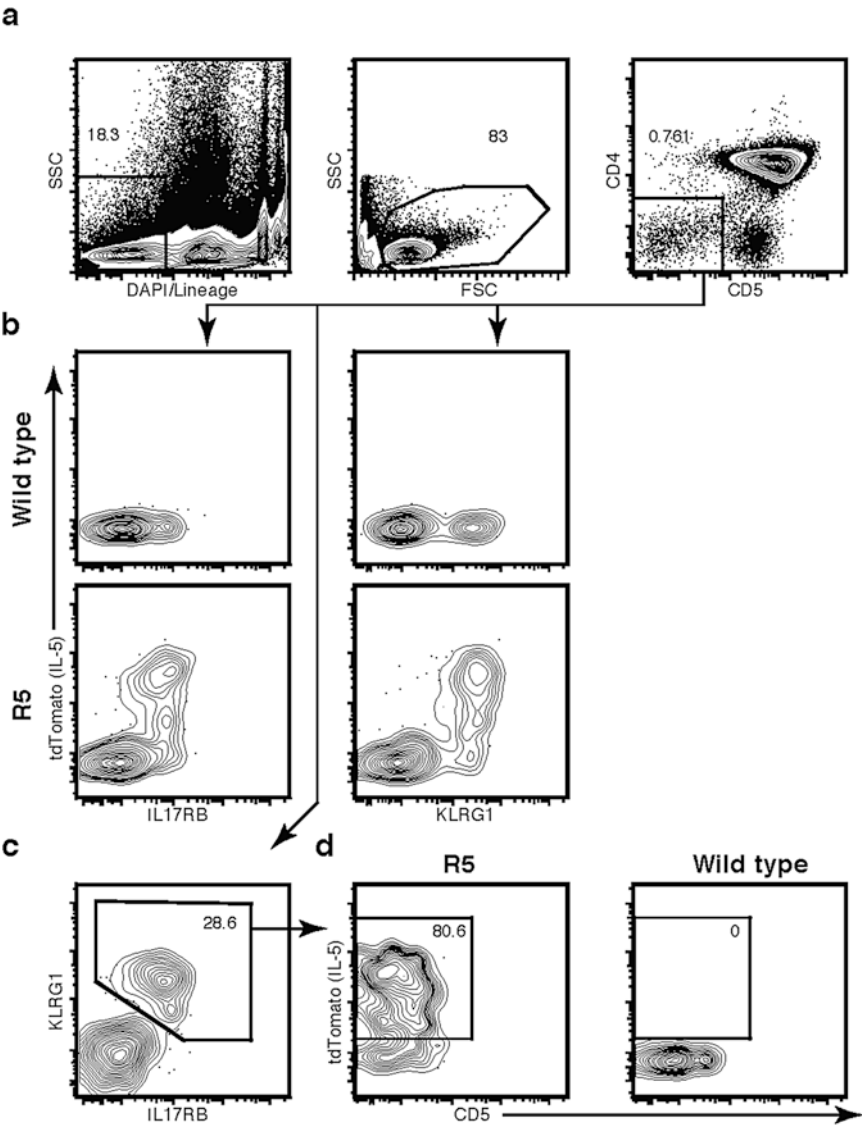


Fig. 2 Flow cytometry plots of mesenteric lymph node single-cell suspensions 10 days after infection with 500 L3 larvae of *N. brasiliensis*. **(a)** Initial gating on live single cells, excluding cells with most lineage markers in the DAP I channel (CD8a, CD19, CD11b, CD11c, NK1.1, Gr-1, NKp46, and F4/80) and subsequent negative gating of T cells (CD4 and CD5). **(b)** Panels show expression of the IL5-RFP (R5) reporter in wild-type and R5/+ heterozygous mice, compared with expression of the indicated markers on the X-axes. **(c)** Subsequent gating from the right panel in **(a)**, showing selection of KLRG1+ IL17RB+ cells. **(d)** Panels show expression of the R5 reporter in wild-type and R5/+ heterozygous mice, previously gated from the right panel in **(c)**

6. All media can be prepared the day before and stored overnight in 4 °C, but the Sputolysin, the DNase, and the Liberase TM should only be added to their respective solutions immediately prior to use.

