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Protocols for studying murine ILC development *in vivo*.

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Abstract

Understanding the origins and developmental trajectory of innate lymphoid cell (ILC) progenitors has been of substantial interest to the fields of ILC biology and immunology. While mature ILC are rare lymphocytes, ILC progenitors represent an even smaller fraction of cells, providing additional challenges in studying them. Moreover, though the approaches to studying these cells are conceptually straightforward, the technical nuances that underlie them can substantially affect the quality of the data. Herein, we provide a detailed protocol for assessing the frequency of ILC progenitors in the bone marrow, their phenotype, and their potential to develop into mature ILC. These methods make up the foundation of *in vivo* investigations into ILC development, and we hope these thorough protocols and associated notes facilitate additional, high-quality inquiries into this fascinating field.

1. INTRODUCTION

Innate lymphoid cells (ILCs) are a heterogeneous group of effector lymphocytes that play a critical role in tissue homeostasis and defense. ILCs are categorized by their developmental relationship and effector functions with the major lineages including group 1, 2, and 3 ILCs (ILC1, ILC2, and ILC3, respectively), natural killer (NK) cells, and lymphoid tissue inducer (LTi) cells. Progression from hematopoietic stem cell to terminally differentiated ILC involves several stepwise progressions, each with defining transcription factors and/or surface markers and with increasingly restricted developmental potential (1). A simplified version of this developmental cascade is shown in Figure 1 (2). All subsets of innate and adaptive lymphocytes derive from lymphoid-primed multipotent progenitors (LMPP) and common lymphoid progenitors (CLP) (3–5). In mice, expression of Id2 is associated with ILC lineage commitment and the ordered cascade of alpha-integrin expressing lymphoid precursors (α LP), early innate lymphoid progenitors (EILP), common helper innate lymphoid progenitors (CHILP), and innate lymphoid cell progenitors (ILCP), each with further restricted lineage potential (6–9). Briefly, α LP can produce all ILC lineages including NK cells whereas the later stage CHILP can only generate ILC1, ILC2, ILC3, and LTi cells (7, 10). ILCP are further restricted, lacking capacity to generate LTi cells (11). Major regulators of these stages are shown in Figure 1 and include TCF-1 (α LP/EILP), ETS1 (CHILP), and PLZF (ILCP) (10–13). This chapter will focus predominantly on the

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evaluation of the “helper-like” ILC lineages—ILC1, ILC2, and ILC3—in mice though the principles can largely be applied to other known and yet to be described ILC populations.

Assessment of ILC development *in vivo* relies heavily on flow cytometric analysis of the major precursor populations, with special attention to both their frequency and phenotype. Herein, we describe our approach to performing flow cytometry for hematopoietic stem cells and ILC precursors in murine bone marrow. A substantial volume of work defining ILC development has been performed in the bone marrow, and evidence suggest that ILC or ILC progenitor trafficking from developmental sites such as the bone marrow may be influential in the perinatal period or during processes that disrupt the tissue homeostasis (14–20). However, at steady-state in adult mice, ILC are a tissue-resident population and ILC progenitors may be embedded in tissues, as is observed in fetal gut, and support ILC populations *in situ* (21–25). Thus, a comprehensive evaluation of ILC development may require evaluation at multiple sites and developmental stages unique to a given study.

Beyond evaluation of frequency and phenotype, lineage potential is a benchmark criterion for establishing the degree of lineage restriction of specific precursor populations and inferring developmental trajectory. In this chapter, we discuss an *in vivo* approach to assessing lineage potential. *In vitro* parallels exist for evaluating lineage potential (6, 7, 10, 11, 26–28) and represent an important methodology for studying ILC development but are beyond the scope of this section (though such methods rely principally on the same flow cytometry and fluorescence activated cell sorting, or FACS, as detailed in this chapter).

