Human innate lymphoid cells

From helper to killer

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Isolation of Human Innate Lymphoid Cells

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Significance Statement

Innate lymphoid cells (ILCs) represent a relatively small group of cells that are the innate counterpart of the adaptive T cells. They contribute to the protective immune response against acute infections, regulation of homeostasis and tissue (re)modelling. ILCs are enriched at mucosal and barrier sites, but are also found in secondary lymphoid organs and the general circulation. Dysregulation of ILCs can result in immune pathology as observed in e.g. asthma, inflammatory bowel disease, and psoriasis. Their low frequency makes the detection and isolation challenging. Furthermore, distinct tissues need to be processed differently to obtain maximum cell yield. Therefore, it is important to use correct and uniform methods for ILC isolation and characterization.

ABSTRACT

Innate lymphoid cells (ILCs) are innate immune cells of lymphoid origin that have important effector and regulatory functions in the first line of defense against pathogens, but also regulate tissue homeostasis, remodeling, and repair. Their function mirrors T helper cells and cytotoxic CD8+ T lymphocytes, but they lack expression of rearranged antigen specific receptors. Distinct ILC subsets are classified in group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s and lymphoid tissue inducer cells), based on the expression of transcription factors and the cytokines they produce. As the frequency of ILCs is low, their isolation requires extensive depletion of other cell types. The lack of unique cell surface antigens further complicates the identification of these cells. Here we describe methods for ILC isolation and characterization from human peripheral blood and different tissues.

Keywords:
Human innate lymphoid cells (ILCs), peripheral blood, tonsil, intestine, nasal polyp, flow cytometry

INTRODUCTION

Innate lymphoid cells (ILCs) represent a relatively small group of cells that are the innate counterpart of adaptive T cells and are particularly enriched at mucosal and barrier sites, but also found in secondary lymphoid organs and the circulation [1]. They contribute to the protective immune response against acute infections, regulation of homeostasis and tissue repair, however, dysregulation of ILCs can result in immune pathology as observed in e.g. asthma, inflammatory bowel disease, and psoriasis [2]. ILCs are classified into three subsets: group 1 ILCs (ILC1s and NK cells), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s) [3,4]. ILC1s are non-cytotoxic IFN-g expressing cells that depend on the transcription factor T-bet, whereas NK cells can display cytotoxicity as well as IFN-g production, are not strictly dependent on T-bet for their development and express Eomes. ILC2s are potent producers of the type 2 cytokines IL-5 and IL-13 and require GATA3. Different subsets of ILC3s have been defined that express RORgt and are potent IL-22 and/or IL-17 producers. ILC3s also encompass lymphoid tissue inducer cells (LTi cells) that function in the development of lymphoid organs by interacting with stromal cells via lymphotoxins.

In contrast to other lymphocytes, ILCs are present at low frequencies and they lack specific cell surface antigens. Consequently, their detection and particularly their isolation requires extra attention in order to study pure populations. Furthermore, distinct tissues need to be processed differently to obtain maximum cell yield. Therefore, it is important to use uniform methods for ILC isolation and characterization. This unit presents general methods for the isolation of mononuclear cells and enrichment for ILCs by either negative or positive selection from human blood (Basic Protocol 1), tonsil (Basic Protocol 2), intestine (Basic Protocol 3), and nasal polyp (Basic Protocol 4). Finally, Basic Protocol 5 describes a method to identify and sort the different ILC subpopulations by flow cytometry. Critical steps are discussed as well as the cell yields to expect from different tissues.

BASIC PROTOCOL 1: ISOLATION OF HUMAN INNATE LYMPHOID CELLS FROM BLOOD

This protocol describes the procedures used for preparation and isolation of human peripheral blood mononuclear cells (PBMCs), followed by enrichment and purification of ILC subsets.

Materials

For isolation of PBMCs

Heparinized blood (after extraction of plasma, generally 10% of original volume)
Phosphate buffered saline (PBS)
Ficoll-HyPaque solution
Pipets
Plastic pasteur pipette
50 ml conical centrifuge tubes
Centrifuge

For ILC enrichment by CD3, CD14, CD16 and CD19 negative selection

Ammonium Chloride Potassium (ACK) lysis buffer (see recipe)
Wash buffer: PBS + 2 mM EDTA + 0.5% BSA
Flow cytometry buffer (FC buffer): PBS with 5% FCS
MojoSort streptavidin Nanobeads (Biolegend, cat nr 480016)
Biotin anti-human CD3 (clone OKT3)
Biotin anti-human CD19 (clone HIB19)
Biotin anti-human CD14 (clone 62D3)
Biotin anti-human CD16 (clone 3G8)
MojoSort Magnet (5 ml)
5 ml polypropylene tube

Protocol steps—Step annotations

Isolation of peripheral blood mononuclear cells (PBMCs):

