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Authors

Strohm, Allyssa N

Doherty, Taylor A

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Detection, Isolation, and Functional Studies of Mouse Pulmonary Group 2 Innate Lymphoid Cells

Allyssa N. Strohm,
Taylor A. Doherty

Abstract

ILC2s are key players in the emergence of type 2 inflammation in many pulmonary diseases. While several phenotypic markers can be used to identify ILC2s, our method utilizes the surface markers CD127 and ST2 to classify a group of type 2 cytokine-producing ILC2s upon activation by the fungal allergen *Alternaria alternata*. Here, we provide our protocol for the detection and isolation of a highly pure population of pulmonary mouse ILCs via flow cytometry and cell sorting. We also describe the methods for in vitro stimulation to assess the functionality of ILC2s.

Keywords

Innate lymphoid cells; ILC2; Type 2 inflammation; Flow cytometry; FACS; ILC2 staining

1 Introduction

As the innate counterpart of T lymphocytes, innate lymphoid cells (ILCs) are key mediators of pulmonary inflammatory diseases such as asthma, pulmonary fibrosis, and COPD [1–3]. Differentiated by the transcription factors expressed and cytokines produced, ILCs are categorized into three subsets: ILC1s which are reliant on the T-box transcription factor Tbet and produce interferon-gamma (IFN-γ); ILC2s reliant on GATA3 and RORα and produce type 2 cytokines; and ILC3s reliant on RORγt and produce IL17 and/or IL22 [4].

Group 2 innate lymphoid cells (ILC2s) play critical roles in type 2 inflammation in mice upon infection with influenza virus or when challenged with allergens such as *Alternaria alternata*, papain, house dust mite, and ovalbumin [5]. Upon allergen exposure, the release of IL33, IL25, and/or thymic stromal lymphopoietin (TSLP) from epithelial cells activates ILC2s to produce type 2 cytokines IL4, IL5, and IL13 [4, 6]. Release of IL4 drives Th2 differentiation and IgE via B cells, IL5 mediates recruitment and activation of eosinophils, and IL13 induces goblet cell hyperplasia and airway hyperresponsiveness [7, 8].

The mouse lung ILC2 population can be greatly expanded through *Alternaria* or cytokine exposure via IL33, IL25, and/or TSLP [6, 9]. The lung can then be harvested and digested carefully to create a single-cell suspension to be either stained for flow cytometry or cultured for stimulation studies [10, 11]. Several groups have established that ILC2s are lineage-negative, c-kit$^+$, Sca-1$^+$, CD127$^+$, Thy1.2$^+$, ST2$^+$, CD25$^+$, and Gata3$^+$, and can also be detected via the markers IL25R and KLRG1 [12–14]. For the following protocol, conventional ILC2s are identified as CD45$^+$, Lin$^-$ Thy 1.2$^+$, ST2$^+$, and CD127$^+$

lymphocytes. To isolate a pure population of ILC2s, sterile fluorescence-activated cell sorting (FACS) can be used and allows for ex vivo cell stimulation to assess functionality and cytokine production [10, 11]. Once isolated, the pure population of ILC2s can also be used for RNA sequencing and qPCR, as previously reported [15, 16].

In this protocol, we describe the methods for the expansion of ILCs in vivo using the fungal allergen *Alternaria alternata*, detection and isolation of ILC2s via flow cytometry, and functional analysis of ILC2s via cell culture. The protocol can be divided into three main sections. The first section describes how to expand the ILC2 population in vivo and how to liberate the cells from the lung tissue (*see* Subheadings 3.1 and 3.2). The second section explains how to create antibody master mixes and how to stain for ILCs through surface staining or intracellular staining (*see* Subheading 3.3). The final section details how to isolate a pure population of ILCs via cell sorting and how to stimulate the cells in cell culture to assess ILC2's ability to produce the type 2 cytokines (*see* Subheading 3.4).

2 Materials

2.1 Expansion of ILCs In Vivo

1. *Alternaria alternata* (*Alternaria*) extract (Greer).
2. Phosphate Buffered Saline (PBS).

2.2 Preparation of ILCs Via Lung Digest

1. RPMI 1640 Medium: Store at 4 °C.
2. Miltenyi Lung Dissociation Kit and Dissociator (Miltenyi Biotec).
3. gentleMACS C tubes (Miltenyi Biotec).
4. Mini LabRoller (Labnet Gentle rotator).
5. 40 μM cell strainer.
6. 50 mL Conical Tubes.
7. Sterile Plunger.

2.3 Staining for ILC2s

1. 5 mL polystyrene round-bottom tubes, 12 × 75 mm—hereby referred to as a FACS tube.
2. FACS Buffer: 500 mL 1 × PBS, 10 mL of Fetal Bovine Serum (FBS), and 5 mL of 2% sodium azide. Store at 4 °C.
3. eBioscience™ FoxP3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific). The kit contains the FoxP3 fixation/permeabilization concentrate (4×), the FoxP3 fixation/permeabilization diluent and the FoxP3 permeabilization buffer concentrate (10×). Make 50 mL of a 1 × FoxP3 permeabilization buffer as per the manufacturer's instructions. This will be used for wash steps as well as in staining master mixes.

4. BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences). The kit contains the Fixation/Permeabilization solution and the Perm/Wash Buffer concentrate (10×). Dilute the Perm/Wash Buffer concentrate 1:10 in distilled water to prepare 1 × BD Perm/Wash buffer.
5. Cell stimulation cocktail plus protein transport inhibitor (CSC) (Invitrogen): phorbol 12-myristate 13-acetate (PMA) at 40.5 μM, ionomycin at 670 μM, brefeldin A at 5.3 mM and monensin at 1 mM in ethanol (500×).
6. Flat bottom polystyrene 96-well plate.
7. T-cell media: 500 mL RPMI, 5 mL of commercially available penicillin/streptomycin stock solution (100×), 5 mL of commercially available glutamine stock solution (100×), 500 μL of 2-mercaptoethanol (1000×), and 50 mL of Fetal Bovine Serum.
8. Required antibodies.
    a. Fc Block: Anti-mouse CD16/32 antibody Clone 93 (Biolegend).
    b. ILC surface stain panel: FITC-conjugated anti-mouse Lineage Cocktail Clones 145–2C11, RB6–8C5, RA3–6B2, TER-119, and M1/70 (Biolegend), FITC-conjugated anti-mouse CD11c Clone N418 (Biolegend), FITC-conjugated anti-mouse NK1.1 Clone PK136 (Biolegend), FITC-conjugated anti-mouse CD5 Clone 53–7.3 (Biolegend), FITC-conjugated anti-mouse FceR1a Clone MAR-1 (Biolegend), FITC-conjugated anti-mouse TcRβ Clone H57–597 (Biolegend), FITC-conjugated anti-mouse TcRγδ Clone GL3 (Biolegend), PerCP/Cyanine5.5-conjugated anti-mouse CD45.2 Clone 104 (Biolegend), Pacific Blue-conjugated anti-mouse CD90.2 (Thy1.2) Clone 53–2.1 (ThermoFisher Scientific); PE-Cy7-conjugated anti-mouse CD127 Clone A7R34 (Biolegend), APC-conjugated anti-mouse IL33Ra Clone DIH9 (Biolegend).
    c. ILC2 GATA3 transcription factor staining: PE-conjugated Gata-3 monoclonal antibody Clone TWAJ (ThermoFisher Scientific), PE-conjugated rat IgG2b, κ Clone eB149/10H5 (Invitrogen).
    d. ILC2 cytokine staining: PE-Conjugated anti-mouse/human IL5 Clone TRFK5 (Biolegend), PE-Conjugated anti-mouse IL13 Clone eBio13A (Invitrogen), PE-Conjugated rat IgG1, κ Clone eBRG1 (Invitrogen).

2.4 ILC Cell Sorting and Culture

1. Red blood cell (RBC) lysis kit (Biolegend).
2. PBS.
3. Sort Buffer: 1% Fetal Bovine Serum (FBS) in Hank’s Balanced Salt Solution (HBSS).

4. T-cell media: 500 mL RPMI, 5 mL of commercially-available penicillin/streptomycin stock solution (100×), 5 mL of commercially available glutamine stock solution (100×), 500 μL of 2-mercaptoethanol, and 50 mL of Fetal Bovine Serum.
5. Interleukin 2 (IL2) (R&D Systems).
6. Interleukin 7 (IL7) (R&D Systems).
7. Interleukin 33 (IL33) (R&D Systems).
8. Supplemented T-cell Media: 10 ng/mL of IL2 and IL7 to T-cell media (as prepared in **step 4** in Subheading 2.4).
9. Zombie Aqua Fixable Viability Kit (Zombie Aqua) (Biolegend).
10. Ultracomp eBeads Compensation beads (Invitrogen).
11. 5 mL Polystyrene round-bottom tube with Cell strainer cap.
12. Flat bottom polystyrene 96-well plate.
13. Required antibodies.
    a. Fc Block: Anti-mouse CD16/32 antibody Clone 93 (Biolegend).
    b. ILC Stain: FITC-conjugated anti-mouse Lineage Cocktail Clones 145–2C11, RB6–8C5, RA3–6B2, TER-119, and M1/70 (Biolegend), FITC-conjugated anti-mouse CD11c Clone N418 (Biolegend), FITC-conjugated anti-mouse NK1.1 Clone PK136 (Biolegend), FITC-conjugated anti-mouse CD5 Clone 53–7.3 (Biolegend), FITC-conjugated anti-mouse FceR1a Clone MAR-1 (Biolegend), FITC-conjugated anti-mouse TcRβ Clone H57–597 (Biolegend), FITC-conjugated anti-mouse TcRγδ Clone GL3 (Biolegend), PerCP/Cyanine5.5-conjugated anti-mouse CD45.2 Clone 104 (Biolegend), Pacific Blue-conjugated anti-mouse CD90.2 (Thy1.2) Clone 53–2.1 (ThermoFisher Scientific).
    c. Fluorescence minus one (FMO) (eFluor 450): FITC-conjugated anti-mouse Lineage Cocktail Clones 145–2C11, RB6–8C5, RA3–6B2, TER-119, and M1/70 (Biolegend), FITC-conjugated anti-mouse CD11c Clone N418 (Biolegend), FITC-conjugated anti-mouse NK1.1 Clone PK136 (Biolegend), FITC-conjugated anti-mouse CD5 Clone 53–7.3 (Biolegend), FITC-conjugated anti-mouse FceR1a Clone MAR-1 (Biolegend), FITC-conjugated anti-mouse TcRβ Clone H57–597 (Biolegend), FITC-conjugated anti-mouse TcRγδ Clone GL3 (Biolegend), PerCP/Cyanine5.5-conjugated anti-mouse CD45.2 Clone 104 (Biolegend).

3 Methods

3.1 ILC2 Expansion In Vivo (see Note 1)

1. Prepare the *Alternaria* reagent for intranasal challenge by adding 50 μg of the lyophilized *Alternaria* extract into 40 μL of PBS per challenge. Protocols for expansion include 3–4 challenges over 3–10 days (*see* Notes 2 and 3).
2. Anesthetize mice with isoflurane. Administer the *Alternaria* reagent intranasally when the mouse's respiratory rate is 1 breath/s.

