Chapter 18

Purification and Adoptive Transfer of Group 3 Gut Innate Lymphoid Cells

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Abstract

Recent studies have identified several related but distinct innate lymphoid cells (ILCs) populations that are relatively enriched in the intestinal mucosal and protect the host from various infections. Among ILCs, group 3 ILCs (ILC3s) produce lymphotoxin and IL-22, and play important roles in the development of the immune system and host–bacteria interactions. Here, we describe methods for the isolation and purification of ILC3s from the mouse intestine, and the adoptive transfer of purified ILC3s into recipient mice.

Key words Group 3 Innate lymphoid cells, Intestine, Purification, *Rag1*, Adoptive transfer

1 Introduction

Innate lymphoid cells (ILCs) represent a novel family of immune cells that resemble adaptive lymphocytes in effector function, yet lack rearranged antigen receptors. Within the past few years, based on cytokine production and transcription factors associated with T helper 1, 2, and 17 cell, three different ILCs population have been defined, including group 1 ILCs, group 2 ILCs, and group 3 ILCs (ILC3s) [1]. Although they are a tiny of lymphoid population, ILCs are enriched in mucosal areas and play essential roles in mucosal homeostasis, initiation of immune responses against pathogens, and tissue repair [2, 3].

ILC3s are Lineage$^-$, RORγt$^+$, IL-7Rα$^+$, and LTα1β2$^+$, with variable expression of NK receptors (NCR), and upon IL-1β and IL-23 stimulation, ILC3s have the ability to produce an array of effector cytokines, including but not limited to IL-17 and IL-22, that correspond tightly with their T helper cell counterpart, Th17 cells. To date, three ILC3 subsets have been described, including lymphoid tissue inducer (LTi) cells, NCR$^-$ ILC3s and NCR$^+$ ILC3s [2, 4]. LTi cells play a critical role in the development of secondary lymphoid organs, such as lymph nodes, Peyer's patches, and isolated lymphoid follicles in the intestine. ILC3s are located in the

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lamina propria of the intestine, have an important role in intestinal homeostasis and host defense against gut pathogens. Defects in the development or function of ILC3s result in increased susceptibility to infection, inflammatory bowel disease, tumor development, and allergies [5–9]. Our and others' recent studies have shown that adoptive transfer of ILC3s could result in homeostatic regulation of the gut microbiota and rescue susceptible mice from the gut pathogen *Citrobacter rodentium* [6, 7, 10]. Therefore, we will describe our detailed protocols for the isolation, purification, and adoptive transfer of ILC3s.

2 Materials

2.1 Animals

C57BL/6 and *Rag1*$^{-/-}$ mice were purchased from Harland Teklad and kept under specific pathogen-free conditions. Animal care and use should be in accordance with protocols approved by the appropriate Institutional Animal Care and Use Committee.

2.2 Reagents (*See* Note 1)

1. Phosphate buffered saline (PBS), pH 7.2–7.4.
2. Washing Buffer I: 1× Hank's buffered salt solution (HBSS) without Ca^{2+} and Mg^{2+}, 10 mM HEPES, 5 mM EDTA, 1 mM Dithiothreitol (DTT) (*see* **Note 2**), 3 % FBS (*see* **Note 3**), pH 7.2–7.4.
3. Washing Buffer II: 1× HBSS without Ca^{2+} and Mg^{2+}, 10 mM HEPES, pH 7.2–7.4.
4. Digestion Buffer: RPMI-1640, 10 mM HEPES, 100 U/ml Penicillin-Streptomycin, 0.1 mg/ml Liberase TL (Roche) (*see* **Note 4**), 0.05 % DNase I (*see* **Note 5**), 3 % FBS, pH 7.2–7.4.
5. RPMI-1640 medium: RPMI-1640, 10 mM HEPES, 100 U/ml Penicillin-Streptomycin, 3 % FBS.
6. DMEM medium: DMEM, 10 mM HEPES, 100 U/ml Penicillin-Streptomycin, 3 % FBS.
7. Complete RPMI-1640 culture medium: RPMI-1640, 10 mM HEPES, 100 U/ml Penicillin-Streptomycin, 20 % FBS.
8. 100 % Percoll solution: Percoll, 10 mM HEPES, 100 U/ml Penicillin-Streptomycin, 10 % 10× HBSS with Ca^{2+} and Mg^{2+}.
9. 80 % Percoll solution: 80 % of 100 % Percoll solution and 20 % of PBS.
10. FACS buffer: 1× PBS, 2 % FBS, 0.02 % Sodium azide (NaN_3) (*see* **Note 6**).
11. Anesthetic Agent: ketamine (60 mg/kg), xylazine (2.5 mg/kg) (*see* **Note 7**).
12. Antibodies: anti-mouse CD16/32 purified (clone number: 2.4G2), anti-mouse CD90.2 (clone number: 30-H12), and