1. Dilute blood 3x with PBS. Add 20 ml of diluted blood per 50 ml tube using a pipette.
2. Slowly add 13 ml of Ficoll-Hypaque underneath the blood using a pipette.
3. Centrifuge 30 min at 340 x *g*, 18-20°C, slow acceleration, no brake.
4. Transfer the mononuclear lymphocyte cell layer to a new 50 ml tube with a plastic Pasteur pipette, wash cells by adding PBS up to 50 ml and centrifuge for 8 min at 470 x *g*, 18-20°C.
   After centrifugation the tube will contain from bottom to top: A red pellet containing erythrocytes and granulocytes, transparent Ficoll-Hypaque solution, a white ring containing the mononuclear lymphocyte layer to be collected and on top of that a suspension consisting of plasma and platelets.
5. Remove supernatant and collect all the cells in one 50 ml tube, add PBS up to 50 ml.

ILC enrichment by CD3, CD14, CD16, and CD19 negative selection:

6. Centrifuge the cell suspension for 5 min at 470 x g, 8°C, remove supernatant by using a pipette. Remove contaminating red blood cells by adding 5 ml ACK lysis buffer to the cell pellet, resuspend the cells and incubate for 10 min on ice.
7. Wash the cells by adding 45 ml PBS, centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant and resuspend the cells in 50 ml PBS and count the cells.
8. Centrifuge the cell suspension for 5 min at 470 x *g*, 8°C, remove supernatant and resuspend cells in cold PBS to obtain a concentration of $1x10^9$ cells / 5 ml. Add 30 µg biotin anti-CD3, 30 µg biotin anti-CD19, 15 µg biotin anti-CD14 and 15 µg biotin anti-CD16 per $1x10^9$ cells, mix well and incubate 15 min at 8°C.
9. Wash with PBS, centrifuge the cell suspension for 5 min at 470 x g, 8°C and remove supernatant by using a pipette, resuspend the cells in cold wash buffer to obtain a concentration of $1x10^8$ cells/ml.
10. Vortex MojoSort streptavidin nanobeads for 5 seconds, add 200 µl / $1x10^9$ cells to the cell suspension, mix well and incubate 15 min at 4°C.
11. Wash with PBS, centrifuge the cell suspension for 5 min at 470 x *g*, 8°C and remove supernatant by using a pipette and add cold wash buffer to obtain a concentration of $1x10^8$ cells / ml, and mix well. Place 4 ml of the cell/beads suspension in a 5 ml tube (polypropylene) and insert to MojoSort magnet, incubate for 3 min at 18-20°C.
12. Without removing the tube from the magnet, pour cell suspension into a collection tube, repeat step 6 for the rest of the cell suspension.
13. Wash the negatively selected cell suspension in the collection tube once again with PBS, centrifuge the cell suspension for 5 min at 470 x g, 8°C, resuspend the cell pellet in 10 ml FC buffer, and count the cells.
    - *If cell numbers are above $1x10^8$ cells, add 1 µl beads per $1x10^7$ cells, and repeat the procedure from step 5.*
    - *Remove debris and remaining beads as much as possible by washing the cell pellet well, as they influence the cell purity.*
14. The cells are ready for staining with antibodies for analysis or sorting by flow cytometry (Basic protocol 5).

ALTERNATE PROTOCOL 1: ILC2 ENRICHMENT FROM PBMC BY CD161 SELECTION

Type 2 ILCs express very high levels of CD161 and if only these cells are to be obtained from peripheral blood (or other tissues), positive selection based on this

marker can be used as an alternative method. ILC1s and ILC3s express lower levels of CD161 and therefore this method results in loss of some of these cell populations.

Materials

PE anti-human CD161 (HP-3G10)
Anti-PE microbeads (Miltenyi, cat 130-048-801)
Wash buffer (PBS + 2 mM EDTA + 0.5% BSA)
Flow cytometry buffer (FC buffer): PBS with 5% FCS
Pre-separation filters (130-041-407, Miltenyi)
LS MACS columns (130-042-401, Miltenyi)
MACS separator magnet
15 ml conical centrifuge tubes

Protocol steps—Step annotations

1. After PBMC isolation (Basic Protocol 1, steps 1-5), count the cells, centrifuge the cell suspension for 5 min at 470 x *g,* 8°C, and resuspend the cells in 200-500 µl PBS.
2. Add 7.5 µg PE anti-CD161 antibody per $1x10^9$ cells, mix well and incubate for 20 min at 4°C with mild agitation.
3. Wash with PBS, centrifuge the cell suspension for 5 min at 470 x *g,* 8°C and remove supernatant.
4. Resuspend the cell pellet in 200-500 µl PBS and add 0.33 µl anti-PE beads per $1x10^6$ cells, mix well and incubate for 20 min at 4°C with mild agitation.
5. Place MACS LS column in the MACS separator magnet, equilibrate the column with 2 ml wash buffer.
6. Wash the cell suspension with PBS, centrifuge for 5 min at 470 x *g,* 8°C, and remove supernatant. Resuspend the cell pellet in 2 ml wash buffer per $5x10^8$ cells.
7. Apply the cell suspension to the column (max $2x10^9$ cells per column). Wait until the suspension has completely run through the column.
8. Wash the column three times with 3 ml wash buffer.
9. Remove the LS column from the magnet, place on a new 15 ml tube. Add 5 ml of wash buffer and push the cells with a plunger from the column into the tube.
10. Centrifuge the cell suspension for 5 min at 470 x *g,* 8°C, resuspend the cells in FC buffer and count the cells. The cells are ready for staining with antibodies for analysis or sorting by flow cytometry (Basic protocol 5).