3.2 Lung Digestion and Processing

1. Euthanize mice in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Collect the whole lung and place it in a FACS tube containing 2 ml of RPMI media.
2. In the gentleMACS C tubes, combine 2.5 mL of the lung digest buffer (diluted to 1× with Deionized/DI Water), 7.5 μL of Enzyme A, and 50 μL of Enzyme D, as per the Miltenyi Lung Dissociation Kit instructions.
3. Add whole lungs to individual gentleMACS C tubes with digest solutions (*see* Note 4).
4. Place gentleMACS C tubes with lung in the gentleMACS dissociator and blend on setting m_Lung_01 (*see* Note 5).
5. Place gentleMACS C tubes with blended lung sample in the mini LabRoller gentle rotator and rotate cells for 30 min at 37 °C (*see* Note 5).
6. Collect tubes after incubation. Place gentleMACS C tubes in the gentleMACS dissociator and blend on setting m_lung_02 (*see* Note 5).
7. Filter each lung cell suspension through a 40 μm cell strainer into a 50 mL conical tube. Use 2 mL of RPMI to rinse the blender top tube and filter the solution through the strainer. Discard the strainer and cap the conical tube containing the lung digest (*see* Note 6).
8. Centrifuge the conical tube with the lung cell suspension at 500 × *g* (~1500 rpm) at 4 °C for 5 min.
9. Aspirate supernatant and add 1 mL of RPMI to each sample.
10. Obtain cell counts using the flow cytometer to approximate the total number of cells for further analysis (*see* Note 7).

3.3 ILC2 Staining

ILC2 staining is effectively accomplished through three separate staining procedures: ILC2 surface stains, ILC2 transcription factor intracellular staining, and ILC2 cytokine intracellular staining for IL5 and IL13. For samples undergoing surface and transcription factor staining, cells can be immediately stained once in a single-cell suspension. For cells

undergoing cytokine intracellular staining, cells should ideally be cultured and stimulated with PMA and ionomycin first before staining occurs (*see* Notes 8 and 9).

3.3.1 Preparation of Master Mixes (Tables 1 and 2)

1. Preparation of a master mix for Fc receptor block: Dilute Fc block in the FACS buffer to a final working concentration of 0.01 μg/μL. All dilutions are in reference to the final staining volume, not the master mix dilution (*see* Note 10). To prepare the master mix, use 50 μL of the FACS buffer per sample, then multiply this volume by the total number of samples. Next, add 1.0 μg of anti-CD16/32 to aliquoted FACS buffer per sample and multiply this amount of antibody by the total number of samples.
2. Preparation of master mixes for ILC2 surface stains.
    a. Preparation of a master mix for complete ILC2 surface stain: Utilize a final staining volume of 100 μL to calculate for the volume of antibody required (*see* Note 10). Enough master mix should be made for all stains, including those undergoing intracellular transcription factor and cytokine staining (*see* Note 10). First aliquot 50 μL of FACS buffer per mouse sample, then multiply this volume by the total number of samples. Next, add antibodies as indicated in Table 1, row “ILC2 surface stain.” Please note that the lineage cocktail is not used at a specific concentration, but rather at a specific volume.
    b. Preparation of a master mix for ILC2 ST2 FMO (surface stain without anti-ST2 antibody): Utilize a final staining volume of 100 μL (*see* Note 10). First, aliquot 50 μL of FACS buffer per mouse sample, then multiply this volume by the total number of samples. Next, add antibodies as indicated in Table 1, row “ILC2: ST2 FMO.”
    c. Preparation of a master mix for ILC2 CD127 FMO (surface stain without anti-CD127 antibody): Utilize a final staining volume of 100 μL (*see* Note 10). First, aliquot 50 μL FACS per mouse sample, then multiply this volume by the total number of samples. Next, add antibodies as indicated in Table 1, row “ILC2: CD127 FMO.”
3. Preparation of master mixes for ILC2 transcription factor and control stains.
    a. Preparation of a master mix for ILC2 transcription factor stain: Aliquot 100 μL of FoxP3 permeabilization buffer (1×) and multiply this volume by the total number of samples. Enough master mix should be made for samples undergoing transcription factor staining. Dilute the PE-conjugated Gata-3 monoclonal antibody to a final working concentration of 0.006 μg/μL, then multiply this amount of antibody by the total number of samples as indicated in Table 1, row “ILC2 Transcription Factor”.
    b. Preparation of a master mix for ILC2 transcription factor isotype control stain: Aliquot 100 μL of FoxP3 permeabilization buffer (1×) and

multiply this volume by the total number of control samples. Dilute the PE-conjugated rat IgG2b, κ isotype to a final working concentration of 0.0006 μg/μL, then multiply this amount of IgG2b by the total number of control samples as indicated in Table 1, row "ILC2 transcription factor isotype."

4. Preparation of master mixes for ILC2 intracellular stain for the IL5 and IL13 cytokines.

    a. Preparation of a master mix for ILC2 intracellular stain for the IL5 cytokine: Aliquot 100 μL of BD Perm/Wash buffer (1×) and multiply this volume by the total number of samples. Dilute the PE-conjugated IL5 to a working concentration of 0.01 μg/μL, then multiply this amount of antibody by the total number of samples as indicated in Table 1, row "ILC2 intracellular IL5 cytokine."

    b. Preparation of a master mix for ILC2 intracellular stain for the IL13 cytokine: Aliquot 100 μL of BD Perm/Wash buffer (1×) and multiply this volume by the total number of samples. Dilute the PE-conjugated IL13 to a working concentration of 0.01 μg/μL, then multiply this amount of antibody by the total number of samples as indicated in Table 1, row "ILC2 intracellular IL13 cytokine."

    c. Preparation of a master mix for ILC2 intracellular stain with an isotype control antibody (control for IL5/IL13 stain): Aliquot 100 μL of BD Perm/Wash buffer (1×) and multiply this volume by the total number of control samples. Dilute the PE-conjugated rat IgG1, κ isotype to a final working concentration of 0.0006 μg/μL, then multiply this amount of antibody by the total number of control samples as indicated in Table 1, row "ILC2 intracellular cytokine stain isotype."

