

Isolation and analysis of group 2 innate lymphoid cells in mice

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Recent studies have identified distinct subsets of innate lymphocytes, collectively called innate lymphoid cells (ILCs), which lack antigen receptor expression but produce various effector cytokines. Group 2 ILCs (ILC2s) respond to epithelial cell-derived cytokines such as interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP), produce large amounts of type 2 cytokines, and have a key role in anti-helminth innate immunity and in the pathophysiology of allergic inflammation. The reported phenotypic characteristics of mouse ILC2s vary, depending on the tissue source and preparation method. This protocol describes improved methods for tissue-specific isolation and analysis of mouse ILC2s of high purity and yield from fat tissue, lung, bronchoalveolar lavage fluid (BALF) and small intestine. These improved methods are the result of our thorough investigation of enzymes used for tissue digestion, methods for the elimination of undesired cells, and a combination of antibodies for the detection and isolation of ILC2s. In addition, this new protocol now enables the isolation of ILC2s of high yield, even from inflamed tissues. Depending on the tissue being analyzed, it takes ~2–4 h for isolation and flow cytometric analysis of ILC2s from the various tissues of a single mouse and ~4–8 h to sort purified ILC2s from pooled tissues of multiple mice.

INTRODUCTION

A coordinated response between the innate and adaptive immune systems is crucial for efficient protection against various invading microbes. Within the past few years, newly identified cytokine-producing ILCs, which are characterized by a lack of cell surface markers associated with other immune cell lineages (including antigen-specific receptors), have been shown to have important roles in immune surveillance, tissue remodeling and in the initiation of immune responses^{1–3}. Three ILC subsets have been proposed by a consortium of experts in the field; these are termed group 1 ILCs, ILC2s and group 3 ILCs on the basis of their ability to produce type 1, type 2 and T helper 17 (T_H17) cell-associated cytokines, respectively⁴.

ILC2s respond to epithelial cell-derived cytokines such as IL-25, IL-33 and TSLP, and they have an important role in anti-helminth innate immunity and in the pathophysiology of allergic inflammation (e.g., asthma, atopic dermatitis and allergic diarrhea)^{5–11}, producing large amounts of type 2 cytokines such as IL-5, IL-6, IL-9 and IL-13, which mediate effector responses through the recruitment and activation of eosinophils and mast cells, as well as goblet cell hyperplasia in the gut and airways¹².

Although intensive studies on mouse ILC2s are currently underway in many laboratories, there are a number of discrepancies in the phenotypic characteristics of ILC2s reported in the literature. ILC2s not only have an important role in the acute response against parasitic infection but also mediate immunopathologic responses in mouse models of allergic asthma^{5–11}. In the lung, many allergens induce lung inflammatory responses through activation of lung epithelial cells that produce IL-25, IL-33 and TSLP, which, in turn, induce type 2 cytokine production and eosinophilic lung inflammation^{13–16}. ILC2s were also described in the lungs of naive mice and were shown to be similar to those described in adipose tissue, although there were some differences^{15–18}. Phenotypic differences may result from differences in the methods of preparation, as well as differences in the tissue source of ILC2s. It is known, for example,

that ILC2s isolated from mice previously administered IL-25 or IL-33 are in an activated state and display different properties compared with naive cells¹⁹. Herein we describe our detailed protocols for the isolation, detection and culture of ILC2s from mesentery, lung, BALF and small intestine, four major tissues in which this cell population is located^{11,15,16,20}.

Establishment of this method and comparison with other methods

There are several methods reported for the isolation of ILC2s from adipose tissues^{11,20}, small intestine^{21–23} and lung^{13–16,18,21}. Some methods describe the isolation of ILC2s, called natural helper (NH) cells, from naive mice^{1,15,16,20}, whereas in others cytokines such as IL-25 and IL-33 were administered to the mice to increase cell number *in vivo*, and the isolated cells were termed nuocytes and innate helper type 2 (I_H2) cells^{24,25}. In addition, various combinations of antibodies have been used to identify ILC2s. Thus, there are discrepancies in the published phenotypic characteristics of this cell population, which may result from differences in the methods of preparation, as well as differences in the tissue source of ILC2s, as noted above. We observed that ILC2s from mice administered cytokines before isolation had less longevity in culture media containing IL-2, IL-7, IL-25, IL-33 or a combination of these cytokines compared with naive ILC2s isolated from untreated mice.

To isolate ILC2s from tissues, the tissues must be digested to liberate the cells. It is crucial that the viability and characteristics of ILC2s, including the expression of surface markers and their ability to respond to extracellular stimuli to produce cytokines, are maintained during tissue digestion. Therefore, we thoroughly examined and optimized conditions for tissue digestion, including selection of enzymes, labware and experimental conditions. In addition, we analyzed various surface markers to determine those that can be used to identify a pure population of ILC2s. On the basis of our thorough review of currently published methods, we established a method to isolate ILC2s of highest

purity and yield from naive mice that we believe can easily be applied to various experiments with ILC2s.

In this protocol, with the exception of ILC2 isolation from BALF, mice are not administered cytokines before cell preparation, and thus changes in cell surface markers of ILC2s upon activation by cytokines can be avoided. Furthermore, major improvements on the previous methods are as follows: first, a combination of killer cell lectin-like receptor subfamily G member 1 (KLRG1) and Sca-1 is used to identify a population of cells in the lineage-negative fraction, nearly 100% of which express trans-acting T cell-specific transcription factor GATA 3 (Gata3). Similarly, almost all Lin[−]Gata3⁺ cells are included in the KLRG1⁺Sca-1⁺ population. Second, collagenase (or a combination of collagenase and dispase, which is used in most of the previously reported methods) digests some cell surface markers such as CD4, class II MHC and T1/ST2, and thus it downregulates

some important markers for ILC2s. We therefore examined various enzymes and chose to use Liberase, which digests tissues and liberates ILC2s without modifying ILC2 markers. We used collagenase in our earlier reports on the isolation of ILC2s from adipose tissues^{11,20}, but we have since found that using Liberase greatly improves ILC2 yield.

In previous methods for the isolation of ILC2s from the lamina propria (LP) of small intestines, tissues were digested in tubes by incubation on a shaker^{20–23}. We now digest tissues in a conical flask with a stir bar and have found that this method greatly increases cell viability and yield. In addition, in the current protocol, digested tissues are passed through a needle using a syringe, which liberates cells from viscous mucus. We have successfully isolated a threefold greater number of cells using this method compared with shaking in tubes. Most importantly, the current protocol can be used to isolate ILC2s from inflamed intestinal

Box 1 | Isolation of ILC2s from the BALF ● TIMING 30 min per mouse (add 15 min per mouse for >1 mouse)

ILC2s are undetectable in BALF of naive mice, but they can be observed under conditions of elevated secretion of IL-25 and/or IL-33, as seen in helminth-infected mice and certain mouse models of asthma.

Collection of ILC2s from BALF. Kill the mouse by cervical dislocation, and surface-sterilize the skin using 70% (vol/vol) ethanol.

1. Make a midline incision on the surface of the skin (**Supplementary Fig. 1a**).
2. Using your hands, retract the skin above the head and below the thighs (**Fig. 1a**).
3. To open the thoracic cavity, first cut along the bottom edge of the sternum, and then along both sides of the rib cage (red and blue lines, **Fig. 1a,b**).

▲ **CRITICAL STEP** It is important to avoid cutting the lung tissue when opening the rib cage. After the initial cut of the sternum, as air enters into the pleural cavity, gently cut the connective tissue on the underside of the rib cage, opening up the cavity and allowing visualization of the lungs.

4. Lay the blade of the dissecting scissors atop the thymus and carefully cut upward, by trimming connective tissue (**Fig. 1b**).
5. Retract the rib cage to fully expose the trachea (**Fig. 1c**).
6. For ease of bronchoalveolar lavage, rotate the mouse 180° so that the head is facing downward.
7. Trim connective tissue surrounding the trachea. The trachea can be distinguished from the esophagus by its striped pattern.
8. To extend the trachea, place forceps (10-cm size) perpendicularly under the trachea and allow them to open (**Fig. 1d**).

▲ **CRITICAL STEP** If the trachea is not sufficiently extended, insertion of the needle and subsequent lavage will be difficult. We have found that the 10-cm-sized dissection forceps open naturally to a length that extends the trachea without rupturing the tissue.

9. Insert a 18-gauge × 1¼-inch plastic needle into the trachea (**Fig. 1e–g**).
10. Ligate the needle to the trachea with a 2-0 type silk braid (**Fig. 1g**).
11. Attach a 1-ml syringe containing 500 µl of PBS. Draw the syringe to 1 ml so that it contains 500 µl of PBS plus 500 µl of air. The air in the syringe will maximize lung inflation to ensure dispersion of PBS.

▲ **CRITICAL STEP** It is important to use a maximum volume of 500 µl of PBS when collecting BALF for cytokine analysis to ensure that the solution is not too dilute and that the cytokines can be detected by ELISA. As 500 µl is a relatively small volume for washing the lung, adding air to the syringe facilitates complete entry of PBS into the lung. Therefore, the air should be in the distal half of the syringe and should go into the lung following infusion of PBS.

12. Infuse 500 µl of PBS plus air intratracheally into the lungs.
13. Gently pump the syringe three times, collect BALF and transfer it to a 1.5-ml tube. We routinely obtain 200–300 µl of lavage fluid.
14. Centrifuge the lavage fluid at 500g for 5 min at 4 °C. Transfer the supernatant to a separate 1.5-ml tube and set it aside for cytokine analyses. Maintain the pellet at 4 °C.
15. To thoroughly collect BALF ILC2s, insert a syringe containing 1 ml of PBS intratracheally, gently pump the syringe about three times to wash lungs and collect the fluid into a 5-ml tube.
16. Repeat the wash twice to collect a total volume of ~3 ml of BALF.

▲ **CRITICAL STEP** It is important to gently infuse the PBS into the lungs and adjust the volume if there is much resistance from lung tissue. Inflamed lung tissue is sensitive to rupture. The volume of PBS used varies according to the size of the mouse. For young mice (6–8 weeks of age), washing three times with <1 ml (~800 µl) is often enough. If the tissue ruptures, as evidenced by leakage of fluid into the pleural cavity, do not proceed with washing the lung, because this will result in contamination of BALF with pleural cavity cells. Collect any remaining fluid within the lung and proceed to Step 18. If the collected BALF contains blood owing to tissue rupture, lyse red blood cells with ACK lysis buffer solution, as described in Step 12A(viii–x) of the main PROCEDURE.