BASIC PROTOCOL 2: ISOLATION OF LEUKOCYTES CONTAINING ILC SUBSETS FROM TONSIL

This protocol describes the preparation and isolation of human mononuclear cells from tonsil, followed by enrichment and purification of ILC subsets.

Materials

For Isolation of mononuclear cells from tonsil
Tonsils
Phosphate buffered saline (PBS)
Ficoll-Hypaque
Scissors
Optional: ACK lysis buffer (see recipe)
Forceps
Metal sieve
Plunger
Small spoon
Pipette
Stomacher
Plastic stomacher bag
Plastic Pasteur pipette
50 ml conical centrifuge tubes
Centrifuge
Ice

For ILC enrichment by CD3 and CD19 negative selection
FITC anti-human CD3 (HP-3G10)
FITC anti-human CD19 (okt-03)
Anti-FITC microbeads (Miltenyi, cat 130-048-701)
Wash buffer (PBS + 2 mM EDTA + 0.5% BSA)
Flow cytometry buffer (FC buffer): PBS with 5% FCS
Pre-separation filters (130-041-407, Miltenyi)
LD MACS columns (130-042-901, Miltenyi)
MACS separator magnet
15 ml conical centrifuge tubes
Centrifuge

Protocol steps—Step annotations

Isolation of mononuclear cells from tonsil:

1. Put tonsils in a petri dish and cut into very small pieces with scissors.
2. Use spoon to transfer pieces into a plastic stomacher bag, add 10 ml PBS and seal the bag after removing air bubbles.
3. Homogenize the sample in the plastic bag for 120 seconds at *high speed.*
4. Transfer the cell suspension into a metal sieve and grind the tissue with a plunger.
5. Add 20 ml of the obtained cell suspension in a 50 ml tube using a pipette.
6. Slowly add 13 ml of Ficoll-Hypaque underneath the cell suspension using a pipette.
7. Centrifuge 30 min at 340 x *g*, 18-20°, slow acceleration, no brake.
8. Transfer the mononuclear lymphocyte cell layer to a new 50 ml tube with a plastic Pasteur pipette, wash cells by adding PBS up to 50 ml and centrifuge for 8 min at 470 x *g*, 18-20°C.
9. Remove supernatant.
   - *Optional (depending on whether or not red blood contamination is present): remove contaminating red blood cells by adding 5 ml ACK lysis buffer to the cell pellet, resuspend the cells and incubate for 10 min on ice.*
10. Wash the cells by adding 45 ml PBS, centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant, resuspend the cells in 50 ml PBS and count the cells.
11. Centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant, and resuspend the cells in 200-500 µl PBS, and proceed with ILC enrichment by negative selection of CD3 and CD19.

ILC enrichment by CD3 and CD19 negative selection (continue from isolation of mononuclear cells from tonsil step 10):

12. Add 10 µg FITC anti-CD3 and 12.5 µg FITC anti-CD19 per $1x10^9$ cells, mix well and incubate for 30 min at 4°C with mild agitation.
13. Wash with PBS, centrifuge the cell suspension for 5 min at 470 x *g,* 8°C, and remove supernatant.
14. Resuspend the cell pellet in 200-500 µl PBS and add 0.33 µl anti-FITC beads per 1 x 10^6 cells, mix well, and incubate for 20 min at 4°C with mild agitation.
15. Place MACS LD column in the MACS separator magnet, equilibrate the column with 2 ml wash buffer.
    - *Do not exceed $5x10^8$ cells per column as this will result in less efficient depletion.*
16. Wash the cell suspension with PBS, centrifuge for 5 min at 470 x *g,* 8°C, and remove supernatant. Resuspend the cell pellet in 2 ml wash buffer per 5 x 10^8 cells.

17. Apply the cell suspension to the column (max $5x10^8$ cells per column). Wait until the suspension has completely run through the column.
18. Wash the column twice with 1 ml wash buffer.
19. The flow through contains ILCs, DCs, NK cells, and granulocytes, which are ready for analysis or sorting by flow cytometry (Basic protocol 5).