Finally, our knowledgebase about ILCs is rapidly expanding and improved techniques continue to refine our understanding of specific ILC precursor populations, their transcriptional regulation, and their lineage potential. In this chapter, we aim to present a set of methods that are fundamental to the study of ILC development yet are adaptable even in light of new discoveries in the field.

2. MATERIALS

2.1 Supplies for bone marrow harvest

1. Surgical tools including sharp scissors, tweezers, and scalpel
2. 70% ethanol in sprayer
3. Delicate task wipes (i.e. Kimwipe)
4. Petri dishes (polystyrene, 60 x 15 mm)
5. RPMI, stored at 4°C
6. 1x phosphate buffered saline (PBS), stored at 4°C
7. 3 mL and 10 mL disposable syringes
8. 18 gauge/1.5 inch and 25 gauge/1 inch needles
9. 15 mL and 50 mL conical tubes
10. Centrifuge compatible with 15 mL and 50 mL conical tubes

11. 70 micron cell strainer for 50 mL conical tubes
12. RBC lysis buffer (Cat. #TNB-4300-L100, Tonbo Biosciences)
13. Trypan blue
14. Hemocytometer

2.2 Supplies for flow cytometry

1. Supplies listed in section 2.1, as appropriate.
2. 5 mL FACS tubes (i.e. Cat. #352052 [non-sterile] or #352054 [sterile], Falcon)
3. Flow staining buffer: 1x PBS with 3% fetal bovine serum, sterile filtered.
4. Fc blocking reagent: anti-CD16/32 antibody (i.e. Purified Anti-Mouse CD16 / CD32 [clone 2.4G2], Cat. #70-0161, Tonbo Biosciences)
5. Flow cytometry-compatible antibodies to identify the desired populations. Fluorophore-conjugated antibodies include c-Kit (clones ACK2 and 2B8), Sca-1 (clone D7), Flt3 (clone A2F10), CD127 (clone SB/199), $\alpha 4\beta 7$ (clone DATK32), PD-1 (clone J43), and CD25 (clone PC61). Biotin-conjugated antibodies contained in the Miltenyi Lineage Cocktail (Cat. #130-092-613) target CD5, CD11b, CD45R, 7-4, Gr-1 (Ly6G/C), and Ter-119 (*see* Note 1). We supplement the Lineage Cocktail with an antibody targeting CD3 (clone 17A2) to ensure the exclusion of T cells. A careful review of the most current literature should be performed regularly to select suitable reagents, as contemporary gating strategies may call for additional or alternative antibodies.
6. If analyzing intracellular markers using fluorescently-tagged antibodies, fixation and permeabilization reagents (i.e. Foxp3/Transcription Factor Staining Buffer Kit, Cat. #TNB-0607-KIT, Tonbo Biosciences)
7. Viability marker: DAPI, propidium iodide, or 7-AAD are good options for non-fixed cells, each with distinct excitation/emission properties. Fixation-compatible reagents are required if cells are fixed and/or permeabilized (i.e. Ghost Dye Fixable Viability Dyes, Tonbo Biosciences).
8. Multi-laser flow cytometer. In this protocol, we use a 5-laser BD LSR II with the following setup: 355nm ultraviolet (2 PMT sensors), 405nm violet (7), 488nm blue (2), 545nm green (5), and 635nm red (3) lasers.
9. Flow cytometry analysis software (i.e. FlowJo, as used in this protocol).

2.3 Supplies for adoptive transfer

1. Supplies listed in sections 2.1 and 2.2, as appropriate
2. MACS buffer: 1x PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, mixed and sterile filtered

¹-This cocktail is optimized for the bone marrow. Additional lineage markers should be considered for other tissues including CD3, CD4, CD8, $\alpha\beta$ TCR, $\gamma\delta$ TCR, NK1.1, and Fc ϵ RI. Of note, NK1.1 is not present on NK cells from BALB/c mice.