5. Preparation of master mixes for ILC sorting and culture.

    a. Preparation of a master mix for Fc receptor block: Dilute Fc block to a final working concentration of 0.03 μg/μL. Utilize a final staining volume of 100 μL to calculate for the volume of antibody required (*see* Note 10). To prepare the master mix, use 50 μL of the sort buffer per sample, then multiply this volume by the total number of samples. Next, add 1.0 μg of anti-CD16/32 to aliquoted sort buffer per sample and multiply this amount of antibody by the total number of samples.

    b. Preparation of a master mix for ILC2 surface stain: Utilize a final staining volume of 100 μL to calculate for the volume of antibody required (*see* Note 10). First aliquot 50 μL sort per mouse sample, then multiply this volume by the total number of tubes that will be sorted. Next, add antibodies as indicated in Table 2, row "ILC surface."

    c. Preparation of a master mix for eFluor450 FMO (stain without eFluor450-labeled antibody): First aliquot 50 μL of sort buffer per mouse sample, then multiply this volume by the total number of tubes

that will be sorted. Next, add antibodies as indicated in Table 2, row "eFluor450 FMO."

3.3.2 ILC2 Surface Staining—All cells must first be stained for ILC2 surface markers before undergoing intracellular transcription factor staining.

1. Utilizing the single-cell suspension from the digested lung tissue, remove the volume containing 1×10^6 cells and place it in a FACS tube.
2. Wash the cells with 400 μL of the FACS buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
3. Aspirate the supernatant.
4. Add 50 μL of the Fc block master mix (Fc Block). Vortex cells gently. Incubate cells for 5 min. at 4 °C (*see* Note 10).
5. As cells are staining, make compensation samples for the flow cytometer. Make one compensation sample for each fluorophore used. Use 1 drop of beads, 100 μL of the FACS buffer, and 1 μL of an antibody with correct fluorophore. Incubate for 30 min at 4 °C (*see* Note 11).
6. Collect tubes with cell samples and directly add 50 μL of the ILC2 surface master mix to each tube. Control tubes should receive the FMO master mixes. Vortex cells gently. Incubate for 30 min at 4 °C (*see* Note 10).
7. Once cells are stained, wash cells and compensation sampleswith 400 μL of the FACS buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
8. Aspirate the supernatant.
9. For cells undergoing further transcription factor staining via permeabilization, set those aside. Resuspend all other samples with 150 μL of the FACS buffer.
10. Samples are ready to be run on the flow cytometer (Fig. 1).

3.3.3 ILC2 Transcription Factor Staining

1. Use the surface-stained cells that were set aside for transcription factor staining (*see* Subheading 3.3.2). These cells should not have any supernatant or FACS buffer.
2. Dilute the FoxP3 fixation/permeabilization concentrate at a 1: 4 dilution of concentrate:diluent. This is the working solution. Vortex well.
3. Add 300 μL of the FoxP3 fixation/permeabilization working solution to all samples undergoing transcription factor staining. Protect cells from light and incubate at room temperature (RT) for 30 min.
4. The fixation/permeabilization step is now complete. Wash all samples with 400 μL of the FoxP3 working solution and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.

5. Aspirate supernatant and add the Gata3 transcription factor master mix onto a cell pellet. Control samples should receive the transcription factor isotype control stain. Incubate 30 min at 4 °C (*see* Note 8).
6. Wash the cells with 400 μL of the FoxP3 permeabilization buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
7. Aspirate the supernatant.
8. Resuspend cells with 150 μL of the FACS buffer and run samples on the flow cytometer (*see* Fig. 2). Utilize parent gating as shown in Fig. 1. Use Lin-Thy1.2+ ILCs to gate for Gata3, as well as analyze ILC subsets (quadrants based on ST2 and CD127 staining). Utilize FMOs to determine gates for ILC subset quadrants. Use Gata3 isotype to confirm where to place the Gata3 gate (Fig. 2).

3.3.4 *ILC2 IL5 and IL13 Cytokine Staining (see* Note 8)

1. Utilizing the single-cell suspension from digested lung tissue, vortex lung suspension and remove the volume containing 10×10^6 cells and place it in a FACS tube. The 10×10^6 cells will eventually be split into 10 wells, with 1×10^6 cells per well.
2. Wash the cells with 400 μL of RPMI and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
3. Aspirate the supernatant and resuspend each cell pellet with 2.1 mL of T-cell media in tissue culture hood.
4. Add 4 μL of CSC to the samples resuspended in T-cell media (diluted 1:500).
5. Add 200 μL of the stimulated cell suspension to each well of a 96-well plate, with 1 lung per row of wells for a total of 10 wells per lung.
6. Incubate the plate at 37 °C for 3 h.
7. After 3 h, remove cells from the incubator.
8. Combine and mix the content of 10 wells (all containing the same sample) by pipetting vigorously and place in a FACS tube. Centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
9. Remove the supernatant and resuspend the pellet in 1 mLof RPMI.
10. Aliquot 450 μL of resuspended cells into a FACS tube for both the IL5 stain and the IL13 stain. Aliquot 25 μL of resuspended cells into a FACS tube for isotype control stain.
11. Wash the cells with 400 μL of the FACS buffer. Centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
12. Aspirate the supernatant.
13. Add 50 μL of the Fc block master mix (Fc Block). Vortex cells gently. Incubate cells for 5 min at 4 °C.