anti-mouse CD45 (clone number: 30-F11). Dilute the antibodies in FACS buffer with one million cells to final concentrations 0.2 μg/100 μl for anti-CD16/32 and CD45 antibodies and 0.1 μg/100 μl for CD90.2 antibody.

13. 7-Aminoactinomycin D (7-AAD) stock solution: 50 μg/ml in PBS with 0.09 % sodium azide, pH 7.2.

2.3 Equipment

1. Dissecting scissors.
2. Forceps.
3. Metal mesh cell strainer or kitchen strainer.
4. 70 μM cell strainer.
5. Constant-temperature incubator/shaker.
6. 6-well plate (for manual dissociation).
7. Centrifuge.
8. Microscope.
9. Hemocytometer.
10. FACS Aria (BD Biosciences) for cell sorting.
11. (Optional) GentleMACS dissociator and gentleMACS C tubes (Miltenyi Biotec).

3 Methods

3.1 Isolation of ILC3s from the Lamina Propria Compartment of the Intestine

Since there are more ILC3s in the small intestine than the colon, this procedure mainly describes the isolation of ILC3s from small intestine. Compared to wild type mice, *Rag1*$^{-/-}$ mice lack adaptive lymphocytes and the total number of ILC3s is greatly increased in the gut. Thus, if adoptive transfer of ILC3s is desired, isolating ILC3s from *Rag1*$^{-/-}$ mice is a good choice (*see* **Note 8**).

1. Make the Washing Buffer II freshly and pre-warm the Washing Buffers I and II in a 37 °C incubator.
2. Euthanize the mouse with CO_2 and surface-sterilize the skin with 70 % ethanol. Open the peritoneal cavity and extract the small intestine. Carefully remove all the mesenteric material and fat. Then remove the Peyer's Patches with dissecting scissors while pushing out fecal material from the intestine. The white Peyer's Patches could be easily seen in contrast to the brown-colored luminal contents within intestine. The total number of Peyer's Patches in wild type C57BL/6 mice is between 6 and 10 and occur in increasing frequencies toward the terminal small intestine. For the preparation of small intestine from *Rag1*$^{-/-}$ mice or colon, there is no need to remove the Peyer's Patches or Colonic Patches respectively (*see* **Note 9**).
3. Cut the intestine open longitudinally and cut into 1 cm pieces.

4. Transfer all the pieces into 50 ml conical tubes with 15 ml 1× PBS buffer in room temperature. Then shake vigorously with a vortex for 30 s to remove the remaining feces and mucus (*see* **Note 10**).
5. Strain the sample through a kitchen strainer or metal mesh strainer, retaining the tissue fragments. Transfer the tissue to 50 ml conical tubes with 15 ml of pre-warmed Washing Buffer I. Then Shake the tubes for 20 min on a platform shaker at 200 rpm at 37 °C to remove the intestinal intraepithelial fraction.
6. Shake vigorously for 10 s using a vortex and strain the sample through a kitchen strainer, retaining the tissue fragments.
7. Repeat **steps 5** and **6**.
8. Transfer the tissue to 50 ml conical tubes with 15 ml Washing Buffer II. Shake for another 20 min on platform shaker at 200 rpm and 37 °C. Shake vigorously for 10 s using a vortex and apply the sample onto a kitchen strainer (*see* **Note 11**).