3. Direct Lineage Cell Depletion Cocktail (Cat. #130-110-470, Miltenyi) containing the following magnetic microbead-conjugated antibodies against the following targets: CD5, CD11b, CD45R (B220), 7-4, Gr-1 (Ly-6G/C), and Ter-119
4. LS Columns (Cat. #130-042-401, Miltenyi)
5. Magnetic column stand (i.e. QuadroMACS Separator, Cat. #130-090-976, Miltenyi)
6. FACS tubes with 35 micron cell strainer caps (Cat. #352235, Falcon)
7. 1 mL disposable syringes
8. 30 gauge/0.5 inch needles

3. METHODS

3.1 Isolation of murine bone marrow cells.

1. To collect tibia and femur from mice, euthanize per institutionally approved animal protocol (*see* Note 2). Wet and sterilize abdomen and legs with 70% ethanol. Make skin incision in the lower abdomen and cut from this point down each leg to expose muscle. Use the blunt aspect of the scissors or forceps to spread the tissue to find the femoroacetabular joint. Cut just above this joint, removing a small piece of the pelvis. Cut along the underside of the leg removing the skin and some bulk muscle. There is no need to excise all of the muscle; it will be removed in a later step. Cut at the bottom of the tibia to remove the leg. There is very little marrow at the distal end of the tibia so you can cut straight through the bone without appreciable marrow loss. Place bones in a 60mm plastic petri dish on ice.
2. Using a scalpel and forceps, briefly scrape along the knee joint to loosen the connective tissue and ligaments. Hyperflex the knee joint to separate the tibia and femur and then cut through the remaining soft issue to fully separate the bones.
3. Using a delicate task wipe (i.e. Kimwipe) for increased grip, pull away the muscle from each of the leg bones. A small amount of muscle can be left on the ends of the bones, as these will be excised in step 4.
4. Cut bones at the metaphysis to expose the marrow. You do not need to modify the distal end of the tibia as this has already been exposed in step 1.
5. Fill a 50 mL conical tube with 10 mL of ice cold RPMI (*see* Note 3). Using a 3 mL syringe with a 25 gauge needle, draw up 3 mL of RPMI from the conical tube, insert syringe needle into the end of the bone, and flush until the bone turns a translucent white color (*see* Notes 4 and 5).

²-From each tibia and femur, we generally recover approximately 15 million live bone marrow cells from female BALB/c mice.

³-In general, try to keep samples on ice (though some steps if performed quickly can be done on the benchtop as long as samples are immediately placed back on ice). For downstream applications that require live cells or maximal cell recovery, pay special attention to keeping all samples and solutions ice cold.

6. Using a 10 mL syringe and an 18 gauge needle, aspirate all of the RPMI in the conical tube, now containing flushed bone marrow, and expel it through the syringe twice for each sample to break up the clumps and generate a single cell suspension.
7. Spin samples in 50 mL conical tubes at 350 x g for 5 minutes at 4°C in a centrifuge. Sample should collect firmly at the bottom of the 50 mL conical tube. Discard supernatant by pouring off into an appropriate biohazard disposal container. This will leave ~250 µl of solution at the bottom of the tube. Resuspend cells by vortexing on medium to medium-high speed (*see* Note 6).
8. Add 3 mL of cold 1x RBC lysis buffer per sample, mix lightly by swirling, and incubate on ice for 3 minutes. Quench by pouring ice cold 1x PBS into the tubes up to the 25 mL mark.
9. Centrifuge samples, discard supernatant, and resuspend cells as in step 7.
10. Adjust volume by adding 3 mL of flow staining buffer (*see* Note 7 if intracellular staining) per one tibia and femur (6 mL for both tibias and femurs).
11. Mix resuspended cells with a brief low speed vortex and pour through a 70 micron cell strainer into new 50 mL conical tubes.
12. Mix samples by lightly vortexing and take an aliquot (10-20 µl) to count the cells. Generally, we dilute 1:10 in trypan blue and manually count on a standard hemocytometer.