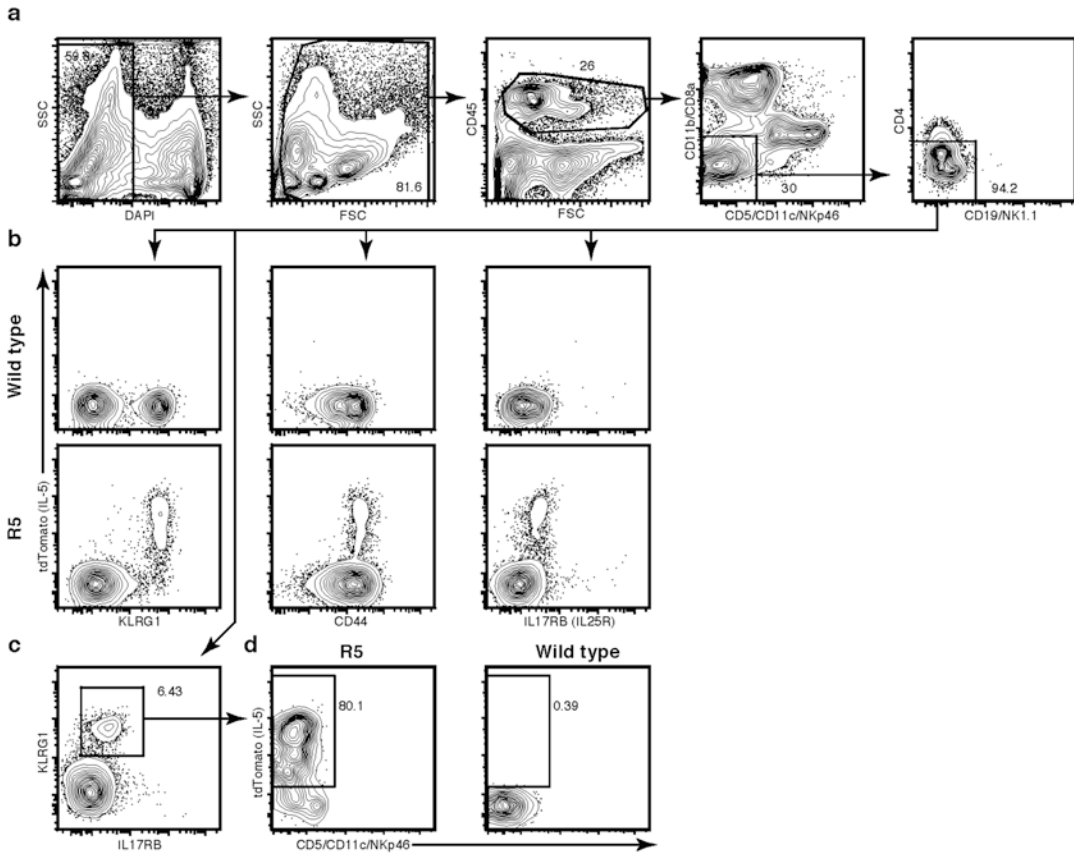


Fig. 3 Flow cytometry plots of small intestine lamina propria single-cell suspensions 28 days after infection with 500 L3 larvae of *N. brasiliensis*. (a) Initial gating on live single CD45+ cells and subsequent negative selection by gating of T cells, B cells, myeloid cells, and NK cells (negative for CD4, CD5, CD8a, CD11b, CD11c, CD19, NKp46, and NK1.1). (b) Panels show expression of the IL5-RFP (R5) reporter in wild-type and R5/+ heterozygous mice, compared with expression of the indicated markers on the X-axes. (c) Subsequent gating from the right panel in (a), showing selection of KLRG1+ IL17RB+ cells. (d) Panels show expression of the R5 reporter in wild-type and R5/+ heterozygous mice, previously gated from the right panel in (c)

7. We have used varying amounts of small intestine tissue, from 10 cm to the entire organ, testing from the duodenum/proximal jejunum down to the distal ileum regions. Using a 12 cm section of the small intestine that starts at the distal duodenum (2 cm from the pylorus) gives a good representative picture of the entire organ; the region of the small intestine that is used does not significantly impact ILC2 surface markers or recovery in naïve mice. However, depending on the immune challenge used, epithelial cell and immune cell populations may vary along the length of the small intestine, and this variable will need to be optimized accordingly.

8. Perfuse gently to flush the fecal material. The intestine should not be allowed to distend or rupture as mechanical stress negatively impacts viable cell yield.
9. This protocol assumes Peyer's patches will not be analyzed. However, they may be collected and mechanically dissociated similar to lymph nodes if desired.
10. This protocol assumes epithelial cells will not be analyzed. However, they may be collected from each of the three epithelial wash incubations in **steps 4** and **5**. Each wash should be centrifuged at $400 \times g$ -force for 5 min, washed with 30 mL FWB and centrifuged again, and resuspended in FWB on ice. The three washes can be combined at the end for epithelial cell analysis.
11. This protocol yields approximately $1\text{--}3 \times 10^6$ total live singlet cells from adult naïve C57BL/6 mouse lungs, $1\text{--}5 \times 10^6$ total live singlet cells from adult naïve C57BL/6 mouse lymph nodes, and $5\text{--}10 \times 10^6$ CD45+ cells from adult naïve C57BL/6 mouse small intestine lamina propria. (Optional: if desired, to determine a precise and consistent cell count for staining, remove 50 μ L of the 5 mL final suspension to a separate FACS tube, incubate with 0.5 μ L anti-mouse CD45 on ice for 10 min, then add 50 μ L of counting beads and 100 μ L of FWB + DAPI, and perform a count.)
12. Supernatant can be removed from FACS tubes by aspiration or by pouring in a single steady motion and, while inverted, blotting once gently on a paper towel. With either method, there should be 50–100 μ L volume remaining in the bottom of the tube in which to resuspend the pellet.
13. The cell yield from challenged mice varies significantly depending on type of challenge, by as much as tenfold. The staining concentrations described in this protocol are high enough to accommodate 5×10^6 live single cells per staining tube, although $0.5\text{--}1 \times 10^6$ cells is recommended. For lungs, depending on the immune challenge, this is usually achieved with a volume between 0.25 mL (for highly inflamed) and 1 mL (for naïve) of the 5 mL single-cell suspension. For intestines, this is usually achieved with 0.5 mL.

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