17. Pool the pelleted ILC2s from steps 15 and 17. Centrifuge the cells at 500g for 5 min at 4 °C.
18. Aspirate the supernatant and proceed to Step 13 of the main PROCEDURE (detection by flow cytometric analysis).

tissues, which was quite difficult using previous methods. Published procedures for ILC2 preparation from the lung also describe digestion of tissues in a tube while shaking^{13–16,18,21}. We now incubate lung tissues in a strainer on a six-well plate without shaking, which increases cell viability and yield.

Experimental design

This protocol can be divided into two major sections. The first section of the main PROCEDURE (Steps 1–12) describes the isolation of ILC2s from the mesentery, and the second section (Step 13) details methods for the detection of ILC2s by flow cytometric analysis and isolation by cell sorting. After the main steps of the PROCEDURE, detailed procedures are provided for the isolation of ILC2s from BALF, lung and small intestine (Boxes 1, 2 and 3, respectively; see also Figs. 1 and 2) The following subsections discuss key aspects of the protocol.

Isolation of ILC2s from fat tissue. Although ILC2s from the mesentery can be detected in very young mice, the number

increases with the age of the animals. We routinely use retired mice (~5 months of age) to maximize recovery of ILC2s for sorting, cell culture and other experimental *in vitro* applications requiring purified ILC2s. If the sole aim of the experiment is flow cytometric analysis, the animal's age is not crucial. It is important to take note of the background strain of the mouse in the isolation of ILC2s. We identify mature ILC2s from various tissues of mice on a C57BL/6N or C57BL/6J background as lineage-negative cells that are positive for the expression of KLRG1 and Sca-1. However, Sca-1 expression is very low in some tissues in BALB/c mice, and therefore it cannot be used as a marker for ILC2s. We discuss alternate options for cell surface staining of ILC2s from BALB/c mice.

We present two options for isolating ILC2s from mesentery, depending on whether you are analyzing ILC2s from an individual mouse with a flow cytometer or pooling fat tissue from many mice and sorting with a cell sorter. We routinely sort ILC2s from pooled mesenteries from 20 mice in our laboratory, and we describe herein the optimal reagent volumes for maximum recovery of ILC2s from this number of animals.

Box 2 | Isolation of ILC2s from the lung tissue ● TIMING 70 min–2 h

1. To remove the lungs, hold the upper end of the trachea with forceps and cut (Fig. 1h).
2. Use forceps to pinch the trachea together with the esophagus, and then lift and trim the tissue along the underside to release the lungs, with the heart and thymus attached (Fig. 1i).
3. Dissect the heart and thymus, as well as the y-shaped tracheas and the esophagus structure, from the lobes of the lung (Fig. 1j,k).
4. Place the two lobes of the lung onto a 40- μ m nylon cell strainer in a six-well culture dish containing 7 ml of RPMI medium with Liberase TM (50 μ g/ml final concentration) and DNase I (1 μ g/ml final concentration) (Fig. 1l).
5. Use sterile scissors prewetted with RPMI to mince the lung tissue into ~2-mm segments.
6. Incubate at 37 °C for 45 min.
7. Transfer the cell homogenate into a 50-ml tube.
8. Place the 40- μ m cell strainer containing the remaining partially digested lung tissue over a 50-ml tube.
9. Homogenize manually using the flat end of the plunger of a 1-ml syringe. Wash the strainer repeatedly with 5 ml of 5% (vol/vol) FBS HBSS and continue to homogenize manually up to a total volume of 20 ml.
10. Centrifuge the homogenate at 500g for 5 min at 4 °C.

Prepare the samples for flow cytometry analysis, depending on whether lung tissue ILC2s were extracted from an individual mouse (option A) or whether they will be pooled from lung tissues of multiple mice and sorted by a cell sorter for a variety of analyses (option B).

(A) Sample preparation for ILC2s extracted from a single mouse

- (i) Aspirate the supernatant and resuspend the pellet with 1 ml of ACK lysis buffer solution to lyse RBCs. Let the tube sit for 2 min at room temperature.
- (ii) Wash the cells with 5% (vol/vol) FBS HBSS by centrifugation at 500g for 5 min at 4 °C.
- (iii) Aspirate the supernatant and proceed to Step 13 of the main PROCEDURE (detection by flow cytometric analysis).

(B) Sample preparation from multiple mice

- (i) Aspirate the supernatant, and pool the pellets from each tube into one 50-ml tube by washing with 5% (vol/vol) FBS HBSS. Cell homogenates from up to ten lungs can be pooled into one tube.
- (ii) Centrifuge the mixture at 500g for 5 min at 4 °C.
- (iii) Resuspend the pellet up to 21 ml total volume with 5% (vol/vol) FBS HBSS.

▲ CRITICAL STEP The centrifuge and reagents used during fractionation with Percoll (step 10B(iii–vii)) should be kept at 25 °C, because treatment with Percoll is a temperature-dependent method.

- (iv) Add 9 ml of 100% Percoll to the suspension to make a 30% (vol/vol) Percoll solution.
- (v) Shake the tube vigorously by hand to ensure that Percoll is well mixed.
- (vi) By using a 9-inch Pasteur pipette, gently pipette 5 ml of 70% Percoll over the bottom layer of the 30% Percoll solution in the 50-ml tube.
- (vii) Centrifuge at 500g for 30 min at 25 °C (accelerator and brake OFF).
- (viii) Aspirate 20 ml of the upper 30% Percoll layer that consists of lung cells.
- (ix) Using a 9-inch pipette, collect cells at the interface between the 30 and 70% layers and transfer them to a new 50-ml tube. Red blood cells will remain under the 70% layer.
- (x) To wash cells, fill the 50-ml tube with 5% (vol/vol) FBS HBSS, shake it well and centrifuge it at 500g for 5 min at 4 °C.
- (xi) Aspirate the supernatant and proceed to Step 12B(xv) of the main PROCEDURE.

Box 3 | Isolation of LP ILC2s from the small intestine ● TIMING 2 h 30 min (plus additional time for >1 mouse)

This procedure describes the depletion of Peyer's Patches (PPs), the IEL plus IEC fractions from the LP compartment and isolation of ILC2s from the small intestine of one mouse. If you are isolating ILC2s from multiple mice by cell sorting, do not pool the intestinal samples until the lymphocytes have been completely isolated (step 26B(iv)).

Depletion of PP, IEL and IEC fractions from the LP compartment

1. In preparation for this procedure, add 50 ml per conical flask of 1 mM EDTA PBS to 100-ml conical flasks, which will be used for depletion of IEL plus IEC fractions, and warm them in a 37 °C incubator.
2. Kill the mouse and surface-sterilize the skin using 70% (vol/vol) ethanol.
3. Make an incision in the peritoneal cavity and extract the small intestine, as described in Step 3 of the main PROCEDURE (Supplementary Fig. 1).
4. Remove the surrounding mesentery, and place the small intestine in a 6-cm culture dish containing 3 ml of 5% (vol/vol) FBS HBSS on ice.
5. Stretch out the intestine on Whatman Benchkote absorbent paper moistened with 5% (vol/vol) FBS HBSS.
6. While applying gentle pressure, stroke the surface of the intestine with the edge of the scissors to push out the feces (Fig. 2a). PPs can be easily seen from the contrast of their white color against the brown-colored intestine containing feces. Remove PPs with dissecting scissors (Fig. 2b), and push the remaining fecal matter out of the intestine. The number of PPs in C57BL/6 and BALB/C mice is ~6–8 and 9–12, respectively.
- ▲ **CRITICAL STEP** As PPs are rich in lymphocytes, it is important to remove these cells in order to obtain an enriched population of lymphoid cells from the LP.
7. Open the small intestine by cutting in a longitudinal direction (Fig. 2c). Remove fecal matter that is stuck to the intestinal lumen by gently patting with a Kimwipe.
8. Cut the small intestine into 7–8-cm pieces of equal length.
9. Transfer the small intestinal pieces into a 10-cm plastic dish containing 7 ml of PBS (Fig. 2d).
10. Wash the remaining fecal matter and mucus from the small intestine by grasping the intestinal pieces with forceps and by shaking vigorously in PBS for 30–60 s. Change PBS and repeat this step twice for a total of three washes or until the solution is clear (Supplementary Video 1).
- ▲ **CRITICAL STEP** It is important to wash off feces and mucus before deletion of the IEL plus IEC fraction. Inflamed small intestines contain a large amount of mucus. If the solution is still cloudy after three washes with PBS, repeat Step 11 until the solution becomes clear.
11. Cut the small intestine into 1-cm pieces, and transfer the pieces into the 100-ml conical flask containing 40 ml of 1 mM EDTA PBS that was prepared in step 1 (Fig. 2e).
12. Place the flask in an incubator and stir the intestinal pieces with a 8 × 40 mm stir bar at 37 °C, 500g for 20 min to deplete the IEL plus IEC fraction.
13. Using forceps, transfer the intestinal segments into a 50-ml tube containing 20 ml of PBS, and shake it vigorously by hand for 20 s. Repeat this step in fresh PBS.
- ▲ **CRITICAL STEP** Small intestine pieces from naive mice will be pink-colored at this stage. If the intestinal tissue was highly inflamed, however, there will be a mixture of pink and white intestinal segments. The white pieces contain dead tissue, and they should be discarded. The proportion of white segments observed will depend on the extent of small intestinal inflammation in the mouse.
14. Transfer the intestinal pieces to a 20-ml conical flask using forceps. Mince into 1–2 mm pieces with surgical scissors.
15. Add 10 ml of prewarmed 2% (vol/vol) FBS RPMI medium containing 20 µg/ml Liberase DH and 50 µg/ml DNase I (Fig. 2f).
16. Place the flask in the incubator and stir it with a 8 × 25 mm stir bar at 37 °C, 500g for 30 min (Fig. 2g).
17. Remove the flask from the incubator. Tilt the flask at a 30° angle and allow it to rest on the laboratory bench for 1 min to allow the intestinal segments to collect at the bottom.
18. Transfer 7 ml of the supernatant to a 15-ml tube and store it on ice.
19. For the remaining 3 ml in the conical flask, repeatedly draw the mixture through an 18-gauge needle using a 2.5-ml syringe to further mince the intestinal pieces (Fig. 2g and Supplementary Video 2).
20. Add 7 ml of fresh prewarmed 2% (vol/vol) FBS RPMI medium containing 20 µg/ml Liberase DH and 50 µg/ml DNase I to the conical flask (Fig. 2h), return it to the incubator and stir it at 37 °C, 500g for 30 min (Fig. 2i).
21. Filter 17 ml of sample (7 ml from step 18 and 10 ml from step 20) through a 70-µm EASY strainer into a new 50-ml tube to remove cellular debris.
22. By holding the strainer over the 50-ml tube, manually homogenize the particulate matter remaining in the strainer using the flat end of the plunger of a 1-ml syringe. Wash the strainer with small volumes of 5% (vol/vol) FBS HBSS (~3 ml) up to a total volume of 50 ml while alternately homogenizing any remaining material in the strainer.
23. Centrifuge at 500g for 5 min at 4 °C. Aspirate the supernatant to the lower line on the 50-ml tube, which corresponds to ~3 ml of remaining liquid, and filter the homogenate through a 37-µm nylon filter into a new 15-ml tube.
24. Repeatedly wash the filter with small volumes of 5% (vol/vol) FBS HBSS (~2 ml) up to a total volume of 15 ml, and centrifuge at 500g for 5 min at 4 °C.
25. Prepare samples for flow cytometry analysis, depending on whether LP ILC2s were extracted from an individual mouse (option A) or pooled from multiple mice (option B).