   *If using gentleMACS C tubes and a gentleMACS Dissociator proceed to **step 10**. If using manual dissociation proceed to **step 9** and skip **step 10**.
9. Transfer the intestinal tissues into a 6 wells plate and mince into 1–2 mm pieces with surgical scissors (about 2 min). Then add 2.5 ml of pre-warmed Digestion Buffer. Shake for 20 min on a platform shaker at 150 rpm, 37 °C. Shake for 30 min for colon ILCs isolation. Very few tissue pieces should remain after digestion and the solution should be a cloudy cell suspension.
10. (Alternatively, if available) Transfer the intestine tissues from **step 8** into the gentleMACS C Tube containing 2.5 ml Digestion Buffer. Shake for 20 min on a platform shaker at 150 rpm, 37 °C. Then tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator. Run the gentleMACS Program m_intestine_01. No tissue pieces should remain and the solution should be a cloudy cell suspension.
11. Add 5 ml of RPMI-1640 medium, and apply the sample suspension to a 70 μm cell strainer placed on a 50 ml conical tube to remove cellular debris. Manually homogenize the tissue pieces in the strainer with the flat end of the plunger from a 1-ml syringe. Wash the strainer once with 5 ml of RPMI-1640 medium. Discard the strainer and centrifuge cell suspension at 300 × *g* for 7 min at room temperature.
12. Aspirate the supernatant completely. Resuspend the cells in 4 ml DMEM medium and add 4 ml of the 80 % percoll solution (for a final percoll concentration of 40 %). Mix well by pipetting and transfer into a new 15 ml conical tubes. Carefully underlay with 4 ml 80 % percoll solution. A clear separation of layers should be observed.

13. Centrifuge at 1200 × *g* for 20 min at room temperature with no break (this is essential). At the end of the spin the ILC3s will appear as an opaque ring between the 40 and 80 % percoll fraction. A top layer of epithelial cells will rest on top of the 40 % fraction. Aspirate and discard top layer of epithelial cells. Carefully collect the lymphoid cells at the interphase using 1 ml pipet and transfer to a fresh 15 ml tube. Wash the cells once with cold FACS buffer and spin down at 400 × *g* for 5 min at 4 °C.
14. Resuspend the cells in FACS buffer and place it on ice. Count the cells with a hemocytometer (*see* **Note 12**).

3.2 Sorting ILC3s from the Lamina Propria Cells by Flow Cytometry

In the intestine, the ILCs are identified as Lineage$^-$, CD45$^+$, and IL-7Rα$^+$. Compared to other ILCs, all ILC3s are positive for RORγt. Because there is not a distinct panel of surface marker to identify ILC3s vs. other ILCs, the sorting of ILC3s mainly relies on *Rorc*$^{gfp/+}$ mice, in which the RORγt$^+$ ILC3s were GFP-positive cells. However, because of extremely low number of ILC3s in wild type mice and the dim GFP of *Rorc*$^{gfp/+}$ mice, it was difficult to obtain enough pure ILC3s for adoptive transfer or other purposes. Our recent study has found that ILC3s from the intestines could be strictly identified by their differential surface expression of bright CD45 and CD90 in both C57BL/6 wild type mice and *Rag1*$^{-/-}$ mice. As shown before and in Fig. 1, the intestinal RORγt$^+$ ILC3s are all CD90^{high} CD45^{low} cells [7].