BASIC PROTOCOL 3: ISOLATION OF LEUKOCYTES CONTAINING ILC SUBSETS FROM INTESTINE:

This protocol describes the preparation of a single cell suspension from human intestinal tissue by enzymatic digestion to be used for the analysis and isolation of ILCs subsets.

Materials

Fresh colon or ileum (± 12 cm^2 when cut open longitudinally)
Phosphate buffered saline (PBS)
500 mM EDTA
Heat inactivated FCS
Digestion medium (see recipe)
ACK lysis buffer (see recipe)
Flow cytometry buffer (FC buffer): PBS with 5% FCS
Scissors
Long forceps (long enough to reach the bottom of a 50 ml tube)
Rotator or shaker
Small magnets
Magnetic stirrer
Plunger
70 µm and 40 µm sieves
50 ml conical centrifuge tubes
Centrifuge
Ice

Protocol steps—Step annotations

Isolation of single cell suspension from intestine:

1. Cut mucosa from underlying muscle tissue and collect mucosa in 50 ml tube.
   *Mucosa can be collected as several stranded pieces. ± 12 cm^2 mucosa will generally result in a cell yield of ± 1,5*10^8 cells. However, inflamed mucosa generally results in a lower cell yield.*

2. Add 40 ml of PBS to mucosa and shake the tube. Transfer mucosa to a clean 50 ml tube and add 40 ml of PBS again, shake. Repeat until mucosa is completely clean.
   - *Shaking will remove residual feces and mucus.*
3. Add 20 ml of PBS + 2% EDTA + 1% FCS to mucosa and incubate on a rotator or shaker at 4°C for 30 min.
   - *This separates the epithelial layer from the lamina propria.*
4. Wash lamina propria as in step 2.
   - *This removes the remaining epithelial cells to improve the purity.*
5. Dry lamina propria on a gauze bandage.
   - *This makes cutting easier. The use of a bandage instead of regular paper tissues prevents fluff formation.*
6. Cut lamina propria into very small pieces and transfer to clean 50 ml tube.
7. Add 5 ml of digestion medium. Add a small magnet and place the tube on a magnetic stirrer at 37°C for 45-60 min.
8. Add 45 ml PBS and filter suspension through a 70 µm sieve and subsequently through a 40 µm sieve. Use a plunger if needed
9. Centrifuge the cell suspension for 5 min at 470 x *g*, 8°C and remove supernatant.
10. Remove contaminating red blood cells by adding 5 ml ACK lysis buffer to the cell pellet, then resuspend the cells and incubate for 10 min on ice.
11. Wash the cells by adding 45 ml PBS, centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant, resuspend the cells in 50 ml PBS, and count the cells.
12. Centrifuge the cell suspension for 5 min at 470 x *g*, 8°C, remove supernatant, and resuspend the cells in FC buffer. The cells are ready for staining with antibodies for analysis or sorting by flow cytometry (Basic protocol 5).

BASIC PROTOCOL 4: ISOLATION OF LEUKOCYTES CONTAINING ILC SUBSETS FROM NASAL POLYP(S):

This protocol describes the preparation of a single cell suspension from human nasal polyp tissue by enzymatic digestion to be used for the analysis and isolation of ILCs subsets.

Materials

Fresh nasal polyp tissue (5-10 grams)
Phosphate buffered saline (PBS)
Digestion medium (see recipe)
ACK lysis buffer (see recipe)

Flow cytometry buffer (FC buffer): PBS with 5% FCS
Scissors
Forceps
Plunger
Rotator or shaker
Small magnets
Magnetic stirrer
Plunger
70 µm and 40 µm sieves
50 ml conical centrifuge tubes
Centrifuge
Ice

Protocol steps—Step annotations

Isolation of single cell suspension from nasal polyp tissue:

1. Put nasal polyps in petri dish and cut into very small pieces with scissors.
   - *5-10 grams of nasal polyp tissue will generally result in a cell yield of ± 5*10^7 cells. However, this can vary considerably among different donors.*
2. Use a spoon to transfer pieces into a 50 ml tube and centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant.
3. Add 5 ml of digestion medium. Add a small magnet and place the tube on a magnetic stirrer at 37°C for 45-60 min.
4. Add 45 ml PBS, centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant, resuspend in 50 ml of PBS and filter suspension through a 70 µm sieve and subsequently through a 40 µm sieve. Use a plunger if needed.
5. Centrifuge the cell suspension for 5 min at 470 x *g*, 8°C and remove supernatant.
6. Remove contaminating red blood cells by adding 5 ml ACK lysis buffer to the cell pellet, resuspend the cells and incubate for 10 min on ice.
7. Wash the cells by adding 45 ml PBS, centrifuge for 5 min at 470 x *g*, 8°C, remove supernatant, resuspend the cells in 50 ml PBS, and count the cells.
8. Centrifuge the cell suspension for 5 min at 470 x *g*, 8°C, remove supernatant, and resuspend the cells in FC buffer. The cells are ready for staining with antibodies for analysis or sorting by flow cytometry (Basic protocol 5).