(continued)

Box 3 | (continued)

(A) Sample preparation for ILC2s extracted from a single mouse

- (i) Aspirate the supernatant.
- (ii) Proceed to Step 13A of the main PROCEDURE.

(B) Sample preparation for sorting ILC2s from pooled samples ● TIMING 40 min

- (i) Aspirate the supernatant.
- (ii) Add 3 ml of 100% Percoll to each tube, and then shake the tubes vigorously by hand to ensure complete mixing.
- (iii) Centrifuge at 500g for 20 min at 25 °C (accelerator and brake OFF).
- (iv) Aspirate the supernatant and pool the cell pellets from multiple tubes into one 15-ml tube (up to 15 ml) with 5% (vol/vol) FBS HBSS, and centrifuge at 500g for 5 min at 4 °C.
- (v) Aspirate the supernatant and proceed to Step 13B of the main PROCEDURE.

However, the procedure can easily be adapted for pooling tissues from different numbers of mice.

The initial step in the isolation of ILC2s from the mesentery is perfusion of the mouse to remove peripheral blood. The purpose of this is twofold: to remove a relatively minor population of Lin⁺c-Kit⁺Sca-1⁺ early T cell progenitors that are known to be present in circulation, and to obtain a cell population that is enriched in mesentery-specific immune cells rather than blood leukocytes, thus increasing the proportion of ILC2s that can be obtained during cell sorting. This step is crucial if the downstream applications with ILC2s are focused on developmental analysis. However, if the sole aim of the experiment is to detect the presence of ILC2s by flow cytometric analysis, this step is unnecessary.

We have found that proper technique for dissection of the mesentery from the small intestine is a critical factor that determines viability and yield of ILC2s. The pancreas, which is enclosed in the proximal portion of the mesentery, must be completely avoided. Contamination with pancreatic enzymes severely reduces viability of the ILC2 population²⁶. **Figure 3** describes the technique for dissection of the mesentery.

We pool mesenteries from multiple animals when sorting ILC2s for cell culture and/or other downstream applications. As ILC2s are a minor cell population in most tissues, to enrich the ILC2 population before sorting, we deplete lineage-positive cells by labeling them with a mixture of anti-mouse biotinylated antibodies for lineage markers and using streptavidin MicroBeads and the autoMACS Pro Separator. In the absence of the autoMACS

Pro Separator, the cells can be sorted by gating out lineage-positive cells using an APC-conjugated anti-streptavidin marker along with antibodies specific for Sca-1 and KLRG1, which are surface markers present on ILC2s. As there are more total cells in the solution, however, the time needed to sort the cells will be increased, and this must be factored into the total timing set aside for the experiment.

Detection of ILC2s by flow cytometric analysis and isolation by cell sorter. Approximately 2×10^4 cells can be recovered from the mesentery, 1×10^4 from the lung, 5×10^3 from BALF and 3×10^4 from the small intestine of one retired mouse. In all of the tissues, mature ILC2s are identified as lineage-negative lymphoid cells that express KLRG1 and Sca-1 (**Fig. 4**). Furthermore, all ILC2s are positive for intracellular expression of GATA-3. We also stain with other antibodies for cell surface molecules expressed on ILC2s including CD45, c-Kit, Thy-1.2, CD25 and T1/ST2 to confirm the identity of these cells and to further characterize their expression in various tissues. However, the expression levels of these additional cell surface markers differ in ILC2s isolated from various tissues, thereby revealing the presence of tissue-specific populations of ILC2s. It is important to note that these

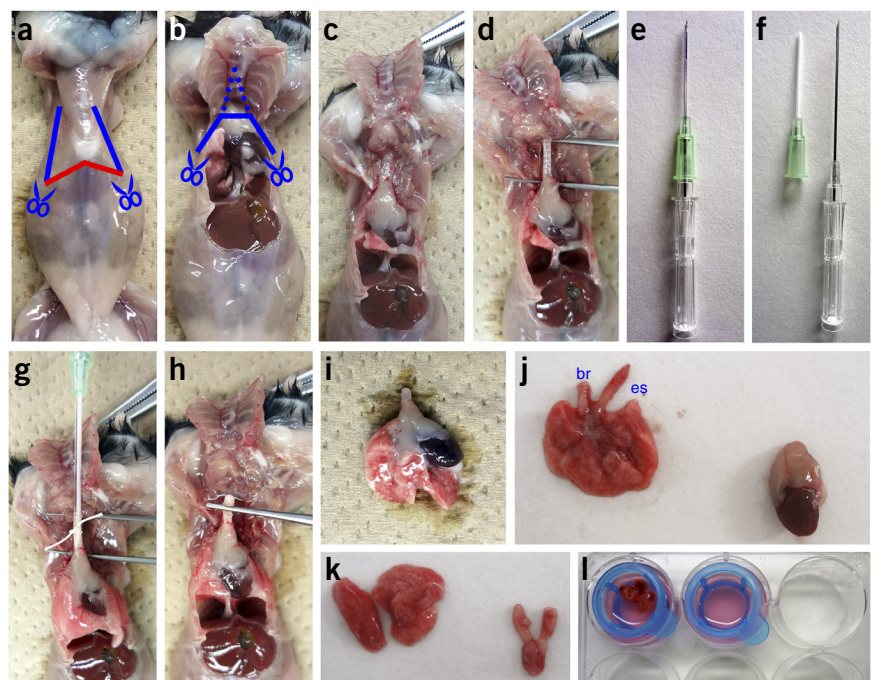
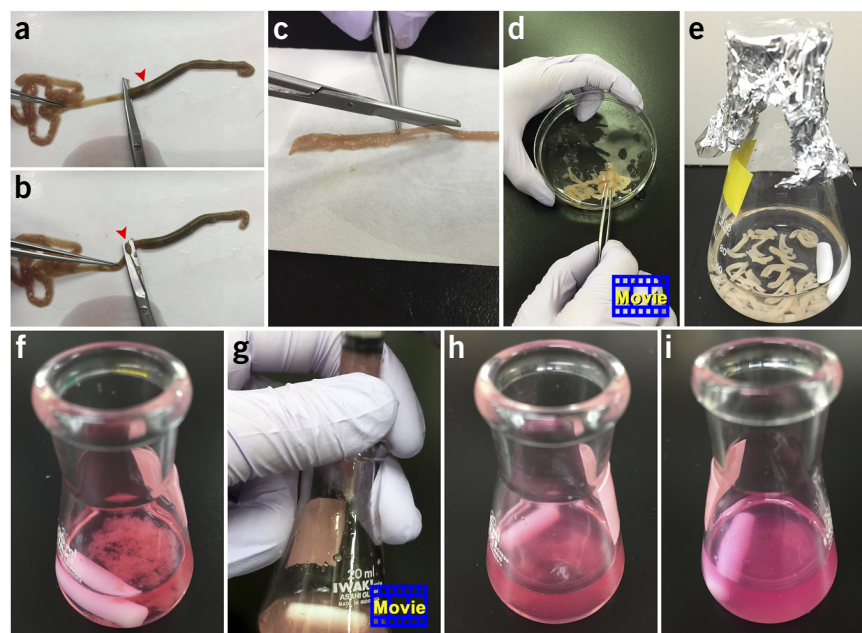


Figure 1 | Stepwise procedure for collection of BALF (steps 1–17, **Box 1**) and lung ILC2s (steps 1–4, **Box 2**). (a–c) Opening of the thoracic cavity. By using surgical scissors, first cut along the bottom edge of the sternum indicated by the red lines, and then along both sides of the rib cage, which is shown by the blue lines. (d–g) Bronchoalveolar lavage for collection of BALF and immune cells. Use a plastic needle to avoid damage to the epithelial lining. (h,i) Cut the upper part of the trachea and proceed with dissection of lungs together with the esophagus, heart and thymus. (j,k) Dissect the lungs from the heart and the thymus. (l) Place the lungs onto a 40-μm filter in six-well dish containing 7 ml of 5% (vol/vol) FBS HBSS. br, bronchi; es, esophagus.

Figure 2 | Stepwise procedure for the isolation of LP ILC2s from the small intestine (steps 1–20, **Box 3**). (a,b) While applying gentle pressure, stroke the surface of the intestine with the edge of the scissors to push out the feces. Dissect out the PPs, which are easily seen from the contrast of their white color against the brown color of the intestine containing feces, and push the remaining fecal matter out of the intestine. Red arrowheads indicate Peyer's patches. (c) Open the intestine by cutting in a longitudinal direction. (d) Transfer the small intestinal pieces into a 10-cm dish containing 7 ml of PBS, and then wash off the remaining fecal matter and mucus by grasping with forceps and shaking vigorously for 30–60 s (**Supplementary Video 1**). (e) Cut the small intestine into 1-cm pieces and transfer the pieces into a conical flask containing 40 ml of prewarmed 1 mM EDTA PBS. (f) Minced intestinal segments in prewarmed 2% (vol/vol) FBS RPMI before digestion with 20 $\mu\text{g}/\text{ml}$ Liberase DH and 50 $\mu\text{g}/\text{ml}$ DNase I. (g) To further digest the intestinal tissue, repeatedly draw the mixture through an 18-gauge needle using a 2.5-ml syringe (**Supplementary Video 2**). (h) Solution containing intestinal tissue before second digestion with 20 $\mu\text{g}/\text{ml}$ Liberase DH and 50 $\mu\text{g}/\text{ml}$ DNase I. (i) Solution containing intestinal tissue after digestion with 20 $\mu\text{g}/\text{ml}$ Liberase DH and 50 $\mu\text{g}/\text{ml}$ DNase I.



antibodies are not essential for the detection of ILC2s. The combination of lineage markers plus Sca-1 and KLRG1 is sufficient to detect mature ILC2s in various tissues of naive mice.