3.2 Flow cytometric assessment of ILC precursors.

1. From section 3.1, add 5 million cells per sample to FACS tubes. In addition to each sample, you need several control tubes—one unstained tube, one tube for single staining for each fluorophore and marker (i.e. viability marker), and any control tubes desired such as isotype controls, FMOs, etc. (*see* Note 8). Spin sample in 5 mL FACS tube at 350 x g for 5 minutes at 4°C in a centrifuge. Cells should collect firmly at the bottom of the 5 mL FACS tube. Discard supernatant by pouring off into an appropriate biohazard disposal container. This will leave ~100 µl of solution at the bottom of the tube. Resuspend cells by vortexing for 5 seconds on moderate speed (*see* Note 9).

⁴. Alternatively, if you are processing bone marrow from a large number of mice, you can consider centrifugation to expel the marrow. Briefly, puncture a hole in the bottom of a 0.5 mL microcentrifuge tube using an 18 gauge needle. Assemble the centrifugation construct by placing the bone and 250 µl cold RPMI inside the punctured 0.5 mL tube and placing this inside a 1.5 mL microcentrifuge tube. Centrifuge to expel marrow and vortex and/or aspirate repeatedly with a pipette to resuspend the cells.

⁵. Generally, complete expulsion of the marrow can be accomplished with one flush but if there appears to be marrow still present, consider additional flushes. You may have to rotate the syringe in the bone or inject through the opposite end of the bone to get all of the marrow.

⁶. If cells are still stuck to the bottom, try tapping the bottom of the tube against a hard surface to dislodge followed by additional vortexing. Over-mixing may affect viability; only mix until cells are resuspended.

⁷. If analyzing intracellular antigens (i.e. transcription factor PLZF), replace flow staining buffer in this step with 1x PBS.

⁸. It is important to recognize that each control type has strengths and limitations reviewed in (29). These limitations can be exacerbated by poor flow cytometry technique such as overstaining due to inadequate antibody titration. Moreover, these controls don't help define "high" versus "low" staining, which is important for certain antigens and populations. Thus, Isotype and FMO controls are best used in conjunction with the published literature and user experience/intuition to assemble gates.

2. Wash cells by adding 2 mL of flow staining buffer (*see* Note 7 if intracellular staining) to each sample. Centrifuge samples, discard supernatant, and resuspend cells as in step 1.
3. For up to 5 million cells (*see* Note 10), add Fc blocking agent (anti-CD16/32 antibody). We use Purified Anti-Mouse CD16 / CD32 (2.4G2) (Cat. #70-0161, Tonbo Biosciences) by mixing 3 μ L antibody into 47 μ L flow staining buffer (*see* Note 7 if intracellular staining) and adding to the sample. If you plan to stain for intracellular antigens, a viability dye needs to be added at this step (including to the single stain control for this viability dye). We use the fixable live/dead dyes from Tonbo Biosciences diluted 1:200 in the Fc block mixture (i.e. add 0.25 μ L fixable live/dead dye and 3 μ L of Fc blocking agent to 46.75 μ L 1x PBS). Mix by vortexing for 1-2 seconds on moderate speed and place samples at 4°C for 15 minutes protected from light.
4. Do not wash between steps 3 and 4. Prepare surface antibody cocktail by diluting antibodies into flow staining buffer (*see* Note 7 if intracellular staining) for a total volume of 50 μ L per sample. Our gating strategy for major ILC precursor populations is listed in Table 1 and shown in Figures 2 and 3, recognizing that different labs evaluate for additional cell populations (i.e. α LP and EILP) or may use variations on these cellular markers. For analysis of multiple samples, make one master mix of antibodies to be applied to all samples. Add 50 μ L of this antibody cocktail to each sample and incubate for 30 minutes at 4°C protected from light (*see* Note 11). Add each antibody individually in 50 μ L to each single stain control tubes as well (one antibody per tube).
5. Wash cells by adding 2 mL of flow staining buffer to each sample. Centrifuge samples, discard supernatant, and resuspend cells as in step 1.
6. If using biotinylated antibodies (i.e. lineage markers, as in our antibody cocktail), add a streptavidin-conjugated fluorophore to the samples by diluting in 100 μ L flow staining buffer. Incubate for 30 minutes at 4°C protected from light.
7. Wash cells by adding 2 mL of flow staining buffer to each sample. Centrifuge samples, discard supernatant, and resuspend cells as in step 1. If staining for intracellular antigens such as PLZF, go to step 8. If only staining for cell surface markers, proceed to step 13.
8. If performing analysis for intracellular antigens such as PLZF, fix and permeabilize the samples (*see* Note 12). We use the Tonbo Foxp3 / Transcription