BASIC PROTOCOL 5: ILC SUBSET ANALYSIS AND/OR ISOLATION BY FLOW CYTOMETRY

This protocol describes the analysis of a single-cell suspension of leukocytes prepared in Basic Protocol 1-4 for the presence of all ILC populations by multicolor flow cytometry. Figure 1 provides examples of the recommended gating strategy for each tissue. ILCs can express a lot of different markers as described in commentary section. Antibodies to detect these markers can be added. Here, we describe a staining strategy with the minimal number of required antibodies to detect all ILC subsets.

Materials

Single cell suspension containing ILCs (Basic Protocol 1-4)
Flow cytometry buffer (FC buffer): PBS with 5% FCS
PBS
Flow cytometer with at least 6 fluorescent channels for ILC analysis
Fluorescence-activated cell sorter with at least 6 fluorescent channels for sorting of ILCs
5 ml polypropylene filter-cap test tubes
Centrifuge
Collection medium for sorted cells

- *No special medium is needed, as after sorting the cells can be transferred to a different medium. It is advisable to add at least 5% of FCS or 1% of NHS to the collection medium.*

Lineage antibodies (all in the same fluorescent color):
anti-human CD3 (OKT3 and UCHT1)
anti-human CD1a (HI149)
anti-human CD4 (RPA-T4)
anti-human CD14 (HCD14)
anti-human CD16 (3G8)
anti-human CD19 (HIB19)
anti-human CD34 (581)
anti-human CD94 (DX22)
anti-human BDCA2 (201A)
anti-human FceRI (AER-37)
Other antibodies in different fluorescent colors:
anti-human CD45 (2D1) (only for tissues)
anti-human CD127 (R34.34)
anti-human CD161 (HP-3G10)

anti-human CD117 (104D2D1)
anti-human CRTH2 (BM16)
anti-human NKp44 (P44-8) (only for tissues)
Viability stain (optional, e.g. Fixable viability dyes for flow cytometry from Thermo Fisher)

Protocol steps—Step annotations

1. Centrifuge single cell suspension obtained in Basic Protocol 1-4 for 5 min at 470 x *g*, 8°C, discard supernatant, and resuspend the cells in 100-200 µl FC buffer. Add all the lineage and other antibodies described in material list above to the cell suspension. Appropriate amounts of antibodies to be used should be determined after titration.
   - *The total cell suspension will be stained with all the antibodies described in the material list. This allows multicolor flow cytometry to identify all ILC subsets within the cell suspension. A recommended staining strategy including fluorochromes is shown in **Table 1**.*
2. Incubate for 30 min at 4°C with mild agitation.
   - *Incubation can also be done at room temperature, in that case the incubation time can be shortened to 15 min.*
   - *Individual titration of the antibodies is highly recommended for the best results.*
3. For sorting, prepare one collection tube (500 µl culture medium in polypropylene tube) per ILC subset to be sorted.
4. Add 1 ml of PBS per stained sample and wash stained cells with PBS, centrifuge for 5 min at 470 x *g*, 8°C, and resuspend cells in 1 ml PBS per $30x10^6$ cells.
5. Filter cells through a filter-capped 5 ml test tube before flow cytometry analysis. Keep cell suspension at 4°C in the dark.
   - *Staining with a viability dye is highly recommended, especially for cell suspensions obtained from tissue.*
6. Analyze or sort ILC subsets on the flow cytometer by using the gating strategies as shown in **Figure 1**.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

ACK lysis buffer (10x concentration)

1.5 M ammonium chloride (NH_4Cl), 82.9 g per liter
0.1 M potassium bicarbonate ($KHCO_3$), 10.01 g per liter

1 mM EDTA, 0.372 g per liter
Dissolve in 1 l deionized, distilled water and set pH to 7.4.
Can be stored for 6 years at 2-8°C.
Dilute 10x in deionized, distilled water before use.