ILC2s from the mesentery can also be sorted as $\text{Lin}^{-}\text{c-Kit}^{+}\text{Sca-1}^{+}$ cells, as described in our initial report on ILC2s in adipose tissue¹¹. The phenotype of surface markers and the ability to produce cytokines were indistinguishable between ILC2s sorted as $\text{Lin}^{-}\text{c-Kit}^{+}\text{Sca-1}^{+}$ cells and $\text{Lin}^{-}\text{Sca-1}^{+}\text{KLRG1}^{+}$ cells. However, when sorting ILC2s from the lung, bone marrow and intestine,

KLRG1 should be used instead of c-Kit, as c-Kit expression in these tissues is lower than that in the mesentery. As mentioned above, Sca-1 cannot be used as a surface marker to identify ILC2s in BALB/c mice. If you are using this strain, we recommend using the combination of KLRG1 and Thy-1 with lineage markers.

Isolation of ILC2s from other tissues. The latter part of the protocol provides methods for the isolation of ILC2s from other tissues. To analyze ILC2s in BALF, as discussed in **Box 1**, animals must either be under conditions of elevated secretion of IL-25 and/or IL-33, as seen in helminth-infected mice and certain mouse models of asthma, or these cytokines must be previously administered to the animals, as ILC2s are undetectable in BALF of naive mice. We routinely administer the cytokines IL-2 + IL-25 or IL-33 alone (500 ng/mouse) intratracheally to mice on days 0 and 2, and we kill the animals on day 4 for the isolation of ILC2s. Lungs from cytokine-treated mice and helminth-infected mice are severely inflamed. Therefore, gentle washing of the lung by infusion with PBS is important to effectively collect BALF cells. If too much pressure is used to infuse PBS, the lung tissue will rupture and it will be very difficult to obtain a pure population of BALF cells. BALF ILC2s can be identified as $\text{CD45}^{+}\text{PI}^{-}$ lymphocytes that express T1/ST2 and CD25. As injection with cytokines does not induce T1/ST2-positive $\text{T}_{\text{H}2}$ cells, ILC2s in BALF can be detected

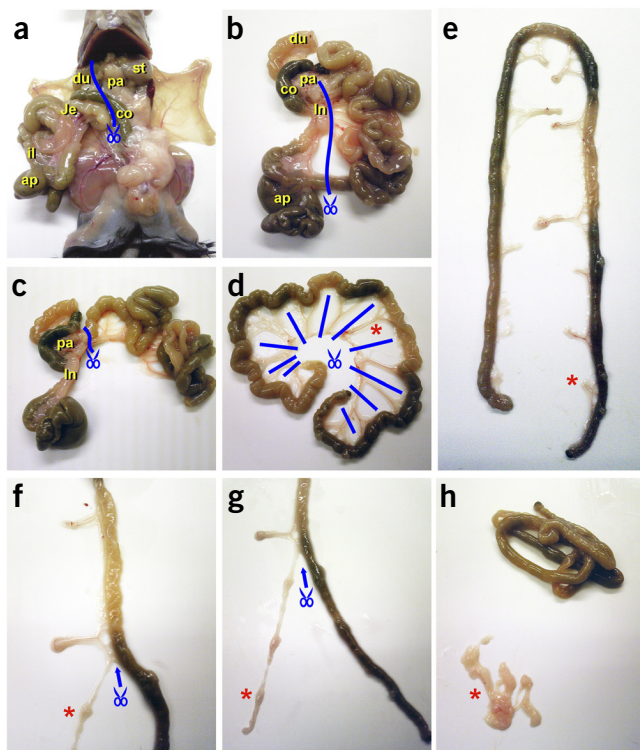
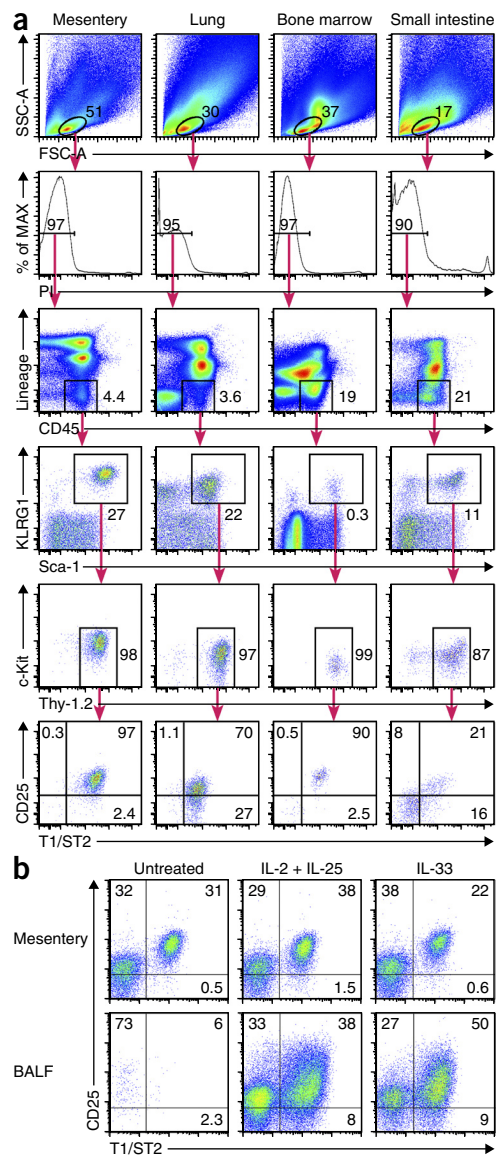


Figure 3 | Stepwise procedure for the dissection of the mesentery (Steps 1–11 of the main PROCEDURE). (a) Extract the small intestine together with mesentery and appendix from the peritoneal cavity. (b,c) Dissect the mesentery and the small intestine from the connecting appendix and lymph nodes, by avoiding the pancreas. (d) Arrange the intestine in a fan-shape and cut the adjoining sections of the mesentery. (e–h) Straighten the intestine and dissect the mesenteric adipose. Blue lines and arrows indicate the method for tissue dissection using surgical scissors. Red asterisks designate mesenteric adipose tissue. st, stomach; du, duodenum; je, jejunum; il, ileum; ap, appendix; co, colon; pa, pancreas; ln, lymph node.

Figure 4 | Representative gating strategy for the identification of ILC2s in various tissues. (a) Analysis of mouse ILC2s from mesentery, lung, bone marrow and small intestine. As ILC2s are slightly larger than typical lymphocytes, gate them with a slightly larger lymphocyte gate on the basis of FSC and SSC properties. When sorting ILC2s, we remove doublet cells and gate for single cells on FSC-W versus FSC-H and SSC-W versus SSC-H plots (plots not shown) after applying the FSC-SSC gate. This is important to obtain a high purity of cells during sorting, but it is not necessary if analysis of this cell population is the sole purpose of the experiment. After selection for live cells with PI, gate CD45⁺Lin⁻ cells. ILC2s can be identified within this gate on the basis of positive expression for KLRG1 and Sca-1. Within this subset of cells, expression of c-Kit, CD25 and T1/ST2 can also be used as cell surface markers for the identification of ILC2s, although these molecules are differentially expressed in various tissues, suggesting the existence of heterogeneous populations of ILC2s. Whereas ILC2s from the mesentery are uniform, exhibiting high expression of all the above surface markers, in the bone marrow, ILC2s have low expression of c-Kit. Both CD25 and T1/ST2 are expressed at low levels in LP ILC2s but show intermediate expression in ILC2s in the lung. Note that Thy-1.2 is expressed at high levels in ILC2s from all tissues, and it can be used along with KLRG1 to identify these cells. This gating strategy is designed for the isolation of ILC2s of high purity. (b) Analysis of mouse ILC2s from the mesentery and BALF of cytokine-treated mice. Mice were treated intratracheally with IL-2 + IL-25 (500 ng each per mouse) or IL-33 (500 ng per mouse) on days 0 and 2 and killed on day 4. After gating on FSC versus SSC, ILC2s were identified as CD45⁺PI⁻ cells (plots not shown) that are positive for the expression of T1/ST2 and CD25. As it is known that injection with these cytokines does not induce T1/ST2⁺ T_H2 cells, flow cytometric analysis was performed without staining for lineage markers. ILC2s are undetectable in BALF of untreated mice, but they are present in large numbers in cytokine-treated mice. In addition, intratracheal administration of cytokines does not affect accumulation of ILC2s in mesentery fat.



by flow cytometry without staining for lineage markers. However, it is important to note that some reagents that are known to induce ILC2s in BALF, such as ovalbumin in ovalbumin-primed mice, also induce T_H2 cells that express T1/ST2. If you are using those reagents, it is important to stain with lineage markers in order to distinguish ILC2s from T_H2 cells. Furthermore, staining with lineage markers should also be performed if you are sorting ILC2s from BALF in order to obtain a cell population of high purity. The isolation and analysis of lung ILC2s from naive mice are discussed in **Box 2**.