⁹Resuspending the cells is critical during preparation for flow cytometry. You may need to increase or decrease your vortex speeds, tap the tubes on the benchtop, or scrape the tubes on a rough surface (i.e. a grate) to loosen the cells.

¹⁰For more than 5 million cells (i.e. *see* section 3.3), multiply the volumes listed in the remainder of the protocol by increments of 5 million cells. For instance, if you have 6 million or 12 million cells, multiply by 2 or 3, respectively.

¹¹Cells will sink to the bottom during lengthy staining steps. If staining for >15 minutes, consider re-mixing the samples every 15 minutes by light vortexing.

¹²Alternatively, many labs use fluorescent reporter mice for intracellular antigens (i.e. PLZF^{GFPcre} (11) or Id2^{RFP} (27) mice). In some cases, investigators have found surrogate surface markers that correlate with the intracellular antigen of interest, such as PD-1 as a surrogate for PLZF in ILCP (12).

Factor Staining Buffer Kit and apply 500 μ l of diluted reagent to each sample for 30 minutes at room temperature protected from light.

9. Wash cells by adding 2 mL of permeabilization buffer to each sample. Centrifuge samples, discard supernatant, and resuspend cells as in step 1.
10. Prepare intracellular antibody cocktail by diluting antibodies into permeabilization buffer for a total volume of 100 μ l per sample. For analysis of multiple samples, make one master mix to be applied to all samples. Add 100 μ l of this antibody cocktail to each sample and incubate for 30 minutes at 4°C protected from light (*see* Note 11). Add each antibody individually in 50 μ l to each single stain control tubes as well (one antibody per tube).
11. Wash cells by adding 2 mL of permeabilization buffer to each sample. Centrifuge samples, discard supernatant, and resuspend cells as in step 1.
12. Wash cells by adding 2 mL of flow staining buffer to each sample. Centrifuge samples, discard supernatant, and resuspend cells as in step 1.
13. If unfixed cells (surface staining only), add 250 μ l of flow staining buffer with a viability stain (including to the single stain control for this viability dye). We use DAPI at a concentration of 25 nM. If cells are fixed to assess for intracellular antigens, a viability dye was used in step 3 and cells can simply be resuspended in 250 μ l of flow staining buffer.
14. Analyze cells on a flow cytometer (*see* Note 13). We primarily use a BD LSR II with a 5 laser configuration as outlined in section 2.2 (*see* Note 14). Export data from the flow cytometer data collection interface and evaluate on your preferred flow cytometric analysis software (i.e. FlowJo, as shown in this protocol). The gating strategies outlined in Table 1 and Figures 2 and 3 provide a template for this analysis (*see* Note 15). Additionally, it may be of interest to study ILC precursor populations in sites outside of the bone marrow. This approach can be applied with appropriate modifications in that setting as well. For instance, we have analyzed ILC2P in the fetal liver (Figure 4).

3.3 Adoptive transfer of ILC precursors.

1. Isolate bone marrow from both the left and right legs (*see* Note 16) of a mouse or multiple mice (*see* Note 17) as described in 3.1 through step 9. Perform this entire protocol under sterile or near-sterile conditions.