Digestion medium

IMDM medium containing:
600 µg/ml collagenase IV
500 µg/ml DNase I
Prepare fresh

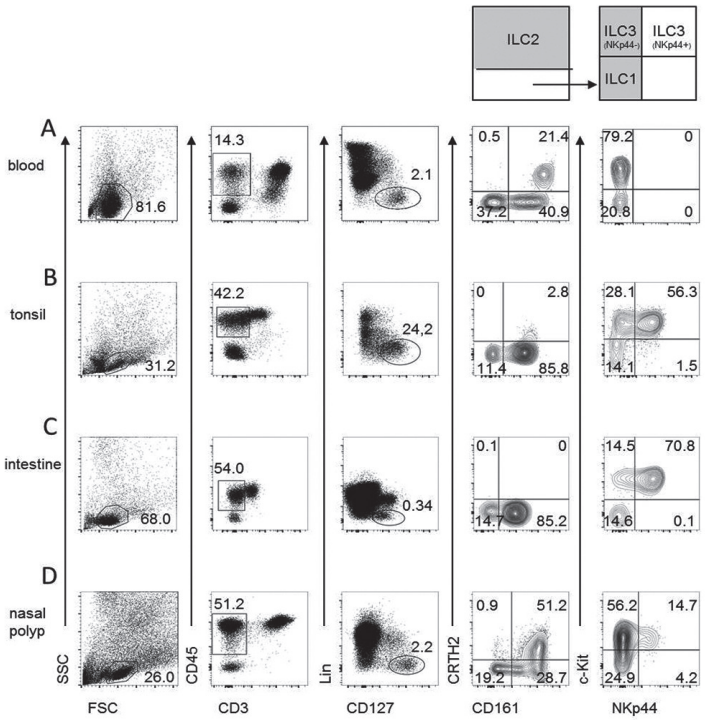

Figure 1. Gating strategy to identify all ILC subpopulations in peripheral blood
(A), tonsil (B), intestine (C), and nasal polyp (D). ILCs are identified as $CD45^+$ lymphocytes that are $CD3^-Lin^-CD127^+$ cells. Within this population a further distinction between ILC2s ($CD161^+$ $CRTH2^+$), ILC1s ($CD161^{+/-}$ c-Kit), and ILC3s ($CD161^{+/-}$ $c\text{-}Kit^+$ $NKp44^{+/-}$) can be made. Numbers in plots represent percentage of gated cells.

Fluorochome	Recommended antibody	Note
FITC	Lineage anti-human CD3 (OKT3 and UCHT1) anti-human CD1a (HI149) anti-human CD4 (RPA-T4) anti-human CD14 (HCD14) anti-human CD16 (3G8) anti-human CD19 (HIB19) anti-human CD34 (581) anti-human CD94 (DX22) anti-human BDCA2 (201A) anti-human FceRI (AER-37)	
AF700	CD45 (2D1)	Only for tissue
PE-CY7	CD127 (R34.34)	
PE-CY5	CD117 (104D2D1)	
PE-CF594	CRTH2 (BM16)	
PE	CD161 (HP-3G10)	
APC	NKp44 (P44-8)	Only for tissue

Table 1. A recommended staining strategy to identify all ILC subsets within a cell suspension.
All antibodies are added to the cell suspension to allow multicolor flow cytometric analysis. Individual titration of each antibody is recommended for the best results. Listed fluorochromes are an example and can be adjusted to suit the instrument used.

COMMENTARY

Background Information

In contrast to other immune cells, the identification of helper ILCs by flow cytometry is more difficult due to the lack of a single ILC-specific surface marker. As such, the current best method to detect ILCs is the exclusion of other immune cells by using an antibody cocktail containing markers specific for monocytes, T cells, B cells, NK cells (cytotoxic ILCs) and granulocytes (lineage); in combination with markers expressed by one or more ILC subsets. Although all helper ILCs express IL7Rα (CD127), it is important to note that the expression is not restricted to ILCs, but ILCs can be identified as $CD45^+$ lymphoid cells that are $lineage^-CD127^+$ cells. In addition, the lymphocyte antigen CD7 is reported to be useful to discriminate ILC population from other cell populations [5,6].

Further identification of ILC subsets is based on subset defining markers. ILC2s express high levels of CD161 and are identified as CRTH2-expressing cells in humans, whereas in mice the main ILC2 surface marker is the IL-33R (ST2) [7]. Although human ILC2s are also responsive to IL-33 and express transcripts for ST2, surface staining for this receptor is difficult with the few antibodies currently available that are specific for this receptor. ILC2s express the transcription factor GATA3 and produce IL-5 and IL-13 in response to stimulation with primarily IL-33, IL-25, TSLP, and IL-1b. CRTH2 is the receptor for prostaglandin D2, which induces migration of and amplifies cytokine production by ILC2s. Human ILC3s are characterized by the lack of CRTH2 and expression of c-Kit and are subdivided into NKp44$^-$ and NKp44$^+$ ILC3s. Although all ILC3s depend on RORgt and are responsive to IL-1b and IL-23, considerable differences have been reported for these subsets. NKp44-expressing ILC3s are only found in tissues and are the main producer of IL-22. The c-Kit$^+$ cells that lack both NKp44 and CRTH2 are a more heterogeneous population and in blood this population was reported to contain a precursor for all ILC subsets [5]. Besides the ILC-identifying markers and activating cytokines mentioned here, ILC2s and ILC3s are regulated by many more soluble factors and interact with other immune cells [3]. ILC1s are CD127$^+$ ILCs that lack the expression of markers generally associated with ILC2s and ILC3s. No ILC1-specific surface markers have been identified. This is further complicated by reports of different ILC1 subsets with heterogeneous expression of the transcription factors T-bet and Eomes. One example is the intra-epithelial ILC1 that lacks CD127 expression, but expresses CD103 and NKp44 [8]. All ILC1 populations produce IFN-g in response to IL-12 and can be distinguished from NK cells by their lack of cytotoxicity [4].