14. Collect tubes and directly add 50 μL of the ILC2 surface antibody master mix. Control samples should receive FMO master mixes. Vortex cells gently. Incubate for 30 min at 4 °C.
15. Surface staining is now complete. Wash cells with 400 μL of the FACS buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
16. Aspirate the supernatant.
17. Add 300 μL of the BD Fixation/Permeabilization solution to each tube. Vortex cells gently. Incubate cells in the dark at 4 °C for 20 min.
18. Wash cells with 400 μL of the 1 × BD Perm/Wash buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
19. Add 100 μL of the ILC2 intracellular cytoplasmic stains to the appropriate tube (either IL5 stain or IL13 stain). Control samples should receive ILC2 intracellular cytokine stain isotype master mix. Vortex cells gently. Incubate cells in the dark at 4 °C for 30 min.
20. Wash cells with 400 μL of the BD Perm/Wash buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
21. Aspirate the supernatant.
22. Resuspend each pellet in 150 μL of the FACS buffer. Cells are ready to be run on the flow cytometer (Fig. 3). Utilize parent gating as shown in Fig. 1. Use Lin-Thy1.2+ ILCs to gate for cytokines, as well as analyze ILC subsets (quadrants based on ST2 and CD127 staining). Utilize FMOs to determine gates for ILC subset quadrants. Gate for IL5 or IL13 expression using the isotype to confirm where to place the gate (Fig. 3).

3.4 ILC Sorting and Culture

ILC population should be expanded in vivo with an intranasal challenge of 50 μg of *Alternaria* delivered 4 times in 10 days. Lung samples can be pooled during collection. For this protocol, lungs are collected and digested in pairs.

1. Euthanize mice in accordance with IACUC policies and protocol.
2. Collect the mouse lung and place in a FACS tube containing 4 mL of RPMI media, placing 2 lungs in one tube.
3. In compliance with the Miltenyi Lung Dissociation Kit instructions, use 100 μL of enzyme D, 15 μL of enzyme A, and 5 mL of the lung digest buffer into each tube (this recipe is for 2 lungs).
4. Digest and incubate the lungs as previously described in Subheading 3.2.
5. After filtering the lungs, combine the samples into one 50 Ml conical tube. Centrifuge at 500 × *g* (~1500RPM) at 4 °C for 10 min.
6. Aspirate the supernatant.

7. Lyse red blood cells by resuspending the pellet in 5 mL of the 1 × RBC lysis buffer. Incubate on ice for 5 min with occasional shaking.
8. Wash the solution with 30 mL of 1 × PBS to stop the reaction. Centrifuge at 500 × *g* (~1500RPM) at 4 °C for 10 min.
9. Aspirate supernatant and resuspend cells in 1 mL of RPMI media.
10. Obtain cell counts (*see* Note 4).
11. Distribute 100 × 10^6 cells per tube for staining. Distribute 1 × 10^6 cells for live/dead compensations and unstained controls.
    a. Unstained cells.
    b. Cells stained with Zombie Aqua (no antibodies).
    c. eFluor450 FMO (with Zombie Aqua).
12. Wash cells with 400 μL of the sort buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
13. Aspirate supernatant.
14. Add 50 μL of the Fc block master mix (Fc Block). Vortex cells gently. Incubate cells for 10 min. at 4 °C.
15. As tubes are staining, create Bead compensation samples (one compensation sample for each fluorophore used). Use 1 drop of beads, 100 μL sort buffer, and 1 μL antibody with correct fluorophore. Incubate for 15 min. or until cells are done staining (*see* Note 11).
16. Collect sample tubes and directly add 50 μL of ILC surface or eFluor450 FMO antibody master mix. Vortex cells gently. Incubate for 30 min at 4 °C.
17. Wash cells and compensation samples with 400 μL of the sort buffer and centrifuge at 500 × *g* (~1500RPM) at 4 °C for 5 min.
18. Resuspend compensation beads in 500 μL of the sort buffer.
19. Resuspend cells in approximately 4 mL of the sort buffer. The final concentration of cells should be ~20 × 10^6 cells/mL.
20. Filter cells into clean FACS tubes using a FACS tube with a cell strainer filter attached at the top.
21. Check pre-sorted ILCs on the flow cytometer by gating for ILCs to confirm in vivo expansion of ILCs. To do this, dilute a sample of the stained cells 1:30 for cell count. Add 5 μL of stained and filtered cells to 145 μL FACS buffer. After this check, cells are ready to be sorted.
22. At the sorter, use 100 μM nozzle (*see* Note 12).
23. Collect cells from the sorter and ask for a purity check, gating for Lineage- Thy1.2+ cells. Compare pre- and post-sort ILC populations (Fig. 4).

24. After sorting, centrifuge cells at 500 × *g* (~1500RPM) at 4 °C for 10 min.
25. Based on post-sort cell count, calculate the number of wells needed for experiment, given 40,000 cells will be seeded per well (*see* Note 13).
26. Aliquot the volume of T-cell media necessary to seed all wells with 200 μL per well. Add 10 ng/mL of IL2 and IL7 to the T-cell media to generate the supplemented T-cell media.
27. Resuspend the cells with the supplemented T-cell media. Seed 40,000 cells in a total of 200 μL of T-cell media per well into a flat-bottom 96-well plate.
28. Rest cells for 36–48 h at 37 °C. (*see* Note 14).
29. After cell rest is complete, centrifuge the plate at 500 × *g* (~1500RPM) at 25 °C for 5 min.
30. Carefully remove 100–150 μL of the supernatant and place in separate wells in a sterile 96-well plate (*see* Note 15).
31. Resuspend each cell pellet with 200 μL of sterile T-cell media per well. Collect cells and pool them together into a FACS tube. Centrifuge at 500 × *g* (~1500RPM) at 25 °C for 10 min.
32. Aspirate the supernatant.
33. Resuspend the cell pellet with 1 mL of the supplemented T-cell media.
34. Perform cell count utilizing a 1:100 dilution. Add 5 μL of cells to 495 μL FACS buffer.
35. Remove the volume required to seed 40,000 cells per well for a total of 10 wells per stimulation condition (see below).
36. Prepare stimulation cocktails. Use 200 μL of a cocktail per well. Cocktails:
    a. The supplemented T-cell media.
    b. The supplemented T-cell media additionally containing 30 ng/mL of IL33.
37. Resuspend aliquoted cells with stimulation cocktail and add200 μL of cell mixture per well to a sterile flat-bottom 96-well plate.
38. Stimulate cells for 24 h.
39. Centrifuge plate at 500 × *g* (~1500RPM) at 25 °C for 5 min.
40. Carefully remove 100–150 μL of the supernatant. This is the post-stimulation supernatant (*see* Note 14).
41. Pre- and post-stimulation supernatant can then be assayed by ELISA for IL5 and IL13 utilizing a 1:10 and 1:100 dilution in T-cell media (*see* Note 16).
42. After removing the supernatant, place cells in 500 μL RNAlater.