¹³ ILC and ILC progenitors are rare cells in the bone marrow and require counting of large numbers of cells. We often collect 500,000-1,000,000 live cell events per sample.

¹⁴ Thoughtful selection of antibodies and fluorophores is crucial for optimal results, particularly given the size of the panels generally required to analyze ILC and ILC precursors. Working backwards from your flow cytometer is good practice, as excitation wavelengths and filter combinations can differ meaningfully between machines altering the “brightness” of different fluorophores and the magnitude of compensation required. Generally, it is best to pair “dimmer” fluorophores with more highly expressed proteins and vice versa. Optimization of your initial cytometry panel will require an investment of time and resources but will provide demonstrably better and more consistent results.

¹⁵ It is important to use singlet gates (i.e. FSC-A vs. FSC-H) and a viability marker to exclude contamination of dead cells our doublets (Figure 2).

¹⁶ Marrow can also be harvested from the hip if it is necessary to maximize per mouse yield.

2. Adjust volume by adding 10 mL of MACS buffer.
3. Mix resuspended cells with a brief low speed vortex and pour through a 70 micron cell strainer into a new 50 mL conical tube.
4. Mix samples by lightly vortexing and take a small aliquot (10-20 μ L) to count the cells. For manual counting with a standard hemocytometer, dilution will be dependent upon the number of mice harvested.
5. Spin samples in 50 mL conical tubes at 350 x g for 5 minutes at 4°C in a centrifuge. Sample should collect firmly at the bottom of the 50 mL conical tube. Discard supernatant by pouring off into an appropriate biohazard disposal container. This will leave ~250 μ L of solution at the bottom of the tube. Resuspend cells by vortexing on medium to medium-high speed (*see* Note 18).
6. Add 90 μ L of MACS buffer per 10 million cells. Then add 10 μ L of Direct Lineage Cell Depletion Cocktail per 10 million cells and mix by gently vortexing.
7. Incubate sample for 15 minutes at 4°C. Then add 1-2 mL of MACS buffer per 10 million cells. Place cap on tube and invert several times to wash the sample.
8. Centrifuge samples and discard supernatant as in step 5. This will leave ~250 μ L of solution at the bottom of the tube. Resuspend cells by pipetting up and down until the cell pellet is no longer visible.
9. Add 500 μ L of MACS buffer per 100 million cells and store at 4°C.
10. Rinse LS Column by placing in magnetic stand and applying 3 mL of buffer. Discard the flow through. Place a new 15 mL conical tube under the tip of the LS Column.
11. Mix sample by pipetting and add up to 100 million cells (500 μ L) to the LS Column (*see* Note 19). Allow entire sample to flow into the column. Then add 500 μ L of MACS buffer to the LS Column and allow to flow into the column.
12. Add 3 mL of MACS buffer to the LS Column and allow to flow through entirely. Repeat this step 2 additional times for a total of 3 flushes.
13. Collect the 15 mL conical tube with flow through; this now contains a population enriched for lineage marker negative cells including lymphoid and ILC progenitors. Spin sample in the 15 mL conical tube at 350 x g for 5 minutes at 4°C in a centrifuge. Cells should collect firmly at the bottom of the 15 mL conical tube. Discard supernatant by pouring off into an appropriate biohazard disposal container. This will leave ~250 μ L of solution at the bottom of the tube.

¹⁷The legs of up to 5 mice can be processed in the same 50 mL Falcon tube. Use an additional 50 mL Falcon tube for each set of up to 5 mice. In our hands, we recover 700-1000 CLP per WT BALB/c mouse.

¹⁸If a significant number of mice are harvested, repeated aspirations and expulsions with a pipette may be necessary to dislodge the pellet.

¹⁹Multiple LS Columns may be required depending on the sample size. Do not exceed 100 million cells per column as this will greatly increase the lineage positive flow through.