Although the surface markers provided in this unit are sufficient to detect and isolate the different ILC subsets, intracellular staining for cytokines and transcription factors specific for each subset can confirm correct isolation and provide more information on their functionality. Moreover, no uniform consensus has been reached on the best identification strategy for human ILCs, which hinders comparisons between different research groups. Here we describe a general protocol to characterize the main human ILC subsets currently identified. The ILC field is still rapidly evolving and novel markers to better identify ILC (subsets) are constantly being found.

Critical Parameters and Troubleshooting

a. Applicable to all tissues described in this unit:

1. If you are isolating ILCs for setting up cultures it is important to work in a biosafety cabinet to prevent contaminations of your samples.

2. It is of critical importance to filter cells before running through the flow cytometer as contaminations with clumps and debris are common in cell suspensions obtained from digested tissues.
3. During flow cytometry analysis and sorting adjust the concentration of the cell suspension so as to allow a flow rate not exceeding 15000 events/s. Large amounts of debris might require lower flow rates.
4. The use of two different anti-CD3 clones during staining for flow cytometry sorting is highly recommended to prevent T cell contamination.
5. CD56 is expressed by part of the ILC3s in all tissues and an antibody against this surface marker should therefore not be included in the antibody mix used to exclude lineage+ cells.
6. CD5+ and CD4+ ILCs represent respectively immature ILCs [9] and a subset of ILC1s [10]; however, extra caution is required to ensure these populations are free of T cell contamination. Note: the expression of CD5 on immature ILCs is one log less than on T cells.

b. Blood specific:

1. It is critical to realize that after positive ILC selection the cells are labeled with CD161-PE and can therefore not be stained in that color for another marker.
2. Take into account that CD161-PE stays on the cell surface for quite some time during cell culture (positive signal observed 5-7 days after CD161-PE isolation), to avoid misinterpretation of the assay result (*e.g.* determination of phenotype or intracellular cytokine production using PE-conjugated antibodies).
3. CD45 staining is not necessary in PBMCs as after the Ficoll-Hypaque step almost all cells within the lymphocyte gate are CD45-expressing cells.
4. NKp44-expressing ILC3s are absent in peripheral blood of healthy individuals and thus the NKp44 antibody is not used for cells obtained from this source.
5. The c-Kit$^+$CRTH2$^-$ fraction was reported to contain circulating ILC-precursors [5].

c. Tonsil specific:

1. Do not exceed the maximum amount of cells per column during negative selection. This will reduce selection efficiency and necessitate a longer sorting time.
2. The use of ACK lysis buffer is optional as there are not always many erythrocytes in the obtained cell suspension after the Ficoll-Hypaque step.

d. Intestine specific:

1. Further purification of the single-cell suspension by positive/negative selection with a column is not possible, due to the slimy state of the cell suspension.

2. Enzymatic digestion can delete surface markers (especially by using LiberaseTM [11]), which is why it is highly recommended to use a less aggressive enzyme during the cell collection procedure (Collagenase IV).
3. The use of ACK Lysis buffer removes debris from the cell suspension, besides removing red blood cells.
4. ILC2s are virtually absent in the (adult) intestine. Therefore, CRTH2 can be added in the lineage to save a fluorescent channel for analysis of another marker.

e. Nasal polyp specific:

1. Further purification of the single-cell suspension by positive/negative selection is not necessary due to a relatively high frequency of ILC(2)s in the nasal polyp tissue.
2. The use of ACK Lysis buffer is required, as the single-cell suspension contains a significant amount of red blood cells.
3. The use of donors with co-morbidities (i.e., allergy or cystic fibrosis) may interfere with ILC subsets distribution.

Understanding Results

Expected ILC frequencies:

This section describes average ILC frequencies per tissue when processed as described above.

If buffy coats (concentrated leukocyte suspensions, usually from 500 ml of blood) are used, after PBMC isolation between 1.0 - 1.5x10^9 cells are obtained. ILCs represent 0.14 ± 0.10% of lymphocytes in blood (**Table 2**). Depletion or selection results in increased ILC frequencies and makes sorting of the cells more feasible. After enrichment by depletion of CD3, CD14, CD16, and CD19-expressing cells between 20 - 100x10^6 cells should remain. CD161-enrichment of ILC2s from PB results in a yield between 20 - 70x10^6 cells.

From a tonsil between 1.0 and 2.0x10^9 mononuclear cells are obtained after the Ficoll-Hypaque step of which ILCs represent 0.52 ± 0.16% (**Table 2**). After depletion of CD3-, and CD19-expressing cells between 12 and 20x10^6 cells remain which are enriched for ILCs.