Box 3 contains a detailed protocol for the isolation of LP ILC2s from the small intestine, in which the tissue is digested with a magnetic stirrer in a conical flask in an incubator at 37 °C. Therefore, an incubator that can house a magnetic stirrer is needed to perform this procedure properly. The incubator that we

use has an opening through which the electric cord of the stirrer can pass through. Unlike most current protocols, our methods allow for the isolation of ILC2s from inflamed intestinal tissue by rigorous washing in PBS to remove mucus before deletion of the intraepithelial lymphocyte (IEL) plus intraepithelial cell (IEC) fraction. However, it should be noted that depending on

Box 4 | *In vitro* culture of ILC2s ● TIMING variable

ILC2s sorted from naive mice can be expanded in cell culture. The seeding density and culture dish can appreciably affect the growth characteristics of these cells. For routine expansion of ILC2s, seed 5,000 cells per well in round-bottomed 96-well plates in 10% (vol/vol) FBS RPMI complete medium containing 10 ng/ml IL-2. Under IL-2 stimulation, ILC2s can expand at a gradual rate for >3 months. An FBS lot check is recommended, as lot-specific differences in FBS influence the expansion of these cells. In our experience, the expansion rate of ILC2s from other tissues, such as bone marrow or lung, in RPMI complete medium containing 10 ng/ml IL-2 is less than that of mesentery-derived ILC2s. ILC2s from the small intestine cannot be maintained in IL-2. For cell activation and induction of rapid growth, we routinely culture ILC2s with 10 ng/ml IL-33 or 10 ng/ml IL-2 + 10 ng/ml IL-25 in 10% (vol/vol) FBS RPMI complete medium. For cell maintenance, culture ILC2s in 10 ng/ml IL-7 in 10% (vol/vol) FBS RPMI complete medium. Half of the medium should be changed every 2 d. We have observed a large variability in the stability and activity of various commercial cytokines and have found those from R&D Systems to be the most stable for culture of ILC2s.

the extent of inflammation in the intestine some of the portions of the small intestine may be damaged, thus containing a greater proportion of dead cells. After incubation of the intestinal segments to deplete the IEL plus IEC fraction followed by vigorous shaking in a 50-ml tube containing PBS, the dead tissue is white and the normal tissue is pink. We generally do not observe white tissue segments when isolating ILC2s from naive mice. At this stage, discard the white tissue and proceed through the remaining steps for digestion using only the pink tissue segments. In addition, as inflamed intestinal tissue contains a large amount of mucus, more extensive washing in PBS will be needed before deletion of the IEL plus IEC fraction. The extra washes should be factored in to the overall timing of the experiment.

The final part of the protocol discusses options for *in vitro* culture of ILC2s sorted from the mesenteries of naive mice (**Box 4**). For routine expansion of ILC2s, we culture them in 10% (vol/vol) FBS RPMI complete medium containing IL-2. In our experience, the expansion rate of ILC2s isolated from the mesentery is greater than that of ILC2s from other tissues. It is important to note,

however, that ILC2s isolated from the small intestine cannot be maintained in medium containing IL-2. The procedures presented in **Box 4** include considerations as to the types of cytokines to use, as well as the best conditions for cell culture of ILC2s.

Applications and limitations of the technique

The methods for the isolation of cells from tissues discussed in this protocol can easily be applied not only to the isolation of ILC2s but also to other types of lymphocytes present in the lung, small intestine and adipose tissues. Indeed, we recently reported on the isolation of regulatory T cells from adipose tissues²⁷. The main limitation of this method is that it results in a low yield of cells from the intestinal intraepithelial fraction. Although the present method (using a stirrer in a conical flask instead of a plastic tube offers a higher yield of cells from the LP fraction of the small intestine (which contains ILC2s and ILC3s), this method is not appropriate for cells in the intestinal intraepithelial fraction, as EDTA contained in the medium kills IECs. Thus, it is not suitable for the isolation of IELs, including ILC1s.

MATERIALS

REAGENTS

Mice **! CAUTION** Mice should be kept under specific pathogen-free conditions. All experiments should be performed in accordance with protocols approved by the appropriate Institutional Animal Care and Use Committee.

- 1× PBS (Sigma-Aldrich, cat. no. P5368)
- RPMI-1640 medium (Sigma-Aldrich, cat. no. R8758)
- Somnopentyl (Kyoritsu Seiyaku, cat. no. 2236; use 5× diluted in saline)
- Saline (Otsuka Pharmaceutical Factory, cat. no. 3311401A2026)
- Heparin (Mochida Pharmaceutical, cat. no. 2508549)
- 10× HBSS (Gibco, cat. no. 14185-052)
- Phenol red (Wako, cat. no. 163-01122)
- FBS (Gibco, cat. no. 10437-028; for use in 5% (vol/vol) FBS/HANKS buffer)
- Sodium azide (NaN₃; Sigma-Aldrich, cat. no. S2002) **! CAUTION** NaN₃ is known to be toxic. Avoid contact with skin, eyes and mucous membranes.
- FBS (Japan Bioserum, cat. no. S1820-500; for use in Complete RPMI-1640 culture medium)
- Penicillin-streptomycin (Gibco, cat. no. 15140-122)
- HEPES buffer solution (Sigma-Aldrich, cat. no. H3537)
- MEM non-essential amino acids (Sigma-Aldrich, cat. no. M7145)
- Sodium pyruvate (Gibco, cat. no. 11360-070)
- 2-Mercaptoethanol (Gibco, cat. no. 21985-023) **! CAUTION** 2-mercaptoethanol is known to be toxic. Avoid contact with skin, eyes and mucous membranes.
- Gentamycin sulfate (Nacalai Tesque, cat. no. 16637-74)
- DMEM medium (Sigma-Aldrich, cat. no. D5796)
- BSA (Sigma-Aldrich, cat. no. A3912)
- NH₄Cl (Sigma-Aldrich, cat. no. A9434)
- KHCO₃ (Wako, cat. no. 166-03275)
- EDTA-2Na (Wako, cat. no. 345-01865)
- Propidium iodide (PI; Sigma-Aldrich, cat. no. 81845)
- Percoll plus (GE Healthcare, cat. no. 17-5445-01)
- Liberase DH, research grade (Roche, cat. no. 05401089001)
- Liberase TM, research grade (Roche, cat. no. 05401127001)
- Dispase (Gibco, cat. no. 17105-041)
- DNase I (Roche, cat. no. 10104159001)
- Antibodies for depletion of lineage-positive cells, flow cytometric analysis and sorting (**Table 1**)
- Streptavidin MicroBeads (Miltenyi Biotec, cat. no. 130-048-101)
- Recombinant mouse IL-2 (R&D Systems, cat. no. 402-ML-100/CF)
- ▲ **CRITICAL** We have observed a large variability in the stability and activity of various commercial cytokines and have found those from R&D Systems to be the most stable for culture of ILC2s.
- Recombinant mouse IL-7 (R&D Systems, cat. no. 407-ML-200/CF; see comment above)

- Recombinant mouse IL-25 (R&D Systems, cat. no. 1399-IL-025/CF; see comment above)
- Recombinant mouse IL-33 (R&D Systems, cat. no. 3626-ML-010/CF; see comment above)

EQUIPMENT

- Flow cytometer (FACSCalibur or FACSCanto (BD Biosciences) for analysis and FACS Aria (BD Biosciences) for cell sorting)
- autoMACS Pro Separator (Miltenyi Biotec, cat. no. 130-092-545)
- Constant-temperature incubator/shaker (for ILC2 isolation from mesentery, BALF and lung)
- Constant-temperature incubator that can house a magnetic stirrer (for ILC2 isolation from small intestine)
- (Optional) GentleMACS dissociator (Miltenyi Biotec, cat. no. 130-093-235) and gentleMACS C tubes (Miltenyi Biotec, cat. no. 130-096-334). These items are optional for ILC2 digestion from adipose tissue. Alternatively, tissues can be digested in a 50-ml plastic tube on an incubator/shaker with intermittent vigorous shaking by hand
- CO₂ incubator
- Tube rotator
- Plastic tubes: 15-ml tube (BD Falcon, cat. no. 352096), 50-ml tube (BD Falcon, cat. no. 352070), 5-ml tube (Bio-bik, cat. no. ST-500), 1.5-ml tube (biomedical sciences, cat. no. BC-MPF-150C) and 1.5-ml screw cap microcentrifuge tube (Sarstedt, cat. no. 72.687.028S)
- 5-ml polystyrene round-bottomed FACS tubes (BD Biosciences, cat. no. 352058)
- 6-cm round culture dishes (Corning, cat. no. 430166)
- 6-well culture plates (Thermo, cat. no. 140675)
- 96-well culture plates (BD Falcon, cat. no. 353077)
- 1-ml syringes (Terumo, cat. no. SS-01T), 2.5-ml syringe (Terumo, cat. no. SS-02SZP) and 50-ml syringe (Terumo, cat. no. SS-50ESZ)
- PSV winged needle set, 20 gauge × 3/4 inches (Nipro, cat. no. 04-020)
- 18 Gauge × 11/4-inch Teflon catheter (Terumo, cat. no. SR-FS1832)
- 40-µm Nylon cell strainer (BD Falcon, cat. no. 352340)
- 100-µm Nylon cell strainer (BD Falcon, cat. no. 352360)
- 70-µm EASY strainer (Greiner Bio-One, cat. no. 542 070)
- 37-µm Nylon mesh (Tokyo Screen, cat. no. N-No.355T)
- Pasteur pipette, 5 3/4 inch (Iwaki, cat. no. IK-PAS-5P)
- Pasteur pipette, 9 inch (Iwaki, cat. no. IK-PAS-9P)
- Tubing for perfusion (NIPRO, cat. no. EX5-45AC)
- Silk braid, 2-0 type (Alfred, cat. no. GE02SW)
- Whatman Benchkote absorbent paper (Whatman, cat. no. 2300-916)
- 8 × 25 mm magnetic bar (IKA, cat. no. 1572300)
- 8 × 40 mm magnetic bar (IKA, cat. no. 1572500)

TABLE 1 | Antibodies for depletion of lineage-positive cells, flow cytometric analysis and sorting of ILC2s.