1. Resuspend the cells from **step 14** in FACS buffer (without azide) containing anti-mouse CD16/CD32 (2.4G2) at a concentration of 1×10^7 cells/ml to block Fc receptor internalization.
2. Stain the ILC3s with fluorescence conjugated antibodies against CD45 and CD90. Incubate the cells for 20 min at 4 °C in the dark. Wash the cells with FACS buffer by centrifugation at 400 *g* for 5 min at 4 °C.
3. Resuspend the cells with FACS buffer and filter the cell suspension using a 70 μm cell strainer to remove cell debris. Add 5 μl of 7-AAD stock solution per million cells in 0.5 ml of FACS buffer to gate out dead cells, and then keep the cells on ice until the cell sorter is ready for separation.
4. To sort ILC3s, first select cells within the lymphocyte gate on the basis of forward-scatter (FSC) and side-scatter (SSC) properties. Then gate out dead cells: 7-AAD-positive cells. ILC3s can be identified as CD90^{high} CD45^{low} lymphoid cells. Place 5 ml collection tubes with cold complete RPMI-1640 culture medium in the appropriate position of the cell sorter and sort ILC3s (*see* **Note 13**).
5. Wash the sorted ILC3s twice with 1× PBS buffer by centrifugation at 400 *g* for 5 min at 4 °C. Resuspend the cells in the