4 Notes

1. Depending on the experimental purpose, the mice can be allergen challenged over a different number of days. To expand the ILC population for ILC2 detection and staining, challenge the mouse with 50 μg of *Alternaria* for 3 days, with a challenge on days 0, 1, and 2. For ILC expansion for isolation of cells via cell sorting, challenge the mouse with 50 μg of *Alternaria* 4 times over 10 days, with a challenge on days 0, 3, 6, and 9. If mice are challenged with *Alternaria* for ≤3 days, ILCs will be activated. Challenging the mice for >3 days will activate and expand ILCs.

2. When working with *Alternaria* in its lyophilized powdered form, always work in the fume hood to prevent exposure to the allergen. Once it is resuspended in PBS, it is safe to use in the open lab environment.

3. Upon successful *Alternaria* challenge, total cell numbers should increase substantially, nearing around a total of 6.5×10^7 cells per single lung. You can expect 2–4% of those cells (around 400,000 cells) will be Lin-Thy1.2+ lymphoid cells. To calculate the number of ILCs, multiply: (total number of cells)(%CD45+)(%Lymphocytes)(%ILCs). Of ILCs, you can expect 40–60% of those cells will be ST2+CD127+ ILC2s (around 160,000 cells) Similarly to calculate the number of ILC2s, multiply: (total number of cells)(%CD45+)(%Lymphocytes)(%ILCs)(%ST2+CD127+).

4. Pour the lungs straight onto a paper towel to absorb the RPMI volume. Use a clean forceps to gently blot the lung and then place in tube filled with digest solutions. Individual lungs are not pooled.

5. To ensure that no leaks will occur, after you have closed the purple top lids, gently tap the top of the lid against a stack of paper towels and look for moisture. If moisture is detected on the paper towel, replace the purple top lid to prevent leakage during incubation.

6. Use a sterile plunger to help push the lung tissue through the strainer.

7. For cell counts, utilize a 1:100 dilution with 5 μL of sample in 495 μL FACS buffer.

8. For the most efficient use of time, master mixes can and should be made the day before the experiment. For surface stain panels, such as the ILC surface stain panel, use FACS buffer to dilute the antibodies to their working concentration. For transcription factor stains that require FoxP3 permeabilization, such as Gata3, use the FoxP3 permeabilization buffer to dilute the antibodies to their working concentration.

9. In order to efficiently accomplish surface, transcription factor, and cytokine staining in one day, put some of your cells into culture first before starting staining protocols. Once your cells are incubating, go back and complete your surface stains and transcription factor staining protocols. Your experiment steps should look as follows: (1) Cells are put into culture for 3 h. (2) During that 3 h,

other cells are surface stained. (3) Cells undergo transcription factor staining. (4) Cells are pulled out of culture and are first surface stained then cytokine stained.

10. When making master mixes for Fc Block and the ILC2 surface stain, the final volume of the FACS buffer per sample should be 50 μL (i.e., 2 μL of antibody into 50 μL FACS buffer). This is because Fc Block and the ILC surface stains will be combined to create a final staining volume of 100 μL per sample.

11. Flow cytometer should be compensated before running stained samples. Once compensation tube is prepared, the compensation can be treated as a sample tube and go through the same wash/centrifuge steps.

12. Once you are at the sorter, you will need to help compensate the machines and create the gating scheme. Utilize the same gating scheme as in Fig. 1. Below is a comprehensive list of items you should bring with you to the sorter.

    a. Unstained cells w/out Zombie Aqua.

    b. Unstained cells w/Zombie Aqua (add after they run the unstained cells).

    c. Stained cells w/Zombie Aqua.

    d. FMO (for APC only) w/Zombie Aqua.

    e. (2) Bead compensation samples w/out Zombie Aqua—includes antibodies from each fluorescence channel used.

    f. Zombie Aqua (to add before running samples).

    g. Sort buffer.

    h. Collection tubes (with 500 μL TCM).

13. When calculating the number of wells, plan to use 10 wells per experimental stimulation condition.

14. Since the ILC2 population was activated and expanded in vivo via allergen challenge, the cells must be rested before they are restimulated.

15. Tilt the plate slightly to help with removing the supernatant and leave a small volume behind because the pellet may be hard to see. Examine the collected supernatant and cells in plate under a microscope to make sure cells were not in the supernatant. Store collected supernatant in 50 μL aliquots at −80 °C (for ELISA as a pre-culture condition).