Ab name	Clone	Supplier	Cat. no.	Concentration of stock (mg/ml)	Dilution in reaction
Blocking of Fc receptor					
Anti-mouse CD16/32 (2.4G2) purified	2.4G2	Hybridoma	—	0.25	1:200
Detection of ILC2s for analysis					
Rat anti-mouse T1/ST2 mAb-FITC conjugated	DJ8	MD Biosciences	101001F	1	1:500
Anti-mouse KLRG1-PECy7	2F1	eBioscience	25-5893-80	0.2	1:1,000
BV421 rat anti-mouse Ly-6A/E (Sca-1)	D7	BD Pharmingen	562729	0.2	1:500
PerCPCy5.5 rat anti-mouse CD25	PC61	BD Pharmingen	551071	0.2	1:100
APCCy7 mouse anti-mouse CD45.2	104	BD Pharmingen	560694	0.2	1:200
PE rat anti-mouse CD117 (c-Kit)	2B8	BD Pharmingen	553355	0.2	1:100
V500 rat anti-mouse CD90.2 (Thy1.2)	53-2.1	BD Pharmingen	561616	0.2	1:400
Detection of ILC2s for analysis and sorting					
FITC rat anti-mouse Ly-6A/E (Sca-1)	E13-161.7	BD Pharmingen	553335	0.5	1:500
PE hamster anti-mouse KLRG1	2F1	BD Pharmingen	561621	0.2	1:200
Deletion of Lineage+ cells					
Biotin hamster anti-mouse CD3ε	145-2C11	BD Pharmingen	553060	0.5	1:100
Biotin rat anti-mouse CD4	GK1.5	BD Pharmingen	553728	0.5	1:500
Biotin rat anti-mouse CD8α	53-6.7	BD Pharmingen	553029	0.5	1:400
Biotin hamster anti-mouse CD11c	HL3	BD Pharmingen	553800	0.5	1:200
Biotin anti-mouse FcεRIα	MAR-1	BioLegend	134304	0.5	1:800
Biotin mouse anti-mouse NK1.1	PK136	BD Pharmingen	553163	0.5	1:400
Biotin rat anti-mouse CD19	1D3	BD Pharmingen	553784	0.5	1:1,000
Biotin rat anti-mouse TER119	TER119	Hybridoma	—	0.55	1:200
Biotin rat anti-mouse CD5	53-7.3	BD Pharmingen	553019	0.5	1:400
Anti-mouse F4/80 antigen biotin	BM8	eBioscience	13-4801-85	0.5	1:800
Biotin rat anti-mouse Ly-6G and Ly-6C (Gr-1)	RB6-8C5	BD Pharmingen	553125	0.5	1:1,000
Second and third Ab for deletion of lineage+ cells					
APC streptavidin	—	BD Pharmingen	554067	0.2	1:400
Streptavidin MicroBeads	—	Miltenyi Biotec	130-048-101	—	0.1 ml for 1 × 10 ⁸ cells

- 100-ml conical flask
- 20-ml conical flask
- Dissecting scissors
- Forceps of various sizes, including 10-cm size
- Hemocytometer

REAGENT SETUP

FBS Heat-inactivate FBS in a water bath at 56 °C for 30 min before use. Heat-inactivated serum can be aliquotted and stored at −30 °C for up to 1 year.

5% (vol/vol) FBS HBSS buffer Prepare a solution containing 5% (vol/vol)

heat-inactivated FBS, 10 µg/ml phenol red and 1× HBSS. Store the solution at 4 °C for 2 weeks.

FACS buffer solution Prepare a solution containing 2% (vol/vol) heat-inactivated FBS, 0.02% (wt/vol) NaN₃ and 1× HBSS. Store the solution at 4 °C for 1 month. **! CAUTION** NaN₃ is known to be toxic. Avoid contact with skin, eyes and mucous membranes.

Complete RPMI-1640 culture medium Supplement RPMI-1640 medium with 10% (vol/vol) heat-inactivated FBS (from Japan BioSerum), 100 U/ml penicillin-streptomycin, 10 mM HEPES buffer solution, 1× MEM

nonessential amino acids, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol and 50 μ g/ml gentamycin sulfate. Store the medium in sterile conditions at 4 °C for up to 2 months. **! CAUTION** 2-mercaptoethanol is known to be toxic. Avoid contact with skin, eyes and mucous membranes.

4% (wt/vol) BSA/DMEM solution Supplement DMEM medium with 4% (wt/vol) BSA. Store the solution at 4 °C for up to 3 months.

0.5% (wt/vol) BSA/PBS solution Supplement 1 $\times$ PBS with 0.5% (wt/vol) BSA. Store the solution at 4 °C for up to 2 months.

ACK lysis buffer solution Prepare a solution containing 0.15 M NH_4Cl , 0.01 M KHCO_3 and 0.1 mM EDTA-2Na in distilled water. Autoclave the solution at 121 °C (200 kPa) for 20 min to sterilize. Store the solution at 4 °C for up to 3 months.

PI/PBS solution Supplement 1 $\times$ PBS with 0.5 μ g/ml PI. Store the solution at 4 °C for up to 3 months.

100% Percoll solution Prepare a 10 $\times$ dilution of Percoll Plus by diluting it with 10 $\times$ HBSS. Store the solution at 4 °C for up to 3 months.

PROCEDURE

Isolation of ILC2s from the mesentery ● **TIMING** 10 min per mouse

1| Administer somnopentyl (5 $\times$ -diluted in saline) to the mouse by i.p. injection. Any anesthetic suitable for use in rodents can be used. Once the animal is deeply anesthetized, place it in a supine position.

2| Surface-sterilize the skin using 70% (vol/vol) ethanol.

3| Make an incision (**Supplementary Fig. 1a–d**) in the abdominal cavity and continue to cut upward through the rib cage and diaphragm so that the peritoneal and thoracic cavities are exposed.

4| Attach a 50-ml syringe containing 10 U of heparin/PBS to a 20 gauge $\times$ 3/4-inch winged needle with extension tubing.

5| While grasping the heart with dissecting forceps, insert the tip of the needle into the left ventricle.

6| Make a small cut in the middle of the right atrium with surgical scissors to allow blood to flow out.

7| (Optional) Perform a transcardial perfusion with 10 U/ml heparin/PBS until the liver becomes pale, which indicates that blood has been eliminated. The optimal flow rate is $\sim$ 20 ml/30 s. If the procedure is performed correctly, 20 ml is sufficient to perfuse one mouse.

▲ CRITICAL STEP Perfusion enables the removal of peripheral blood from the mesentery, which has an abundant supply of blood vessels. This step removes blood leukocytes (including a relatively minor population of Lin[−]c-Kit⁺Sca-1⁺ early T cell progenitors), leading to a cell population that is enriched in mesentery-specific immune cells. It is important to remove these cells if the intended downstream application of the ILC2s involves investigation of developmental analysis. If the sole aim of the experiment is to detect the presence of ILC2s by flow cytometric analysis, perfusion is unnecessary. In that case, the mouse can be killed by cervical dislocation, and the mesentery can be dissected as described from Step 8 of the main PROCEDURE.

8| Extract the small intestine and mesentery together with the appendix and mesenteric lymph nodes by cutting at the colon and at the adjoining point of the duodenum to the stomach (**Fig. 3a**). If tissues from many mice will be pooled for sorting of ILC2s, extract the small intestine and mesentery together from the peritoneal cavity of each mouse and pool in 5% (vol/vol) FBS HBSS until perfusion of each mouse is completed. Long-term incubation (>1 h) of the mesentery with the intestine results in reduced viability of ILC2s, and it should be avoided.

9| Starting from the distal end, dissect the mesentery and small intestine from connecting appendix and lymph nodes by cutting along the border of the appendix, alongside the mesenteric lymph nodes (**Fig. 3b**). Cut at the proximal end of the mesentery, excluding the pancreas (**Fig. 3c**).

10| Arrange the mesentery in a fan shape and cut the adjoining tissue sections (**Fig. 3d,e**).

11| To dissect the mesentery, gently pull fat tissue to one side using forceps and cut upward along the border of the intestine starting from the distal end (**Fig. 3f–h**).

▲ CRITICAL STEP When you are dissecting the mesentery from the small intestine, it is crucial to completely avoid dissection of the pancreas, which is enclosed in the proximal portion of the mesentery. Contamination of the mesentery with pancreatic tissue results in severely reduced viability of the ILC2 population, as described by Caesar and Drevon²⁶. The pancreas can be distinguished from the adipose tissue as a pale yellow-colored glandular tissue.

Preparation of ILC2s

12| Prepare ILC2s from an individual mouse for analysis or from multiple mice for sorting, as described in Steps 12A and B, respectively.

(A) Preparation of ILC2s from an individual mouse for analysis ● **TIMING 90 min (add 10 min per mouse for >1 mouse)**

- (i) Place the mesentery into a 15-ml tube containing 7 ml of 4% (wt/vol) BSA in DMEM medium on ice.
- (ii) Tilt the tube at a 45° angle to separate fat from liquid, and mince the tissue with surgical scissors prewetted with 4% (wt/vol) BSA/DMEM (**Fig. 5a,b**).
- (iii) Add Liberase DH (20 µg/ml final concentration) and DNase I (1 µg/ml final concentration) to the tube.
- (iv) Shake the tube vigorously by hand to ensure dispersion of Liberase and DNase I.
- (v) Preincubate the tubes in a 37 °C water bath for 5 min.
- (vi) Incubate the tubes at 37 °C at 150 r.p.m. for 60 min. Shake the tubes vigorously by hand for 10 s halfway through the incubation (after 30 min) and after 1 h.
▲ CRITICAL STEP We have found that shaking the tubes by hand intermittently during this incubation stage greatly increases the digestion of adipose tissue and subsequent recovery of viable ILC2s.
- (vii) Centrifuge the tubes at 500g for 5 min at 4 °C. Before centrifugation, the solution should be homogeneous and pink-colored, as in **Figure 5c**, indicating complete digestion of the adipose tissue. After centrifugation, the adipocytes will be in the upper floating layer. The pellet consists of the stromal vascular fraction that includes immune cells (**Fig. 5d**).
? TROUBLESHOOTING
- (viii) Aspirate the upper layer of adipocytes and supernatant, and resuspend the pellet with 1 ml of ACK lysis buffer solution to lyse RBCs. If the mouse was perfused with 10 U/ml heparin/PBS before dissection of the mesentery, skip this step. ACK lysis is not necessary, and you can proceed directly to flow cytometric analysis. Let the tube sit for 2 min at room temperature (20–25 °C).
- (ix) Wash the cells with 5% (wt/vol) FBS HBSS by centrifugation at 500g for 5 min at 4 °C.
- (x) Aspirate the supernatant and proceed directly to flow cytometric analysis (Step 13A).