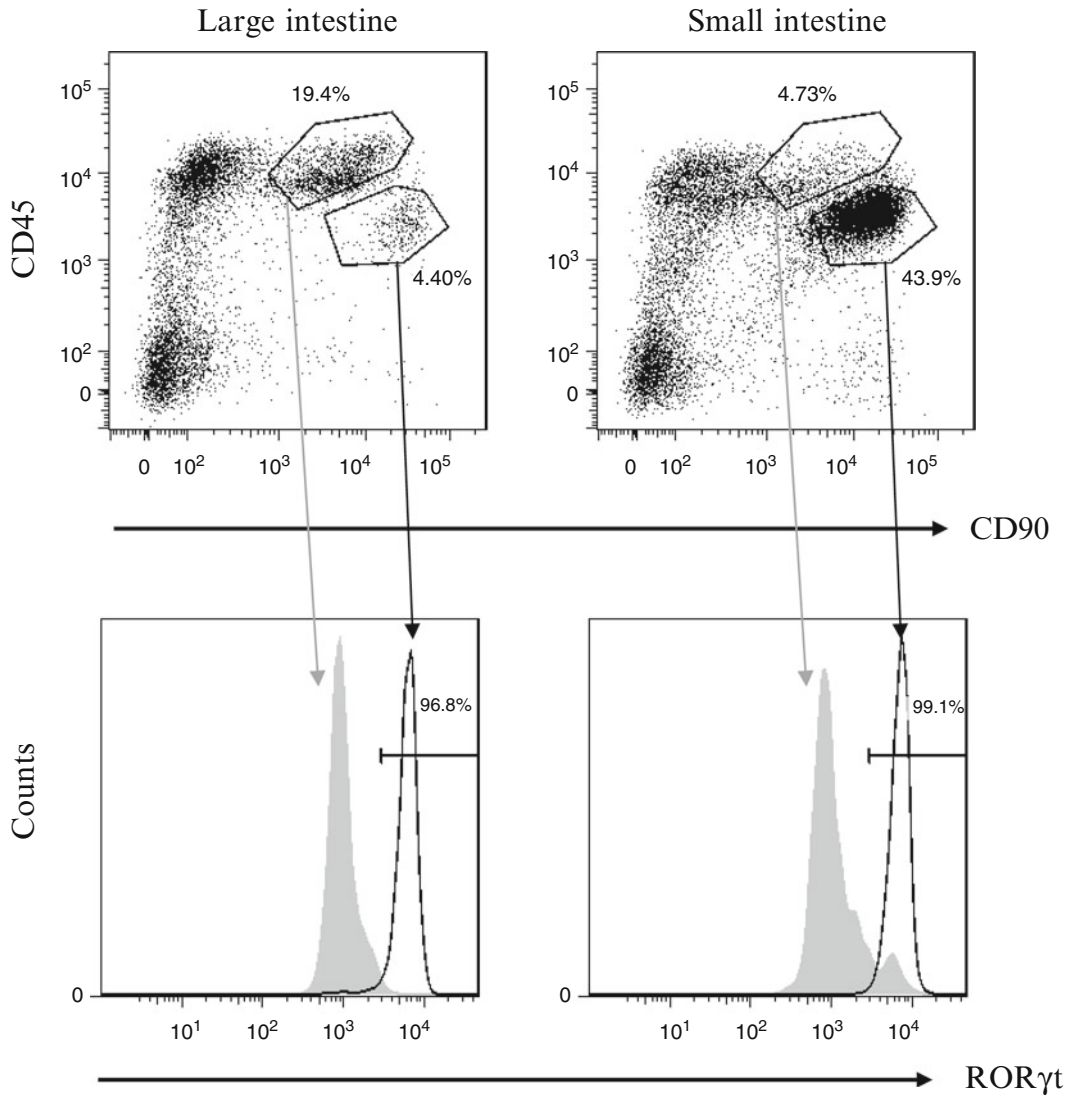

Fig. 1 Intestinal CD90^{hi}CD45^{lo} LPLs are RORγt^{+} ILC3s. LPLs were isolated from both the large intestine and small intestine of *Rag1*$^{-/-}$ mice and gated by CD45 and CD90 expression. Two separate populations, CD90^{high}CD45^{low} and CD90^{low}CD45^{high} were further analyzed with the expression of RORγt

PBS buffer at a concentration of 2×10^6 cells/ml. It might be important to have confirmation of purity or identity of the cells while keeping the cells on ice until adoptive transfer.

3.3 Adoptive Transfer of ILC3s into Recipient Mice

1. Anaesthetize the recipient mouse with ketamine (60 mg/kg) and xylazine (2.5 mg/kg) solution via intraperitoneal injection.
2. Wait until the mouse is fully anaesthetized and then adoptive transfer 2×10^5 ILC3s (100 μl per mouse) via retro-orbital injections.

3. After the injection is complete, place the mouse back into its cage for observing recovery.
4. Confirm and compare the efficacy of adoptive transfer by flow cytometry analysis or functional study.

4 Notes

1. If sorting and adoptive transfer of cells is desired, prepare all the solutions under sterilize conditions and perform all the experiments after **step 11** in a sterile space such as biosafety cabinet.
2. Make a stock solution of DTT and add it freshly into the washing buffer immediately before use.
3. Heat-inactivate FBS in a water bath at 56 °C for 30 min before use.
4. If Librase is not available, it can be replaced by Collagenase D (0.5 mg/ml) and Dispase (0.5 mg/ml).
5. DNase I could remove the DNA from digested samples to prevent the clumping of cells.
6. NaN3 is toxic. Avoid contact with skin, eyes, and mucous membranes.
7. Access to anesthetic is regulated by US state and federal law. The anesthetics should be locked up and the using of anesthetics should be recorded.
8. This method describes isolation of ILC3s from the small intestine of one mouse. If you are using multiple mice, do not pool the intestines until you get the lamina propria leukocytes at **step 13**.
9. Peyer's Patches are enriched in adaptive lymphocytes, thus it is necessary to remove Peyer's Patches in order to obtain an enriched population of ILCs from the lamina propria.
10. It is important to remove luminal contents and mucus, which may influence the effect of the digestion buffer.
11. After this step, the small intestine pieces should be pink in color. If the intestinal tissue contains white segments, which suggest dead tissues, you should discard the white tissues.
12. Usually, total $3–10 \times 10^6$ lamina propria leukocytes could be isolated from the small intestine of one naïve C57BL/6 mouse, and about 2×10^6 cells from *Rag1*$^{-/-}$ mouse.
13. Usually, around 1×10^4 ILC3 can be sorted from the small intestine of one naïve C57BL/6 mouse, and about 5×10^5 cells from one *Rag1*$^{-/-}$ mouse.

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