16. T-cell media can also be used as a blank or as ELISA diluent, as needed.

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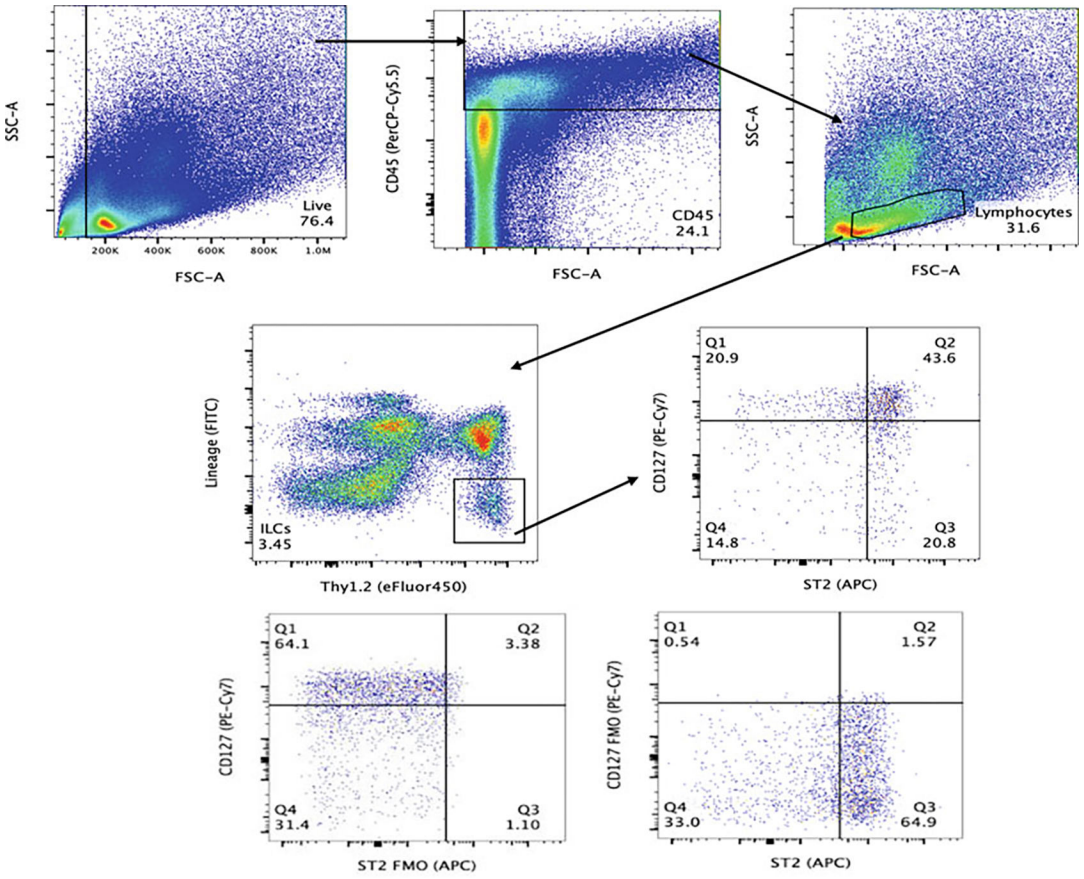

Fig. 1.

Parent gating for ILC2s. ILCs were gated as CD45+ Lineage- Thy1.2+ lymphocytes. Subsets of ILCs were identified through the expression of ST2 and CD127, with ILC2s identified as ST2+CD127+ cells. FMOs were used to determine gating

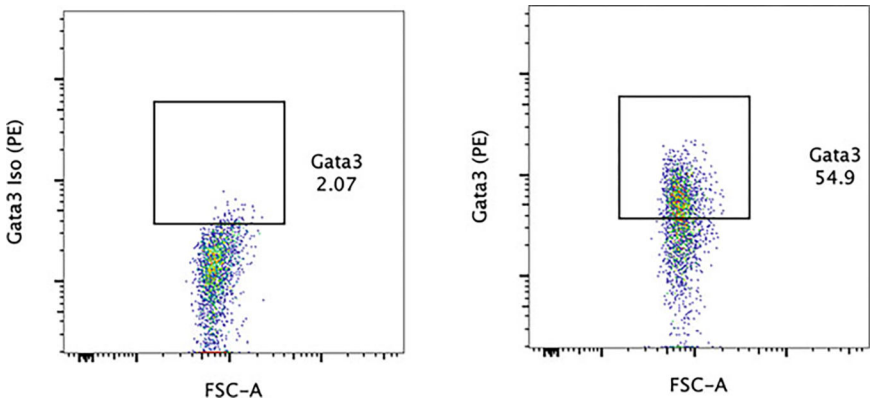

Fig. 2.

ILC2 transcription factor staining. ILCs were gated as CD45+ Lineage- Thy1.2+ lymphocytes. GATA3 expression in ILCs was determined utilizing an isotype (Iso)-stained sample as a control sample

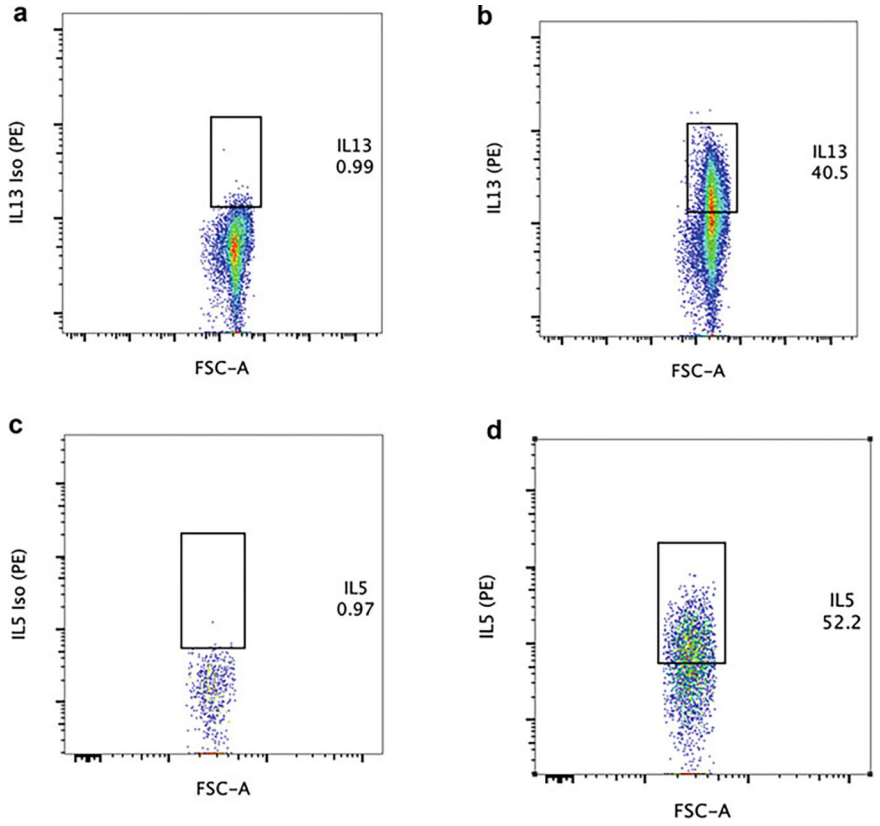

Fig. 3.