- Resuspend cells by pipetting up and down until the cell pellet is no longer visible.
14. Add 1 mL of flow staining buffer and transfer sample to a 5 mL FACS tube. Vortex for 3-5 seconds on moderate speed to mix the cells. Take a 10-20 μ L aliquot, mix 1:1 with trypan blue, and count using a standard hemocytometer.
 15. Spin sample in 5 mL FACS tube at 350 x g for 5 minutes at 4°C in a centrifuge. Cells should collect firmly at the bottom of the 5 mL FACS tube. Discard supernatant by pouring off into an appropriate biohazard disposal container. This will leave ~100 μ L of solution at the bottom of the tube. Resuspend cells by vortexing for 5 seconds on moderate speed.
 16. Perform Fc block and cell surface staining (*see* Note 20) as outlined in section 3.2 ensuring cells are kept cold and handled with maximum sterility. Resuspend cells at a concentration of 10-20 million cells per mL of flow staining buffer, or adjust to the optimal concentration for your sorting machine. Include a viability dye (i.e. DAPI) at this stage.
 17. Following the final wash and viability stain, filter samples through a FACS tube cell strainer cap (35 μ m) by pipetting sample into the strainer cap on top of a new 5 mL FACS tube and centrifuging at 350 x g for 2 minutes at 4°C.
 18. Perform FACS and collect precursor populations of interest in a new 5 mL FACS tube with 1 mL of cold sterile RPMI (*see* Note 21). Record the number of cells that were sorted from the machine.
 19. Centrifuge samples, discard supernatant, and resuspend cells as in step 15 (*see* Note 22).
 20. Resuspend progenitor cells at a concentration of 1500 cells per 100 μ L of RPMI (*see* Note 23). Vortex gently for 2-3 seconds to ensure sample is mixed.
 21. Using a 1 mL syringe with 0.1 mL gradations and a 30 gauge needle, aspirate 100 μ L of sample per mouse to inject.
 22. Anesthetize recipient mice (*see* Note 24) per approved institutional animal protocol. Once anesthetized, inject 100 μ L of cells intravenously. We prefer the retroorbital route of delivery for ease and reproducibility of delivery, but tail vein is another common site for injection. Perform all procedures in accordance with approved institutional animal protocols.

²⁰. MACS is an important step to enrich for lineage negative cells and therefore significantly increase the efficiency of your FACS. Even with optimal MACS procedure, a percentage of lineage positive cells will flow through the column. Therefore, it is still important to surface stain at this step with lineage markers using fluorescently tagged antibodies for exclusion of contaminating lineage positive cells during FACS.

²¹. Keep samples chilled during the sort if your FACS machine has this capability.

²². Cell number will likely be too few to visually observe in the tube. Do not be alarmed if this is the case. Proceed to the next step.

²³. Titration of cells to be transferred is important. Most published studies use 500-3000 cells per mouse, depending on the lab and the cell population being transferred.

²⁴. Common recipient mice include mice genetically engineered to lack all lymphocytes including ILC such as *Rag2*^{-/-} *Il2rg*^{-/-} mice or CD45 allelic mismatch mice (i.e. CD45.1⁺ BALB/c or C57BL/6 mice when injecting CD45.2⁺ cells) that have been exposed to sublethal doses of radiation.

23. Allow 3-12 weeks for reconstitution from the transferred progenitor cells, depending on the application. Analyze immunologic compartments of interest by preferred method (i.e. flow cytometry, immunofluorescence, etc.) to assess reconstitution of desired subsets of ILC and lineage potential of transferred progenitors (Figure 5).

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Figure 1: ILC Development.