The ILC subset distribution in blood and the mentioned tissues are shown in **Table 3**.

	ILCs (% CD45$^+$ lymphocytes)		
	Before enrichment	**CD3/CD14/CD16/CD19 depletion***	**CD161 selection**
Peripheral blood	0.14 ± 0.10	1.33 ± 0.98	0.47 ± 0.25
Tonsil	0.52 ± 0.16	12.10 ± 5.05	N.A.
Intestine	0.86 ± 0.24	N.A.	N.A.
Nasal polyp	2.08 ± 0.94	N.A.	N.A.

Table 2. Average frequencies with standard deviation of total helper ILCs within total CD45$^+$ lymphocytes calculated as Lin$^-$CD127$^+$ cells.

*For tonsil only CD3 and CD19 are used for depletion.

	Blood	**Tonsil**	**Intestine**	**Polyp**
ILC1	21.03 ± 11.87	5.62 ± 4.67	3.11 ± 1.39	10.54 ± 4.14
ILC2	17.48 ± 10.43	3.53 ± 2.87	0	56.24 ± 7.07
NKp44$^-$ ILC3	56.38 ± 11.77	37.4 ± 12.82	22.63 ± 13.0	20.12 ± 6.44
NKp44$^+$ ILC3	0	52.64 ± 15.96	74.26 ± 13.4	13.10 ± 4.13

Table 3. Average frequencies with standard deviation of helper ILC subsets in blood and tissues calculated as percentage of total CD45$^+$CD3$^-$Lin$^-$CD127$^+$ population.

ILC frequencies are altered in disease

As ILCs are highly responsive to changes in their environment, e.g. changing cytokine milieu, in many inflammatory diseases the ILC frequency is altered. The feature of ILC plasticity further adds to the shift in ILC subset distribution. Below we mention some examples.

1. Enhanced frequencies of ILC1s are found in the gut of patients with inflammatory bowel disease [6,12]. ILC1s are also enriched in the blood and lungs of patients with chronic obstructive pulmonary disease [13,14]. This can at least partly be explained by the induction of ILC2s and ILC3s transdifferentiation into ILC1s in response to IL-12.
2. Enhanced frequencies of ILC2s are found in blood of asthma patients [15]. Nasal polyps are enriched for ILC2s as compared to healthy nasal turbinate tissue. This is a result of the high TSLP and IL-4 levels that were found in these polyps [13].
3. Whereas NKp44-expressing ILC3s are not found in the blood of healthy donors, they are present in the blood of psoriasis patients [16].

Time Considerations

This section summarizes the time frames to complete full protocols for ILC isolation from different tissues. However, indicated times frames depend on processing experience and donor to donor variations.

Blood

The isolation of mononuclear cells from blood (buffy coat): 1.5-2 hrs
ILC enrichment by negative selection: 1.5-2 hrs
ILC enrichment by positive selection: 1.5-2 hrs
ILC purification by cell sorting: 0.5-2 hrs (per buffy coat, smaller volumes of blood can be sorted considerably faster).

With sufficient practice, ILCs can be purified from 3 buffy coats within one day. If this is not manageable, a convenient point to store cells is after negative or positive selection, before cell staining for FACS analysis or sorting. Cells can be kept overnight in the fridge in medium with FCS. However, this will negatively influence the final ILC yield.

Tonsil

Isolation of mononuclear cells from tonsil: 1-2 hrs
ILC enrichment by negative selection: 1.5-2.5 hrs
ILC purification by cell sorting: 0.5-2 hrs

With sufficient practice, ILCs can be purified from 2 tonsils within one day. If this is not manageable, a convenient point to store cells would be after the Ficoll-Hypaque step, before negative selection; or after negative selection, but before cell staining for analysis or sorting. However, this will negatively influence the final ILC yield.

Intestine

Isolation of mononuclear cells from intestine: 3-4 hrs
ILC purification by cell sorting: 2-3 hrs

Indicated time frames are highly dependent on the size of the intestinal resection specimen and are here described for ± 12 cm^2 mucosal tissue. A convenient stopping point for this protocol would be after isolation of mononuclear cells from intestine with enzymatic digestion and before staining for cell sorting or analysis. It is crucial to wash away all enzymes from the cell suspension. However, this will negatively influence the final ILC yield.

Nasal Polyp

Isolation of mononuclear cells from nasal polyp: 1-2 hrs
ILC purification by cell sorting: 1-2 hrs

Indicated time frames are highly dependent on the size of the nasal polyp specimen and are here described for 5-10 grams of the nasal mucosa tissue. A convenient stopping point for this protocol would be after isolation of mononuclear cells from nasal polyps with enzymatic digestion and before staining for cell sorting or analysis. It is crucial to wash away all enzymes from the cell suspension. However, this will negatively influence the final ILC yield.

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