ILC2 cytokine staining. ILCs were gated as CD45+ Lineage- Thy1.2+ lymphocytes. Cytokine expression was assessed in Lin-Thy1.2+ cells. IL13 and IL5 cytokine expression (b and d) was determined utilizing an isotype/Iso-stained sample (**a** and **c**) as a control sample

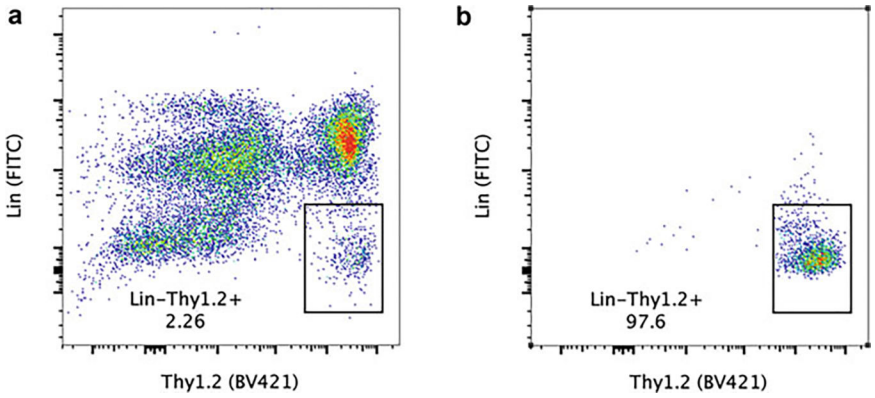

Fig. 4.

Pre- and post-sort ILC populations. Representative FACS plot of Lin-Thy1.2+ ILCs: (**a**) pre-sort and (**b**) post-sort

Table 1

Master mix stain sheet for ILC staining. Given are the amounts of each antibody in micrograms to be added to the appropriate volume of a buffer per sample

Stain	Antibodies (per tube)
Fc Block	Fc block (1.0 μg) FACS buffer (50 μL)
ILC2 surface	FITC: Lin (2.5 μL) FITC: CD11c (0.25 μg) FITC: NK1.1 (0.25 μg) FITC: CD5 (0.25 μg) FITC: FcER1 (0.25 μg) FITC: TCRβ (0.25 μg) FITC: TCRγδ (0.25 μg) PerCP-Cy5.5: CD45.2 (0.4 μg) Pacific Blue: Thy1.2 (0.4 μg) PE-Cy7: CD127 (0.4 μg) APC: IL33Rα (ST2) (0.4 μg) FACS buffer (50 μL)
ILC2: ST2 FMO	FITC: Lin (2.5 μL) FITC: CD11c (0.25 μg) FITC: NK1.1 (0.25 μg) FITC: CD5 (0.25 μg) FITC: FcER1 (0.25 μg) FITC: TCRβ (0.25 μg) FITC: TCRγδ (0.25 μg) PerCP-Cy5.5: CD45.2 (0.4 μg) Pacific Blue: Thy1.2 (0.4 μg) PE-Cy7: CD127 (0.4 μg) FACS buffer (50 μL)
ILC2: CD127 FMO	FITC: Lin (2.5 μL) FITC: CD11c (0.25 μg) FITC: NK1.1 (0.25 μg) FITC: CD5 (0.25 μg) FITC: FcER1 (0.25 μg) FITC: TCRβ (0.25 μg) FITC: TCRγδ (0.25 μg) PerCP-Cy5.5: CD45.2 (0.4 μg) Pacific Blue: Thy1.2 (0.4 μg) APC: IL33Rα (ST2) (0.4 μg) FACS buffer (50 μL)
ILC2 transcription factor	PE: Gata3 (0.6 μg) FoxP3 wash buffer (100 μL)
ILC2 transcription factor isotype	PE: rat IgG2b, κ (0.06 μg) FoxP3 wash buffer (100 μL)
ILC2 intracellular IL5 cytokine	PE: IL5 (1.0 μg) BD wash buffer (100 μL)
ILC2 intracellular IL13 cytokine	PE: IL13 (1.0 μg) BD wash buffer (100 μL)
ILC2 intracellular cytokine stain isotype	PE: rat IgG1, κ (0.06 μg) FoxP3 buffer (100 μL)

Table 2

Master Mix stain sheet for ILC Cell sorting. Given are the amounts of antibody in micrograms to be added to the stated volume of an appropriate staining buffer. These amounts are optimized for maximal detection and isolation of ILCs

Stain	Antibodies (per tube)
FC Block	Fc block (3.0 μg) Sort buffer (50 μL)
ILC surface	PerCP-Cy5.5: CD45.2 (1.2 μg) eFlour450: Thy1.2 (1.2 μg) FITC: Lin (7.5 μL) FITC: CD11c (0.75 μg) FITC: NK1.1 (0.75 μg) FITC: CD5 (0.75 μg) FITC: FcER1 (0.75 μg) FITC: TCRβ (0.75 μg) FITC: TCRγδ (0.75 μg) Sort buffer (50 μL)
eFluor450 FMO	CD45.2 (0.4 μg) Lin (2.5 μL) CD11c (0.25 μg) NK1.1 (0.25 μg) CD5 (0.25 μg) FcER1 (0.25 μg) TCRβ (0.25 μg) TCRγδ (0.25 μg) Sort buffer (50 μL)