Innate lymphoid cell (ILC) development and effector functions. ILCs develop from lymphoid-primed multipotent progenitor (LMPP) or common lymphoid progenitor (CLP) cells similar to T and NK (NK) cells in the bone marrow and fetal liver. ILC development proceeds through several stepwise, ILC-committed progenitors and requires a number of transcription factors that are expressed at unique points throughout development. Mature ILC1s, ILC2s, and ILC3s represent the major lineages of ILCs with unique transcriptional governance, stimuli, and cytokine production. These are primarily found at mucosal sites, in

adipose tissue, and in limited quantities in secondary lymphoid organs (SLO). α LP, α -integrin-expressing lymphoid precursor; CHILP, common helper innate lymphoid progenitor; IFN, interferon; IL, interleukin; ILCP, innate lymphoid cell progenitor; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; PGD₂, prostaglandin D₂; TSLP, thymic stromal lymphopoietin. Image adapted and reprinted from *Annals of Allergy, Asthma & Immunology*, 119(6), Stier et al., “Innate lymphoid cells and allergic disease”, 480-488, Dec. 2017, with permission from Elsevier (2).

Figure 2: Flow cytometric exclusion of “doublets” and dead cells.

Bone marrow was harvested from 8-12 week old BALB/c female mice and processed for analytical flow cytometry as described in sections 3.1 and 3.2. Doublets were excluded using 1:1 gates for FSC-A/FSC-H and SSC-A/SSC-H. Dead cells were excluded as DAPI⁺.

Figure 3: Gating strategy for LMPPs, CLPs, CHILPs, and ILCPs in the bone marrow.

Bone marrow was harvested from 8-12 week old BALB/c female mice and processed for analytical flow cytometry as described in sections 3.1 and 3.2. Doublets and dead cells excluded as indicated in Figure 2. Values within flow plots indicate percentage of the parent population. Image reproduced from Stier et al., *Journal of Experimental Medicine*, 2018 January 2;215(1):263-281 with permission from Rockefeller University Press (14).

Figure 4: Analysis of ILC2P in fetal liver and adult bone marrow.

Fetal liver leukocytes were isolated by a brief collagenase/DNase digestion, manual disruption, and RBC lysis. Flow cytometric analysis for ILC2P was performed on these fetal liver leukocytes and compared to ILC2P in adult bone marrow. ILC2P were pre-gated for viable CD45⁺ singlets (not shown). Values within flow plots indicate percentage of the parent population.

Figure 5: Reconstitution of donor lineages after adoptive transfer of CLP.

Donor CLP were isolated per the methods section 3.3 from adult female CD45.2⁺ BALB/c mice and 1500 donor CLP were adoptively transferred via retroorbital injection into sublethally irradiated (5 Gy) adult female CD45.1⁺ BALB/c recipient mice. Mice were rested for 3 weeks to allow for partial leukocyte reconstitution. Mice were euthanized and splenocytes were evaluated for (A) donor CD45.2⁺ cells. (B) Subset analysis demonstrated reconstitution of donor CLP-derived adaptive lymphocytes (B cells, pre-gated for CD45.2⁺ CD3⁻) and innate lymphocytes (NK cells, pre-gated CD45.2⁺ CD3⁻ CD19⁻) but not myeloid cells (neutrophils, pre-gated CD45.2⁺), consistent with known CLP lineage restriction. Values within flow plots indicate percentage of the parent population.

Table 1:

Gating Strategy for ILC Progenitors.

Population	Gating Strategy
LMPP	Lin ⁻ CD25 ⁻ Flt3 ⁺ CD127 ⁻ Sca-1 ^{hi} c-Kit ^{hi}
CLP	Lin ⁻ CD25 ⁻ Flt3 ⁺ CD127 ⁺ Sca-1 ^{int} c-Kit ^{int}
CHILP	Lin ⁻ CD25 ⁻ Flt3 ⁻ CD127 ⁺ $\alpha_4\beta_7$ ^{hi}
ILCP	Lin ⁻ CD25 ⁻ Flt3 ⁻ CD127 ⁺ c-Kit ⁺ $\alpha_4\beta_7$ ⁺ PD-1 ⁺
ILC2P	Lin ⁻ IL-25R ⁺ CD25 ⁺ CD127 ⁺