(B) Preparation of ILC2s from multiple mice for sorting (mesentery pooled) ● **TIMING 2 h 40 min**

- (i) Pool mesenteries from 20 mice into a 50-ml tube containing ~40 ml of 4% (wt/vol) BSA/DMEM medium. Keep the tube on ice until the tissues have been dissected from all mice (**Fig. 6a,b**).
▲ CRITICAL STEP The volumes of reagents described throughout this option in the procedure have been optimized for the preparation of ILC2s from adipose tissue of 20 mice and should be adjusted accordingly if you are pooling tissues from different numbers of animals.
- (ii) Use forceps to transfer the adipose tissue to one gentleMACS C tube (**Fig. 6c,d**).
- (iii) Mince the tissue using surgical scissors prewetted with 4% (wt/vol) BSA/DMEM. If you are combining samples from >20 mice, divide the adipose tissue into two or three gentleMACS C tubes to a maximum of 5 ml per tube (**Fig. 6e**). Wash fat from the scissors and bring the total volume to 10 ml with 4% (wt/vol) BSA/DMEM medium. If gentleMACS C tubes are not available, transfer the adipose tissue to a 50- ml tube, mince it and bring up to a total volume of 30 ml with 4% (wt/vol) BSA/DMEM.
- (iv) Add Liberase DH to a final concentration of 20 µg/ml.
- (v) Preincubate the tubes in a 37 °C water bath for 5 min.
- (vi) Incubate the tubes at 37 °C at 150 r.p.m. for 30 min.
- (vii) Remove the gentleMACS C tubes from the incubator, and shake them on the gentleMACS dissociator (program: m_brain_01_02 C tube setting). If you are using 50-ml tubes, remove the tubes from the shaker every 15 min and shake them vigorously by hand.
- (viii) Return the tubes to the incubator at 37 °C at 150 r.p.m. for another 30 min.
- (ix) Remove the tubes and shake them on the gentleMACS dissociator (program B.01 C tube setting). Before centrifugation, the solution should be homogeneous as in **Figure 6f**, indicating complete digestion of the adipose tissue.
- (x) Centrifuge the tubes at 500g for 5 min at 4 °C.
- (xi) Discard the cap, and aspirate the upper layer of adipocytes and supernatant to the lower line on the gentleMACS C tube, which corresponds to ~1.5 ml of remaining medium.

Figure 5 | Digestion of the mesentery and recovery of the stromal vascular fraction from a single mouse (Step 12A(i–vii) of the main PROCEDURE). **(a,b)** Result of mincing fat with surgical scissors in 4% (wt/vol) BSA/DMEM. **(c)** Homogenate obtained after digestion with 20 µg/ml Liberase DH and 1 µg/ml DNase I at 37 °C at 150 r.p.m. for 60 min. **(d)** Result of centrifugation of the homogenate at 500g for 5 min. The upper layer contains adipocytes (blue arrowhead) and the bottom layer consists of the stromal vascular fraction of adipose tissue, which contains immune cells (red arrowhead).

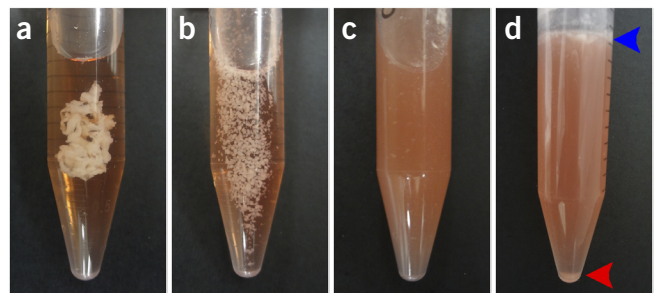
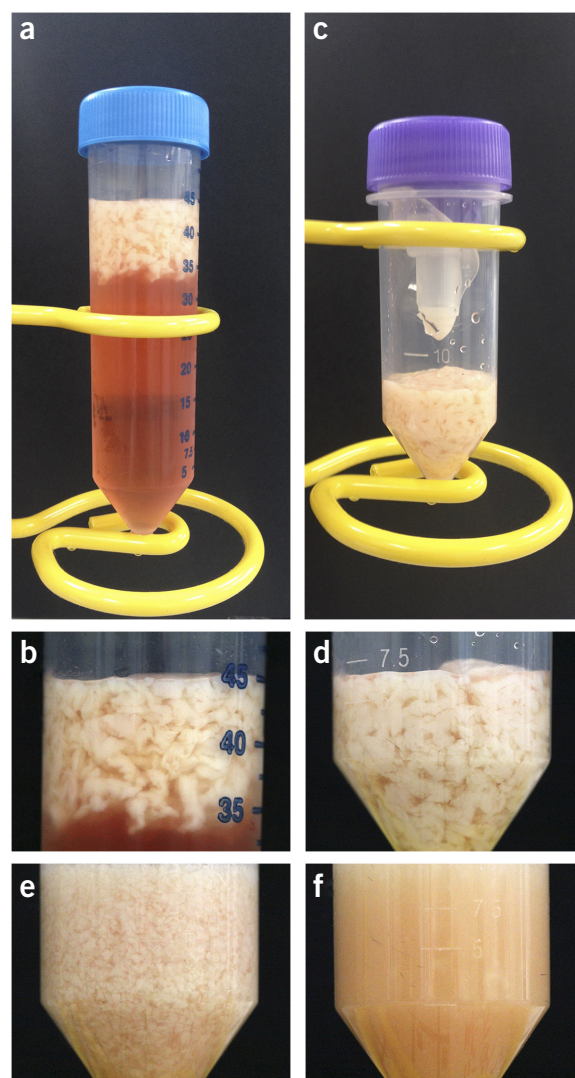


Figure 6 | Digestion of mesenteric adipose pooled from 20 mice in a gentleMACS C tube (Step 12B(i–viii) of the main PROCEDURE). (a,b) Pool adipose tissue into a 50-ml tube. (c,d) Transfer the adipose tissue into a gentleMACS C tube. (e) Mince the adipose tissue with surgical scissors and bring up the total volume to 10 ml with 4% (wt/vol) BSA/DMEM. (f) Homogenate obtained after digestion with 20 µg/ml Liberase DH at 37 °C at 150 r.p.m. for 30 min followed by homogenization on a gentleMACS dissociator, a further incubation at 37 °C at 150 r.p.m. for 30 min and final dissociation on the gentleMACS dissociator.



- (xii) Resuspend the pellet with a 5 3/4-inch Pasteur pipette and with gentle repeated pipetting, and then filter the homogenate through a 100-µm nylon cell strainer into a 50-ml tube to remove cellular debris. Pool the homogenates from multiple C-tubes into one 50-ml tube.
- (xiii) Filter the homogenate through a 40-µm nylon cell strainer into a 15-ml tube.
- (xiv) To prevent cell loss, wash the C-tube with 3 ml of 5% (wt/vol) FBS HBSS, and filter the wash through the 100- and 40-µm cell strainers before adding them to the filtrate. Repeat this step twice. With repeated pipetting, carefully filter the washes through the cell strainers.

? TROUBLESHOOTING

- (xv) Centrifuge the tube at 500g for 5 min at 4 °C.
- (xvi) Aspirate the supernatant and resuspend the cells in 0.5 ml of FACS buffer solution containing anti-mouse CD16/CD32 mAb (2.4G2) to block nonspecific binding to Fc receptors, and a mix of anti-mouse biotinylated antibodies for lineage markers (CD3ε, CD4, CD8α, FcεR1α, CD11c, CD19, NK1.1, F4/80, TER119, Gr-1 and CD5) using the dilutions shown in **Table 1**. Use 0.5 ml of antibody cocktail per 20 mice.
- (xvii) Incubate the cells for 20 min on ice.
- (xviii) Wash the cells with 0.5% (wt/vol) BSA/PBS by centrifugation at 500g for 5 min at 4 °C, and proceed through Step 12B(xviii–xxvi) for depletion of lineage-positive cells on an autoMACS Pro separator. If this equipment is not available, wash the cells with FACS buffer solution and proceed to Step 13B of the main PROCEDURE.
- (xix) Resuspend the cells in 1 ml of 0.5% (wt/vol) BSA/PBS containing 100 µl of streptavidin MicroBeads (1:10 dilution according to the manufacturer's protocol). Use 1 ml of this streptavidin MicroBeads solution per 20 mice.
- (xx) Refrigerate the cells for 15 min (4–8 °C). Do not place them on ice.
- (xxi) Wash the cells with 0.5% (wt/vol) BSA/PBS by centrifugation at 500g for 5 min at 4 °C.
- (xxii) Aspirate the supernatant and resuspend the cells in 1 ml of 0.5% (wt/vol) BSA/PBS.
- (xxiii) Filter the cell suspension through a 37-µm nylon mesh, and wash the mesh with 2 ml of 0.5% (wt/vol) BSA/PBS for a total cell suspension volume of 3 ml.
- (xxiv) Insert the tube on the autoMACS Pro separator, and run the 'DEPLETES' program according to the manufacturer's protocol.
- (xxv) Collect the unlabeled cell fraction.
- (xxvi) Wash the cells with 5% (wt/vol) FBS HBSS by centrifugation at 500g for 5 min at 4 °C.
- (xxvii) Aspirate the supernatant.

Detection of ILC2s by flow cytometric analysis and isolation by cell sorter

13 | This procedure describes flow cytometric analysis of ILC2s from an individual mouse (option A) and isolation of mouse ILC2s by cell sorting of pooled samples (option B). To save time, we recommend that the tubes for collection of ILC2s during cell sorting be prepared before setting up the flow cell sorter.

(A) Flow cytometry analysis of ILC2s from an individual mouse ● TIMING 90 min

- (i) Resuspend the cells in 50 µl of FACS buffer solution containing anti-mouse CD16/CD32 (2.4G2) and the following biotin-conjugated anti-mouse monoclonal antibodies for staining of lineage-positive cells, using the dilutions shown in **Table 1**: CD3ε, CD4, CD8α, CD11c, FcεR1α, NK1.1, CD19, TER119, CD5, F4/80 and Gr-1.

- (ii) Incubate the cells for 20 min at 4 °C. Wash the cells with FACS buffer solution by centrifugation at 500g for 5 min at 4 °C.
- (iii) Resuspend the cells in 50 µl of FACS buffer solution containing the following monoclonal anti-mouse antibodies: APCy7-conjugated CD45.2, PECy7-conjugated KLRG1, BV421-conjugated Ly-6A/E (Sca-1), PE-conjugated CD117 (c-Kit), V500-conjugated CD90.2 (Thy-1.2), PerCPy5.5-conjugated CD25, FITC-conjugated T1/ST2 and APC-conjugated streptavidin using the dilutions shown in **Table 1**. If the flow cytometry detectors are limited, the combination of Sca-1 and KLRG1 with lineage markers is enough to discriminate ILC2s from other cells.
- (iv) Incubate the cells for 20 min at 4 °C in the dark.
- (v) Wash the cells with FACS buffer solution by centrifugation at 500g for 5 min at 4 °C.
- (vi) Aspirate the supernatant and resuspend the cells in ~100 µl of FACS buffer solution.
- (vii) Filter the cell suspension through a round-bottomed FACS tube with a filter cap.
- (viii) Add 100 µl of PI PBS to gate out dead cells.
- (ix) Analyze the cells by flow cytometry by first selecting cells within the lymphocyte gate on the basis of forward-scatter (FSC) and side-scatter (SSC) properties. Gate out dead cells on the basis of PI staining. ILC2s can be identified as CD45⁺Lin⁻ lymphoid cells that express KLRG1 and Sca-1 (**Fig. 4**).

(B) Isolation of mouse ILC2s by cell sorting (pooled samples) ● TIMING 1 h 50 min

- (i) To prepare tubes for collection of ILC2s during sorting, add 500 µl of sterile complete RPMI-1640 culture medium to a 1.5-ml screw-cap microcentrifuge and spin it gently on a cell rotator (~30 r.p.m.) for at least 1 h before sorting. The number of tubes needed depends on the expected yield of ILC2s based on the number of mice used. We routinely sort $\sim 3.5 \times 10^5$ cells into one tube.

? TROUBLESHOOTING

- (ii) Resuspend the unlabeled cell fraction obtained after separation on the autoMACS Pro separator, in ~500 µl of FACS staining buffer solution containing FITC-conjugated anti-mouse Sca-1 and PE-conjugated anti-mouse KLRG1 for staining of ILC2s and APC-conjugated anti-streptavidin for staining the remaining lineage-positive cells using the dilutions shown in **Table 1**.
- (iii) Incubate the cells for 20 min at 4 °C in the dark.
- (iv) Wash the cells with FACS buffer solution by centrifugation at 500g for 5 min at 4 °C.
- (v) Aspirate the supernatant and resuspend the cells in FACS buffer solution. Use 300 µl of FACS buffer solution for every 10 mice.
- (vi) Keep the cells on ice until the cell sorter is ready for separation.
- (vii) Place FBS-coated 1.5-ml collection tubes containing 500 µl of 10% (wt/vol) FBS RPMI complete medium, prepared in Step 13B(i), in the appropriate position of the cell sorter.
- (viii) Filter the cell suspension through a round-bottomed FACS tube with a filter cap. Add PI (100 µl/10 mice) to gate out dead cells, and then separate the cells on a cell sorter.
- (ix) To sort ILC2s, set the sorting gate to include live lymphoid cells in the Lin⁻Sca-1⁺KLRG1⁺gate.
- (x) For *in vitro* culture of ILC2s, spin the tubes at 500g for 5 min at 4 °C and proceed to the steps described in **Box 4**.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12A(vii)	Color of the solution obtained after adipose tissue digestion is yellow	Most of the cells are dead. The most likely cause is contamination with pancreatic enzymes	Completely avoid pancreas tissue during dissection of the mesentery from the intestine, as shown in Figure 3
12B(xiv)	Low yield of ILC2s	Poor washing and collection of filtrates after adipose tissue digestion (Step 12B(xiii))	With repeated pipetting, carefully filter the washes through the cell strainers, as described
13B(i)	Low yield of ILC2s	Binding of cells to collection tubes used for sorting	To ensure that the 1.5-ml collection tubes are coated with FBS before sorting, add 500 µl of 10% (vol/vol) FBS with RPMI complete medium and spin on a cell rotator for at least 1 h before sorting

● TIMING

The protocol for isolation and detection of ILC2s can be completed within 1 d. The amount of time needed varies according to tissue type, the number of mice and whether cells will be isolated for analysis or sorting on the FACS Aria. The following breakdown describes how much time is required for each step:

Steps 1–11, isolation of ILC2s from the mesentery: 10 min per mouse

Step 12A, preparation of ILC2s from an individual mouse for analysis: 90 min (add 10 min per mouse for >1 mouse)

Step 12B, preparation of ILC2s from multiple mice for sorting (mesentery pooled): 2 h 40 min

Step 13A, flow cytometry analysis of ILC2s from an individual mouse: 90 min, depending on the expertise of the researcher and the number of tubes being analyzed

Step 13B, isolation of mouse ILC2s by cell sorting of pooled samples: 1 h 50 min for pooled mesenteries from 20 mice.

Sorting time will increase if tissues are pooled from >20 mice

Box 1, isolation of ILC2s from BALF: 30 min/mouse (add 15 min per mouse for >1 mouse)

Box 2, steps 1–10, isolation of ILC2s from lung tissue: 70 min (steps 1–10A(iii)) and 2 h (steps 1–10B(xi))

Box 3, isolation of LP ILC2s from the small intestine: 2 h 30 min (add 20 min per mouse for >1 mouse; steps 1–25A) and 40 min (step 25B)

Box 4, *in vitro* culture of ILC2s: variable

ANTICIPATED RESULTS

Isolation of ILC2s

Proper dissection of the mesentery from the intestine and subsequent digestion with Liberase DH and DNase I results in a homogeneous pink-colored solution, as shown in **Figure 5c**, indicating complete digestion of the adipose tissue (Steps 8–12A(i–vii)). After centrifugation, the adipocytes will be located in the upper floating layer (indicated by the blue arrowhead in **Fig. 5d**), and the immune cells contained in the stromal vascular fraction will be pelleted at the bottom of the tube (red arrowhead, **Fig. 5d**). If the solution is yellow, most of the cells are dead. Please refer to **Table 2** to address this problem.

When adipose tissue from multiple mice are pooled (Step 12B(i–viii)), digestion with Liberase DH results in a creamy homogeneous solution, as shown in **Figure 6f**, which indicates complete digestion of the adipose tissue. This is observed regardless of whether gentleMACS C tubes or 50-ml plastic tubes are used for digestion. If the adipose tissue is not completely digested (pieces of adipose tissue are still visible), the adipose tissue may need to be further minced into smaller pieces and placed on the shaker for a longer time.

When you are isolating ILC2s from inflamed small intestine, it is important to be aware that, after incubation of the intestinal segments to deplete the IEL plus IEC fraction followed by vigorous shaking in a 50-ml tube containing PBS, there may be both white and pink tissue segments. The white segments are damaged intestinal tissue pieces containing dead cells. At this stage, discard the white tissue and proceed through the remaining steps for digestion using only the pink tissue segments. In addition, as inflamed intestinal tissue contains a large amount of mucus, more extensive washing in PBS will be needed before deletion of the IEL plus IEC fraction.

Analysis of ILC2s from various tissues

Approximately 2×10^4 cells can be recovered from the mesentery of one retired mouse and 1×10^4 from the lung, 5×10^3 from BALF and 3×10^4 from the small intestine of one 8-week-old mouse. **Figure 4** shows typical results of flow cytometric analysis of ILC2s from various tissues. As shown at the top of **Figure 4a**, the lymphocyte gate containing ILC2s, based on FSC and SSC properties, is slightly larger than the lymphocyte gates typically used for T cells; it is closer in size to gates used for NK cells. When sorting ILC2s, we remove doublet cells and gate for single cells on FSC-W versus FSC-H and SSC-W versus SSC-H plots after FSC-A versus SSC-A (A, area; H, height; W, width). This step is used to obtain a high purity of cells during sorting, but it is not necessary if analysis of the ILC2 population is the sole purpose of the experiment. We use PI to gate out dead cells, but 7-AAD can also be used. The viability of lymphocytes obtained after tissue digestion is >90% in all tissues. If cell viability is much lower than this, it is most likely owing to poor dissection techniques, as described in **Table 2**.

In all tissues, ILC2s can be identified as CD45⁺Lin[−] lymphoid cells that express KLRG1 and Sca-1 (**Fig. 4a**). Furthermore, all ILC2s are positive for intracellular expression of GATA-3 (data not shown). CD45 is a useful marker for the detection of ILC2s, particularly in the lung and BM, because both tissues contain many Lin[−]CD45[−] nonimmune cells. Differential expression levels of the cell surface markers c-Kit, Thy-1.2, CD25 and T1/ST2 in ILC2s isolated from adipose tissue, lung, bone marrow and small intestine reveal the presence of tissue-specific populations of ILC2s (**Fig. 4**). The combination of antibodies used depends largely on the purpose of the experiment. In the mesentery, the ILC2 population is fairly homogeneous with high expression of the above-mentioned cell surface markers. Thus, any combination of these markers can be used to identify ILC2s in adipose tissue. The ILC2 populations in other tissues are more heterogeneous. c-Kit expression in ILC2s from the lung, BM and intestine is lower than that in the mesentery and should not be used when sorting ILC2s from these tissues. Furthermore, it is evident from **Figure 4a** that, unlike ILC2s in the other tissues, a large proportion of these

cells in the small intestine have low/negative expression of CD25 and T1/ST2. Although staining for c-Kit, Thy-1.2, CD25 and T1/ST2 helps confirm the population of cells, these antibodies are not essential for the detection of ILC2s. The combination of lineage markers plus Sca-1 and KLRG1 is sufficient to detect ILC2s in various tissues. **Figure 4b** shows representative plots of ILC2s in the mesentery and BALF of untreated mice and in mice treated intratracheally with the combination of IL-2 plus IL-25 or IL-33. Lymphocytes were gated by FSC versus SSC, and ILC2s were identified as CD45⁺PI⁻ cells that express T1/ST2 and CD25. ILC2s are undetectable in the BALF of untreated mice, but they are present in large numbers after injection with IL-2+IL-25 or IL-33. ILC2s are also present in the BALF of IL-25-treated mice (K.M., unpublished data), but the cell number is significantly lower than that observed with IL-2 plus IL-25 injection. Intratracheal administration of cytokines does not affect ILC2s in the mesentery. As it is known that injection with IL-2 plus IL-25 or IL-33 alone does not induce T1/ST2-positive T_H2 cells, ILC2s can be detected by flow cytometry without staining for lineage markers. Unlike ILC2s in the mesentery, ILC2s from the BALF of cytokine-injected mice show a wide range of expression of T1/ST2 and CD25.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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